REVIEW ARTICLE

RTX proteins: a highly diverse family secreted by a common mechanism

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Abstract

Repeats-in-toxin (RTX) exoproteins of Gram-negative bacteria form a steadily growing family of proteins with diverse biological functions. Their common feature is the unique mode of export across the bacterial envelope via the type I secretion system and the characteristic, typically nonapeptide, glycine- and aspartate-rich repeats binding Ca2+ ions. In this review, we summarize the current state of knowledge on the organization of rtx loci and on the biological and biochemical activities of therein encoded proteins. Applying several types of bioinformatic screens on the steadily growing set of sequenced bacterial genomes, over 1000 RTX family members were detected, with the biological functions of most of them remaining to be characterized. Activities of the so far characterized RTX family members are then discussed and classified according to functional categories, ranging from the historically first characterized pore-forming RTX leukotoxins, through the large multifunctional enzymatic toxins, bacteriocins, nodulation proteins, surface layer proteins, up to secreted hydrolytic enzymes exhibiting metalloprotease or lipase activities of industrial interest.

Introduction

With molecular cloning and DNA sequencing taking grounds in bacteriology labs, it has become clear since 1987 that a novel family of large secreted cytolytic toxins of Gram-negative pathogens emerged. Summarizing the similarities of the first five characterized determinants in a seminal MicroReview in 1991, Rodney A. Welch first introduced the concept of the RTX family of proteins characterized by the presence of arrays of glycine- and aspartate-rich nonapeptide repeats. He predicted that this was a broadly disseminated family, while at that time it was difficult to imagine how broad and variable it could be.

RTX proteins are produced by a variety of Gram-negative bacteria and exhibit two common features. The first is the presence of repetitions of glycine- and aspartate-rich sequences, typically nonapeptides, which are located in the carboxy-terminal portion of the protein. These form numerous sites for the binding of Ca2+ ions and are at the origin of the historical name of the protein family, where RTX stands for repeats in toxin (Welch, 2001). The second key feature is the unique mode of secretion of RTX proteins via the type I secretion system (TISS). Protein translocation occurs through an oligomeric secretion channel spanning across the entire Gram-negative bacterial cell envelope (i.e. cytoplasmic membrane, periplasmic space and outer membrane). These dedicated ATP-binding cassette (ABC) transporter-based secretion apparatus recognize uncleavable C-terminal secretion signals and mediate a single-step translocation of the RTX polypeptides from bacterial cytosol across both the inner and the outer bacterial membranes, directly into the extracellular space and without a periplasmic secretion intermediate. The rtx genes and genes needed for secretion are mostly located within a single larger rtx locus.
RTX proteins represent a family of proteins that exhibit a wide range of activities and molecular masses from 40 to > 600 kDa. A prominent and historically first described group of RTX proteins consists of toxins, mostly exhibiting a cytotoxic pore-forming activity, often first detected as a hemolytic halo surrounding bacterial colonies grown on blood agar plates (Goebel & Hedgpeth, 1982; Muller et al., 1983; Felmlee et al., 1985; Welch, 1991).

While the word ‘toxin’ is, for historical reasons, embodied in the name of the RTX protein family, a broad class of RTX proteins also comprises secreted proteases and lipases. These can act as synergistic virulence factors causing tissue damage and/or by eliciting the production of inflammatory mediators. Recently, a subgroup of very large RTX proteins (> 3200 amino acid residues) with multiple activities [multifunctional autoprocessing RTX toxins (MARTX)] was discovered. For example, the prototype *Vibrio cholerae MARTX Vc* was shown to cause rounding of epithelial cells by catalyzing covalent cross-linking of cellular actin. RTX proteins can further act as bacteriocins or contribute to defense against environmental aggression by forming protective bacterial surface (S)-layers. Some RTX proteins were also found to play a role in plant nodulation or Cyanobacteria motility, while the biological role of most RTX proteins remains unknown. Bioinformatic mining of the explosively growing database of bacterial genomes indicates that RTX proteins form a large and diverse family of proteins, with a broad spectrum of biological and biochemical activities.

**RTX repeats**

The requirement for calcium ions in RTX toxin activities was first documented for *Escherichia coli* α-hemolysin (Short & Kurtz, 1971) and *Bordetella pertussis* CyaA (Hanski & Farfel, 1985). Binding of calcium ions to the repeats of RTX toxins occurs only upon secretion, as the intracellular cytoplasmic calcium concentration in bacteria is quite low (< 100 nM) (Gangola & Rosen, 1987). The RTX protein needs to unfold or remains in a floppy conformation before translocation out of the cell through the TISS (Kenny et al., 1991; Koronakis et al., 2000). Calcium binding to the nonapeptide repeats in the C-terminal portions of these toxins then appears to promote folding and imposes adoption of a functional conformation on the secreted RTX proteins in the extracellular environment (Felmlee & Welch, 1988; Ludwig et al., 1988; Rose et al., 1995; Rhodes et al., 2001).

Analysis of the three-dimensional structure of *Pseudomonas aeruginosa* alkaline protease possessing six of the RTX motifs with a consensus sequence X-(L/I/F)-X-G-G-X-G-(N/D)-D, where X means any residue, revealed that the repeated sequences constitute a new type of calcium-binding structure (Fig. 1), called a parallel β-helix or a parallel β-roll (Baumann et al., 1993). In this structure, the first six residues of each motif form a turn that binds calcium, and the remaining three residues build a short β-strand. The...
arrangement of consecutive turns and β-strands builds up a right-handed helix of parallel β-strands, where one turn of this helix consists in two consecutive nonapeptides. Calcium is then bound within two consecutive turns by the conserved aspartic acids.

The numbers of RTX repeats vary among RTX proteins from $< 10$ to $> 40$. While RTX proteases and lipases typically have a single block of seven to eight RTX nonapeptide repeats, very extensive RTX repeat domains were found recently in the large MARTX or in putative RTX proteins encoded in some sequenced genomes. Somewhere in the middle between the extremes is the RTX domain of CyaA from *B. pertussis*. For example, this is organized in five successive blocks, containing about eight nonapeptide RTX motifs each, which are separated by linkers of variable lengths (Glaser *et al*., 1988a; Osicka *et al*., 2000). The repeat domain of CyaA was then shown to harbor a small number (three to five) of high-affinity ($K_D < 1 \text{nM}$) and about 40 low-affinity ($K_D \sim 0.5–0.8 \text{mM}$) binding sites for $\text{Ca}^{2+}$ ions (Rose *et al*., 1995; Rhodes *et al*., 2001).

**TISS**

Gram-negative bacteria have evolved several (I–VI) pathways for protein secretion beyond the outer membrane to the extracellular environment (for reviews, see Saier *et al*., 2008; Fronzes *et al*., 2009). The RTX proteins contain an ~60-residue-long C-terminal secretion signal that is not processed during secretion (Gentschev *et al*., 1990; Stanley *et al*., 1991; Sebo & Ladant, 1993). This is recognized by the sec-independent TISS, which mediates the translocation of proteins directly from the cytoplasmic compartment into the extracellular space through a channel spanning the entire cell envelope (Fig. 2).

Type I secretion across the cell wall depends on three specific proteins: a polytopic inner membrane protein with a cytoplasmic ATPase domain operating as an ABC exporter, a membrane fusion protein (MFP) and an outer membrane protein (OMP). The MFP spans out from the inner membrane into the periplasm and contacts both the inner membrane ABC exporter and the OMP. The paradigm of the type I secretion pathway is based on the analysis of the mechanism of secretion of the *E. coli* α-hemolysin (HlyA). The Hly exporter was also shown to promote to some extent the secretion of a number of heterologous RTX proteins expressed in *E. coli*, including the CyaA of *B. pertussis* (Sebo & Ladant, 1993), LtkA of *Aggregatibacter* (formerly *Actinobacillus*, *Haemophilus*) *actinomycetemcomitans* (Lally *et al*., 1989), PaxA of *Pasteurella aerogenes* (Kuhnert *et al*., 2000) or FrpA of *Neisseria meningitidis* (Thompson & Sparling, 1993).

The *hlyCABD* operon (Fig. 3a) codes for the toxin activation protein (HlyC), the hemolysin itself (HlyA), the ABC transporter (HlyB) and the MFP protein (HlyD) (Wagner *et al*., 1983). The outer membrane component (TolC), a multifunctional protein, is encoded outside of the...
The hly operon on *E. coli* chromosome (Wandersman & Delepelaire, 1990) and is part of the mar-sox regulon (Aono et al., 1998). In some other species, however, the gene for a TolC homologue, such as *cyaE* of *B. pertussis*, is comprised in the rtx operon (Glaser et al., 1988b). TolC forms a trimeric export channel in the outer membrane and its presence plays a critical role in type I protein secretion (Delepelaire, 2004; Koronakis et al., 2004).

The structure of TolC was determined by X-ray crystallography (Koronakis et al., 2000). It has been proposed that the trimeric accessory protein HlyD forms a substrate-specific complex with the inner membrane protein HlyB, which subsequently recognizes the C-terminal signal peptide of HlyA. Upon binding of HlyA, the HlyD trimer interacts with the trimeric TolC protein of the outer membrane, inducing its conformational change and export of HlyA (Andersen et al., 2001). This complex appears to be transient, with the inner membrane complex of HlyB, HlyD and TolC disengaging and reverting to a resting state once the substrate has been transported (Thanabalu et al., 1998). The energy necessary for the secretion process depends not only on ATP hydrolysis mediated by HlyB but also on the proton motive force on the inner membrane (Koronakis et al., 1991, 1993, 1995).

However, the work on secretion of *Serratia marcescens* hemoprotein HasA and *Erwinia chrysanthemi* metalloproteases B and C indicated a slightly different order of events (Letoffe et al., 1996), where the ABC transporter and MFP would associate only upon substrate binding.

It is generally assumed that type I secretion involves the translocation of unfolded proteins (Young & Holland, 1999). Secretion of the HasA protein of *S. marcescens* was, indeed, shown to depend on the binding of the chaperone SecB. Moreover, HasA cannot be transported if allowed to fold in the cytoplasm (Delepelaire & Wandersman, 1998; Wolff et al., 2003). Intriguingly, contact with HlyD was suggested to affect directly or indirectly the folding of HlyA following or during its transit through the translocator (Pimenta et al., 2005).

An atypical TISS has been described for the large MARTX proteins in *Vibrio*. Here, the apparati consists of four proteins, an analogue of HlyB (RtxB), an analogue of HlyD (RtxD), a TolC-like protein and an additional ATP-binding protein RtxE, where both ABC exporter proteins, RtxB and RtxE, appear to be necessary for MARTX protein secretion (Boardman & Satchell, 2004; Lee et al., 2008).

A gene for a TolC-like OMP is also present in the protease and lipase secretion loci, but is absent from most other...
operons encoding the RTX toxin family TISS, except for B. pertussis (cya) or Moraxella bovis (mbx) loci (Glaser et al., 1988b; Angelos et al., 2003). A unique genetic organization of TISS for RTX proteins was revealed in N. meningitidis (Wooldridge et al., 2005). In contrast to the cistronic organization of the secretion genes for most other rtx operons, homologues of hlyD and tolC genes are flanked by genes normally associated with mobile genetic elements and do not form an operon with the hlyB gene. Furthermore, the three genes were shown to be expressed independently and mutation at either locus resulted in the inability to secrete the FrpC and FrpC-like (FrpC2) RTX proteins.

Initially, the gene encoding a TolC homologue had not been recognized in the genomes of A. actinomycetemcomitans HK1651 (http://www.genome.ou.edu/act.html) and Mannheimia haemolytica PHL213 (Gioia et al., 2006). Crosby & Kachlany (2007), however, found an ORF in A. actinomycetemcomitans that encodes the TdeA protein of predicted structural properties similar to TolC and showed that inactivation of the tdeA gene resulted in a mutant unable to secrete LtxA.

**Classes of RTX proteins**

**RTX cytotoxins**

Cytotoxic RTX proteins are produced by a broad range of Gram-negative pathogens (Table 1) including the genera *Escherichia*, Bordetella, Proteus, Morganella, Moraxella, Vibrio and Kingella, and members of the Pasteurellaceae family (Mannheimia, Pasteurella and Aggregatibacter). RTX cytotoxins can be roughly divided into two families: the conventional and broadly studied pore-forming leukotoxins and the more recently discovered very large MARTX. The best-studied prototype of the MARTX group is VcRtxA (or MARTX<sub>Nb</sub>) from *V. cholerae*, an over 4500-amino-acid-residue-long protein, which causes depolymerization of F-actin stress fibers in a broad range of cell types (Fullner & Mekalanos, 2000).

**Pore-forming RTX cytotoxins**

Pore-forming RTX cytotoxins represent a unique class of bacterial proteins that share (1) the requirement for post-translational activation through amide-linked fatty acylation of internal lysine residues; (2) possess a hydrophobic domain that was shown or is presumed to form cation-selective pores in target cell membranes; (3) are exported by TISSs; and (4) upon secretion are activated for exerting biological activity by binding calcium ions within the acidic glycine- and aspartate-rich nonapeptide repeats.

Based on the most obvious and/or historically first characterized activity, RTX toxins were divided into hemo-
lysins and leukotoxins. The so-called RTX ‘hemolysins’ were initially found to exhibit a limited target cell and species specificity, while the activity of leukotoxins was considered to be species and cell-type specific (Welch, 1991; Coote, 1992). For example, *E. coli* α-hemolysin (HlyA) appears to be rather promiscuous, exhibiting a well-detectable cytotoxic activity on a wide spectrum of cells from various species, including erythrocytes, granulocytes, monocytes, endothelial cells or renal epithelial cells from mice, ruminants and primates (Gadeberg & Orskov, 1984; Keane et al., 1987; Bhakdi et al., 1989, 1990; Mobley et al., 1990; Suttorp et al., 1990; Crosby & Kachlany, 2007). In turn, the leukotoxins of *A. actinomycetemcomitans* (LtxA) and *M. haemolytica* (LktA) appear to be quite selective and cytotoxic only to a restricted group of cell types in a species-specific manner (Shewen & Wilkie, 1982; Taichman et al., 1984, 1987; Strathdee & Lo, 1989).

However, this traditional classification as ‘hemolysins’ (pore-forming cytotoxins) and leukotoxins appears to be somewhat obsolete. Even the ‘promiscuous’ α-hemolysin of *E. coli* (HlyA) and the ‘hemolytic’ adenylate cyclase (AC) toxin (CyaA) of *Bordetella* were now found to preferentially bind and target leukocytes expressing the β<sub>2</sub>-integrins LFA-1 and Mac-1, respectively (Lally et al., 1997; Guermonprez et al., 2001). It appears more appropriate to assume that all pore-forming RTX toxins are primarily leukotoxins, with selectivity for leukocytes being, at least for some of them, eroded at supraphysiological toxin concentrations. The residual activity of the most potent cytolytic (pore-forming) leukotoxins, with a less narrow host spectrum, would then be readily detected as cytolytic activity towards a broader variety of cell types, including erythrocytes. In contrast to these ‘hemolysins,’ the true ‘leukotoxins’ would lack any obvious activity on other cell types other than leukocytes from a certain host species.

**Post-translational activation of RTX cytotoxins by covalent fatty acylation**

The cytolytic (pore-forming) RTX leukotoxins are synthesized as inactive protoxins that undergo post-translational activation before export from the toxin-producing bacteria. This consists in post-translational modification of ε-amino groups of internal lysine residues within conserved acylation sites by covalent attachment of amide-linked fatty acyl residues (Fig. 3b). This reaction is catalyzed by the RtxC acyltransferases expressed together with the protoxins (Goebel & Hedgpeth, 1982; Barry et al., 1991; Sebo et al., 1991). The mechanism of this novel type of protein acylation was analyzed in substantial detail for the prototype RTX toxin-activating and acyl-ACP-dependent protein acyltransferase HlyC, which converts the *E. coli* proHlyA to mature α-hemolysin toxin HlyA (Issartel et al., 1991; Stanley et al.,
Characterized members of the RTX toxin family

| RTX toxin | Bacterium | rtxA gene product size (kDa) | Cell type cytotoxicity and host specificity | Operon structure* | References |
|-----------|-----------|-----------------------------|---------------------------------------------|------------------|------------|
| HlyA      | Uropathogenic *Escherichia coli* | 110                          | Broad                                       | > CABD/tolC      | Goebel & Hedgpeth (1982) |
| EhxA      | Enterohemorrhagic *Escherichia coli* | 107                          | Human and bovine erythrocytes, leukocytes   | > CABD/tolC      | Schmidt et al. (1995)   |
| CyaA      | *Bordetella pertussis*              | 177                          | Primarily human CD11b+ myeloid phagocytes, activity detectable on all cell types | C < / > ABDE1    | Glaser et al. (1988a)   |
| LktA      | *Mannheimia haemolytica*            | 102                          | Bovine leukocytes and platelets, weak hemolytic activity | > CABD/tolC      | Lo et al. (1987)         |
| PlLktA    | *Mannheimia varigena*               | 102                          | Porcine leukocytes                         | > CABD/tolC      | Chang et al. (1993)      |
| PaxA      | Pasteurella aerogenes               | 107.5                        | Cohemolytic activity2                     | > CABD/tolC      | Kuhnert et al. (2000)    |
| PvxA      | Proteus vulgaris                    | 110                          | Erythrocytes                               | > CABD/tolC      | Welch (1987)              |
| MmxA      | *Morganella morganii*               | 110                          | Erythrocytes                               | > CABD/tolC      | Koronakis et al. (1987)  |
| LtxA      | *Aggregatibacter actinomycetemcomitans* | 114                        | Human and primate leukocytes               | > CABD/tolC      | Lally et al. (1989)       |
| ApxA      | *Actinobacillus pleuropneumoniae*   | 110                          | Broad                                      | > CABD/tolC      | Frey et al. (1991)        |
| ApxD      | *Actinobacillus pleuropneumoniae*   | 102.5                        | Narrow against porcine leukocytes, weak hemolytic activity | > CA/tolC4       | Chang et al. (1989)       |
| ApxIII A | *Actinobacillus pleuropneumoniae*   | 120                          | Porcine leukocytes, cohemolytic activity2   | > CABD/tolC      | Jansen et al. (1993)      |
| ApxIVA    | *Actinobacillus pleuropneumoniae*   | 200                          | Weak hemolytic activity                    | > ORF1/IVA6      | Schaller et al. (1999)    |
| ApxI      | *Actinobacillus suis*               | 110                          | Horse and porcine lymphocytes, erythrocytes | > CABD/tolC      | Schaller et al. (2000)    |
| ApxII     | *Actinobacillus suis*               | 102.5                        | Horse and porcine lymphocytes, erythrocytes | > CA/tolC       | Burrows & Lo (1992)       |
| ApxII     | *Actinobacillus porcitonsillarum*   | 102.5                        | Lymphocytes, erythrocytes                  | > CABD/tolC      | Kuhnert et al. (2005)     |
| AqxA      | *Actinobacillus equuli*             | 110                          | Horse and porcine lymphocytes, erythrocytes | > CABD/tolC      | Berthoud et al. (2002)    |
| VcRtxA    | *Vibrio cholerae*                   | 484                          | Monkey kidney fibroblasts, human laryngeal epithelial cells** | ACchp < / > BDE/tolC11 | Lin et al. (1999)         |
| VvRtxA    | *Vibrio vulnificus*                 | 550                          | Human intestinal epithelial cells           | ACchp < / > BDE/tolC11 | Chen et al. (2003)        |
| MbxA      | *Moraxella bovis*                   | 99                           | Bovine erythrocytes, leukocytes             | > CABD/tolC      | Angelos et al. (2003)     |
| RTX cytotoxin | *Kingella kingae*              | 7                            | Respiratory epithelial, synovial, macrophage-like cells | > CABD/tolC      | Kehl-Fie & St Geme (2007) |

* A-structural gene, BDE/tolC-components of the type I secretion apparatus, C-acyltransferase. With the exception of the cyaA and mbxA gene clusters, the CABD/tolC operon structure is in a 5'–3' gene order of CABD with toIC unlinked and encoded at a distant locus. The transcriptional organization of the RTX operons is indicated by arrowheads.
2 cyaA is homologous to tolC from *Escherichia coli*.
3 The CAMP test for cohemolytic activity was performed on blood agar plates using a β-hemolytic *Staphylococcus aureus* strain (Christie et al., 1944).
4 Not in an operon with type I secretion genes B and D. The secretion of ApxxA is mediated by ApxxB and ApxxD.
5 ORF1 appears to be required for ApxxV activity. Nothing is known about the secretion of ApxxV.
6 The rtxA-like genes from insect pathogens *Photorhabdus luminescens* and *Xenorhabdus bovienii* (Duchaud et al., 2003; Venter et al., 2004) were identified by genome sequence analysis.
7 No cytolytic activity, causes depolymerization of F-actin by cross-linking of G-actin.
8 The ORF named chp encodes a conserved hypothetical protein; the rtxE gene encodes additional ATPase that is related to rtxB.
9 RtxA toxin from *Vibrio vulnificus* lacks the actin cross-linking domain (Sheahan et al., 2004).
10 Product size unknown.
HlyC uses the fatty acyl residues carried by acyl-ACP to form a covalent acyl-HlyC intermediate, which transfers the fatty acyl residues to the ε-amino groups of Lys^{564} and Lys^{690} residues of proHlyA (Worsham et al., 2001, 2005). Several residues, including Ser^{22} and His^{23}, were identified as being potentially involved in the catalysis of acyl transfer by HlyC (Issartel et al., 1991; Trent et al., 1998, 1999a, b, c). Acyl-ACPs carrying various fatty acyl residues, including the palmitic (C16:0) and palmitoleic (C16:1) residues most common in E. coli, could be efficiently used in vitro as acyl donors for modification of HlyA, while acyl-CoA is not used as a substrate by HlyC (Issartel et al., 1991; Trent et al., 1998). In vivo, however, HlyC exhibits a high selectivity for C14:0 myristic acid, which was found to constitute about 68% of the acyl chains covalently linked to Lys^{564} and Lys^{690} of native HlyA (Lim et al., 2000). Surprisingly, the extremely rare odd carbon-saturated C15:0 and C17:0 fatty-acyl residues were found to constitute the rest of the in vivo acylation of HlyA from two different clinical E. coli isolates (Lim et al., 2000). The biological relevance of the use of odd-carbon acyl residues for activation of HlyA as well as the mechanism by which HlyC selects these acyl-ACP loaded by the extremely rare acyl residues remains to be clarified.

The role of acylation in toxin activity was analyzed for B. pertussis CyaA, where the extent of fatty acylation in vivo was found to depend on the producing strain. Initially, the Bp-CyaA extracted from a Tohama I-type B. pertussis 338 was found to be monoacylated by a single palmitoylation at the Lys^{983} residue only (Hackett et al., 1994). Further work confirmed that the acylation of Lys^{983} was necessary and sufficient for the activation of CyaA (Basar et al., 2001; Masin et al., 2005). The CyaA sequence, however, comprises two characteristic acylation sites conserved in the RTX cytolyin family, suggesting that CyaA can also be acylated on a second lysine residue, Lys^{980}. The recombinant r-Ec-CyaA toxin produced in the presence of CyaC in E. coli was, indeed, found to bear a second acylation at Lys^{980} (Hackett et al., 1995). Moreover, recombinant r-Bp-CyaA protein overproduced by a B. pertussis 18323/pBN strain was also later found to be acylated on both Lys^{980} and Lys^{983} residues (Havlicek et al., 2001). The reduced specific hemolytic activity of r-Ec-CyaA was then attributed to the modification by mainly the unsaturated palmitoleic (cis9 C16:1) fatty-acyl groups when produced in E. coli, while exclusively saturated C16:0 palmitoyl residues were found attached to Bp-CyaA in B. pertussis (Havlicek et al., 2001). Furthermore, acylation of each of the Lys^{980} or Lys^{983} residues alone was necessary and sufficient for conferring CyaA a full capacity to tightly bind its α_{2b}β_{2} integrin receptor (CD11b/CD18). The mutant CyaA-K983R, acylated only on Lys^{980}, still exhibited a fairly high (~20%) cytotoxic activity towards murine monocytic cells expressing CD11b/CD18, when compared with CyaA-K860R mutant monoacylated on the Lys^{983} residue alone, or to intact r-Ec-CyaA acylated on both Lys^{980} and Lys^{983} residues (Masin et al., 2005). Acylation of Lys^{983} appears, in turn, to be absolutely essential for the residual cytolytic activity of CyaA on cells lacking CD11b/CD18 (Basar et al., 1999, 2001).

Pore-forming RTX toxins require fatty acylation for all known cytotoxic activities. However, the exact role of the post-translational modification in the mechanism of action is not truly understood. The nonacylated proHlyA and proCyaA form pores in planar lipid bilayers with a much reduced propensity, but the formed pores have quite similar properties as the pores generated by acylated toxin (Ludwig et al., 1996; Masin et al., 2005). Both nonacylated proHlyA and proCyaA are also quite active in penetrating a naked liposome membrane (Soloaga et al., 1996; Masin et al., 2004), suggesting that the acyl residues are not essential for toxin penetration into the membrane lipid bilayer. Recent evidence indicated that fatty-acylation status and nature may modulate toxin oligomerization and is essential for productive binding of RTX toxins to target cell receptors, allowing the cytotoxic action to occur (Sun et al., 1999; El-Azami-El-Idrissi et al., 2003; Thumbikat et al., 2003; Masin et al., 2005).

Pore-forming activity and interaction with the cell membrane in the absence of a specific cell receptor

The highly potent and less specific cytolytic RTX leukotoxins, such as E. coli HlyA or Bordetella CyaA, also exhibit a readily detectable activity on cells other than leukocytes. Their interaction with the target cell membrane devoid of a specific proteinaceous receptor appears to occur in two steps, starting with a reversible adsorption of the toxin that is sensitive to electrostatic forces, which is then followed by an irreversible membrane insertion (Bakas et al., 1996; Ostołaza et al., 1997). Adsorption of RTX toxins is detectable on both toxin-sensitive cells and on certain toxin-resistant cells (Iwase et al., 1990). Studies with the isolated calcium-binding domain of HlyA revealed that this part of the protein may adsorb on the membrane in the early stages of HlyA–membrane interaction (Sanchez-Magraner et al., 2007). Recent results with CyaA, HlyA and LtxA showed that these toxins exhibit a weak lectin activity and recognize and bind the N-linked oligosaccharides of their β_2 integrin receptors (Morová et al., 2008). This raises the possibility that the initial unsaturable binding of RTX cytotoxins to various cells might occur through the recognition of glycosylated membrane components, such as glycoproteins and gangliosides.

Whether proteinaceous receptors are involved in binding of RTX toxins to cell types other than leukocytes remains an open question. For example, earlier dose–response binding
assays indicated an upper limit of 4000 HlyA binding sites per erythrocyte, implying at least some degree of specificity (Bauer & Welch, 1996b). Eberspächer et al. (1989), however, did not observe any saturability of binding of HlyA to erythrocytes, suggesting that binding was receptor independent. These data are compatible with later observations of Cortajarena et al. (2001, 2003), showing that HlyA can use the abundant glycophorin protein as a high-affinity receptor on erythrocytes. HlyA binding and action on erythrocytes was blocked by antibodies binding glycophorin, by a competing peptide comprising residues 914–936 of HlyA or upon glycophorin digestion with trypsin.

Once HlyA has inserted into the cell membrane, it appears to undergo an irreversible conformational change (Moayeri & Welch, 1997), after which it cannot be recovered from the membrane without the use of detergents (Bhakdi et al., 1986). However, the mechanism of membrane insertion and pore formation by RTX toxins remains poorly understood. Several studies confirmed that the hydrophobic domain of the N-terminal half of the pore-forming RTX leukotoxins is critical for their ability to form transmembrane pores (Ludwig et al., 1987; Glaser et al., 1988a; Cruz et al., 1990; Osickova et al., 1999; Basler et al., 2007). Other studies also showed that the hydrophobic region of E. coli HlyA was responsible for the insertion of the toxin into the target membrane (Hyland et al., 2001; Schindel et al., 2001). Biophysical studies demonstrated that RTX toxins form cation-selective pores of a defined size and with short lifetimes of only a few seconds (Menestrina et al., 1987, 1996; Benz et al., 1989, 1994; Szabo et al., 1994; Lear et al., 1995; Maier et al., 1996; Schmidt et al., 1996; Karakelian et al., 1998). At higher toxin concentrations, however, these pores may change subunit stoichiometry over time and aggregate into larger lesions in the cell membrane (Moayeri & Welch, 1994).

Whether pore formation by the RTX leukotoxins depends on the toxin oligomerization step remained a matter of controversy. HlyA has been recovered from target membranes only as a monomer (Menestrina et al., 1987; Eberspächer et al., 1989; Stanley et al., 1993). On the other hand, the dose–response analyses indicated that the lytic activity on target cells was a highly cooperative function of toxin concentration, suggesting that oligomerization was involved in RTX toxin pore formation (Cavaliere & Snyder, 1982; Simpson et al., 1988; Bhakdi et al., 1989; Taichman et al., 1991b; Betsou et al., 1993; Szabo et al., 1994; Bauer & Welch, 1996a; Gray et al., 1998; Osickova et al., 1999). Moreover, in vitro complementation within pairs of individually inactive deletion variants of E. coli HlyA or B. pertussis CyaA allowed to restore, at least in part, the hemolytic and cytotoxic activities, suggesting that two or more toxin molecules had aggregated to form a pore. This substantiated the view that oligomerization was involved in pore formation by HlyA or CyaA (Ludwig et al., 1993; Iwaki et al., 1995; Bejerano et al., 1999). Recent results obtained with CyaA revealed the presence of rather unstable (dynamic stoichiometry) CyaA oligomers in the erythrocyte membrane, revealing a correlation between oligomerization of CyaA mutants in the membrane and their pore-forming capacity (Vojtova-Vodolanova et al., 2009).

Binding through cell-specific receptors

Recently, it has become increasingly clear that even the more ‘promiscuous’ RTX leukotoxins bind leukocytes through specific proteinaceous receptors of the β2 integrin family. For example, CyaA of B. pertussis was shown to use the αMβ2 (CD11b/CD18) integrin (known also as a complement receptor 3 or Mac-1) as a target-cell specific receptor (Guermontrez et al., 2001). HlyA of E. coli was also found to specifically bind leukocytes (Welch, 1991) and to interact with the β2 integrin CD11a/CD18 at low concentrations (Lally et al., 1997). Other studies, however, indicated that the binding of HlyA to cells occurred in a nonsaturable manner and the toxin did not interact with a specific protein receptor on granulocytes (Valeva et al., 2005). These contradictory results await definitive clarification and it is possible that too low concentrations of HlyA were used in the latter study and saturation of the abundant CD11a receptor was not reached. Moreover, A. actinomycetemcomitans and M. haemolytica leukotoxins (LtxA and LktA, respectively) are specific for human and bovine leukocytes, respectively, and were also found to interact with CD11a/CD18 (Lally et al., 1997; Ambagala et al., 1999; Li et al., 1999). The initial interaction of RTX leukotoxins with β2 integrins then appears to rely on the recognition of N-linked glycans, as revealed for A. actinomycetemcomitans LtxA, E. coli HlyA and B. pertussis CyaA (Morova et al., 2008).

Elevation of calcium concentration in target cells

Elevation and modulation of free cytosolic calcium concentrations are basic strategies of host cell manipulation by pathogens (TranVan Nhieu et al., 2004). Cytosolic calcium levels are tightly controlled and their modulation is part of most prominent cellular signalling pathways that regulate many cellular processes (Berridge et al., 1998, 2000). The initial observation that HlyA of E. coli was responsible for significant calcium influx into cells was made by Jørgensen et al. (1983). This was corroborated by demonstrating that pore-forming RTX toxins, such as M. haemolytica LktA, A. actinomycetemcomitans LtxA or B. pertussis CyaA, also caused unregulated calcium influx into target cells (Taichman et al., 1991b; Sun et al., 1999; Fiser et al., 2007). It has even been proposed that cell killing by RTX toxins was due to unregulated calcium influx, which would initiate cytoskeletal destruction and cell lysis (Welch, 2001). This mechanism may underlie the action of A. actinomycetemcomitans LtxA that was
reported to promote Ca\(^{2+}\) changes in T-cells and to initiate a series of events that involve the activation of calpain, talin cleavage, mobilization of \(\beta_2\) integrin molecules into membrane lipid rafts and subsequent cell lysis (Fong et al., 2006). Other findings indicate that the calcium influx induced by sublytic doses of RTX toxins accounts for the induction of inflammatory responses (Hsuan et al., 1999; Uhlen et al., 2000; Cudd et al., 2003b).

There persists, nevertheless, a controversy on the mechanisms by which specifically the RTX family of toxins would promote calcium influx into cells. Uhlen et al. (2000) reported that sublytic doses of \textit{E. coli} HlyA stimulated oscillatory calcium responses in renal epithelial cells through the activation of L-type voltage-gated calcium channels and the subsequent response of IP\(_3\) receptor channels in the endoplasmic reticulum. Moreover, \textit{M. haemolytica} LktA appears to increase the cytoplasmic Ca\(^{2+}\) concentration in bovine alveolar macrophages and neutrophils both by the influx of extracellular Ca\(^{2+}\) through voltage-gated channels (Ortiz-Carranza & Czuprynski, 1992; Hsuan et al., 1998; Cudd et al., 2003a) as well as by promoting the release of Ca\(^{2+}\) from stores in the endoplasmic reticulum (Cudd et al., 2003a). However, working with different concentrations of HlyA on different cell lines, others have reported that HlyA induces an increase in cytoplasmic [Ca\(^{2+}\)]\(_i\), by allowing a passive influx of Ca\(^{2+}\) into cells through toxin pores (Valeva et al., 2005; Koschinski et al., 2006). Moreover, Fiser et al. (2007) reported that \textit{B. pertussis} CyaA caused an increase of [Ca\(^{2+}\)]\(_i\) in monocytes by a mechanism that is independent of its pore-forming activity or of Ca\(^{2+}\) release from intracellular stores and depends on membrane translocation of the N-terminal cell-invasive domain polypeptide, but not on its enzymatic activity. The translocating AC domain, as such, appears, indeed, to participate in the formation of a novel and transient Ca\(^{2+}\) influx path in the host cell membrane (Fiser et al., 2007).

These seemingly contradictory results may be reconciled in part by the observed positive feedback effect on initial cell membrane permeabilization due to toxin insertion. As shown for HlyA, ATP leakage and Ca\(^{2+}\) influx, accompanying membrane insertion of HlyA, induces the activation of purinergic receptors and pannexin channels that are permeable for monovalent cations and Ca\(^{2+}\). This further potentiates the influx of extracellular Ca\(^{2+}\) and contributes to cell lysis (Skals et al., 2009).

**Broadly cytolytic RTX leukotoxins (hemolysins)**

RTX leukotoxins are typically produced by Gram-negative pathogens and commensals of respiratory, gastrointestinal or reproductive tracts or oral cavities of animals and humans. The characterized broadly cytolytic RTX leukotoxins, thus far classified as ‘hemolysins’, are listed in Table 1. These toxins exhibit a hemolytic activity in \textit{vitro} that is revealed by cultivating corresponding bacteria on sheep blood agar plates. \textit{In vivo}, these toxins induce the production of inflammatory mediators or display cytotoxic and cytolytic effects on host immune cells of different species, thus inducing necrosis, apoptosis and inflammation (Czuprynski \\& Welch, 1995; Welch, 2001; Frey \\& Kuhnert, 2002).

**HlyA of \textit{E. coli}**

Among the best-characterized RTX ‘hemolysins’ is HlyA, the single polypeptide (107 kDa) \(\alpha\)-hemolysin secreted by uropathogenic as well as many commensal fecal isolates of \textit{E. coli} (Fig. 3). The N-terminal 200 amino acid hydrophobic domain of HlyA is predicted to contain nine amphipathic \(\alpha\)-helices (Hyland et al., 2001), while the C-terminal Ca\(^{2+}\)-binding domain contains 11–17 of the glycine- and aspartate-rich nonapeptide \(\beta\)-strand repeats (depending on the stringency of the criterium for consensus motif conservation). It is assumed that membrane interaction of HlyA occurs mainly through the amphipathic \(\alpha\)-helical domain. However, it has been proposed recently that both major domains of HlyA are directly involved in the membrane interaction of HlyA, the Ca\(^{2+}\)-binding domain being responsible for the early stages of HlyA docking to the target membrane (Sanchez-Magraner et al., 2007). Similarly, Masin et al. (2004) showed that both the pore-forming and the acylation domain of CyaA are required for membrane interaction. Cortajarena et al. (2001) observed that a short sequence from the C-terminal domain (amino acid 914–936) was the main HlyA segment binding the \(\alpha\)-glycoporphin on erythrocytes.

A homologous EhxA protein is produced by the enterohemorrhagic \textit{E. coli} O157:H7 from a gene located on a 90 kbp plasmid (pO157). EhxA exhibits 61% identity to HlyA, but displays a more narrow target cell specificity and binds erythrocytes less efficiently (Bauer \\& Welch, 1996b; Stanley et al., 1998), exhibiting virtually no activity against human leukocytes (Bauer \\& Welch, 1996b).

**MmxA, MbxA and PvxA of \textit{Enterobacteriaceae} and MbxA of \textit{Moraxellaceae}**

‘Hemolysins’ homologous to HlyA were identified by Koronakis et al. (1987) as MmxA of \textit{Morganella morganii} and PvxA of \textit{Proteus vulgaris}, where MmxA also exhibited cytotoxic activity towards human polymorphonuclear leukocytes (Eberspach et al., 1990). The MbxA secreted by \textit{M. bovis} is implicated in the pathogenesis of infectious bovine keratoconjunctivitis (Brown et al., 1998), where it promotes corneal epithelial cell damage (Rogers et al., 1987; Beard \\& Moore, 1994). The \textit{mbxCABD} genes form a classical rtX operon that is absent from nonhemolytic strains (Angelos et al., 2003) and is part of a mobile genetic element designated as a pathogenicity island of \textit{M. bovis} (Hess \\& Angelos, 2006).
A large group of homologous cytolytic RTX ‘hemolysins’ is secreted by bacteria of the genus Pasteurellaceae. These include ApxIA, ApxIIA, ApxIIIA, AqxA and PaxA of Pasteurella aerogenes (Chang et al., 1989; Rycroft et al., 1991; Frey & Kuhnert, 2002) or PaxA of Actinobacillus equuli (Berthoud et al., 2002) or PaxA of Pasteurella aerogenes (Frey & Kuhnert, 2002). In fact, genetic analysis suggests that RTX determinants might have evolved in Pasteurellaceae and spread to other Gram-negative bacteria by horizontal gene transfer.

Studies involving apx deletion mutants and trans-complementation experiments show that ApxIIA is essential in the pathogenesis of porcine pleuropneumonia and that the combination of ApxIA and ApxIIA, secreted by certain serotypes of Actinobacillus pleuropneumoniae, accounts for the severe course of the disease with a fatal outcome (Reimer et al., 1995). The ApxIIIA protein (Rycroft et al., 1991; Frey et al., 1993a, b) as well as PaxA of P. aerogenes (Kuhnert et al., 2000) are nonhemolytic on erythrocytes, but show a significant CAMP reaction, a cohemolytic reaction dependent on the sphinogmyelinase from β-hemolytic Staphylococcus aureus (Frey et al., 1994; Kuhnert et al., 2000). ApxIIIA was shown to be highly cytotoxic against macrophages and was previously called the macrophage toxin (Lally et al., 1989; MacDonald & Rycroft, 1992). The cytotoxic activity of PaxA, also produced by Pasteurella mairi, has not yet been analyzed in detail (Frey & Kuhnert, 2002), but all P. aerogenes and P. mairi isolated from aborted feta or neonatal septicemia of pigs produced PaxA, while strains devoid of the paxA gene are isolated as opportunistic pathogens or commensals (Kuhnert et al., 2000). In general, there are little molecular and functional data available on the role in virulence and host or target cell specificity of most of the RTX toxins from animal pathogens.

Species-specific RTX leukotoxins

The sensu stricto RTX leukotoxins appear to be active only on a restricted group of cell types in a species-specific manner (Frey & Kuhnert, 2002; Henderson et al., 2003; Zecchinon et al., 2005). For example, the biological effect of LktA produced by M. haemolytica is largely restricted to ruminant leukocytes and platelets (Kaehler et al., 1980; Shewen & Wilkie, 1982; Strathdee & Lo, 1987; Brown et al., 1997), whereas A. actinomyctetemcomitans LtxA only kills lymphocytes and granulocytes from humans, the Great Ape and Old World monkeys (Taichman et al., 1984, 1987). Both LktA and LtxA, however, also exhibit a detectable hemolytic activity on erythrocytes (Shewen & Wilkie, 1982; Taichman et al., 1991a; Balashova et al., 2006).

LktA plays a role in the pathogenesis of bovine and ovine pneumatic Pasteurellosis (Jeyaseelan et al., 2002), whereas A. actinomyctetemcomitans LtxA is the main virulence factor of localized aggressive periodontitis in humans (Henderson et al., 2003; Fine et al., 2006; Haubek et al., 2006). These toxins can inhibit the mobility, chemotaxis and respiratory burst of neutrophils, release inflammatory mediators from granulocytes or macrophages, prevent phagocytosis by polymorphonuclear cells and disrupt the phagosome, thus allowing bacterial invasion of the phagocyte. Altogether, these effects strongly reduce the immune response of the host. The A. actinomyctetemcomitans LtxA was further reported to interact with Cu, Zn superoxide dismutase, which may protect both the bacteria and the LtxA from reactive oxygen species produced by host inflammatory cells (Balashova et al., 2007).

Cytolytic RTX leukotoxins (Table 1) are proteins of a typical molecular mass of 100–120 kDa that possess similar structural and functional domains and are encoded within similar rtx operons as the RTX ‘hemolysins’ (Fig. 3) (Chang et al., 1987; Lo et al., 1987; Lally et al., 1989). LtxA shares 40–50% amino acid homology with the E. coli HlyA and M. haemolytica LktA. Similarly, both the nonacylated proLktA and proLtxA were in turn able to bind target cells without increasing the intracellular calcium concentration or inducing cytolysis (Sun et al., 1999; Highlander et al., 2000; Thumbikat et al., 2003; Balashova et al., 2009). The proLktA can be acylated to mature LktA by E. coli HlyC, Bordetella CyaC and Actinobacillus LtxC (Lally et al., 1994; Westrop et al., 1997). Substitution of Lys-554 to Thr or Cys reduced the lytic activity of LktA against bovine lymphocytes by only 40%, indicating the presence of a second functionally redundant acylation site (Pellett & Welch, 1996). Recently, two internal lysine residues of A. actinomyctemcomitans LtxA (Lys-562 and Lys-687) have been identified as targets for covalent fatty acid modification by the ltxC gene product (Balashova et al., 2009). Interestingly, LtxA is the only RTX toxin that has a basic pI of 8.9. Also, the RTX domains of both LktA and LtxA are shorter than that of HlyA and contain only seven and eight calcium-binding nonameric repeats, respectively (Lally et al., 1991b; Kuhnert et al., 1997). Their hydrophobic domain in the N-terminal part was proposed as the region that spans the target cell membrane and deletions in this region impair the cytolytic and pore-forming activities (Cruz et al., 1990). While the region of LtxA required for the recognition of human target cells appears to span the glycine-rich repeats (Lally et al., 1994), the segment determining specificity for ruminant cells has been associated with the N-terminal portion of LktA (Forestier & Welch, 1991; Thumbikat et al., 2003).

The production of leukotoxins is increased under anaerobic conditions (Spitznagel et al., 1995; Hritz et al., 1996; Kolodrubetz et al., 2003) and is regulated by environmental cues, such as the availability of iron (Balashova et al., 2006).
2006), temperature (Marciel & Highlander, 2001), sugar levels (Inoue et al., 2001) and the concentration of signalling molecules of the quorum-sensing system (Fong et al., 2001).

A difference in the release of LtxA into the culture medium and retention on the cell surface was observed between nonadherent (smooth) and adherent (rough) strains of A. actinomycetemcomitans (Tsai et al., 1984; Lally et al., 1991a; Ohta et al., 1991; Berthold et al., 1992; Kachlany et al., 2000) and LtxA release into the medium could be related to mutations in the tad gene involved in the tight nonspecific adherence of A. actinomycetemcomitans (Kachlany et al., 2001). Recently, A. actinomycetemcomitans LtxA was also found to be released within outer-membrane vesicles (Kato et al., 2002; Diaz et al., 2006). These are likely to deliver LtxA to host cells (Demuth et al., 2003), similar to the delivery of E. coli heat-labile enterotoxin into mammalian cells (Kesty et al., 2004).

The quite narrow host range of leukotoxins appears to be due to cell-specific binding through the β2 integrins. The requirement for β2 integrins in cytotoxic action was first observed for M. haemolytica LktA, which was not cytotoxic to neutrophils isolated from animals suffering from a bovine leukocyte adhesion deficiency. This deficiency is due to a single point mutation (D128G) in the highly conserved N-terminal 128 amino acid residues of human CD11a that are missing in the murine LFA-1 counterpart. However, the leukotxin species selectivity of LtxA by showing that it recognizes the extracellular region of bovine CD18 (Shantha-lingam & Srikrumaran (2009) have recently shown that the LktA-binding site is formed by amino acids 5–17 of CD18, which, surprisingly, comprise most of the amino acids of the signal peptide that remains intact on mature CD18 molecules on the cell surface.

Leukotxin activities against target cells are highly dose dependent. At sublytic concentrations, leukotoxins induce an increase in [Ca2+]i, in cells and activate neutrophils and mononuclear cells to undergo oxidative burst and degranulation (Czuprynski et al., 1991; Maheswaran et al., 1992; Stevens & Czuprynski, 1995; Balashova et al., 2009). This is accompanied by the production of several lipid mediators and proinflammatory cytokines from human macrophages (Johansson et al., 2000; Kelk et al., 2005, 2008). Pulmonary mast cells were shown to release histamine in response to LktA (Adusu et al., 1994) and LktA has also been shown to inhibit bovine lymphocyte blastogenesis induced by concanavalin A and pokeweed mitogen (Majury & Shewen, 1991). Mannheimia haemolytica LktA also affects the adhesion of platelets, which gives rise to fibrin deposits in lung alveoli of cattle with pasteurellosis (Cheryk et al., 1998; Nyarko et al., 1998). As the concentration of leukotoxins increases, target cells are stimulated to undergo apoptosis, involving LFA-1 signalling through protein kinase C and adverse effects on the mitochondria (Kato et al., 1995; Korostoff et al., 2000; Kelk et al., 2003; Atapattu & Czuprynski, 2005). At higher leukotoxin concentrations, the apoptotic mechanisms are exceeded and necrosis occurs due to pore formation. LktA-mediated cell permeabilization was shown to cause a rapid leakage of the intracellular potassium and cell swelling (Clinkenbeard et al., 1989a; Clinkenbeard & Upton, 1991). In vitro studies showed that LtxA formed voltage-gated ion channels of large conductance in the planar lipid bilayer, with an approximate functional diameter of 0.96 nm (Iwase et al., 1990; Lear et al., 1995). A pore size of about 1.2 nm was, in turn, deduced for LktA from osmotic protection experiments with raffinose (Clinkenbeard et al., 1989b). In addition, metabolites from phospholipase C activation (arachidonic acid) appear to contribute to LktA-induced cytolysis significantly (Jeyaseelan et al., 2001).

AC toxin – a bifunctional toxin with a cell-invasive enzymatic and pore-forming activity

The 1706-residue-long CyaA is unique among RTX leukotoxins by being a bifunctional toxin in which a cell-invasive
mediated endocytosis of the toxin (Gentile et al., 1994); (2) an activation domain, where the post-translational palmitoylation of lysine residues 860 and 983 of CyaA occurs (Hackett et al., 1994, 1995); and (3) a typical calcium-binding RTX domain, harboring the nonapeptide repeats of a consensus sequence X-(L/I/F)-X-G-G-X-G-(N/D)-D, which form numerous (~40) calcium-binding sites (Rose et al., 1995; Rhodes et al., 2001).

CyaA primarily targets and paralyzes with high efficacy the leukocytes expressing the α4β2 integrin (CD11b/CD18). With reduced efficiency, however, the toxin can also penetrate and deliver the AC enzyme into a variety of cells lacking the CD11b/CD18 (Hanksi, 1989; Bellalou et al., 1990). Unlike most other enzymatically active toxins, which penetrate into cell cytosol from endosomes, several reports showed that the AC is delivered directly across the cytoplasmic membrane of cells without the need for receptor-mediated endocytosis of the toxin (Gentile et al., 1988; Gordon et al., 1988; Guermonprez et al., 1999; Schlecht et al., 2004; Basler et al., 2006). In target cytosol, the N-terminal AC domain of CyaA binds intracellular calmodulin, whereupon its specific enzymatic activity is increased by ~10000-fold and catalyzes uncontrolled conversion of cellular ATP to cAMP, a key second messenger signalling molecule (Wolff et al., 1980; Confer & Eaton, 1982). This subverts the signalling of protein kinase A and essentially instantaneously ablates the bactericidal functions of phagocytes, such as oxidative burst and phagocytosis, and induces the secretion of immunomodulatory cytokines (Vojtova et al., 2006). Recently, the crystal structure of the AC domain of CyaA in the complex with the C-terminal fragment of calmodulin was resolved by Guo et al. (2005).

The main segment of CyaA required for binding to the α4β2 integrin was located in the glycine-rich repeat region between residues 1166 and 1281 of CyaA (El-Azami-El(Idrissi et al., 2003). However, cooperation and structural integrity of all domains of the ‘hemolysin’ moiety of CyaA appear to be critical for membrane insertion and translocation of the N-terminal AC enzyme domain into cell cytosol (Bellalou et al., 1990; Iwaki et al., 1995). Translocation itself, but not the mere insertion of CyaA into the cytoplasmic membrane of cells, is driven by a negative membrane potential (Otero et al., 1995). Fiser and colleagues showed that the translocating AC polypeptide inserted into the cell membrane participates in the formation of a novel type of membrane path for Ca2+ influx by calpain and enables mobilization of CyaA with CD11b/CD18 into lipid rafts, where the cholesterol-enriched lipid environment supports the translocation of the AC domain across membrane (Bumba et al., 2010). Moreover, translocation of the AC domain and oligomerization into cation-selective pores appear to represent two independent and parallel/competing activities of the membrane-inserted form of CyaA. Either activity can be upmodulated at the expense of the other by specific substitutions of key glutamate residues forming pairs in the predicted transmembrane segments between residues 500 and 700 of CyaA. These were found to play a critical role in cell binding, formation of cation-selective pores and translocation of the AC domain into the cells (Osicikova et al., 1999, 2010; Basler et al., 2007; Fiser et al., 2007; Vojtova-Vodolanova et al., 2009). The ‘hemolysin’ pores formed by CyaA have a diameter of only about 0.6–0.8 nm and the specific ‘hemolytic’ activity of CyaA is relatively low, compared with HlyA for example (Bellalou et al., 1990). It appears, nevertheless, to synergize with the invasive AC enzyme activity in maximizing the overall cytotoxic potency of the toxin on CD11b+ cells (Basler et al., 2006; Hewlett et al., 2006).

The role of CyaA in the interaction of Bordetella with cells of the respiratory epithelia and in the modulation of the host immune response through the induction of proinflammatory cytokine secretion remains poorly explored. Recent work has suggested that CyaA activity may account for the induction of IL-6 in tracheal epithelia colonized by B. pertussis (Basset et al., 2004). CyaA contributes to numerous pathologic effects in the murine model of lung infection, such as efficient pulmonary colonization, induction of histopathological lesions in lungs, recruitment of inflammatory leukocytes and induction of lethality (Weiss et al., 1984; Gueirard et al., 1998). Low concentrations (~1–5 ng mL$^{-1}$) of CyaA have recently been shown to effectively inhibit complement-mediated opsonophagocytosis, which is a crucial defense mechanism of naive unimmunized hosts (Kamanova et al., 2008). In fact, CyaA-mediated intoxication by cAMP was also found to inhibit the phagocytosis of B. pertussis cells via Fc receptors by neutrophils (Weingart & Weiss, 2000). CyaA activity further causes a loss of chemotactic and oxidative burst capacities required for the bactericidal activity of leukocytes (Friedman et al., 1987). Moreover, the toxin can induce macrophage apoptosis (Khelif et al., 1993; Khelif & Guiso, 1995) by a mechanism involving disruption of the membrane potential of mitochondria (Bachelet et al., 2002). In many Bordetella isolates, the CyaA protein remains attached to the bacterial surface following secretion, due to interaction with the filamentous hemagglutinin (Zaretzky et al., 2002). Most of the cell-attached CyaA appears, however, to be aggregated and unable to act as a ‘contact weapon’, because only the newly secreted CyaA was found to be capable of penetrating target cells and increasing the intracellular cAMP levels (Gray et al., 2004).
Use of CyaA in research and vaccine applications

Because Gram-negative bacteria generally do not express calmodulin homologues and the AC domain exhibits only residual enzyme activity in the absence of calmodulin, the AC domain of CyaA could elegantly be used as a reporter enzyme for tracing protein translocation into the eukaryotic cell cytosol (Sory & Cornelis, 1994). The AC (Cya) reporter protein is fused to an effector protein secreted through the type III secretion pathway (TTSS), such as the Yersinia Yop proteins. While the fusion exhibits only very low AC enzyme activity in the bacterial cell and/or the culture supernatants, once it is injected through the TTSS pathway into a eukaryotic host cell, the AC enzyme is activated > 1000-fold by host calmodulin and catalyzes the rapid conversion of ATP to cAMP. Use of the AC (Cya) fusion reporter has now become a standard technique for the demonstration of contact-dependent direct translocation of TTSS effector proteins into animal and plant host cells by a number of Gram-negative species.

Another original application makes use of the fact that the residual activity of the AC domain in the absence of calmodulin requires the physical interaction of the T25 and T18 fragments of the AC domain. This could be well exploited to develop a bacterial two-hybrid system for the detection of protein–protein interactions (Karimova et al., 2004). This allowed the use of CyaA for antigen delivery and induction of strong Th1-polarized and epitope-specific cytotoxic T-cell responses, effective in prophylactic and therapeutic vaccination against viruses and in immunotherapy of certain tumors (Sebo et al., 1995; Fayolle et al., 1996, 1999, 2001; Saron et al., 1997; Dadaglio et al., 2000; Loucka et al., 2002; Preville et al., 2005; Mackova et al., 2006). Phase I/II human clinical trials aimed at exploring this exciting application of CyaA for cervical cancer and melanoma immunotherapy are currently in preparation.

A quite different and recently discovered division of the RTX-toxin family is a group of very large toxins that differ from all previously known RTX proteins by the molecular structure and rtx gene cluster organization (Table 1, Fig. 3). These MARTX have thus far been identified in several different Vibrio species (Vibrio sp. RC385, V. cholerae, Vibrio splendidus, Vibrio anguillarum, Vibrio vulnificus) and are also present in Aeromonas hydrophila, Yersinia enterocolitica and Yersinia kristensenii, Proteus mirabilis, Photobahdus luminescens and Photorhabdus asymbiotica (Supporting Information, Tables S1 and S2) (Satchell, 2007; Li et al., 2008). The best-studied prototype of MARTX is the V. cholerae RtxA (VcRtxA), renamed to MARTXVc (Satchell, 2007).

All rtxA-like MARTX genes encode proteins that range from 3212 to 5206 amino acid residues in length. In contrast to other toxins, the C-terminal repeats of MARTX proteins exhibit an 18-residue-long consensus sequence X(V/I)XXGXX(X/V/I)XXGXXDX. These share a common G7X-GXXN central motif, instead of the typical nonapeptide repeat (Lin et al., 1999; Satchell, 2007). Moreover, MARTX proteins possess additional N-terminal repeats, which fall in two classes. The first has a 20-residue consensus sequence GXXG(N/D)(L/I)(T/S)FXGAG(A/G) XNX(L/I)X(RH) and the second has a 19-residue consensus sequence GXXG(N/D)(L/I)XXGDGXDX. These share a common G7X-GXXN central motif, instead of the typical nonapeptide repeat (Lin et al., 1999; Satchell, 2007). Moreover, MARTX proteins possess additional N-terminal repeats, which fall in two classes. The first has a 20-residue consensus sequence GXXG(N/D)(L/I)(T/S)FXGAG(A/G) XNX(L/I)X(RH) and the second has a 19-residue consensus sequence GXXG(N/D)(L/I)XXGDGXDX. These share a common G7X-GXXN central motif, instead of the typical nonapeptide repeat (Lin et al., 1999; Satchell, 2007).
transferase) and a conserved hypothetical gene (rtxH) of unknown function, which is found only in the marxt gene clusters. The divergent operon contains three more genes that encode homologues of the ATP-binding transporter protein RtxB, the MFP RtxD and a second ATPase, RtxE (Boardman & Satchell, 2004). The latter also appears to be involved in MARTX secretion by TISS, as rtxE gene disruption in *V. vulnificus* and *V. anguillarum* blocked the secretion of MARTX<sub>Vv</sub> (MARTX<sub>Vv</sub>, respectively) and induced a significant reduction in bacterial cytotoxic activity against epithelial cells *in vitro* (Lee et al., 2008; Li et al., 2008). The necessary homologues of the OMP tolC are then found outside the rtx loci. This atypical four-component TISS seems to be a conserved feature across the entire MARTX family (Boardman & Satchell, 2004).

The production of MARTX TISS components is regulated by the growth phase. The repressor regulating rtxBDE expression is encoded outside the rtx locus and is not directly linked to quorum sensing, while *V. cholerae* may apparently couple the regulation of the rtx locus to the detection of stress (Boardman et al., 2007). The rtxH, rtxC and rtxA genes are coordinately expressed on a single mRNA (Boardman et al., 2007).

The prototype rtxA gene was found in both clinical and environmental isolates of *V. cholerae*, but not in the O1 classical biotypes (Lin et al., 1999; Chow et al., 2001). The deduced MARTX<sub>Vc</sub> protein is 4545 residues long, with a predicted molecular mass of > 485 kDa (Lin et al., 1999). In contrast to pore-forming RTX leukotoxins, the MARTX<sub>Vc</sub> does not appear to disrupt membrane integrity or cause cell death. Rather, MARTX<sub>Vc</sub> activity contributes to the severity of acute inflammatory responses in the pathology of cholerla by inducing alteration of permeability of the paracellular tight junctions. This results from the capacity of MARTX<sub>Vc</sub> to induce cell rounding and depolymerization of the actin cytoskeleton in a broad range of cell types, and yet the cells remain viable (Cordero et al., 2006). Concurrent with actin stress fiber disassembly, actin monomers are covalently cross-linked into dimers, trimers and higher multimers by the actin cross-linking domain (ACD), which utilizes G-actin as a substrate and hydrolyzes one molecule of ATP per cross-linking event (Kudryashov et al., 2008a).

The ACD of MARTX<sub>Vc</sub> located between residues 1963 and 2375, catalyzes a unique reaction consisting of the formation of an intermolecular iso-peptide bond between the γ-carboxyl group of glutamic acid residue 270 and the ε-amino group of lysine residue 50 of actin (Kudryashov et al., 2008b). Contrary to expectation, however, deletion of the ACD did not ablate the cell-rounding activity of MARTX<sub>Vc</sub>, revealing that the large toxin carried a second cell-rounding activity (Lin et al., 1999; Fullner & Mekalanos, 2000; Fullner et al., 2001, 2002; Sheahan et al., 2004). This targets the regulation of the small Rho GTPases, Rho, Rac and Cdc42, rather than the Rho GTPase proteins directly, and a 548-residue-long Rho inactivation domain (RID) of MARTX<sub>Vc</sub> was recently found to inactivate the Rho GTPases by a mechanism distinct from other Rho-modifying bacterial toxins (Shehan & Satchell, 2007).

The MARTX<sub>Vc</sub> toxin was found to insert into the host cell plasma membrane and is supposed to directly translocate the ACD to the cytosol of cells in a way involving insertion of the N- and C-terminal repeat regions into the cell membrane. Inside the cell, the ACD is released into cell cytosol (Sheahan et al., 2004) through self-processing catalyzed by a conserved cysteine protease domain (CPD), which cleaves MARTX<sub>Vc</sub> between residues L<sup>3428</sup> and A<sup>3429</sup>. Three additional CPD-dependent processing sites were identified, all at leucine residues delimiting the ACD, RID and α/β junction domains (Prochazkova et al., 2009; Shen et al., 2009). Autoprocessing of MARTX<sub>Vc</sub> thereupon results in the release of individual activity domains into target cytosol. Autoprocessing activity of the CPD is induced by binding inositol hexakisphosphate (InsP<sub>6</sub>), which is abundant at the inner surface of the cell membrane (Prochazkova & Satchell, 2008), and the CPD activation mechanism was recently characterized in molecular detail (Prochazkova et al., 2009). Because InsP<sub>6</sub> is exclusive to eukaryotes and is present at cytosolic concentrations > 10 μM, the evolution of a proteolytic biosensor responding to InsP<sub>6</sub> appears to be an ingenious strategy for ensuring that the functional activation of a secreted toxin occurs only once it has reached the host cell cytosol (Lupardus et al., 2008).

The sequence of the *V. vulnificus* RTX toxin (VvRtxA, MARTX<sub>Vv</sub>) was reported in 2003 (Chen et al., 2003), showing that it consists of 5206 residues and has a predicted molecular mass of 556 kDa (Lee et al., 2007). The deduced primary amino acid sequence of MARTX<sub>Vv</sub> is ~80–90% identical throughout most regions to that of MARTX<sub>Vc</sub>. However, no ACD is present in MARTX<sub>Vv</sub>, which also does not cause actin cross-linking (Sheahan et al., 2004) and possesses only the Rho-inactivating activity associated with an RID (Sheahan & Satchell, 2007). This is followed by a CPD located towards the C-end of MARTX<sub>Vv</sub> (Prochazkova & Satchell, 2008).

Unlike MARTX<sub>Vc</sub>, the MARTX<sub>Vv</sub> may be able to disrupt membranes by the predicted segments homologous to those of pore-forming RTX toxins. This would go well with the difference in the virulence of the pathogens, where *V. vulnificus* is exceedingly more destructive and cytolytic, as compared to *V. cholerae* (Lee et al., 2007). Indeed, MARTX<sub>Vv</sub> appears to be crucial for *V. vulnificus* virulence and cytotoxicity (Kim et al., 2007; Lee et al., 2007; Liu et al., 2007).

The RtxA of *V. anguillarum* appears to play a major role in the virulence of the agent causing vibriosis in fish. The rtx operon encodes a second hemolysin gene cluster in *V. anguillarum* M93Sm, which also has a hemolysin gene,
A particularly novel class of RTX proteins of *Vibrio* Cadherin domain proteins

by the presence of more than one cadherin domain and were represented by RtxL1 and RtxL2, which are characterized by *et al.* (Duchaud et al., 2003). The function of these genes in pathogenesis was not analyzed, but the actin cross-linking domain was not found in MARTX sequences from *Photobacterium*, which appear to have evolved unique cellular activities through the acquisition of a different genetic material (Sheahan et al., 2004).

Cadherin domain proteins

A particularly novel class of RTX proteins of *Vibrio* is represented by RtxL1 and RtxL2, which are characterized by the presence of more than one cadherin domain and were first identified in *V. cholerae* N16961 (Chatterjee et al., 2008). The *rtxl1* and *rtxl2* genes are arranged in tandem, which is different from the arrangement of the *V. cholerae* RTX gene cluster (Lin et al., 2003). RtxL1 and RtxL2 belong to the RTX family of hemolysins/leukotoxins, exhibiting hemolytic activity on human erythrocytes, but also appear to play a role in adherence and biofilm formation by *V. cholerae* N16961.

Both *rtxl1* and *rtxl2* genes are expressed in all *V. cholerae* isolates belonging to O1 (strains N16961, O395 and 569B), O139 (strain SG24) and non-O1-nonO139 (strains VCE232 and VCE309) serovars under natural and in vitro conditions and appear to play a role in virulence in a mouse model (Chatterjee et al., 2008).

Another member of the RTX-toxin group with cadherin domains was found as a novel RTX-like hemagglutinin (FrHa) of the *V. cholerae* O1 classical strain O395, the O1 El Tor strain A1552 and the O1 El Tor strain P27459 (Syed et al., 2009). FrHa expression is positively regulated by the flagellar regulatory hierarchy. It mediates adherence to chitin and epithelial cells, enhances biofilm formation and is involved in intestinal colonization in infant mice.

RTX proteases

The RTX proteases belong to microbial zinc metalloproteases (Hooper, 1994; Miyoshi & Shinoda, 2000) and form a group of approximately 50 kDa proteolytic enzymes secreted by a variety of pathogens. These proteases consist of an N-terminal proteolytic domain and a C-terminal calcium-binding RTX domain and are synthesized as zymogens that are activated by processing upon secretion.

The RTX proteases belong to the subgroup of metzincin metalloendopeptidases (Stock et al. 1995; Stocker et al., 1995) that contains an extended zinc-binding motif HEXXHXXGXXH, of which the three histidine residues are involved in binding the catalytically essential zinc ion, while the glutamic acid residue was postulated to take part in the catalytic activity. In addition, the metzincins all share a conserved methionine, which is located on a turn near the catalytic site, some 40–60 residues towards the C-terminus (the Met-turn).

To date, these proteases with characteristics of the RTX protein family were discovered and identified in six bacterial genera (Table 2): *Serratia* (Nakahama et al., 1986), *Erwinia* (Wandersman et al., 1987; Dahler et al., 1990; Letoff et al., 1990; Zhang et al., 1990), *Pseudomonas* (Guzzo et al., 1990; Duong et al., 1992; Liao & McCallus, 1998; Chabeaud et al., 2001; Woods et al., 2001), *Proteus* (Wassif et al., 1995; Walker et al., 1999), *Caulobacter* (Umelo-Njaka et al., 2002) and *Photobacterium* (Bowen et al., 2003).

Genetic organization of RTX protease loci

The RTX protease locus of *P. aeruginosa* alkaline protease AprA was first identified by Duong in 1992 (Duong et al., 1992). It consists of five ORFs, with aprD, aprE and aprF encoding the TISS proteins, the structural aprA gene for the protease and aprI encoding a protease inhibitor (inh).

The genetic organization of RTX protease operons varies. In *Pseudomonas fluorescens* CY091 (Liao & McCallus, 1998), the protease gene is located upstream of the inh and aprDEF transporter genes. The same organization of the locus was shown in *P. luminescens* (Bowen et al., 2003), starting with the structural *prtA* gene for the protease, and followed by a gene encoding a putative protease inhibitor, *inh* and then the three members of an associated TISS, **prtB**, **prtC** and **prtD** (Fig. 4).

| Bacterium                        | Described RTX proteases |
|---------------------------------|-------------------------|
| *Serratia marcescens*           | PrtSM                   |
| *Erwinia chrysanthemi*          | PrtG, PrtB, PrtC, PrtA  |
| *Erwinia amylovora*             | PrtA                    |
| *Pseudomonas aeruginosa*        | AprA                    |
| *Pseudomonas fluorescens*       | AprX                    |
| *Pseudomonas brassicaeaeorum*   | AprA                    |
| *Proteus mirabilis*             | ZapA, ZapE              |
| *Caulobacter crescentus*        | Sap                     |
| *Photobacterium*                | PrtA                    |
The genetic organization of RTX protease operons appears to depend on the number of secreted proteases. For example, *E. chrysanthemi*, a phytopathogenic enterobacterium, secretes four RTX proteases, where the first gene in the operon is the structural gene for *prtG* (Ghigo & Wandersman, 1992). This is followed by a gene encoding a putative protease inhibitor, *inh*, and then the three genes encoding TISS components, *prtD*, *prtE* and *prtF*. Structural genes for the other three RTX proteases *prtB*, *prtC* and *prtA* are adjacent to and belong to independent transcription units (Delepelaire & Wandersman, 1989; Dahler et al., 1990; Ghigo & Wandersman, 1992).

Unlike *E. chrysanthemi*, the other phytopathogenic species, *Erwinia amylovora*, secretes a single protease (Zhang et al., 1999), while *P. mirabilis* produces two RTX proteases: ZapA and ZapE (Walker et al., 1999). ZapE shares homology with ZapA and other RTX proteases, but is larger (687 residues).

In some bacterial species, the RTX protease operon is combined with the genes for RTX lipases or other proteins. For example, in *P. fluorescens* B52, an *aprX*-lipA operon contains the protease (*aprX*) and the lipase (*lipA*) genes encoded at opposite ends of a contiguous set of genes (Woods et al., 2001). This also includes the protease inhibitor, TISS genes and two autotransporter genes (*aprX*-inh-aprDEF-prtAB-lipA), constituting an operon (Ahn et al., 1999).

**Structure–function relationships of RTX proteases**

The typical size of RTX proteases is around 480 amino acid residues and 50 kDa, with the theoretical isoelectric point of most RTX proteases being about 4.0–4.6. Delepelaire & Wandersman (1989) first showed that the extracellular proteases B and C of *E. chrysanthemi* were synthesized as inactive zymogens proB and proC that were activated by autoprocessing of 16 or 18 amino acid residues (~2 kDa) from the N-termini in the external medium containing divalent cations. As other zinc metalloproteases, the RTX proteases are inhibited by the general metalloprotease inhibitor EDTA, as well as by *o*-phenanthroline, a specific zinc metalloprotease inhibitor (Zhang et al., 1999).

Three-dimensional structures of the alkaline proteases of *P. aeruginosa* (AprA) and PrtSM of *S. marcescens* were the first RTX protein structures resolved (Baumann et al., 1993; Baumann, 1994). Both proteases have a very similar two-domain structure. The N-terminal part is the proteolytic domain with a folding topology very similar to astacin, the archetypical metzincin protease (Bode et al., 1996). The C-terminal RTX domain consists of a 21-strand β-sandwich. Within this domain, a ‘parallel β roll’ structure was first described, in which successive β strands are wound in a right-handed spiral and Ca$^{2+}$ ions are bound within the turns between strands by a repeated GGXGXG sequence, thus setting a paradigm for the RTX portions of the whole protein family.
RTX protease inhibitors

The RTX protease inhibitor from E. chrysanthemi (Inh) was shown to be synthesized as a 12 kDa polypeptide having a signal peptide of 19 residues, which is cleaved off during Sec-dependent secretion into the periplasm (Letofte et al., 1989). The mature 10 kDa inhibitor is entirely located in the periplasm of E. chrysanthemi and its presumed physiological role consists in protecting periplasmic proteins against proteases that might potentially leak out from the TISS channel assembly. Inh forms a 1 : 1 complex with proteases A, B and C from E. chrysanthemi, AprA from P. aeruginosa and PrtSM from S. marcescens (Letofte et al., 1989). In the crystal structure of the complex of S. marcescens PrtSM with Inh from E. chrysanthemi (Baumann et al., 1995), the Inh was found to fold into a compact eight-stranded antiparallel β-barrel structure, interacting with the protease via five N-terminal residues that insert into the active site cleft of PrtSM.

Similar inhibitors have been characterized for other RTX proteases, such as P. aeruginosa (Feltzer et al., 2000), S. marcescens (Kim et al., 1995) and Photobacterium (Valens et al., 2002; Bowen et al., 2003). The SmaPI inhibitor of S. marcescens, however, shows a very high protease specificity, while the protease inhibitor of P. aeruginosa (APRin) exhibits a significantly higher inhibitory activity (K_D of 4 pM) compared with the inhibitors of E. chrysanthemi and S. marcescens (K_D values from 1 to 10 μM).

Biological activity of RTX proteases

Pseudomonas aeruginosa AprA was shown to hydrolyze fibrin and fibrinogen, with specific activities similar to plasmin (Shibuya et al., 1991). AprA also exhibits an anticoagulant activity in human plasma, which was attributed to its direct fibrinogenolytic function. This may account, at least in part, for the most characteristic pathologic feature of P. aeruginosa, its direct fibrinogenolytic function. This may account, at least in part, for the most characteristic pathologic feature of P. aeruginosa, which was attributed to its direct fibrinogenolytic function. This may account, at least in part, for the most characteristic pathologic feature of P. aeruginosa, which was attributed to its direct fibrinogenolytic function. This may account, at least in part, for the most characteristic pathologic feature of P. aeruginosa, which was attributed to its direct fibrinogenolytic function.

RTX bacterial lipases

Extracellular lipases of Gram-negative bacteria have been extensively characterized, being considered as valuable tools for a variety of biotechnological, biomedical and food industry applications (Jaeger et al., 1994). Until now, the genera Pseudomonas and Serratia were reported to produce RTX lipases of the I.3 subfamily. These do not have cysteine residues, do not require any additional gene products for activity and are secreted through TISS (Duong et al., 1994; Akatsuka et al., 1995; Ahn et al., 1999). Studies were conducted on lipases from P. fluorescens strain B52, SIK W1, no. 33, LS107d2, HU380 (Chung et al., 1991; Johnson et al., 1992; Tan & Miller, 1992; Kumura et al., 1998; Kojima & Shimizu, 2003; Kojima et al., 2003; Jiang et al., 2005), S. marcescens strains Sm6, Sr41 (Akatsuka et al., 1994; Li et al., 1995), Pseudomonas brassicacearum (Chabeaud et al., 2001) and Pseudomonas sp. strains MIS38 and KB700A (Amada et al., 2000; Rashid et al., 2001). The polyester polyurethanes PueA and PueB of Pseudomonas chlororaphis are also classified as lipases (Stern & Howard, 2000).
Lipases from *P. fluorescens* show remarkable sequence similarity to that of *S. marcescens*, with an identity of about 65% over virtually the entire length of the sequence (Li et al., 1995). The *S. marcescens* SM6 and *Pseudomonas* sp. MIS38 enzymes are 613 and 617 residues long, respectively, and compared with *P. fluorescens* lipase, which is 476 amino acids in length, appear to bear an extra domain consisting of 138 and 135 amino acid residues inserted between residues Asn⁴⁰⁵ and Thr⁴⁰⁶ of the *P. fluorescens* lipase backbone (Amanda et al., 2000).

Lipase production by *P. fluorescens* B52 was shown to be repressed by iron and is regulated by temperature. Optimal lipase production occurs well below the optimum growth temperature (Woods et al., 2001). Lipase production is also regulated by the homologue of the *E. coli* EnvZ-OmpR two-component osmoregulatory system and its secretion is reduced by NaCl (McCarthy et al., 2004). The lipase production by *P. brassicacearum* was, in turn, shown to be under the control of phase variation (McCarthy et al., 2004). In *S. marcescens*, the lipA gene is not linked to TISS component genes for LipB (ABC protein), LipC (MFP) and LipD (OMP), which, besides secreting LipA, can also promote the secretion of the metalloprotease, PrtA (Nakahama et al., 1986), and of the S-layer protein homologue SlaA (Kawai et al., 1998), and of the S-layer protein homologue SlaA (Kawai et al., 1998). In *P. fluorescens* SIK W1, the RTX lipase gene *tliA* is situated downstream of the ABC exporter genes *tliDEF*, with genes *ptrA* and *inh*, for an RTX protease and a protease inhibitor, being located upstream of *tliDEF* (Ahn et al., 1999). In *P. fluorescens* strain no. 33, *lipA* is clustered with an *aprA* gene for an alkaline protease, *aprDEF* genes for the TISS and *pspA* and *pspB* genes for two homologues of *Serratia* serine proteases (Kawai et al., 1999). In the *P. fluorescens* B52, *lipA* is situated downstream of the *aprX-inh-aprDEF-prtAB* operon (Woods et al., 2001). The organizations of the various gene clusters are depicted in Fig. 5.

**Fig. 5.** The schematic representation of *rtx* lipase gene clusters of lipases. The arrows represent coding regions and transcriptional directions of the genes deposited under the GenBank accession numbers: *Serratia marcescens* (D49826), *Pseudomonas fluorescens* SIK W1 (AF083061), *P. fluorescens* no. 33 (AB015053), *Pseudomonas brassicacearum* (AF286062).

**RTX bacteriocins**

Bacteriocins are structurally and functionally diverse bacterial toxins inhibiting the growth of other bacterial strains (Jacob et al., 1973). Plasmid-borne genes for bacteriocins belonging to the RTX protein family (Table 3) were found in Gram-negative plant endosymbionts and pathogens, such as *Rhizobium leguminosarum*, *Bradyrhizobium elkanii*, *Xyella fastidiosa*, *Xanthomonas oryzae* or *Agrobacterium tumefaciens*, respectively (Oresnik et al., 1999; Simpson et al., 2000; Venter et al., 2001; Watson et al., 2001; Cherif et al., 2006; Sugawara et al., 2007). The mechanism of action of these putative bacterial toxins remains to be characterized. Nodulation competition experiments with mutants lacking the RTX bacteriocin activity indicated a role in the competitiveness of *R. leguminosarum* 248 (Oresnik et al., 1999).

**S-layer RTX proteins**

S-layer proteins form regularly arranged two-dimensional crystalline arrays covering the entire outer surface of a broad spectrum of bacteria and archaea. These are often composed of a single protein or glycoprotein species of 40–200 kDa, which is endowed with the ability to assemble on the supporting envelope layer, thus representing one of the simplest self-assembly systems (Sleytr & Beveridge, 1999; Sleytr et al., 2007).

Several RTX proteins have been identified among S-layer proteins of pathogenic as well as nonpathogenic bacteria and cyanobacteria (see the section on RTX proteins involved in the motility of *Cyanobacteria* for more details). S-layer proteins have an acidic pH, lack cysteines and are produced in large quantities (10–12% of cell protein). They usually possess two structurally different domains, with the N-terminal part typically showing similarity to other S-layer proteins and the C-terminal part containing the
characteristic RTX structures and an uncleaved TISS signal, respectively. The RTX repeats of S-layer proteins were shown to bind calcium, which was proposed to mediate proper S-layer crystallization.

The best-studied S-layer RTX protein is the 98 kDa RsaA that forms a hexagonal lattice on the cell surface of *C. crescentus*. Six copies of RsaA form a ring-like subunit that interconnects with other subunits to form a two-dimensional array consisting of approximately 40,000 RsaA units and with a porosity predicted to exclude molecules larger than ~17 kDa (Smit *et al*., 1992). Some RsaA monomers are anchored to the outer bacterial membrane via interaction with smooth lipopolysaccharide, while the others are presumed to remain surface-associated by interaction with already attached RsaA monomers (Bingle *et al*., 1997a; Awram & Smit, 2001; Ford *et al*., 2007). Calcium appears to mediate proper RsaA crystallization and perturbations in RsaA at or near the RTX repeats result in the shedding of RsaA (Walker *et al*., 1994; Beveridge *et al*., 1997; Nomellini *et al*., 1997; Ford *et al*., 2007). It is presumed that a key function of the S-layer is to form a selective porosity barrier protecting the bacterium from a variety of predatorial assaults in the complex environments of bacterial biofilms. The S-layer appears to provide a barrier protecting against attack by the predatory *Bdellovibrio*-like bacterium (*Koval & Hynes*, 1991).

The N-terminal part of the 98 kDa RsaA protein shows the highest homology score to *Campylobacter fetus* S-layer protein, while its C-terminal portion contains five RTX repeats (Gilchrist *et al*., 1992). RsaA is produced constitutively to make up 10–12% of the total cell proteins, whereby it largely exceeds the secretion levels of any other type of RTX proteins. The ABC and MFP components of the TISS complex are encoded by rsaD and rsaE genes, located immediately downstream of the rsaA gene (Awram & Smit, 1998), while the rsaF<sub>a</sub> gene encodes one of two alternative OMP components of the RsaA secretion machinery and is located several kilobases downstream of the rsaA gene. The rsaF<sub>b</sub> gene encoding a second OMP component is completely unlinked. This is the only known example of a TISS that can utilize either of the two OMPs for the secretion of the same protein. Both OMPs, however, appear to be needed to handle the large amounts of RsaA produced, as neither one of the OMPs alone could induce wild-type secretion levels of RsaA (Toporowski *et al*., 2004).

Because of high secretion levels, cell surface location and geometrical packing of the RsaA protein, and thanks to the ease of genetic manipulation, the *Caulobacter* S-layer system has been exploited for biotechnology applications. These comprise the secretion of large quantities of ‘passenger’ proteins of economic and research interest into culture media (Bingle *et al*., 2000), or surface presentation of heterologous protein inserts on the the S-layer, such as S-layer-mediated display of the IgG-binding domain of streptococcal protein G (Bingle *et al*., 1997b; Umelo-Njaka *et al*., 2001; Nomellini *et al*., 2007).

Three other S-layer RTX proteins, Crs (1361 residues), CsxA (1123 residues) and CsxB (1238 residues), have also been identified in *Campylobacter rectus*, a Gram-negative bacterium associated with several forms of human periodontal disease (Miyamoto *et al*., 1998; Wang *et al*., 1998; Braun *et al*., 1999; LaGier & Threadgill, 2008). These proteins appear to be the potential virulence factors involved in the evasion of host defense, such as phagocytic uptake and bactericidal activity of serum (Okuda *et al*., 1997; Thompson, 2002).

SlaA, another S-layer family RTX protein of 1002 residues partially similar to the *C. crescentus* RsaA protein, was found in *S. marcescens* (Kawai *et al*., 1998). The 101 kDa protein appears to be exported by the Lip TISS system and a sequence dissimilarity in the N-terminal regions of SlaA has been observed among different strains of *S. marcescens*, which may be related to the antigenic variation of *S. marcescens* (Kawai *et al*., 1998).

### Table 3. Known members of the RTX bacteriocin family

| ORF     | Bacterium                                      | Molecular weight (kDa) | Number of RTX repeats |
|---------|------------------------------------------------|------------------------|-----------------------|
| ORF     | *Rhizobium leguminosarum* strain 248          | 102                    | 18                    |
| RzcA    | *Rhizobium leguminosarum* bv. *viciae* strain 306 | 439                    | 18                    |
| RtxA    | *Bradyrhizobium elkanii*                      | 88                     | NA                    |
| XF2407  | *Xylella fastidiosa*                          | 219                    | NA                    |
| XF2759  | *Xylella fastidiosa*                          | 139                    | NA                    |
| RtxA    | *Xanthomonas oryzae pv. oryzae*               | 48                     | NA                    |
| RzcA    | *Agrobacterium tumefaciens* strain C58       | 204                    | 54                    |

**RTX proteins involved in the motility of Cyanobacteria**

RTX proteins appear to be abundant in cyanobacteria, while the function of most of them remains elusive. Quite unexpectedly, however, some of the described cyanobacterial RTX proteins have been shown to be involved in cell motility, including SwmA of *Synechococcus* sp. strain...
WH8102, oscillin of Phormidium uncinatum or hemolysin-like protein SI1951 of Synechocystis sp. strain PCC 6803.

The RTX protein SwmA was suggested to be part of the S-layer and to be required for the swimming motility of the marine unicellular cyanobacterium Synechococcus sp. strain WH8102 (Brahamsha, 1996; McCarran et al., 2005). Swimming Synechococcus strains are observed to rotate around their longitudinal axis, as they translocate at speeds of up to 25 μm s⁻¹ and once fortuitously attached to a microscope slide, they rotate around the point of attachment. Intriguingly, cells with an insertionally inactivated swmA gene lack the S-layer and are nonmotile, and yet still rotate around the point of attachment. Thus, SwmA is somehow required for the generation of thrust, but not torque. However, the mechanism by which the SwmA and the S-layer function in motility remains elusive (Brahamsha, 1996; McCarran et al., 2005).

SwmA is a 130 kDa polypeptide that appears to be glycosylated and contains glycine- and aspartate-rich repeats. It is assumed that RTX repeats of SwmA function in calcium ion bridging to the outer membrane and thereby mediate the anchoring of the S-layer. Treatment of Synechococcus sp. strain WH8102 with the chelator EDTA, indeed, removes the outer membrane and solubilizes SwmA (Brahamsha, 1996; McCarran et al., 2005).

The surface fibrils on the top of P. uncinatum S-layer consist of a single rod-shaped RTX protein of 646 residues with 46 repeats, the oscillin. Its structure appears to favor gliding, a relatively slow and smooth surface-associated translocation (Hoiczyk & Baumeister, 1997). As proposed by Hoiczyk and colleagues, the highly glycosylated surface of oscillin fibrils possesses ideal physicochemical properties for the temporary adhesion of the slime necessary for the generation of thrust and the helical arrangement of oscillin fibrils might guide the rotation of the P. uncinatum filament (Hoiczyk & Baumeister, 1997; Hoiczyk, 2000).

The hemolysin-like RTX protein SI1951 of 1741 residues produced by a unicellular freshwater cyanobacterium Synechocystis appears to be related to the elimination of motility, although the mechanism of its action remains largely unknown (Sakiyama et al., 2006).

Nodulation RTX proteins
A 30 kDa RTX protein, NodO, was identified in R. leguminosarum bv. viciae and was shown to play a role in pea and vetch nodulation (Economou et al., 1990). The exact mechanism of the action of NodO in the process of the formation of nitrogen-fixing nodules on legume roots remains to be clarified. NodO was shown to form cation-selective channels in planar lipid bilayers (Sutton et al., 1994), and two hypotheses were proposed on how this might enhance nodulation. One assumes that NodO pores in root cell membrane facilitate the passage of lipooligosaccharide nodulation factors. Another possibility could be the synergy of signalling resulting from cation fluxes through NodO channels across the plasma membrane, such as Ca²⁺ entry into root cells, thus amplifying the response induced by lipooligosaccharide nodulation factors (Sutton et al., 1994). The pores formed by NodO in planar lipid bilayers are relatively large (> 2 nm) and remain stably open for rather long, but are not voltage gated.

The prsDE genes for the TISS used by NodO appear to be conserved in all members of the Rhizobiaceae tested, even though these strains do not contain a nodO gene. The TISS, however, appears to be required for the secretion of several other Ca²⁺-binding proteins that are involved in the formation and the nitrogen-fixing capacity of nodules induced by R. leguminosarum bv. viciae (Finnie et al., 1997). It also mediates the secretion of non-RTX glycanases PlyA and PlyB involved in the processing of Rhizobium exopolysaccharide (Finnie et al., 1998).

RTX proteins of unknown biological function
Initially, two partially homologous proteins, FrpA and FrpC, possessing the characteristic carboxy-proximal repetitions of the RTX nonapeptide motif, were discovered in N. meningitidis, a commensal of the human nasopharynx that occasionally causes invasive meningococcal disease (Thompson et al., 1993a, b). Biological activity of the Frp (Fe-regulated protein) proteins remains unknown. However, their secretion under iron-limited growth conditions, which mimic the condition in body fluids, and the elevated titers of antibodies against Frp proteins found in convalescent sera of patients from meningococcal disease, suggest a potential role of the FrpC-like proteins in meningococcal carriage or virulence (Oswicka et al., 2001).

Meningococci appear to carry a whole polymorphic family of frpC-like genes that code for proteins sharing large portions of identical sequence and varying in the number of C-terminal RTX repeat blocks and/or in insertions/deletions in the N-terminal portions. For example, the 122 kDa FrpA harbors 13 copies of nonapeptide repeats, while the 198 kDa FrpC has 43 copies. The N-terminal 293 residues of FrpA and the 407 N-terminal residues of FrpC do not exhibit any sequence homology, apparently due to the foreign DNA inserted at the 5'-end of the frpA gene (Thompson et al., 1993b; Parkhill et al., 2000; Tettelin et al., 2000).

The prototype FrpC protein of N. meningitidis Fam20 or MC58 strains is an 1829-residue-long protein with an amino-terminal portion of 876 residues and a carboxy-terminal RTX moiety of 953 residues. The N-terminal portion of FrpC (residues 1–414) does not exhibit significant homology to any known proteins. However, it binds with very high affinity the FrpD lipoprotein, which is expressed from a gene located immediately upstream of the
frpC gene in a predicted iron-regulated frpDC operon (Prochazkova et al., 2005). Recently, a unique 'clip-and-link' self-processing module (SPM) located between residues 414 and 657 of FrpC was characterized. It exhibits a high degree of sequence homology to segments of several RTX proteins of unknown function that are encoded in genomes of plant and animal pathogens (Osicka et al., 2004). Upon binding of calcium ions, the SPM produces a novel type of autocatalytic cleavage of the peptide bond between residues Asp414 and calcium ions, the SPM produces a novel type of autocatalytic cleavage of the peptide bond between residues Asp414 and Pro415 of FrpC. Moreover, the newly generated N-terminal fragment of FrpC can be covalently linked to another protein molecule by a novel type of Asp-Lys isopeptide bond, which forms between the carboxyl group of the C-terminal Asp414 residue of the thus generated fragment and the ε-amino group of an internal lysine of another protein molecule (Osicka et al., 2004). This defines a novel class of autoprocessing RTX proteins of unknown biological function, as the same type of calcium-dependent processing and cross-linking activity was also demonstrated for the purified ApxIVA protein of A. pleuropneumoniae (Osicka et al., 2004).

ApxIVA is specific to the species of A. pleuropneumoniae (Schaller et al., 1999) and is genetically quite distant from other known RTX toxins. It is produced by A. pleuropneumoniae only during infections in vivo. When expressed as a recombinant protein in E. coli, it shows slight hemolytic activity and a distinct cohemolytic effect (CAMP) reaction. The apxIV determinant lacks the activator C gene and the type I secretion genes B and D, which are found in loci for the production of additional Apx toxins (ApxI–ApxIII). However, upstream of apxIVA, an ORF can be found that is necessary for the hemolytic and CAMP activity of ApxIVA (Schaller et al., 1999; Frey & Kuhnert, 2002). ApxIVA was shown to be required for the full virulence of A. pleuropneumoniae, although its mechanism of action remains unclear (Liu et al., 2009).

Recently, a system for single-step affinity chromatography purification of untagged recombinant proteins based on the SPM of N. meningitidis was developed (Sadilkova et al., 2008). The N-terminus of the SPM is fused to a target protein of interest and the C-terminus to an affinity tag. Upon loading of cell lysate and binding of the fusion protein to an affinity matrix, contaminating proteins are washed away and site-specific cleavage of the Asp-Pro bond linking the target protein to the self-excision module is induced by the addition of calcium ions. This results in the release of the target protein with only a single aspartic acid residue added at the C-terminus, while the self-excising affinity module remains trapped on the affinity matrix (Sadilkova et al., 2008).

Identification of novel RTX proteins

Currently, almost 1000 prokaryotic genomes are fully sequenced and > 2000 genome sequencing projects are in progress. We downloaded 840 fully sequenced bacterial genomes available in February 2009 and the putative RTX proteins were identified using three different methods: (1) pattern search; (2) Hidden Markov Model (HMM) search by HMMER 2.3.2 (Eddy, 1998); and (3) rps-BLAST (Marchler-Bauer et al., 2002).

For the identification of RTX proteins by pattern search, we tested the Prosite pattern PS00330 (DX[L/I] XXXXGD(D/E)X[L/I]XGGXXXD) (Hulo et al., 2006), the pattern proposed for the AC toxin GGXXGNDX[L/I/F] (Bauche et al., 2006), and a universal short pattern for the calcium-binding site GGXXGD. Matches to a database of all bacterial proteins were identified by the program preg, which is a part of the EMBoss package (Rice et al., 2000). We analyzed > 2.75 million sequences of a total length of 865 million amino acid residues. As expected, the longer the tested pattern was, the fewer the proteins that contained a positive hit. In total, 2598 hits to the Prosite pattern were found in 773 proteins. Shorter patterns were identified 5337 and 34027 times, respectively, in 1210 and 20963 proteins, respectively. To assess how many hits could be expected at random, we calculated an average amino acid composition of the bacterial protein database and generated a database of proteins of a random sequence, but with the same average composition. The average protein composition was calculated by pepstats and random sequences were generated by makeproteq from the EMBoss package (Rice et al., 2000). The overall size of the randomized database was equal to the size of the database containing all bacterial proteins. The randomized database was tested for the presence of the aforementioned patterns and randomization and testing was repeated 100 times. On average, only 1.6 hits to the Prosite pattern were found in the random database. Not surprisingly, the other two shorter patterns were more frequent, with 373 and 20504 hits on average for GGGXXGNDX(L/I/F) and GGXXGD motifs, respectively. The specificity of shorter patterns in identifying RTX proteins could be improved by searching for multiple hits in a single protein. For the purpose of this review, we only considered proteins containing a hit to the Prosite pattern as being a putative RTX protein.

The alternative approach to identification of calcium-binding sites common to RTX proteins was to use the HMM-based model for hemolysin-type calcium-binding repeats, the PF00353 from Pfam database release 23.0 (Finn et al., 2006), which is an 18-residue-long model of two calcium-binding sites. We used HMMER 2.3.2 (Eddy, 1998) for the identification of proteins containing these calcium-binding sites. One thousand and thirteen proteins exhibited significant homology to the PF00353 Pfam model. On average, only 0.1 hits were found in the above-described random database. Thus, this approach appeared to be highly specific and generated virtually no false-positive hits.
The last used approach was to predict RTX proteins with RPS-BLAST (Marchler-Bauer et al., 2002). We searched the bacterial protein database for homology to the 510-amino-acid-long COG2931, position-specific scoring matrix (PSSM) that represents RTX toxins and the related Ca\(^{2+}\) binding proteins. In contrast to Pfam models, there was no cutoff e-value defined for PSSM; therefore, we tested e-value cutoffs lower from 10, 1, 0.1 and 0.01. An e-value cutoff equal to or higher than 1 was not specific enough and >15 proteins were identified even in the random sequence database. Using an e-value of 0.1 as a cutoff, >400 proteins were identified in the bacterial database, compared with only 1.25 in the random sequence database. We therefore considered a cutoff of e-value equal to or <0.1 as being sufficiently stringent for the purpose of this review.

The combination of results from the three above-described methods revealed 1024 putative RTX proteins (Table S1) in 251 bacterial species. In bacterial genomes containing at least one putative RTX protein, components of the TISS were identified by HMMER (Eddy, 1998). Towards this aim, the following models from the TIGRFAM database version 8.0 (Haft et al., 2003) were used: (1) TIGR01842, TIGR01846 and TIGR03375 for ABC transporters (PrtD, HlyB and LssB-like); (2) TIGR01843 for MFP (HlyD-like); and (3) TIGR01844 for OMP (TolC-like) (Table S2).

**Predicted RTX proteins**

Bacteria harboring RTX protein genes were mostly **Gamma-proteobacteria** (100 strains), **Alphaproteobacteria** (56 strains) and **Betaproteobacteria** (32 strains). Cyanobacteria were represented 25 times and RTX proteins appear, indeed, to be most abundant in cyanobacteria, with **Trichodesmium erythraeum** IMS101 bearing genes for 35 putative RTX proteins and up to 28 RTX proteins being encoded in the **Acaryochloris marina** MBIC11017 genome.

Surprisingly, putative RTX protein genes were also detected in seven Gram-positive bacteria genomes, with six **Actinobacteria** and one **Firmicutes** (**Streptococcus sanguinis** SK36), respectively. None of the **Actinobacteria** genomes, however, harbored any significant homologues of TISS components. Interestingly, the **S. sanguinis** SK36 genome contains an operon encoding an RTX protein, a HlyB-like protein and a HlyD-like protein. The TolC protein was not identified. A TolC homologue would, however, not be needed for Type I RTX secretion in a Gram-positive bacterium, which lacks the outer membrane. Whether the RTX protein and TISS components are really produced and functional in **S. sanguinis** remains to be determined.

RTX proteins identified by the aforementioned approaches vary significantly in length and range from <100 residues (protein NP_437763 of **Sinorhizobium melloti**) to 36,800 residues (protein YP_378930 of **Chlorobium chloratii**). Ninety percent of the putative RTX proteins fit to the range of 300–5000 residues, with an average of about 1600 residues. With the exception of three proteins, all RTX proteins appear to be acidic, with a theoretical pI in the range from 3.2 to 4.9 for 90% of the putative RTX proteins (Fig. 6).

We further predicted a number of possible Ca\(^{2+}\)-binding sites in the identified RTX proteins. The following consensus sequences were considered as potential binding sites for Ca\(^{2+}\): (1) GGXGX(D/N), (2) GXXGND, (3) GDXGXD, (4) GDAXXN, (5) GXGGXD, (6) GEAGDD and (7) GAGRVD, respectively. The highest number of almost 200 and 170 calcium-binding sites was predicted for the putative 4334-residue-long RTX protein of **Chlorobium limicola** and the 5107-residue-long protein from **Pseudomonas syringae pv. phaseolicola**, respectively.

Independent of the primary annotation, the prediction of functions of the putative RTX proteins was attempted. We used RPS-BLAST ver 2.2.20 (Altschul et al., 1997) to detect significant homologies of putative RTX proteins to PSSMs in the CDD database version 2.16 (Marchler-Bauer et al., 2002), which contains almost 32,000 domain models from Pfam, COG, SMART and KOG databases. Based on detected homologies to the CDD database, we were able to assign putative functions to about 30% of the identified RTX proteins (Table S1). The largest group would consist of proteases (112) and adhesins (113), the latter falling into two major classes of cadherins (52) and vWA domain.

**Fig. 6.** Comparison of size and pI distribution of the bulk of bacterial proteins and of the sum of characterized and putative RTX proteins. Molecular weight (MW) and pI was calculated for the bulk of bacterial proteins (about 2.75 mil., shown in black) and for the identified and predicted RTX proteins (1024 proteins, shown as red crosses) using the program pepstat, a part of the EMBOSS package (Rice et al., 2000). MW was log base 10 transformed (y-axis) and plotted against calculated pI (x-axis) in statistical package R version 2.9.0 (http://www.r-project.org/).
proteins, respectively (46). A further 20 lipases and 16 peroxidases were predicted.

About 20 enzymes were predicted to be involved in the degradation of saccharides and polysaccharides as glycosyl hydrolases, polysaccharide hydrolases and endoglucanases. About 20 putative RTX proteins might be involved in the degradation of DNA as endonucleases, nucleotidases and phosphodiesterases. A further four might act as phytases, one as a sulfatase, three as cyclophilin-like peptidylprolyl cis–trans isomerases and five as phosphatases, respectively.

Interestingly, three β-lactamases and one putative lysozyme were also predicted among the computationally detected putative RTX proteins. A further nine putative RTX proteins appear to exhibit weak homology to Hedgehog/Intein domain proteins, suggesting that they might undergo autoprocessing and splicing.

Conclusions

Being first recognized as a group of pore-forming bacterial leukotoxins two decades ago (Welch, 1991), the RTX family nowadays comprises a particularly broad range of exoproteins that play important roles in the colonization of various habitats and hosts by Gram-negative bacteria (Fig. 7). Bioinformatic mining of the growing database of bacterial genomes indicates that a large spectrum of biological and biochemical activities of RTX proteins still remains to be characterized. The unique and central overarching feature of RTX proteins is the presence of variable numbers of the calcium-binding glyine- and aspartate-rich C-terminal repeats. These separate the unprocessed C-terminal secretion signals from the specific bodies of individual proteins. By being intrinsically unstructured at the very low calcium concentrations found within bacterial cytoplasm, the repeats maintain an unfolded or a loosely folded state of the RTX protein that enables recognition of its secretion signal by a dedicated type I secretion ATPase assembly. This function of RTX repeats allows a single-step passage of extremely large proteins through the entire Gram-negative cell envelope. Binding of extracellular calcium ions to repeats emerging at the bacterial surface then ‘turns on’ structuration of the repeats, pulling the protein out of the...

Fig. 7. Use of a simple RTX building unit in generation of complex biological functionalities. By maintaining an unfolded state which allows a post-translational secretion from the calcium-depleted cytoplasm, and by promoting protein folding upon the binding of calcium ions (yellow balls) in the extracellular environment, the C-terminal assemblies of glycine- and aspartate-rich nonapeptide RTX repeat units first assist in the translocation of even very large RTX proteins. These transit across the entire Gram-negative bacterial cell envelope in a single step mediated by the dedicated the type I secretion machinery recognizing unprocessed C-terminal secretion signals. Proteins using this secretion pathway exhibit a very broad range of biological functions in colonizing diverse host environments. RTX proteins were found to exert activities like structural proteins involved in protective S-layer formation and motility of bacteria, in colonization of root nodules of plants by symbiotic bacteria, serving as bacteriocins on other bacteria, exerting hydrolase activities, or playing a prominent role as essential colonization and virulence factors of bacteria in animal hosts, respectively. Besides of a large group of pore-forming leukotoxins a particular sophistication of function is observed for the very large (thousands of residues long) RTX toxins consisting of multiple domains exhibiting enzymatic and cytotoxic activities (MARTX).
cells and driving folding of the rest of the protein. Moreover, the RTX repeats themselves support an amazingly vast array of biological activities, be it a role in the formation of S-layers, bacterial adherence/motility or host–receptor interaction and membrane penetration of RTX proteins. Hence, through a simple variation of number and block arrangement of a small repeat unit, an elegant construction of sophisticated biological functions is achieved.

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References

Adusu TE, Conlon PD, Shewen PE & Black WD (1994) Pasteurella haemolytica leukotoxin induces histamine release from bovine pulmonary mast cells. Can J Vet Res 58: 1–5.

Ahn JH, Pan JG & Rhee JS (1999) Identification of the nilDEF ABC transporter specific for lipase in Pseudomonas fluorescens SIK W1. J Bacteriol 181: 1847–1852.

Akatsuka H, Kawai E, Omori K, Komatsubara S, Shibatani T & Tosa T (1994) The lipA gene of Serratia marcescens which encodes an extracellular lipase having no N-terminal signal peptide. J Bacteriol 176: 1949–1956.

Akatsuka H, Kawai E, Omori K & Shibatani T (1995) The three genes lipB, lipC, and lipD involved in the extracellular secretion of the Serratia marcescens lipase which lacks an N-terminal signal peptide. J Bacteriol 177: 6381–6389.

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W & Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25: 3389–3402.

Amada K, Haruki M, Imanaka T, Morikawa M & Kanaya S (2000) Overproduction in Escherichia coli, purification and characterization of a family L3 lipase from Pseudomonas sp. MIS38. Biochir Biochir Acta 1478: 201–210.

Ambagala TC, Ambagala AP & Srikumaran S (1999) The leukotoxin of Pasteurella haemolytica binds to beta(2) integrins on bovine leukocytes. FEMS Microbiol Lett 179: 161–167.

Andersen C, Hughes C & Koronakis V (2001) Protein export and drug efflux through bacterial channel–tunnels. Curr Opin Cell Biol 13: 412–416.

Angelos JA, Hess JF & George LW (2003) An RTX operon in hemolytic Monaxella bovis is absent from nonhemolytic strains. Vet Microbiol 92: 363–377.

Aono R, Tsukagoshi N & Yamamoto M (1998) Involvement of outer membrane protein TolC, a possible member of the mar-sox regulon, in maintenance and improvement of organic solvent tolerance of Escherichia coli K-12. J Bacteriol 180: 936–944.

Atapattu DN & Czuprynski CJ (2005) Mannheimia haemolytica leukotoxin induces apoptosis of bovine lymphoblastoid cells (BL-3) via a caspase-9-dependent mitochondrial pathway. Infect Immun 73: 5504–5513.

Awram P & Smit J (1998) The Caulobacter crescentus paracrystalline S-layer protein is secreted by an ABC transporter (type I) secretion apparatus. J Bacteriol 180: 3062–3069.

Awram P & Smit J (2001) Identification of lipopolysaccharide O antigen synthesis genes required for attachment of the S-layer of Caulobacter crescentus. Microbiology 147: 1451–1460.

Bachelet M, Richard MJ, Francois D & Polla BS (2002) Mitochondrial alterations precede Bordetella pertussis-induced apoptosis. FEMS Immunol Med Mic 32: 125–131.

Bakas L, Ostolaza H, Var WL & Goni FM (1996) Reversible adsorption and nonreversible insertion of Escherichia coli alpha-hemolysin into lipid bilayers. Biophys J 71: 1869–1876.

Balashova NV, Diaz R, Balashov SV, Crosby JA & Kachlany SC (2006) Regulation of Aggregatibacter (Actinobacillus) actinomycetemcomitans leukotoxin secretion by iron. J Bacteriol 188: 8658–8661.

Balashova NV, Park DH, Patel JK, Figurski DH & Kachlany SC (2007) Interaction between leukotoxin and Cu,Zn superoxide dismutase in Aggregatibacter actinomycetemcomitans. Infect Immun 75: 4490–4497.

Barry EM, Weiss AA, Ehrmann IE, Gray MC, Hewlett EL & Goodwin MS (1991) Bordetella pertussis adenylyl cyclase toxin and hemolytic activities require a second gene, cyaC, for activation. J Bacteriol 173: 720–726.

Basar T, Havlicek V, Bezouskova S, Halada P, Hackett M & Sebo P (1999) The conserved lysine 860 in the additional fatty-acylation site of Bordetella pertussis adenylyl cyclase is crucial for toxin function independently of its acylation status. J Biol Chem 274: 10777–10783.

Basar T, Havlicek V, Bezouskova S, Hackett M & Sebo P (2001) Acylation of lysine 983 is sufficient for toxin activity of Bordetella pertussis adenylyl cyclase. Substitutions of alanine 140 modulate acylation site selectivity of the toxin acyltransferase CyaC. J Biol Chem 276: 348–354.

Basler M, Masin J, Osicka R & Sebo P (2006) Pore-forming and enzymatic activities of Bordetella pertussis adenylyl cyclase toxin synergize in promoting lysis of monocytes. Infect Immun 74: 2207–2214.
Benz R, Maier E, Ladant D, Ullmann A & Sebo P (1994) Crystal structure of the 50 kDa metalloprotease from Serratia marcescens. J Mol Biol 242: 244–251.

Baumann U (1994) Crystal structure of the 50 kDa metalloprotease of Serratia marcescens. J Mol Biol 242: 244–251.

Baumann U, Wuu S, Flaherty KM & McKay DB (1993) 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: 3357–3364.

Baumann U, Bauer M, Letoffe S, Delepelaire P & Wandersman C (1995) Crystal structure of a complex between Serratia marcescens metallo-protease and an inhibitor from Erwinia chrysanthemi. J Mol Biol 248: 653–661.

Beard MK & Moore LJ (1994) Reproduction of bovine keratoconjunctivitis with a purified haemolytic and cytotoxic fraction of Moraxella bovis. Vet Microbiol 42: 15–33.

Bejermano M, Nisan I, Ludwig A, Goebel W & Hanski E (1999) Characterization of the C-terminal domain essential for toxic activity of adenylate cyclase toxin. Mol Microbiol 31: 381–392.

Bellalou J, Sakamoto H, Ladant D, Geoffroy C & Ullmann A (1993) Three-dimensional structure of the alkaline protease of Pseudomonas aeruginosa. EMBO J 12: 3357–3364.

Baumann U, Bauer M, Letoffe S, Delepelaire P & Wandersman C (1995) Crystal structure of a complex between Serratia marcescens metallo-protease and an inhibitor from Erwinia chrysanthemi. J Mol Biol 248: 653–661.

Beard MK & Moore LJ (1994) Reproduction of bovine keratoconjunctivitis with a purified haemolytic and cytotoxic fraction of Moraxella bovis. Vet Microbiol 42: 15–33.

Bejermano M, Nisan I, Ludwig A, Goebel W & Hanski E (1999) Characterization of the C-terminal domain essential for toxic activity of adenylate cyclase toxin. Mol Microbiol 31: 381–392.

Bellalou J, Sakamoto H, Ladant D, Geoffroy C & Ullmann A (1993) Three-dimensional structure of the alkaline protease of Pseudomonas aeruginosa. EMBO J 12: 3357–3364.

Baumann U, Bauer M, Letoffe S, Delepelaire P & Wandersman C (1995) Crystal structure of a complex between Serratia marcescens metallo-protease and an inhibitor from Erwinia chrysanthemi. J Mol Biol 248: 653–661.

Beard MK & Moore LJ (1994) Reproduction of bovine keratoconjunctivitis with a purified haemolytic and cytotoxic fraction of Moraxella bovis. Vet Microbiol 42: 15–33.
proteins from Campylobacter rectus. *J Bacteriol* **181**: 2501–2506.

Brown JE, Leite F & Czuprynski CJ (1997) Binding of Pasteurella haemolytica leukotoxin to bovine leukocytes. *Infect Immun* **65**: 3719–3724.

Brown MH, Brightman AH, Fenwick BW & Rider MA (1998) Infectious bovine keratoconjunctivitis: a review. *J Vet Intern Med* **12**: 259–266.

Bumba L, Masin J, Fiser R & Sebo P (2010) Bordetella adenylate cyclase toxin mobilizes its b2 integrin receptor into lipid rafts to accomplish translocation across target cell membrane in two steps. *PLoS Pathog* **6**: e1000901.

Burrows LL & Lo RY (1992) Molecular characterization of an RTX proteins.

Cavalieri SJ & Snyder IS (1982) Effect of *Escherichia coli* alpha-hemolysin on human peripheral leukocyte function in vitro. *Infect Immun* **37**: 966–974.

Chabaud P, de Groot A, Bitter W, Tommassen J, Heulin T & Achouak W (2001) Phase-variable expression of an oopron encoding extracellular alkaline protease, a serine protease homolog, and lipase in *Pseudomomas brasiliaccearaum*. *J Bacteriol* **183**: 2117–2120.

Chang YF, Young R, Post D & Struck DK (1987) Identification and characterization of the Pasteurella haemolytica leukotoxin. *Infect Immun* **55**: 2348–2354.

Chang YF, Young R & Struck DK (1989) Cloning and characterization of a hemolysin gene from *Actinobacillus (Haemophilus) pleuroneumoniae*. *DNA* **8**: 635–647.

Chang YF, Ma DP, Young R & Struck DK (1993) Cloning, sequencing and expression of a Pasteurella haemolytica A1 gene encoding a PurK-like protein. *DNA Sequence* **3**: 357–367.

Chatterjee R, Nag S & Chaudhuri K (2008) Identification of a new RTX-like gene cluster in *Vibrio cholerae*. *FEMS Microbiol Lett* **284**: 165–171.

Chen CY, Wu KM, Chang YC et al. (2003) Comparative genome analysis of *Vibrio vulnificus*, a marine pathogen. *Genome Res* **13**: 2577–2587.

Cheong TG, Chan M, Kurunathan S et al. (2010) Construction and characterization of rtxA and rtxC mutants of auotphorous O139 *Vibrio cholerae*. *Microb Pathogen* **48**: 85–90.

Cherif A, Rezgwi R, Daffonchio D & Boudabous A (2006) Characterization and partial purification of entomocin 110, a newly identified bacteriocin from *Bacillus thuringiensis* subsp. *Entomocidus* HD110. *Microbiol Res* **163**: 684–692.

Cheryk LA, Hooper-McGreevy KE & Gentry PA (1998) Alterations in bovine platelet function and acute phase proteins induced by Pasteurella haemolytica A1. *Can J Vet Res* **62**: 1–8.

Chow KH, Ng TK, Yuen KY & Yam WC (2001) Detection of RTX toxin gene in *Vibrio cholerae* by PCR. *J Clin Microbiol* **39**: 2594–2597.

Christie R, Atkins NE & Munch-Petersen E (1944) A note on a lytic phenomenon shown by group B streptococci. *Aust J Exp Biol Med* **22**: 197–200.

Chung GH, Lee YP, Jeohn GH, Yoo OJ & Rhee JS (1991) Cloning and nucleotide sequence of thermostable lipase gene from *Pseudomonas fluorescens* SIK W1. *Agr Biol Chem* **55**: 2359–2365.

Clinkenbeard KD & Upton ML (1991) Lysis of bovine platelets by *Pasteurella haemolytica* leukotoxin. *Am J Vet Res* **52**: 453–457.

Clinkenbeard KD, Mosier DA & Confer AW (1989a) Effects of *Pasteurella haemolytica* leukotoxin on isolated bovine neutrophils. *Toxicon* **27**: 797–804.

Clinkenbeard KD, Mosier DA, Timko AL & Confer AW (1989b) Effects of *Pasteurella haemolytica* leukotoxin on cultured bovine lymphoma cells. *Am J Vet Res* **50**: 271–275.

Confer DL & Eaton JW (1982) Phagocyte impotence caused by an invasive bacterial adenylate cyclase. *Science* **217**: 948–950.

Coote JG (1992) Structural and functional relationships among the RTX toxin determinants of gram-negative bacteria. *FEMS Microbiol Rev* **8**: 137–161.

Cordero CL, Kudryashov DS, Reisler E & Satchell KJ (2006) The actin cross-linking domain of the *Vibrio cholerae* RTX toxin directly catalyzes the covalent cross-linking of actin. *J Biol Chem* **281**: 32366–32374.

Cortijarena AL, Goni FM & Ostolaza H (2001) Glycophorin as a receptor for *Escherichia coli* alpha-hemolysin in erythrocytes. *J Biol Chem* **276**: 12513–12519.

Cortijarena AL, Goni FM & Ostolaza H (2003) A receptor-binding region in *Escherichia coli* alpha-haemolysin. *J Biol Chem* **278**: 19159–19163.

Crosby JA & Kachlany SC (2007) TdETa, a TolC-like protein required for toxin and drug export in *Aggregatibacter (Actinobacillus) actinomycetemcomitans*. *Gene* **388**: 83–92.

Cruz WT, Young R, Chang YF & Struck DK (1990) Deletion analysis resolves cell-binding and lytic domains of the Pasteurella leukotoxin. *Mol Microbiol* **4**: 1933–1939.

Cudd L, Clarke C & Clinkenbeard K (2003a) Contribution of intracellular calcium stores to an increase in cytosolic calcium concentration induced by Mannheimia haemolytica leukotoxin. *FEMS Microbiol Lett* **225**: 23–27.

Cudd L, Clarke C & Clinkenbeard K (2003b) *Mannheimia haemolytica* leukotoxin-induced increase in leukotriene B4 production by bovine neutrophils is mediated by a sustained and excessive increase in intracellular calcium concentration. *FEMS Microbiol Lett* **224**: 85–90.

Czuprynski CJ & Welch RA (1995) Biological effects of RTX toxins: the possible role of lipopolysaccharide. *Trends Microbiol* **3**: 480–483.

Czuprynski CJ, Noel EJ, Ortiz-Carranza O & Srikumaran S (1991) Activation of bovine neutrophils by partially purified *Pasteurella haemolytica* leukotoxin. *Infect Immun* **59**: 3126–3133.

Dadaglio G, Moukrim Z, Lo-Man R, Sheshko V, Sebo P & Leclerc C (2000) Induction of a polarized Th1 response by insertion of multiple copies of a viral T-cell epitope into adenylate cyclase of Bordetella pertussis. *Infect Immun* **68**: 3867–3872.
Dahler GS, Barras F & Keen NT (1990) Cloning of genes encoding extracellular metalloproteases from *Erwinia chrysanthemi* EC16. *J Bacteriol* **172**: 5803–5815.

Dassanayake RP, Maheswaran SK & Srikumaran S (2007) Monomeric expression of bovine beta2-integrin subunits reveals their role in Mannheimia haemolytica leukotoxin-induced biological effects. *Infect Immun* **75**: 5004–5010.

Delepelaire P (2004) Type I secretion in gram-negative bacteria. *Biochim Biophys Acta* **1694**: 149–161.

Delepelaire P & Wandersman C (1989) Protease secretion by *Erwinia chrysanthemi*. Proteases B and C are synthesized and secreted aszymogens without a signal peptide. *J Biol Chem* **264**: 9083–9089.

Delepelaire P & Wandersman C. (1998) The SecB chaperone is involved in the secretion of the *Serratia marcescens* HasA protein through an ABC transporter. *EMBO J* **17**: 936–944.

Demuth DR, James D, Kowashi Y & Kato S (2003) Interaction of Actinobacillus actinomyctecomitans outer membrane vesicles with HL60 cells does not require leukotoxin. *Cell Microbiol* **5**: 111–121.

Deshpande MS, Ambagala TC, Ambagala AP, Kehri ME Jr & Srikumaran S (2002) Bovine CD18 is necessary and sufficient to mediate *Mannheimia* (*Pasteurella*) haemolytica leukotoxin-induced cytolysis. *Infect Immun* **70**: 5058–5064.

Diaz R, Ghofaily LA, Patel J, Balashova NV, Freitas AC, Labib I & Delepelaire P (2006) Characterization of leukotoxin from a clinical strain of *Actinobacillus actinomyctecomitans*. *Microb Pathogenesis* **40**: 48–55.

Dileepan T, Thumbikat P, Walcheck B, Kannan MS & Maheswaran SK (2005) Recombinant expression of bovine LFA-1 and characterization of its role as a receptor for *Mannheimia haemolytica* leukotoxin. *Microb Pathogenesis* **38**: 249–257.

Dileepan T, Kachlany SC, Balashova NV, Patel J & Maheswaran SK (2007a) Human CD18 is the functional receptor for *Aggregatibacter actinomyctecomitans* leukotoxin. *Infect Immun* **75**: 4851–4856.

Dileepan T, Kannan MS, Walcheck B & Maheswaran SK (2007b) Integrin-EGF-3 domain of bovine CD18 is critical for *Mannheimia haemolytica* leukotoxin species-specific susceptibility. *FEMS Microbiol Lett* **274**: 67–72.

Duchaud E, Rusniok C, Frangeul L et al. (2003) The genome sequence of the entomopathogenic bacterium *Photorhabdus luminescens*. *Nat Biotechnol* **21**: 1307–1313.

Duong F, Lazardnus A, Cami B & Murgier M (1992) Sequence of a cluster of genes controlling synthesis and secretion of alkaline protease in *Pseudomonas aeruginosa*: relationships to other secretory pathways. *Gene* **121**: 47–54.

Duong F, Soscia C, Lazardnus A & Murgier M (1994) The *Pseudomonas fluorescens* lipase has a C-terminal secretion signal and is secreted by a three-component bacterial ABC-exporter system. *Mol Microbiol* **11**: 1117–1126.

Eberspächer B, Hugo F & Bhakdi S (1989) Quantitative study of the binding and hemolytic efficiency of *Escherichia coli* hemolysin. *Infect Immun* **57**: 983–988.

Eberspächer B, Hugo F, Pohl M & Bhakdi S (1990) Functional similarity between the haemolysins of *Escherichia coli* and *Morganella morganii*. *J Med Microbiol* **33**: 165–170.

Economou A, Hamilton WD, Johnston AW & Downie JA (1990) The *Rhizobium* nodulation gene nodO encodes a Ca2(+) binding protein that is exported without N-terminal cleavage and is homologous to haemolysin and related proteins. *EMBO J* **9**: 349–354.

Eddy SR (1998) Profile hidden Markov models. *Bioinformatics* **14**: 755–763.

El-Azami-El-Idrissi M, Bauche C, Loucka J, Osicka R, Sebo P, Ladant D & Leclerc C (2003) Interaction of *Bordetella pertussis* adenylate cyclase with CD11b/CD18: role of toxin acylation and identification of the main integrin interaction domain. *J Biol Chem* **278**: 38514–38521.

Fayolle C, Sebo P, Ladant D, Ullmann A & Leclerc C (1996) *In vivo* induction of CTL responses by recombinant adenylate cyclase of *Bordetella pertussis* carrying viral CD8+ T cell epitopes. *J Immunol* **156**: 4697–4706.

Fayolle C, Ladant D, Karimova G, Ullmann A & Leclerc C (1999) Therapy of murine tumors with recombinant *Bordetella pertussis* adenylate cyclase carrying a cytotoxic T cell epitope. *J Immunol* **162**: 4157–4162.

Fayolle C, Osicka A, Osicka R et al. (2001) Delivery of multiple epitopes by recombinant detoxified adenylate cyclase of *Bordetella pertussis* induces protective antiviral immunity. *J Virol* **75**: 7330–7338.

Felmlee T & Welch RA (1988) Alterations of amino acid repeats in the *Escherichia coli* hemolysin affect cytolytic activity and secretion. *P Natl Acad Sci USA* **85**: 5269–5273.

Felmlee T, Pellett S & Welch RA (1985) Nucleotide sequence of an *Escherichia coli* chromosomal hemolysin. *J Bacteriol* **163**: 94–105.

Feltzer RE, Gray RD, Dean WL & Pierce WM Jr (2000) Alkaline proteinase inhibitor of *Pseudomonas aeruginosa*. Interaction of native and N-terminally truncated inhibitor proteins with *Pseudomonas* metalloproteinases. *J Biol Chem* **275**: 21002–21009.

Fetzter AE, Werner AS & Hagstrom JW (1967) Pathologic features of pseudomonal pneumonia. *Am Rev Respir Dis* **96**: 1121–1130.

Fine DH, Kaplan JB, Kachlany SC & Schreiner HC (2006) How we got attached to *Actinobacillus actinomyctecomitans*: a model for infectious diseases. *Periodontal 2000* **2000**: 114–157.

Finn RD, Mistry J, Schuster-Bockler B et al. (2006) Pfam: clans, web tools and services. *Nucleic Acids Res* **34**: D247–D251.

Finnie C, Hartley NM, Findlay KC & Downie JA (1997) The *Rhizobium leguminosarum* prsDE genes are required for secretion of several proteins, some of which influence nodulation, symbiotic nitrogen fixation and exopolysaccharide modification. *Mol Microbiol* **25**: 135–146.

Finnie C, Zorreguieta A, Hartley NM & Downie JA (1998) Characterization of *Rhizobium leguminosarum* exopolysaccharide glycans that are secreted via a type I mechanism.
RTX proteins

exporter and have a novel heptapeptide repeat motif. J Bacteriol 180: 1691–1699.
Fiser R, Masin J, Basler M, Krusek J, Spulaková V, Konopasek I & Sebo P (2007) Third activity of Bordetella adenylate cyclase (AC) toxin-hemolysin. Membrane translocation of AC domain polypeptide promotes calcium influx into CD11b+ monocytes independently of the catalytic and hemolytic activities. J Biol Chem 282: 2808–2820.

Fong KP, Chung WO, Lamont RJ & Denmuth DR (2001) Intracellular and interspecies regulation of gene expression by Actinobacillus actinomycetemcomitans LuxS. Infect Immun 69: 7625–7634.

Fong KP, Pacheco CM, Otis LL et al. (2006) Actinobacillus actinomycetemcomitans leukotoxin requires lipid microdomains for target cell cytotoxicity. Cell Microbiol 8: 1753–1767.

Ford MJ, Nomellini JF & Smit J (2007) Intra-membrane anchoring and localization of an S-layer associated protease in Caulobacter crescentus. J Bacteriol 189: 2226–2237.

Forestier C & Welch RA (1991) Identification of RTX toxin target cell specificity domains by use of hybrid genes. Infect Immun 59: 4212–4220.

Frey J & Kuhnt D P (2002) RTX toxins in Pasteurellaceae. Int J Med Microbiol 292: 149–158.

Frey J, Meier R, Gygi D & Nicolet J (1991) Nucleotide sequence of the hemolysin I gene from Actinobacillus pleuropneumoniae. Infect Immun 59: 3026–3032.

Frey J, Beck M, Stucki U & Nicolet J (1993a) Analysis of hemolysin operons in Actinobacillus pleuropneumoniae. Gene 123: 51–58.

Frey J, Bosse JT, Chang YF et al. (1993b) Actinobacillus pleuropneumoniae RTX-toxins: uniform designation of haemolysins, cytolsins, pleurotoxin and their genes. Genetica 92: 1723–1728.

Frey J, Kuhn R & Nicolet J (1994) Association of the CAMP phenomenon in Actinobacillus pleuropneumoniae with the RTX toxins ApxI, ApxII and ApxIII. FEMS Microbiol Lett 124: 245–251.

Friedman RL, Fiederlein RL, Glasser L & Galgiani JN (1987) Bordetella pertussis adenylate cyclase: effects of affinity-purified adenylate cyclase on human polymorphonuclear leukocyte functions. Infect Immun 55: 135–140.

Fronzes R, Christie PJ & Waksman G (2009) The structural biology of type IV secretion systems. Nat Rev Microbiol 7: 703–714.

Fullner KJ & Mekalanos JJ (2000) In vivo covalent cross-linking of cellular actin by the Vibrio cholerae RTX toxin. EMBO J 19: 5315–5323.

Fullner KJ, Lencer WI & Mekalanos JJ (2001) Vibrio cholerae-induced cellular responses of polarized T84 intestinal epithelial cells are dependent on production of cholera toxin and the RTX toxin. Infect Immun 69: 6310–6317.

Fullner KJ, Boucher JC, Hanes MA et al. (2002) The contribution of accessory toxins of Vibrio cholerae O1 El Tor to the proinflammatory response in a murine pulmonary cholera model. J Exp Med 195: 1455–1462.

Gadeberg OV & Orskov I (1984) In vitro cytotoxic effect of alpha-hemolysin Escherichia coli on human blood granulocytes. Infect Immun 45: 255–260.

Gangola P & Rosen BP (1987) Maintenance of intracellular calcium in Escherichia coli. J Biol Chem 262: 12570–12574.

Gentile E, Raptis A, Knipling LG & Wolff J (1988) Bordetella pertussis adenylate cyclase. Penetration into host cells. Eur J Biochem 175: 447–453.

Gentschev I, Hess J & Goebel W (1990) Change in the cellular localization of alkaline phosphatase by alteration of its carboxy-terminal sequence. Mol Gen Genet 222: 211–216.

Ghigo JM & Wandersman C (1992) Cloning, nucleotide sequence and characterization of the gene encoding the Erwinia chrysanthemi B374 PrtA metalloprotease: a third metalloprotease secreted via a C-terminal secretion signal. Mol Gen Genet 236: 135–144.

Gilchrist A, Fisher JA & Smit J (1992) Nucleotide sequence analysis of the gene encoding the Caulobacter crescentus paracrystalline surface layer protein. Can J Microbiol 38: 193–202.

Gioia J, Qin X, Jiang H et al. (2006) The genome sequence of Mannheimia haemolytica A1: insights into virulence, natural competence, and Pasteurellaceae phylogeny. J Bacteriol 188: 7257–7266.

Glaser P, Ladant D, Sezer O, Pichot F, Ullmann A & Danchin A (2002) The genome sequence of Pasteurellaceae: insights into virulence, natural competence, and Pasteurellaceae phylogeny. J Bacteriol 188: 195–200.

Glaser P, Sakamoto H, Bellalou J, Ullmann A & Danchin A (1988b).Secretion of cyclophilin, the calmodulin-sensitive adenylate-cyclase-hemolysin bifunctional protein of Bordetella pertussis. EMBO J 7: 3997–4004.

Goebel W & Hedgpeth J (1982) Cloning and functional characterization of the plasmid-encoded hemolysin determinant of Escherichia coli. J Bacteriol 151: 1290–1298.

Gopinath RS, Ambagala TC, Deshpande MS, Donis RO & Gadeberg OV & Orskov I (1984) Covalent cross-linking of Bordetella pertussis adenylate cyclase-hemolysin in alveolar macrophage apoptosis model. J Exp Med 195: 1455–1462.
during *Bordetella pertussis* infection *in vivo*. *Infect Immun* **66**: 1718–1725.

Guerronprez P, Ladant D, Karimova G, Ullmann A & Leclerc C (1999) Direct delivery of the *Bordetella pertussis* adenylate cyclase toxin to the MHC class I antigen presentation pathway. *J Immunol* **162**: 1910–1916.

Guerronprez P, Khelef N, Blouin E et al. (2001) The adenylate cyclase toxin of *Bordetella pertussis* binds to target cells via the alpha(M)beta(2) integrin (CD11b/CD18). *J Exp Med* **193**: 1035–1044.

Guo Q, Shen Y, Lee YS, Gibbs CS, Mrksich M & Tang WJ (2005) Structural basis for the interaction of *Bordetella pertussis* adenylyl cyclase toxin with calmodulin. *EMBO J* **24**: 3190–3201.

Guzzo J, Murgier M, Filloux A & Lazuñski A (1990) Cloning of the *Pseudomonas aeruginosa* alkaline protease gene and secretion of the protease into the medium by *Escherichia coli*. *J Bacteriol* **172**: 942–948.

Hackett M, Guo L, Shabanowitz J, Hunt DF & Hewlett EL (1994) Internal lysine palmitoylation in adenylate cyclase toxin from *Bordetella pertussis*. *Science* **266**: 433–435.

Hackett M, Walker CB, Guo L et al. (1995) Hemolytic, but not cell-invasive activity, of adenylyl cyclase toxin is selectively affected by differential fatty-acylation in *Escherichia coli*. *J Biol Chem* **270**: 20250–20253.

Haft DH, Selengut JD & White O (2003) The TIGRFAMs database of protein families. *Nucleic Acids Res* **31**: 371–373.

Hanski E (1989) Invasive adenylate cyclase toxin of *Bordetella pertussis*. *Trends Biochem Sci* **14**: 459–463.

Hanski E & Farfel Z (1985) *Bordetella pertussis* invasive adenylyl cyclase. Partial resolution and properties of its cellular penetration. *J Biol Chem* **260**: 5526–5532.

Haubek D, Havemose-Poulsen A & Westergaard J (2006) Aggressive periodontitis in a 16-year-old Ghanaian adolescent, the original source of *Actinobacillus actinomycetemcomitans* strain HK1651 – a 10-year follow up. *Int J Paediatr Dent* **16**: 370–375.

Havlicek, V, Higgins L, Chen W, Halada P, Sebo P, Sakamoto H & Hackett M (2001) Mass spectrometric analysis of recombinant adenylyl cyclase toxin from *Bordetella pertussis* strain 18323/pHSP9. *J Mass Spectrom* **36**: 384–391.

Heck LW, Morihara K & Abrahamson DR (1986) Degradation of soluble laminin and depletion of tissue-associated basement membrane laminin by *Pseudomonas aeruginosa* elastase and alkaline protease. *Infect Immun* **54**: 149–153.

Henderson B, Nair SP, Ward JM & Wilson M (2003) Molecular pathogenicity of the oral opportunistic pathogen *Actinobacillus actinomycetemcomitans*. *Annu Rev Microbiol* **57**: 29–55.

Hess JF & Angelos JA (2006) The *Moraxella bovis* RTX toxin locus mbx defines a pathogenicity island. *J Med Microbiol* **55**: 443–449.

Hewlett EL, Donato GM & Gray MC (2006) Macrophage cytotoxicity produced by adenylyl cyclase toxin from *Bordetella pertussis* more than just making cyclic AMP!. *Mol Microbiol* **39**: 447–459.

Highlander SK, Fedorova ND, Dusek DM, Panchiera R, Alvarez LE & Rinehart C (2000) Inactivation of *Pasteurella* (*Mannheimia*) *haemolytica* leukotoxin causes partial attenuation of virulence in a calf challenge model. *Infect Immun* **68**: 3916–3922.

Hoiczyk E (2000) Gliding motility in cyanobacteria: observations and possible explanations. *Arch Microbiol* **174**: 11–17.

Hoiczyk E & Baumeister W (1997) Oscillin, an extracellular, Ca2+-binding glycoprotein essential for the gliding motility of cyanobacteria. *Mol Microbiol* **26**: 699–708.

Hong YQ & Ghebrehiwet B (1992) Effect of *Pseudomonas aeruginosa* elastase and alkaline protease on serum complement and isolated components C1q and C3. *Clin Immunol Immunopath* **62**: 133–138.

Hooper NM (1994) Families of zinc metalloproteases. *FEBS Lett* **354**: 1–6.

Horvat RT & Parmely MJ (1988) *Pseudomonas aeruginosa* alkaline protease degrades human gamma interferon and inhibits its bioactivity. *Infect Immun* **56**: 2925–2932.

Hritz M, Fisher E & Demuth DR (1996) Differential regulation of the leukotoxin operon in highly leukotoxic and minimally leukotoxic strains of *Actinobacillus actinomycetemcomitans*. *Infect Immun* **64**: 2724–2729.

Hsuan SL, Kannan MS, Jeyaseelan S, Prakash YS, Sieck GC & Maheswaran SK (1998) *Pasteurella haemolytica* A1-derived leukotoxin and endotoxin induce intracellular calcium elevation in bovine alveolar macrophages by different signaling pathways. *Infect Immun* **66**: 2836–2844.

Hsuan SL, Kannan MS, Jeyaseelan S et al. (1999) *Pasteurella haemolytica* leukotoxin and endotoxin induced cytokine expression in bovine alveolar macrophages requires NF-kappaB activation and calcium elevation. *Microb Pathogenesis* **26**: 263–273.

Hulo N, Bairoch A, Bulliard V et al. (2006) The PROSITE database. *Nucleic Acids Res* **34**: D227–D230.

Hyland C, Vuillard L, Hughes C & Koronakis V (2001) Membrane interaction of *Escherichia coli* hemolysin: flotation and insertion-dependent labeling by phospholipid vesicles. *J Bacteriol* **183**: 5364–5370.

Inoue T, Tanimoto I, Tada T, Ohashi T, Fukui K & Ohta H (2001) Fermentable-sugar-level-dependent regulation of leukotoxin synthesis in a variably toxic strain of *Actinobacillus actinomycetemcomitans*. *Microbiology* **147**: 2749–2756.

Issartel JP, Koronakis V & Hughes C (1991) Activation of *Escherichia coli* prohaemolysin to the mature toxin by acyl carrier protein-dependent fatty acylation. *Nature* **351**: 759–761.

Iwaki M, Ullmann A & Sebo P (1995) Identification by *in vitro* complementation of regions required for cell-invasive activity of *Bordetella pertussis* adenylyl cyclase toxin. *Mol Microbiol* **17**: 1015–1024.

Iwase M, Lally ET, Berthold PH, Korchak HM & Taichman NS (1990) Effects of cations and osmotic protectants on cytolytic
activity of Actinobacillus actinomycetemcomitans leukotoxin. Infect Immun 58: 1782–1788.

Jacob F, Blobel H & Scharmann W (1973) [Typing of Pseudomonas aeruginosa by means of titrated pyocines (author’s trans)]. Zentralbl Bakteriol [Orig A] 224: 472–477.

Jaeger KE, Ransac S, Dijkstra BW, Colson C, van Heuvel M & Misset O (1994) Bacterial lipases. FEMS Microbiol Rev 15: 29–63.

Jansen R, Braiée J, Kamp EM, Gielkens AL & Smits MA (1993) Cloning and characterization of the Actinobacillus pleuropneumoniae-RTX-toxin III (ApxIII) gene. Infect Immun 61: 947–954.

Jeyaseelan S, Kannan MS et al. (2000) Lymphocyte function-associated antigen 1 is a receptor for Pasteurella haemolytica leukotoxin in bovine leukocytes. Infect Immun 68: 72–79.

Jeyaseelan S, Kannan MS, Hsuan SL, Singh AK, Walseth TF & Maheswaran SK (2002) Role of Mannheimia haemolytica leukotoxin in the pathogenesis of bovine pneumoniae. Anim Health Res Rev 3: 69–82.

Jiang Z, Zheng Y, Luo Y, Wang G, Wang H, Ma Y & Wei D (2005) Cloning and expression of a novel lipase gene from Pseudomonas fluorescens B52. Mol Biotechnol 31: 95–101.

Johansson A, Claesson R, Hanstrom L, Sandstrom G & Kalfas S (2000) Polymorphonuclear leukocyte degranulation induced by leukotoxin from Actinobacillus actinomycetemcomitans. J Periodontal Res 35: 85–92.

Johnson LA, Beacham IR, MacRae IC & Free ML (1992) Degradation of triglycerides by a pseudomonad isolated from milk: molecular analysis of a lipase-encoding gene and its expression in Escherichia coli. Appl Environ Microbiol 58: 1776–1779.

Jorgensen SE, Mulcahy PF, Wu GK & Louis CF (1983) Calcium accumulation in human and sheep erythrocytes that is induced by Escherichia coli hemolysin. Toxicon 21: 717–727.

Kachlany SC, Fine DH & Figurski DH (2000) Secretion of RTX leukotoxin by Actinobacillus actinomycetemcomitans. Infect Immun 68: 6094–6100.

Kachlany SC, Planet PJ, DeSalle R, Fine DH & Figurski DH (2001) Genes for tight adherence of Actinobacillus actinomycetemcomitans: from plaque to plaque to pond scum. Trends Microbiol 9: 429–437.

Kaehler KL, Markham RJ, Muscoplat CC & Johnson DW (1980) Evidence of species specificity in the cytotoxic effects of Pasteurella haemolytica. Infect Immun 30: 615–616.

Kamanova J, Kofronova O, Masin J et al. (2008) Adenylate cyclase toxin subverts phagocyte function by RhoA inhibition and unproductive ruffling. J Immunol 181: 5587–5597.

Karakelian D, Lear JD, Lally ET & Tanaka JC (1998) Characterization of Actinobacillus actinomycetemcomitans leukotoxin pore formation in HL60 cells. Biochim Biophys Acta 1406: 175–187.

Karinova G, Pidoux J, Ullmann A & Ladant D (1998) A bacterial two-hybrid system based on a reconstituted signal transduction pathway. P Natl Acad Sci USA 95: 5752–5756.

Kato S, Muro M, Akifusa S et al. (1995) Evidence for apoptosis of murine macrophages by Actinobacillus actinomycetemcomitans infection. Infect Immun 63: 3914–3919.

Kato S, Kowashi Y & Demuth DR (2002) Outer membrane-like vesicles secreted by Actinobacillus actinomycetemcomitans are enriched in leukotoxin. Microb Pathogenesis 32: 1–13.

Kawai E, Akatsuka H, Idei A, Shibatani T & Omori K (1998) Serratia marcescens S-layer protein is secreted extracellularly via an ATP-binding cassette exporter, the Lip system. Mol Microbiol 27: 941–952.

Kawai E, Idei A, Kumura H, Shimazaki K, Akatsuka H & Omori K (1999) The ABC-exporter genes involved in the lipase secretion are clustered with the genes for lipase, alkaline protease, and serine protease homologues in Pseudomonas fluorescens no. 33. Biochim Biophys Acta 1446: 377–382.

Keane WF, Welch R, Gekker G & Peterson PK (1987) Mechanism of Escherichia coli alpha-hemolysin-induced injury to isolated renal tubular cells. Am J Pathol 126: 350–357.

Kehl-Fie TE & St Gene JW III (2007) Identification and characterization of an RTX toxin in the emerging pathogen Kingella kingae. J Bacteriol 189: 430–436.

Kelk P, Johansson A, Claesson R, Hanstrom L & Kalfas S (2003) Caspase 1 involvement in human monocyte lysis induced by Actinobacillus actinomycetemcomitans leukotoxin. Infect Immun 71: 4448–4455.

Kelk P, Claesson R, Hanstrom L, Lerner UH, Kalfas S & Johansson A (2005) Abundant secretion of bioactive interleukin-1beta by human macrophages induced by Actinobacillus actinomycetemcomitans leukotoxin. Infect Immun 73: 453–458.

Kelk P, Claesson R, Chen C, Sjostedt A & Johansson A (2008) IL-1beta secretion induced by Aggregatibacter (Actinobacillus) actinomycetemcomitans is mainly caused by the leukotoxin. Int J Med Microbiol 298: 529–541.

Kenny B, Haigh R & Holland IB (1991) 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: 2557–2568.

Kesty NC, Mason KM, Reedy M, Miller SE & Kuehn MJ (2004) Enterotoxigenic Escherichia coli vesicles target toxin delivery into mammalian cells. EMBO J 23: 4538–4549.

Khelef N & Guiso N (1995) Induction of macrophage apoptosis by Bordetella pertussis adenylate cyclase-hemolysin. FEMS Microbiol Lett 134: 27–32.

Khelef N, Zychlinsky A & Guiso N (1993) Bordetella pertussis induces apoptosis in macrophages: role of adenylate cyclase-hemolysin. Infect Immun 61: 4064–4071.

Kieba IR, Feng KP, Tang HY, Hoffman KE, Speicher DW, Klickstein LB & Lally ET (2007) Aggregatibacter actinomycetemcomitans leukotoxin requires beta-sheets 1 and 2
of the human CD11a beta-propeller for cytotoxicity. *Cell Microbiol* 9: 2689–2699.

Kim KS, Kim TU, Kim JI, Byun SM & Shin YC (1995) Characterization of a metalloprotease inhibitor protein (SmaPI) of *Serratia marcescens*. *Appl Environ Microb* 61: 3035–3041.

Kim YR, Lee SE, Kook H et al. (2007) *Vibrio vulnificus* RTX toxin kills host cells only after contact of the bacteria with host cells. *Cell Microbiol* 10: 848–862.

Kojima Y & Shimizu S (2003) Purification and characterization of the lipase from *Pseudomonas fluorescens* HU380. *J Biosci Bioeng* 96: 219–226.

Kojima Y, Kobayashi M & Shimizu S (2003) A novel lipase from *Pseudomonas fluorescens* HU380: gene cloning, overproduction, renaturation-activation, two-step purification, and characterization. *J Biosci Bioeng* 96: 242–249.

Kolodrubetz D, Phillips L, Jacobs C, Burgum A & Kraig E (2003) Anaerobic regulation of *Actinobacillus actinomycetemcomitans* leukotoxin transcription is ArcA/FnrA-independent and requires a novel promoter element. *Res Microbiol* 154: 645–653.

Koronakis E, Hughes C, Milisavl J & Koronakis V (1995) Protein exporter function and *in vitro* ATPase activity are correlated in ABC-domain mutants of HlyB. *Mol Microbiol* 16: 87–96.

Koronakis V, Cross M, Senior B, Koronakis E & Hughes C (1987) The secreted hemolysins of *Proteus mirabilis*, *Proteus vulgaris*, and *Morganella morganii* are genetically related to each other and to the alpha-hemolysin of *Escherichia coli*. *J Bacteriol* 169: 1509–1515.

Koronakis V, Hughes C & Koronakis E (1991) Energetically distinct early and late stages of HlyB/HlyD-dependent secretion across both *Escherichia coli* membranes. *EMBO J* 10: 3263–3272.

Koronakis V, Hughes C & Koronakis E (1993) ATPase activity and ATP/ADP-induced conformational change in the soluble domain of the bacterial protein translocator HlyB. *Mol Microbiol* 8: 1163–1175.

Koronakis V, Sharff A, Koronakis E, Luisi B & Hughes C (2000) Crystal structure of the bacterial membrane protein TolC central to multidrug efflux and protein export. *Nature* 405: 914–919.

Koronakis V, Eswaran J & Hughes C (2004) Structure and function of TolC: the bacterial exit duct for proteins and drugs. *Annu Rev Biochem* 73: 467–489.

Korostoff J, Yamaguchi N, Miller M, Kiaa I & Lally ET (2000) Perturbation of mitochondrial structure and function plays a central role in *Actinobacillus actinomycetemcomitans* leukotoxin-induced apoptosis. *Microb Pathogenesis* 29: 267–278.

Koschinski A, Repp H, Unver B et al. (2006) Why *Escherichia coli* alpha-hemolysin induces calcium oscillations in mammalian cells – the pore is on its own. *FASEB J* 20: 973–975.

Koval SF & Hynes SH (1991) Effect of paracryalline protein surface layers on predation by *Bdellovibrio bacteriovorus*. *J Bacteriol* 173: 2244–2249.

Kreger AS & Gray LD (1978) Purification of *Pseudomonas aeruginosa* proteases and microscopic characterization of pseudomonal protease-induced rabbit corneal damage. *Infect Immun* 19: 630–648.

Kudryashov DS, Cordero CL, Reisler E & Satchell KJ (2008a) Characterization of the enzymatic activity of the actin cross-linking domain from the *Vibrio cholerae* MARTX toxin. *J Biol Chem* 283: 445–452.

Kudryashov DS, Durer ZA, Ytterberg AJ et al. (2008b) Connecting actin monomers by iso-peptide bond is a toxicity mechanism of the *Vibrio cholerae* MARTX toxin. *P Natl Acad Sci USA* 105: 18337–18342.

Kuhnhart P, Heyberger-Meyer B, Burnens AP, Nicolet J & Frey J (1997) Detection of RTX toxin genes in gram-negative bacteria with a set of specific probes. *Appl Environ Microb* 63: 2258–2265.

Kuhnhart P, Heyberger-Meyer B, Nicolet J & Frey J (2000) Characterization of PaxA and its operon: a cohemolytic RTX toxin determinant from pathogenic *Pasteurella aerogenes*. *Infect Immun* 68: 6–12.

Kuhnhart P, Schlatter Y & Frey J (2005) Characterization of the type I secretion system of the RTX toxin ApxII in *Actinobacillus porcitonsillarum*. *Vet Microbiol* 107: 225–232.

Kumura H, Hirose S, Sakurai H, Mikawa K, Tomita F & Shimazaki K (1998) Molecular cloning and analysis of a lipase gene from *Pseudomonas fluorescens* No. 33. *Biosci Biotech Bioch* 62: 2233–2235.

LaGier MJ & Threadgill DS (2008) Identification of novel genes in the oral pathogen *Campylobacter rectus*. *Oral Microbiol Immun* 23: 406–412.

Lally ET, Golub EE, Kiaa IR et al. (1989) Analysis of the *Actinobacillus actinomycetemcomitans* leukotoxin gene. Delineation of unique features and comparison to homologous toxins. *J Biol Chem* 264: 15451–15456.

Lally ET, Golub EE, Kiaa IR et al. (1991a) Structure and function of the B and D genes of the *Actinobacillus actinomycetemcomitans* leukotoxin complex. *Microb Pathogenesis* 11: 111–121.

Lally ET, Kiaa IR, Taichman NS et al. (1991b) *Actinobacillus actinomycetemcomitans* leukotoxin is a calcium-binding protein. *J Periodontal Res* 26: 268–271.

Lally ET, Golub EE & Kiaa IR (1994) Identification and immunological characterization of the domain of *Actinobacillus actinomycetemcomitans* leukotoxin that determines its specificity for human target cells. *J Biol Chem* 269: 31289–31295.

Lally ET, Kiaa IR, Sato A et al. (1997) RTX toxins recognize a beta2 integrin on the surface of human target cells. *J Biol Chem* 272: 30463–30469.

Lear JD, Furburl UG, Lally ET & Tanaka JC (1995) *Actinobacillus actinomycetemcomitans* leukotoxin forms large conductance, voltage-gated ion channels when incorporated into planar lipid bilayers. *Biochim Biophys Acta* 1238: 34–41.
Lee BC, Lee JH, Kim MW et al. (2008) Vibrio vulnificus rtxE is important for virulence, and its expression is induced by exposure to host virulence. Infect Immun 76: 1509–1517.

Lee JH, Kim MW, Kim BS, Kim SM, Lee BC, Kim TS & Choi SH (2007) Identification and characterization of the Vibrio vulnificus txaA essential for cytotoxicity in vitro and virulence in mice. J Microbiol 45: 146–152.

Leite F, Brown JF, Sylte MJ, Briggs RE & Czuprynski CJ (2000) Recombinant bovine interleukin-1beta amplifies the effects of partially purified Pasteurella haemolytica leukotoxin on bovine neutrophils in a beta(2)-integrin-dependent manner. Infect Immun 68: 5581–5586.

Letoffe S, Delepelaire P & Wandersman C (1989) Characterization of a protein inhibitor of extracellular proteases produced by Erwinia chrysanthemi. Mol Microbiol 3: 79–86.

Letoffe S, Delepelaire P & Wandersman C. (1990) Protease secretion by Erwinia chrysanthemi: the specific secretion functions are analogous to those of Escherichia coli alpha-haemolysin. EMBO J 9: 1375–1382.

Letoffe S, Delepelaire P & Wandersman C. (1996) 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: 5804–5811.

Li J, Clinkenbeard KD & Ritchey JW (1999) Bovine CD18 identified as a species specific receptor for Pasteurella haemolytica leukotoxin. Vet Microbiol 67: 91–97.

Li L, Rock JL & Nelson DR (2008) Identification and characterization of a repeat-in-toxin gene cluster in Vibrio anguillarum. Infect Immun 76: 2620–2632.

Li X, Telling S, Winkler UK, Jaeger KE & Benedik MJ (1995) Gene cloning, sequence analysis, purification, and secretion by Escherichia coli of an extracellular lipase from Serratia marcescens. Appl Environ Microb 61: 2674–2680.

Liao CH & McCallus DE (1998) Biochemical and genetic characterization of an extracellular protease from Pseudomonas fluorescens CY091. Appl Environ Microb 64: 914–921.

Lim KB, Walker CR, Guo L et al. (2000) Escherichia coli alpha-haemolysin (HlyA) is heterogeneously acylated in vivo with 14-, 15-, and 17-carbon fatty acids. J Biol Chem 275: 36698–36702.

Lin W, Fullner KJ, Clayton R et al. (1999) Identification of a Vibrio cholerae RTX toxin gene cluster that is tightly linked to the cholera toxin prophage. P Natl Acad Sci USA 96: 1071–1076.

Liu J, Chen X, Tan C et al. (2009) In vivo induced RTX toxin ApxIVA is essential for the full virulence of Actinobacillus pleuropneumoniae. Vet Microbiol 137: 282–289.

Liu M, Alice AF, Naka H & Cosa JH (2007) The HlyU protein is a positive regulator of rtxA, a gene responsible for cytotoxicity and virulence in the human pathogen Vibrio vulnificus. Infect Immun 75: 3282–3289.

Lo RY, Strathdee CA & Shewen PE (1987) Nucleotide sequence of the leukotoxin genes of Pasteurella haemolytica A1. Infect Immun 55: 1987–1996.

Loomes LM, Kerr MA & Senior BW (1993) The cleavage of immunoglobulin G in vitro and in vivo by a proteinase secreted by the urinary tract pathogen Proteus mirabilis. J Med Microbiol 39: 225–232.

Louka J, Schlecht G, Vodolanova J, Leclerc C & Sebo P (2002) Delivery of a MalE CD4(+)-T-cell epitope into the major histocompatibility complex class II antigen presentation pathway by Bordetella pertussis adenylate cyclase. Infect Immun 70: 1002–1005.

Ludwig A, Vogel M & Goebel W (1987) Mutations affecting activity and transport of haemolysin in Escherichia coli. Mol Gen Genet 206: 238–245.

Ludwig A, Jarchau T, Benz R & Goebel W (1988) The repeat domain of Escherichia coli haemolysin (HlyA) is responsible for its Ca2+-dependent binding to erythrocytes. Mol Gen Genet 214: 553–561.

Ludwig A, Benz R & Goebel W (1993) Oligomerization of Escherichia coli haemolysin (HlyA) is involved in pore formation. Mol Gen Genet 241: 89–96.

Ludwig A, Garcia F, Bauer S, Jarchau T, Benz R, Hoppe J & Goebel W (1996) Analysis of the in vivo activation of hemolysin (HlyA) from Escherichia coli. J Bacteriol 178: 5422–5430.

Lupardus PJ, Shen A, Bogoy M & Garcia KC (2008) Small molecule-activated allosteric activation of the Vibrio cholerae RTX cysteine protease domain. Science 322: 265–268.

MacDonald J & Rycroft AN (1992) Molecular cloning and expression of ptxA, the gene encoding the 120-kilodalton cytotoxin of Actinobacillus pleuropneumoniae serotype 2. Infect Immun 60: 2726–2732.

Mackova J, Stasikova J, Kutinova L et al. (2006) Prime/boost immunotherapy of HPV16-induced tumors with E7 protein delivered by Bordetella adenylate cyclase and modified vaccinia virus Ankara. Cancer Immunol Immun 55: 39–46.

Maheswaran SK, Weiss DJ, Kannan MS, Townsend EI, Reddy KR, Whiteley LO & Srikumaran S (1992) Effects of Pasteurella haemolytica A1 leukotoxin on bovine neutrophils: degranulation and generation of oxygen-derived free radicals. Vet Immunol Immunop 33: 51–68.

Maier E, Reinhard N, Benz R & Frey J (1996) Channel-forming activity and channel size of the RTX toxins ApxI, ApxII, and ApxIII of Actinobacillus pleuropneumoniae. Infect Immun 64: 4415–4423.

Majury AL & Shewen PE (1991) The effect of Pasteurella haemolytica A1 leukotoxic culture supernate on the in vitro proliferative response of bovine lymphocytes. Vet Immunol Immunop 29: 41–56.

Marchler-Bauer A, Panchenko AR, Shoemaker BA, Thiessen PA, Geer LY & Bryant SH (2002) CDD: a database of conserved domain alignments with links to domain three-dimensional structure. Nucleic Acids Res 30: 281–283.

Marcel AM & Highlander SK (2001) Use of operon fusions in Mannheimia haemolytica to identify environmental and cis-acting regulators of leukotoxin transcription. Infect Immun 69: 6231–6239.
Masin J, Konopasek I, Svobodova J & Sebo P (2004) Different structural requirements for adenylate cyclase toxin interactions with erythrocyte and liposome membranes. Biochim Biophys Acta 1660: 144–154.

Masin J, Basler M, Knapp O et al. (2005) Acylation of lysine 860 allows tight binding and cytotoxicity of Bordetella adenylate cyclase on CD11b-expressing cells. Biochemistry 44: 12759–12766.

McCarren J, Heuser J, Roth R, Yamada N, Martone M & Brahamsha B (2005) Inactivation of swmA results in the loss of an outer cell layer in a swimming s. strain. J Bacteriol 187: 224–230.

McCarthy CN, Woods RG & Beacham IR (2004) Regulation of the aprX-lipA operon of Pseudomonas fluorescens B52: differential regulation of the proximal and distal genes, encoding protease and lipase, by ompR-envZ. FEMS Microbiol Lett 241: 243–248.

Menestrina G, Mackman N, Holland IB & Bhakdi S (1987) Escherichia coli haemolysin forms voltage-dependent ion channels in lipid membranes. Biochim Biophys Acta 905: 109–117.

Menestrina G, Pederzolli C, Dalla Serra M, Bregante M & Gamvale F (1996) Permeability increase induced by Escherichia coli hemolysin A in human macrophages is due to the formation of ion pores: a patch clamp characterization. J Membrane Biol 149: 113–121.

Miyamoto M, Maeda H, Kitanaka M, Kokeguchi S, Takashiba S & Murayama Y (1998) The S-layer protein from Campylobacter rectus: sequence determination and function of the recombinant protein. FEMS Microbiol Lett 166: 275–281.

Miyoshi S & Shinoda S (2000) Microbial metalloproteases and pathogenesis. Microbes Infect 2: 91–98.

Moayeri M & Welch RA (1994) Effects of temperature, time, and toxin concentration on lesion formation by the Escherichia coli hemolysin. Infect Immun 62: 4124–4134.

Moayeri M & Welch RA (1997) Prelytic and lytic conformations of erythrocyte-associated Escherichia coli hemolysin. Infect Immun 65: 2233–2239.

Mobley HL, Green DM, Trifillis AL et al. (1990) Pyelonephritogenic Escherichia coli and killing of cultured human renal proximal tubular epithelial cells: role of hemolysin in some strains. Infect Immun 58: 1281–1289.

Morova J, Osicka R, Masin J & Sebo P (2008) RTX cytolysins recognize beta2 integrin receptors through N-linked oligosaccharides. P Natl Acad Sci USA 105: 5355–5360.

Muller D, Hughes C & Goebel W (1983) Relationship between plasmid and chromosomal hemolysin determinants of Escherichia coli. J Bacteriol 153: 846–851.

Nagahata H (2004) Bovine leukocyte adhesion deficiency (BLAD): a review. J Vet Med Sci 66: 1475–1482.

Nakahama K, Yoshimura K, Marumoto R, Kikuchi M, Lee IS, Hase T & Matsubara H (1986) Cloning and sequencing of Serratia protease gene. Nucleic Acids Res 14: 5843–5855.

Nomellini JF, Cupcu S, Sleytr UB & Smit J (1997) Factors controlling in vitro recrystallization of the Caulobacter crescentus paracrystalline S-layer. J Bacteriol 179: 6349–6354.

Ohta H, Kato K, Kokeguchi S, Hara F, Fukui K & Murayama Y (1991) Nuclease-sensitive binding of an Actinobacillus actinomycetemcomitans leuckotoxin to the bacterial cell surface. Infect Immun 59: 4599–4605.

Okuda K, Kigure T, Yamada S et al. (1997) Role for the S-layer of Campylobacter rectus ATCC33238 in complement mediated killing and phagocytic killing by leukocytes from guinea pig and human peripheral blood. Oral Dis 3: 113–120.

Oresnik II, Twelker S & Hynes MF (1999) Cloning and characterization of a Rhizobium leguminosarum gene encoding a bacteriocin with similarities to RTX toxins. Appl Environ Microb 65: 2833–2840.

Ortiz-Carranza O & Czuprynski CJ (1992) Activation of bovine neutrophils by Pasteurella haemolytica leuckotoxin is calcium dependent. J LeukocYTE Biol 52: 558–564.

Osicka R, Osickova A, Basar T, Guermonprez P, Rojas M, Leduc C & Sebo P (2000) Delivery of CD8(+ ) T-cell epitopes into major histocompatibility complex class I antigen presentation pathway by Bordetella pertussis adenylate cyclase: delineation of cell invasive structures and permissive insertion sites. Infect Immun 68: 247–256.

Osicka R, Kalmusova J, Krizova P & Sebo P (2001) Neisseria meningitidis RTX protein FprC induces high levels of serum antibodies during invasive disease: polymorphism of FprC alleles and purification of recombinant FprC. Infect Immun 69: 5509–5519.

Osicka R, Prochazkova K, Suli M, Linhartova J, Havlicek V & Sebo P (2004) A novel ‘clip-and-link’ activity of repeat in toxin (RTX) proteins from gram-negative pathogens. Covalent protein cross-linking by an Asp-Lys isopeptide bond upon calcium-dependent processing at an Asp-Pro bond. J Biol Chem 279: 24944–24956.

Osickova A, Osicka R, Maier E, Benz R & Sebo P (1999) An amphipathic alpha-helix including glutamates 509 and 516 is crucial for membrane translocation of adenylate cyclase toxin and modulates formation and cation selectivity of its membrane channels. J Biol Chem 274: 37644–37650.

Osickova A, Masin J, Fayolle C et al. (2010) Adenylate cyclase toxin translocates across target cell membrane without forming a pore. Mol Microb 75: 1550–1562.

Ostolaza H, Bakas L & Goni FM (1997) Balance of electrostatic forces of membrane channels. J Biol Chem 272: 137–145.
Otero AS, Yi XB, Gray MC, Szabo G & Hewlett EL (1995) Membrane depolarization prevents cell invasion by Bordetella pertussis adenylate cyclase toxin. *J Biol Chem* **270**: 9695–9697.

Parkhill J, Achtman M, James KD et al. (2000) Complete DNA sequence of a serogroup A strain of *Neisseria meningitidis* Z2491. *Nature* **404**: 502–506.

Pellett S & Welch RA (1996) *Escherichia coli* hemolysin mutants with altered target cell specificity. *Infect Immun* **64**: 3081–3087.

Pimenta AL, Racher K, Jamieson L, Blight MA & Holland IB (2005) Mutations in HlyD, part of the type 1 translocator for Bordetella pertussis carrying a CD8+ T cell epitope from lymphohcyt choriomeningitis virus. *P Natl Acad Sci USA* **94**: 3314–3319.

Preville X, Ladant D, Timmerman B & Leclerc C (2005) Eradication of established tumors by vaccination with recombinant Pertussis toxin adenylate cyclase toxin carrying the human papillomavirus 16 E7 oncoprotein. *Cancer Res* **65**: 641–649.

Prochazkova K & Satchell KJ (2008) Structure-function analysis of inositol hexaphosphate-induced autoprocessing of the Vibrio cholerae multifunctional-autoprocessing RTX toxin. *J Biol Chem* **335**: 23656–23664.

Prochazkova K, Osicka R, Linihartova I, Halada P, Sule M & Sebo P (2005) The *Neisseria meningitidis* outer membrane lipoprotein FprD binds the RTX protein FprC. *J Biol Chem* **280**: 3251–3258.

Prochazkova K, Shuvalova LA, Minasov G, Voburka Z, Anderson WF & Satchell KJ (2009) Structural and molecular mechanism for autoprocessing of MARTX toxin of *Vibrio cholerae* at multiple sites. *J Biol Chem* **284**: 26557–26568.

Rashid N, Shimada Y, Ezaki S, Atomi H & Imanaka T (2001) Low-temperature lipase from psychrotrophic *Pseudomonas* sp. strain KB700A. *Appl Environ Microb* **67**: 4064–4069.

Reimer D, Frey J, Jansen B, Veit HP & Inzana TJ (1995) Molecular investigation of the role of ApxI and ApxII in the virulence of *Actinobacillus pleuropneumoniae* serotype 5. *Microb Pathogenesis* **18**: 197–209.

Rhodes CR, Gray MC, Watson JM, Muratore TL, Kim SB, Hewlett EL & Grisham CM (2001) Structural consequences of divalent metal binding by the adenyl cyclase toxin of Bordetella pertussis. *Arch Biochem Biophys* **395**: 169–176.

Rice P, Longden I & Blesa A (2000) EMBOS: the European molecular biology open software suite. *Trends Genet* **16**: 276–277.

Rogers DG, Cheville NF & Pugh GW Jr (1987) Pathogenesis of corneal lesions caused by *Moraxella bovis* in gnotobioc calves. *Vet Pathol* **24**: 287–295.

Rose T, Sebo P, Bellalou J & Ladant D (1995) Interaction of calcium with Bordetella pertussis adenylate cyclase toxin. Characterization of multiple calcium-binding sites and calcium-induced conformational changes. *J Biol Chem* **270**: 26370–26376.

Rycroft AN, Williams D, Cullen JM & Macdonald J (1991) The cytotoxin of Actinobacillus pleuropneumoniae (pleurotoxin) is distinct from the haemolysin and is associated with a 120 kDa polypeptide. *J Gen Microbiol* **137**: 561–568.

Sadilkova L, Osicka R, Sulc M, Linihartova I, Novak P & Sebo P (2008) Single-step affinity purification of recombinant proteins using a self-excising module from Neisseria meningitidis FprC. *Protein Sci* **17**: 1834–1843.

Saier MH, Ma CH, Rodgers L, Tamang DG & Yen MR (2008) Protein secretion and membrane insertion systems in bacteria and eukaryotic organelles. *Adv Appl Microbiol* **65**: 141–197.

Sakiyama T, Ueno H, Homma H, Numata O & Kuwabara T (2006) Purification and characterization of a hemolysin-like protein, Slr1951, a nontoxic member of the RTX protein family from the cyanobacterium *Synechocystis* sp. strain PCC 6803. *J Bacteriol* **188**: 3535–3542.

Sanchez-Magraner L, Viguera AR, Garcia-Pacios M et al. (2007) 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**: 11827–11835.

Saron MF, Fayolle C, Sebo P, Ladant D, Ullmann A & Leclerc C (1997) Anti-viral protection conferred by recombinant adenylate cyclase toxins from Bordetella pertussis carrying a CD8+ T cell epitope from lymphohcyt choriomeningitis virus. *P Natl Acad Sci USA* **94**: 3314–3319.

Satchell KJ (2007) MARTX, multifunctional autoprocessing repeats-in-toxin toxins. *Infect Immun* **75**: 5079–5084.

Schaller A, Kuhn R, Kuhnert P et al. (1999) Characterization of apdIVA, a new RTX determinant of Actinobacillus pleuropneumoniae. *Microbiology* **145**: 2105–2116.

Schaller A, Kuhnert P, de la Puente-Redondo VA, Nicolet J & Frey J (2000) Apx toxins in *Pasteurellaceae* species from animals. *Vet Microbiol* **74**: 365–376.

Schindel C, Zitzer A, Schulte B et al. (2001) Interaction of Escherichia coli hemolysin with biological membranes. A study using cysteine scanning mutagenesis. *Eur J Biochem* **268**: 800–808.

Schlecht G, Loucka J, Najar H, Sebo P & Leclerc C (2004) Antigen targeting to CD11b allows efficient presentation of CD4+ and CD8+ T cell epitopes and in vivo Th1-polarized T cell priming. *J Immunol* **173**: 6089–6097.

Schmidt H, Beutin L & Karch H (1995) Molecular analysis of the plasmid-encoded hemolysin of Escherichia coli O157:H7 strain EDL 933. *Infect Immun* **63**: 1055–1061.

Schmidt H, Maier E, Karch H & Benz R (1996) Pore-forming properties of the plasmid-encoded hemolysin of enterohemorrhagic Escherichia coli O157:H7. *Eur J Biochem* **241**: 594–601.

Sebo P & Ladant D (1993) Repeat sequences in the Bordetella pertussis adenylate cyclase toxin can be recognized as alternative carboxy-proximal secretion signals by the Escherichia coli alpha-hemolysin translocator. *Mol Microbiol* **9**: 999–1009.

Sebo P, Glaser P, Sakamoto H & Ullmann A (1991) High-level synthesis of active adenylate cyclase toxin of Bordetella pertussis in a reconstructed Escherichia coli system. *Gene* **104**: 19–24.

Sebo P, Fayolle C, d’Andria O, Ladant D, Leclerc C & Ullmann A (1995) Cell-invasive activity of epitope-tagged adenylate cyclase of Bordetella pertussis allows in vitro presentation of a...
foreign epitope to CD8+ cytotoxic T cells. *Infect Immun* **63**: 3851–3857.

Shanthalingam S & Srikumaran S (2009) Intact signal peptide of CD18, the beta-subunit of beta2-integrins, renders ruminants susceptible to *Mannheimia haemolytica* leukotoxin. *P Natl Acad Sci USA* **106**: 15448–15453.

Sheahan KL & Satchell KJ (2007) Inactivation of small Rho GTases by the multifunctional RTX toxin from *Vibrio cholerae*. *Cell Microbiol* **9**: 1324–1335.

Sheahan KL, Cordero CI & Satchell KJ (2004) Identification of a domain within the multifunctional *Vibrio cholerae* RTX toxin that covalently cross-links actin. *P Natl Acad Sci USA* **101**: 9798–9803.

Shen A, Lupardus PJ, Albrow VE, Guzzetta A, Powers JC, Garcia SH & Baumeister W (1992) Killing of human leukocyte adhesion deficiency in holstein cattle. *Infect Immun* **53**: 1324–1335.

Sheehan KL, Cordero CI & Satchell KJ (2004) Identification of a domain within the multifunctional *Vibrio cholerae* RTX toxin that covalently cross-links actin. *P Natl Acad Sci USA* **101**: 9798–9803.

Shen A, Lupardus PJ, Albrow VE, Guzzetta A, Powers JC, Garcia SH & Baumeister W (1992) Killing of human leukocyte adhesion deficiency in holstein cattle. *Infect Immun* **53**: 1324–1335.

Shen A, Lupardus PJ, Albrow VE, Guzzetta A, Powers JC, Garcia SH & Baumeister W (1992) Killing of human leukocyte adhesion deficiency in holstein cattle. *Infect Immun* **53**: 1324–1335.

Shen A, Lupardus PJ, Albrow VE, Guzzetta A, Powers JC, Garcia SH & Baumeister W (1992) Killing of human leukocyte adhesion deficiency in holstein cattle. *Infect Immun* **53**: 1324–1335.

Shen A, Lupardus PJ, Albrow VE, Guzzetta A, Powers JC, Garcia SH & Baumeister W (1992) Killing of human leukocyte adhesion deficiency in holstein cattle. *Infect Immun* **53**: 1324–1335.

Shen A, Lupardus PJ, Albrow VE, Guzzetta A, Powers JC, Garcia SH & Baumeister W (1992) Killing of human leukocyte adhesion deficiency in holstein cattle. *Infect Immun* **53**: 1324–1335.

Shen A, Lupardus PJ, Albrow VE, Guzzetta A, Powers JC, Garcia SH & Baumeister W (1992) Killing of human leukocyte adhesion deficiency in holstein cattle. *Infect Immun* **53**: 1324–1335.

Shen A, Lupardus PJ, Albrow VE, Guzzetta A, Powers JC, Garcia SH & Baumeister W (1992) Killing of human leukocyte adhesion deficiency in holstein cattle. *Infect Immun* **53**: 1324–1335.

Shen A, Lupardus PJ, Albrow VE, Guzzetta A, Powers JC, Garcia SH & Baumeister W (1992) Killing of human leukocyte adhesion deficiency in holstein cattle. *Infect Immun* **53**: 1324–1335.

Shen A, Lupardus PJ, Albrow VE, Guzzetta A, Powers JC, Garcia SH & Baumeister W (1992) Killing of human leukocyte adhesion deficiency in holstein cattle. *Infect Immun* **53**: 1324–1335.

Shen A, Lupardus PJ, Albrow VE, Guzzetta A, Powers JC, Garcia SH & Baumeister W (1992) Killing of human leukocyte adhesion deficiency in holstein cattle. *Infect Immun* **53**: 1324–1335.

Shen A, Lupardus PJ, Albrow VE, Guzzetta A, Powers JC, Garcia SH & Baumeister W (1992) Killing of human leukocyte adhesion deficiency in holstein cattle. *Infect Immun* **53**: 1324–1335.

Shen A, Lupardus PJ, Albrow VE, Guzzetta A, Powers JC, Garcia SH & Baumeister W (1992) Killing of human leukocyte adhesion deficiency in holstein cattle. *Infect Immun* **53**: 1324–1335.

Shen A, Lupardus PJ, Albrow VE, Guzzetta A, Powers JC, Garcia SH & Baumeister W (1992) Killing of human leukocyte adhesion deficiency in holstein cattle. *Infect Immun* **53**: 1324–1335.

Shen A, Lupardus PJ, Albrow VE, Guzzetta A, Powers JC, Garcia SH & Baumeister W (1992) Killing of human leukocyte adhesion deficiency in holstein cattle. *Infect Immun* **53**: 1324–1335.

Shen A, Lupardus PJ, Albrow VE, Guzzetta A, Powers JC, Garcia SH & Baumeister W (1992) Killing of human leukocyte adhesion deficiency in holstein cattle. *Infect Immun* **53**: 1324–1335.

Shen A, Lupardus PJ, Albrow VE, Guzzetta A, Powers JC, Garcia SH & Baumeister W (1992) Killing of human leukocyte adhesion deficiency in holstein cattle. *Infect Immun* **53**: 1324–1335.

Shen A, Lupardus PJ, Albrow VE, Guzzetta A, Powers JC, Garcia SH & Baumeister W (1992) Killing of human leukocyte adhesion deficiency in holstein cattle. *Infect Immun* **53**: 1324–1335.

Shen A, Lupardus PJ, Albrow VE, Guzzetta A, Powers JC, Garcia SH & Baumeister W (1992) Killing of human leukocyte adhesion deficiency in holstein cattle. *Infect Immun* **53**: 1324–1335.
Suttrop N, Floer B, Schnittler H, Seeger W & Bhakdi S (1990) Effects of Escherichia coli hemolysin on endothelial cell function. Infect Immun 58: 3796–3801.

Syed KA, Beyhan S, Correa N et al. (2009) The Vibrio cholerae flagellar regulatory hierarchy controls expression of virulence factors. J Bacteriol 191: 6555–6570.

Szabo G, Gray MC & Hewlett EL (1994) Adenylate cyclase toxin from Bordetella pertussis produces ion conductance across artificial lipid bilayers in a calcium- and polarity-dependent manner. J Biol Chem 269: 22496–22499.

Taichman NS, Jenker BJ, Tsai CC, Glickman LT, Baehni PC, Stevens R & Hammond BF (1984) Cytopathic effects of Actinobacillus actinomycetemcomitans on monkey blood leukocytes. J Periodontal Res 19: 133–145.

Taichman NS, Simpson DL, Sakurada S, Cranfield M, DiRienzo J & Slots J (1987) Comparative studies on the biology of Actinobacillus actinomycetemcomitans leukotoxin in primates. Oral Microb Immun 2: 97–104.

Taichman NS, Iwase M, Korchak H, Berthold P & Lally ET (1991a) Membranolytic activity of Actinobacillus actinomycetemcomitans leukotoxin. J Periodontal Res 26: 258–260.

Taichman NS, Iwase M, Lally ET, Shattil SJ, Cunningham ME & Korchak HM (1991b) Early changes in cytosolic calcium and membrane potential induced by Actinobacillus actinomycetemcomitans leukotoxin in susceptible and resistant target cells. J Immunol 147: 3587–3594.

Tan Y & Miller KJ (1992) Cloning, expression, and nucleotide sequence of a lipase gene from Pseudomonas fluorescens B52. Appl Environ Microb 58: 1402–1407.

Tettelin H, Saunders NJ, Heidelberg J et al. (2000) Complete genome sequence of Neisseria meningitidis serogroup B strain MC58. Science 287: 1809–1813.

Thanabalu T, Koronakis E, Hughes C & Koronakis V (1998) Genome sequence of a lipase gene from Neisseria meningitidis synthesizes an S-layer-editing metalloprotease possessing a domain sharing sequence similarity with its paracrystalline S-layer protein. J Bacteriol 180: 2709–2718.

Valens M, Brouillet AC, Lefebvre M & Blight MA (2002) A zinc metalloprotease inhibitor, Inh, from the insect pathogen Photorhabdus luminescens. Microbiology 148: 2427–2437.

Venter AP, Twelker S, Oresnik IJ & Hynes MF (2001) Analysis of the biochemistry of hemolysin toxin activation: characterization of HlyC, an internal protein acyltransferase. Biochemistry 37: 4644–4652.

Trent MS, Worsham LM & Ernst-Fonberg ML (1998) The biochemistry of hemolysin toxin activation: characterization of HlyC, an internal protein acyltransferase. Biochemistry 37: 4644–4652.

Trent MS, Worsham LM & Ernst-Fonberg ML (1999a) HlyC, the internal protein acyltransferase that activates hemolysin toxin: role of conserved histidine, serine, and cysteine residues in enzymatic activity as probed by chemical modification and site-directed mutagenesis. Biochemistry 38: 3433–3439.

Trent MS, Worsham LM & Ernst-Fonberg ML (1999b) HlyC, the internal protein acyltransferase that activates hemolysin toxin: the role of conserved tyrosine and arginine residues in enzymatic activity as probed by chemical modification and site-directed mutagenesis. Biochemistry 38: 8831–8838.

Trent MS, Worsham LM & Ernst-Fonberg ML (1999c) HlyC, the internal protein acyltransferase that activates hemolysin toxin: roles of various conserved residues in enzymatic activity as probed by site-directed mutagenesis. Biochemistry 38: 9541–9548.

Tsai CC, Shenker BJ, DiRienzo JM, Malamud D & Taichman NS (1994) Extraction and isolation of a leukotoxin from Actinobacillus actinomycetemcomitans with polymyxin B. Infect Immun 63: 700–705.

Uhlen P, Laestadius A, Jahnukainen T et al. (2000) Alpha-haemolysin of uropathogenic E. coli induces Ca2+ oscillations in renal epithelial cells. Nature 405: 694–697.

Umelo-Njaka E, Nomellini JF, Bingle WH, Glasier LG, Irvin RT & Smit J (2001) Expression and testing of Pseudomonas aeruginosa vaccine candidate proteins prepared with the Caulobacter crescentus S-layer protein expression system. Vaccine 19: 1406–1415.

Umelo-Njaka E, Bingle WH, Borchani F et al. (2002) Caulobacter crescentus synthesizes an S-layer-editing metalloprotease possessing a domain sharing sequence similarity with its paracrystalline S-layer protein. J Bacteriol 184: 2709–2718.

Toporowski MC, Nomellini JF, Awram P & Smit J (2004) Two outer membrane proteins are required for maximal type I secretion of the Caulobacter crescentus S-layer protein. J Bacteriol 186: 8000–8009.

TranVan Nhieu G, Clair C, Grompone G & Sansonetti P (2004) Calcium signalling during cell interactions with bacterial pathogens. Biol Cell 96: 93–101.

Trent MS, Worsham LM & Ernst-Fonberg ML (1998) The biochemistry of hemolysin toxin activation: characterization of HlyC, an internal protein acyltransferase. Biochemistry 37: 4644–4652.
Vojtova J, Kamanova J & Sebo P (2006) Bordetella adenylate cyclase toxin: a swift saboteur of host defense. *Curr Opin Microbiol* 9: 69–75.

Vojtova-Vodolanova J, Basler M, Osicka R et al. (2009) Oligomerization is involved in pore formation by Bordetella adenylate cyclase toxin. *FASEB J* 23: 2831–2843.

Wagner W, Vogel M & Goebel W (1983) Transport of hemolysin across the outer membrane of *Escherichia coli* requires two functions. *J Bacteriol* 154: 200–210.

Walker KE, Moghaddame-Jafari S, Lockatell CV, Johnson D & Belas R (1999) ZapA, the IgA-degrading metalloprotease of *Proteus mirabilis*, is a virulence factor expressed specifically in swarmer cells. *Mol Microbiol* 32: 825–836.

Walker SG, Karunaratne DN, Ravencroft N & Smit J (1994) Characterization of mutants of *Caulobacter crescentus* defective in surface attachment of the paracrystalline surface layer. *J Bacteriol* 176: 6312–6323.

Wandersman C & Delepelaire P (1990) TolC, an extracellular protease: cloning and expression of the protease genes in *Escherichia coli*. *J Bacteriol* 169: 5046–5053.

Wang B, Kraig E & Kolodrubetz D (1998) Identification of two different hemolysin substrates. *Biochemistry* 37: 14037–14046.

Weingart CL & Weiss AA (2000) *Bordetella pertussis* virulence factors affect phagocytosis by human neutrophils. *Infect Immun* 68: 1735–1739.

Weiss AA, Hewlett EL, Myers GA & Falkow S (1984) Pertussis toxin and extracytoplasmic adenylate cyclase as virulence factors of *Bordetella pertussis*. *J Infect Dis* 150: 219–222.

Welch RA (1987) Identification of two different hemolysin determinants in uropathogenic *Proteus* isolates. *Infect Immun* 55: 2183–2190.

Welch RA (1991) Pore-forming cytolysins of gram-negative bacteria. *Mol Microbiol* 5: 521–528.

Welch RA (2001) RTX toxin structure and function: a story of numerous anomalies and few analogies in toxin biology. *Curr Top Microbiol Immunol* 257: 85–111.

Westrop G, Hormoz K, da Costa N, Parton R & Coote J (1997) Structure-function studies of the adenylate cyclase toxin of *Bordetella pertussis* and the leukotoxin of *Pasteurella haemolytica* by heterologous C protein activation and construction of hybrid proteins. *J Bacteriol* 179: 871–879.

Wolff J, Cook GH, Goldhammer AR & Berkowitz SA (1980) Calmodulin activates prokaryotic adenylate cyclase. *P Natl Acad Sci USA* 77: 3841–3844.

Wolff N, Sapriel G, Bodenreider C, Chaffotte A & Delepelaire P (2003) Antifolding activity of the SecB chaperone is essential for secretion of HasA, a quickly folding ABC pathway substrate. *J Biol Chem* 278: 38247–38253.

Woods RG, Burger M, Beven CA & Beacham IR (2001) The aprX-lipA operon of *Pseudomonas fluorescens* B52: a molecular analysis of metalloprotease and lipase production. *Microbiology* 147: 345–354.

Wooldridge KG, Kizil M, Wells DB & Al’aldeen DA (2005) Unusual genetic organization of a functional type I protein secretion system in *Neisseria meningitidis*. *Infect Immun* 73: 5554–5567.

Worsham LM, Trent MS, Earls L, Jolly C & Ernst-Fonberg ML (2001) Insights into the catalytic mechanism of HlyC, the internal protein acyltransferase that activates *Escherichia coli* hemolysin toxin. *Biochemistry* 40: 13607–13616.

Worsham LM, Langston KG & Ernst-Fonberg ML (2005) Thermodynamics of a protein acylation: activation of *Escherichia coli* hemolysin toxin. *Biochemistry* 44: 1329–1337.

Young J & Holland IB (1999) ABC transporters: bacterial exporters-revisited five years on. *Biochim Biophys Acta* 1461: 177–200.

Zaretzky FR, Gray MC & Hewlett EL (2002) Mechanism of association of adenylate cyclase toxin with the surface of *Bordetella pertussis*: a role for toxin-filamentous haemagglutinin interaction. *J Bacteriol* 177: 1521–1526.

Zecchinon L, Fett T & Desmecht D (2005) How *Campylobacter rectus* S-layer protein family: characterization of the crs gene from *Campylobacter rectus*. *Infect Immun* 73: 3631–3636.

Zhang Y, Bak DD, Heid H & Geider K (1999) Molecular characterization of a protease secreted by *Erwinia amylovora*. *J Mol Biol* 289: 1239–1251.

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**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Predicted RTX proteins identified by bioinformatic screening of sequence databases.

**Table S2.** Bacterial genomes containing *rtx* genes, as identified by sequence database screening.

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