Introduction
Nosocomial infections with *Clostridium difficile* often occur during antibiotic therapy. The *C. difficile*-associated diseases, namely antibiotic-associated diarrhoea and pseudomembranous colitis, are an increasing problem in health care, especially since hypervirulent strains (NAP1/027) have emerged recently (McDonald et al., 2005). The pathogenicity of *C. difficile* is based upon the action of at least one of the two major exotoxins produced and secreted by the bacterium, named toxin A (TcdA) and toxin B (TcdB), which belong to the family of clostridial glucosylating toxins (Sullivan et al., 1982; Rifkin et al., 1977; Bartlett et al., 1977; Voth & Ballard, 2005; Just & Gerhard, 2004). In addition, an actin-ADP-ribosylating toxin, CDT, has been identified in some strains of *C. difficile*, the pathological role of which is not clear so far (Popoff et al., 1988). Notably, the hypervirulent strain NAP1/027 is characterized by production of large amounts of glucosylating toxins, resistance against fluoroquinolones and the presence of CDT (Warry et al., 2005). Toxins A and B are encoded by *tcdA* and *tcdB*, which are found in a 19.6 kb pathogenicity locus. Three additional genes (*tcdC, tcdD* and *tcdE*) encoding negative (*tcdC*) and positive (*tcdD*) regulators as well as a holin-like pore-forming protein (*tcdE*) are part of this locus (Hammond & Johnson, 1995; Hundsberger et al., 1997; Mani & Dupuy, 2001). Variations in the structure of the pathogenicity locus are the basis for more than 20 toxinotypes (Rupnik et al., 1997, 1998; Torres, 1991). The emerging highly virulent *C. difficile* strain NAP1/027 is characterized by a deletion in the *tcdC* locus and this may cause high toxin A and B production (McDonald et al., 2005).

A hallmark of these large protein toxins (toxin A, 308 kDa; toxin B, 269 kDa) is a modular, tripartite composition (von Eichel-Streiber et al., 1996; Jank et al., 2007b). The N-terminal catalytic domain (aa 1–543), also called the ‘A’ domain, possesses full biological activity (Hofmann et al., 1997; Faust et al., 1998). The C-terminal domain consists of repetitive oligopeptides, involved in receptor binding (Tucker & Wilkins, 1991; Wren, 1991; Frisch et al., 2003; Ho et al., 2005). The central domain is by far the largest part of the proteins and is characterized by a small hydrophobic stretch (aa 956–1128) which is thought to mediate membrane insertion during translocation processes. The central ‘translocation’ domain and the C-terminal ‘binding’ domain are classically referred to as one unit, the ‘B’ domain (von Eichel-Streiber, 1992; Just & Gerhard, 2004). Notably, only the N-terminal ‘A’ domain is translocated into the cytosol of target cells (Pfeifer et al., 2003; Rupnik et al., 2005). Therefore, a controlled and limited proteolysis is an essential step in the uptake of clostridial glucosylating toxins.

Toxins A and B possess glucosyltransferase activity and inactivate Rho GTPases
Intracellular targets of the bacterial glucosyltransferases are small GTPases of the Rho family (Just et al., 1995), which comprise a family of about 20 GTP-binding proteins. Rho proteins function as molecular switches and are involved in multiple cellular signalling processes, including regulation of the actin cytoskeleton, adhesion, migration and cell polarity. They control enzyme activities, gene transcription, cell cycle progression and apoptosis (Etienne-Manneville & Hall, 2002). The toxins catalyse the mono-O-glucosylation of the Rho GTPases at a threonine residue (Thr35/37), which is essential for the switch function of the GTPases (Just et al., 1995). Glucosylation blocks the activation of Rho GTPases by their activators (guanine nucleotide exchange factors, GEFs), inhibits interaction with their effectors (e.g. protein kinases and adaptor proteins), blocks their membrane–cytosol cycling and favours membrane binding. The structural basis of the inhibiting effects on
Rho functions is probably a blockade of the active conformation of Rho GTPases by glucosylation (Sehr et al., 1998; Vetter et al., 2000; Geyer et al., 2003). This leads, amongst others, to the depolymerization of the actin cytoskeleton, cell rounding and finally apoptosis (Just & Gerhard, 2004; Voth & Ballard, 2005).

**N-terminus: the catalytic centre**

The biologically active domain, which is delivered into the cytosol, comprises the first 543 aa (Rupnik et al., 2005). The recently solved 3D-structure of this fragment revealed that it was closely related to other bacterial glucosyltransferases belonging to the GT-A family (Reinert et al., 2005). The catalytic core consists of 234 aa and is formed by a mixed α/β-fold with mostly parallel β-strands as the central part. The more than 300 additional residues are mainly helices, of which the first four N-terminal helices are most probably involved in membrane association, therefore assuring close proximity of the enzyme with its substrates. Characteristic for GT-A family members is the DXD motif involved in complexation of manganese ions, UDP and glucose. Mutation of these essential aspartate residues leads to inactivation of the toxin (Busch et al., 1998). The cosubstrate for the bacterial glucosyltransferases is UDP-glucose; only α-toxin from *Clostridium novyi* utilizes UDP-N-acetylglucosamine (UDP-GlcNAc) (Seltzer et al., 1996).

This difference in cosubstrate specificity is based on sterical hindrance by bulky amino acids (e.g. Ile<sup>383</sup>/Gln<sup>385</sup> in toxin B) blocking the catalytic pocket for the larger UDP-GlcNAc. In α-toxin, small serine and alanine residues at the corresponding positions allow UDP-GlcNAc to enter the catalytic cleft (Jank et al., 2005). Little is known so far about the molecular/structural determinants underlying the differences in substrate recognition by different glucosylating toxins. Based on crystallographic and biochemical data, a preliminary docking model has been proposed where the GTPases bind to the glucosyltransferases with the same consensus region and in a comparable manner to how they normally bind to effector molecules (Dvorsky & Ahmadian, 2004; Jank et al., 2007a).

**The C-terminal region mediates receptor binding**

The C-terminus of the ‘B’ domain consists of clostridial repetitive oligopeptides, which are involved in receptor binding. The nature of the receptor has still not been solved, but there are hints for a role of carbohydrate structures in toxin binding. In the case of toxin A, binding to, for example, a galactose- and N-acetylglucosamine glucoprotein, a membranous sucrose-isomaltase glucoprotein and Galα1-3Galβ1-4GlcNAc in different animal model systems has been reported (Rolfe & Song, 1993; Pothoulakis et al., 1996; Krivan et al., 1986; Tucker & Wilkins, 1991). However, since these structures are absent in a wide variety of sensitive cells and also α-anomeric galactose bonds are absent in human tissue (Larsen et al., 1990), these carbohydrate structures are unlikely to be or cannot be part of the intestinal receptor in humans. Nevertheless, the proposed role of carbohydrates was supported by the recently solved structure of two C-terminal fragments of toxin A and the co-crystallization of toxin A with an artificial trisaccharide (Ho et al., 2005; Greco et al., 2006). These data showed that the C-terminus possesses a solenoid-like structure, consisting of 7 large repeats with 30 residues and 32 small repeats with 15–21 residues. The large and small peptide repeats have single β-hairpin structures with antiparallel β-strands of 5–6 residues. The β-hairpins are connected by loops of 7–10 residues in short repeats, and by 18 residues in long repeats. Each hairpin is rotated by 120°, resulting in a screw-like structure. However, since the identified amino acid residues participating in carbohydrate binding are not conserved in other clostridial glucosylating toxins, the receptor(s) still remains to be identified.

**The central translocation domain**

The large middle part of the protein toxins makes up more than 50 % of the total size, but little is known about its exact functions. It is characterized by a hydrophobic stretch which is most probably responsible for membrane penetration (transmembrane prediction) (von Eichel-Streiber et al., 1992). Therefore, this region is referred to as the ‘translocation domain’. Deletion studies proved the importance of the hydrophobic region for toxin activity (Barroso et al., 1994). The same report also indicated a large impact of specific residues located inside the translocation domain but outside the hydrophobic region on the cytotoxic activity of the protein. For example, exchange of cysteine 698 to serine or histidine 653 to glutamine in toxin B reduced the cytotoxic titre by about 90 or 99 %, respectively. At that time, no molecular explanation for these observations was available, but it was already proposed that these residues may be involved in the uptake and processing of the toxins.

**Uptake of clostridial glucosylating toxins**

Clostridial glucosylating toxins enter eukaryotic target cells according to the ‘short trip model’ of bacterial exotoxin uptake (Sandvig et al., 2004). Following receptor-mediated endocytosis, the acidification of early endosomes by the vesicular H<sup>+</sup>-ATPase induces a conformational change characterized by an increase in hydrophobicity (Florin & Thelestam, 1983; Barth et al., 2001; Qa’Dan et al., 2000). This is probably due to a surface exposure of the hydrophobic region, which then enables the corresponding part of the toxin to insert into the membrane and to build a pore through which the catalytic domain can translocate into the cytosol. Pore formation under acidic conditions has been demonstrated for *C. difficile* toxin A and toxin B (Barth et al., 2001; Giesemann et al., 2006). As mentioned above, solely the N-terminal catalytic domain (aa 1–543) is then released from the early endosomes and reaches the cytosol of eukaryotic cells. This translocation of the ‘A’
domain across the cellular membrane and the release into the cytosol still remains enigmatic. One essential step is the secession of the first 543 aa from the protein under controlled conditions. Where this separation takes place is not clear, neither is the exact nature of the proteolytic activity involved in this process. Just recently, it was demonstrated that this cutting may be accomplished by an intrinsic activity of the toxin itself. Two independent studies identified autoprolysis activated by dithiothreitol (DTT) and/or myo-inositol hexakisphosphate (InsP₆). One of these studies ascribed the function to a putative aspartate protease domain located in the C-terminal part of the translocation domain (Reineke et al., 2007). The second study reports that the proteolytic activity is based on an intrinsic cysteine protease domain (CPD) located adjacent to the autocleavage site in the N-terminal part of the translocation domain (Egerer et al., 2007).

Identification and biochemical characterization of an intrinsic CPD

The primary sequence of toxin B aa 544–955, a fragment bordered by the N-terminal glucosyltransferase domain (‘GT’) and the hydrophobic, putative transmembrane region (‘HR’), aa 956–1128; see Fig. 1, upper panel) displays a striking sequence similarity to repeat in toxin (RTX) protein toxins and autotransporter adhesins from, for example, Vibrio cholerae and Vibrio vulnificus/Vibrio splendidus. Although overall similarity is relatively low, ranging from 23 to 25 %, the sequence identities concentrate in specific clusters resembling a putative catalytic triad of a cysteine protease (see Fig. 1, lower panel; D587H653C698). This assumption is strengthened by the recent identification and characterization of the corresponding intrinsic CPD within V. cholerae RTX (Sheahan et al., 2007).

According to the prediction of an intrinsic proteolytic activity, the degradation of the holotoxin into GT domain and binding/translocation domain can be induced by factors and/or conditions as found in the cytosol. For example, under reducing conditions, toxin A and toxin B show a split product at about 63 kDa, a size corresponding to the isolated N-terminal glucosyltransferase domain. MALDI-TOF analysis confirmed the expected degradation of the holotoxin into the corresponding domains. The onset of autoprolysis under the influence of DTT points to an intramolecular disulfide bond within toxin B544–955 implicated in the regulation of the CPD.

Data that clearly indicate an essential role of cysteine residues in this process come from the utilization of N-ethylmaleimide (NEM), a common inhibitor of cysteine proteases. When NEM is added after onset of proteolytic cleavage achieved by low DTT concentrations, further degradation is inhibited. InsP₆ induces autoprolysis of toxin B in a comparable manner, but InsP₆ is more efficient than DTT. Proteolysis starts at lower concentrations and is faster compared to proteolysis with DTT. Interestingly, when varying concen-

![Fig. 1.](image_url) Clostridial glucosylating toxins: primary structure and partial sequence alignment of the catalytic triad of the cysteine protease domain. Upper panel: model of the primary structure of Clostridium difficile toxin B with the N-terminal glucosyltransferase domain (GT), the newly identified intrinsic cysteine protease domain (CPD), the central translocation domain, including a hydrophobic region (HR), and the N-terminal receptor binding domain, consisting of clostridial repetitive oligopeptides (CROPs). Due to the multifunctionality of the toxins, which is mirrored by the multidomain structure, we suggest amending the classical denomination ‘AB toxins’ to ‘ABCD toxins’, with ‘A’ for biological activity, ‘B’ for binding, ‘C’ for cutting and ‘D’ for delivery. Lower panel: partial sequence alignment of the putative catalytic triad of toxin B CPD with RTX toxin and autotransporter adhesins from Vibrio spp. (as indicated). The alignment is based on a BLAST search with a fragment of toxin B (aa 544–955). Depicted here are typical consensus sequences representing the catalytic triad of a cysteine protease.
trations of InsP$_6$ are combined with low DTT concentrations, a synergistic effect on the proteolysis is observable.

The exchange of single residues of the putative catalytic triad (D587H653C698) in a fragment of toxin B encompassing aa 1–955 proved the importance of each of these residues for autoproteolysis. When the corresponding $^{35}$S-labelled polypeptides are produced by 
in vitro transcription/translation, wild-type toxin B 1–955 undergoes autoproteolysis during or right after translation. In contrast, the point mutants D587N, H653A and C698A are stabilized. Of these, toxin B1–955 C698A and H653A

**Fig. 2.** Biochemical characterization of the autoproteolytic processing of toxin B by an intrinsic cysteine protease activity. (a) Autoproteolysis of toxin B is activated under reducing conditions and inhibited by N-ethylmaleimide (NEM), an inhibitor of cysteine proteases. One microgram of native toxin B was incubated in Tris/ HCl buffer (pH 7.5) with 0.5 mM DTT for 30 min at RT to induce onset of autoproteolysis before 10 mM NEM was added (as indicated). DTT at a final concentration of 20 mM was added either simultaneously with NEM or after another 30 min incubation (1., 2., 3.=order of application, accordingly). Simultaneous addition leads to inactivation of NEM by DTT. All reactions were incubated overnight at RT. The inhibitory effect of NEM was irreversible, since an excess amount of DTT did not reanimate autoproteolysis. (b) DTT and InsP$_6$ induce autoproteolytic cleavage of toxin B in a synergistic manner. One microgram of native toxin B was incubated with increasing concentrations of InsP$_6$ with or without additional 0.5 mM DTT (as indicated). Samples were incubated for 30 min at RT. Proteolysis started at ~2–10 mM InsP$_6$ and was faster compared to proteolysis with DTT (10–30 min versus 1–2 h). The combination of varying concentrations of InsP$_6$ with 0.5 mM DTT resulted in a synergistic effect on proteolysis. This is mirrored by the onset of proteolysis at 1 mM InsP$_6$ plus 0.5 mM DTT in contrast to the case with 1 mM InsP$_6$ alone, where no significant proteolysis is observable under the same conditions. (a, b) All reactions were stopped by heating the samples at 95 °C in Laemmli buffer. Samples were subjected to SDS-PAGE and immunoblotting utilizing a toxin B-specific monoclonal antibody recognizing the catalytic domain at a dilution of 1 : 100 000 followed by an anti-mouse IgG horseradish peroxidase-labelled secondary antibody. Corresponding bands were detected using enhanced chemiluminescence. (c) Verification of the catalytic triad D587H653C698 of toxin B by site-directed mutagenesis. Fragments of toxin B encompassing aa 1–955 (toxin B1–955 'wt'), the corresponding point mutants D587N, H653A, C698A and C595A and the double mutant L543A/G544A were produced as $^{35}$S-labelled proteins by 
in vitro transcription/translation utilizing pET28a constructs as described recently by Egerer *et al.* (2007). Wild-type (wt) toxin B1–955 undergoes complete proteolysis in the lysate utilized in this assay. In contrast, the point mutants D587N, H653A and C698A are stabilized. Exchange of C595 has no stabilizing effect and mutation of the autocleavage site (toxin B1–955 L543A/G544A) also results in a stabilized protein. (d) Impact of autoproteolysis on cytotoxicity. Recombinant toxin B and the point mutant C698A were produced as recombinant glutathione S-transferase fusion proteins as described by Egerer *et al.* (2007). Wild-type recombinant toxin B displays limited proteolysis during purification, which is absent in the C698A mutant (see Coomassie-stained SDS-PAGE gels, upper panel). For cell intoxication, HeLa cells were cultivated in Dulbecco's minimal Eagle's medium in 12-well cell culture dishes. Recombinant toxin B (rec. toxin B) and toxin B point mutant (C698A) (1 μg ml$^{-1}$) were applied for 4–6 h at 37 °C. Wild-type toxin B induced complete cell rounding within 4–5 h. The C698A mutant showed a significantly diminished cytotoxic potential.
show no degradation at all, whereas toxin B\(^{1-955}\) D587N is not completely insensitive to degradation. This is probably based on other aspartate and glutamine residues neighbouring D587 which can in part substitute D587 in the catalytic triad. Notably, exchange of an unrelated cysteine (C595) has no stabilizing effect and the mutation of the autocleavage site (L\(^{242}\)/G\(^{344}\)) results in a stabilized protein comparable to the catalytic triad point mutants.

Autoproteolysis is essential for the cytotoxic potential of toxin B. When the catalytic cysteine 698 is mutated in recombinant holotoxin, the corresponding toxin variant is stabilized (comparable to toxin B\(^{1-955}\) C698A) and loses its cytotoxicity almost completely. This effect is in line with former observations concerning toxin B mutagenesis (Barroso et al., 1994).

These data (summarized in Fig. 2; see also Egerer et al., 2007) indicate that the translocation domain of clostridial glucosylating toxins comprises a CPD. This intrinsic activity is responsible for the autocalytic processing of the toxins. The proteolysis, which is essential for cytotoxic activity, is activated by reducing conditions and/or InsP\(_6\).

**Conclusions**

*C. difficile* toxins are large multidomain proteins. Their action depends on a complex uptake mechanism including proteolytic processing (Aktories, 2007). Although the theoretical model of toxin uptake (schematically outlined in Fig. 3) is generally accepted (Sandvig et al., 2004), the precise molecular mechanisms have not been well characterized. However, a major step forward was made with the finding of an intrinsic proteolytic activity of the toxins. This autoproteolytic activity is induced by InsP\(_6\) and/or DTT and is responsible for the separation of the catalytic domain from the holotoxin (Reineke et al., 2007; Egerer et al., 2007). These findings are in line with reports on RTX toxin from *V. cholerae*, which also undergoes autocatalytic processing during uptake (Sheahan et al., 2007). The striking similarity between clostridial glucosylating toxins and RTX toxins is limited to the CPD of RTX and concentrates around the putative catalytic residues, e.g. D\(^{587}\)/H\(^{655}\)/C\(^{698}\) of toxin B. The importance of these residues for autocatalytic processing was shown by site-directed mutagenesis. Notably, these residues are conserved in all clostridial glucosylating toxins. Processing does not require the holotoxin, but is also detectable with fragments comprising only the first 955 aa. This suggests against the hypothesis of an intrinsic aspartate protease domain located around a DXG motif at position D\(^{686}\) in close vicinity to the C-terminal polypeptide repeats domain (Reineke et al., 2007). In this context, it is noteworthy that the DXG motif is absent in *C. novyi* \(\alpha\)-toxin, although this toxin is also autocalytically cleaved under the same conditions. Furthermore, the localization of the CPD in toxin B adjacent to the GT domain is comparable to the CPD flanking the actin-cross-linking domain in RTX toxins. Since, in the case of toxin B, InsP\(_6\) seems to be the physiologically relevant inducer of autoproteolysis, this close proximity of the CPD and GT domain makes sense with regard to the uptake process (see Fig. 3). Here, a cotranslocation of the GT domain and the CPD would guarantee access of the CPD to cytosolic InsP\(_6\) and therefore a controlled onset of proteolysis after complete translocation. The exact role of InsP\(_6\) in this process is still unclear, but since this highly charged, multifunctional molecule seems to have diverse structural effects, an impact on the stability by modulation of toxin conformation seems to be a plausible assumption (Shears, 2001).

Considering the fast propagation of severe nosocomial infections with hypervirulent strains of *C. difficile*, new strategies for therapy are needed (Bartlett & Perl, 2005). Since the pathogenicity of *C. difficile* depends on its glucosylating protein toxins and severity seems to correlate with toxin amount, these molecules may be targets for

---

**Fig. 3.** Theoretical model of clostridial glucosylating toxin uptake. A simplified model of the primary structure of toxin B is shown that considers the multidomain composition of the protein (‘ABCD model’; see also Fig. 1). Uptake starts with the binding of the binding (‘B’) domain to a yet unknown receptor, followed by receptor-mediated endocytosis. Acidification of the endosomal lumen by a vesicular H\(^+\)-ATPase results in a refolding of the toxin characterized by an increase in hydrophobicity. This exposure of the hydrophobic region allows membrane penetration and simultaneous pore formation by the delivery ‘D’ domain. The catalytically active ‘A’ domain and eventually the adjacent cysteine protease domain (‘C’; cutting) are translocated through the pore. Cytosolic InsP\(_6\) can approach the translocated part and induce autocleavage of the toxin, resulting in free biologically active ‘A’ domain.
therapeutic strategies. Development of glycan-mimicking compounds blocking the receptor-binding domain is a potential approach (Greco et al., 2006). Moreover, the 3D-structure of the catalytic domain of various glucosyltransferases may allow the design of membrane-permeable inhibitors targeting the catalytic domain (Reinert et al., 2005). In any case, further progress in understanding the molecular mechanisms involved in the actions of C. difficile toxins will certainly provide new perspectives for development of new strategies against the pathogen and its toxins.

References

Aktories, K. (2007). Self-cutting to kill: new insights into the processing of Clostridium difficile toxins. ACS Chem Biol 2, 228–230.

Barroso, L. A., Moncrief, J. S., Lyerly, D. M. & Wilkins, T. D. (1994). Mutagene
essis of the Clostridium difficile toxin B gene and effect on cytotoxic activity. Microb Pathog 16, 297–303.

Barth, H., Pfeifer, G., Hofmann, F., Maier, E., Benz, R. & Aktories, K. (2001). Low pH-induced formation of ion channels by Clostridium difficile toxin B in target cells. J Biol Chem 276, 10670–10676.

Bartlett, J. G. & Perl, T. M. (2005). The new Clostridium difficile – what does it mean? N Engl J Med 353, 2503–2505.

Bartlett, J. G., Onderdonk, A. B., Cisneros, R. L. & Kasper, D. L. (1977). Clindamycin-associated colitis due to a toxin-producing species of Clostridium in hamsters. J Infect Dis 136, 701–705.

Busch, C., Hofmann, F., Selzer, J., Munro, J., Jeckel, D. & Aktories, K. (1998). A common motif of eukaryotic glycosyltransferases is essential for the enzyme activity of large clostridial cytotoxins. J Biol Chem 273, 19566–19572.

Dvorsky, R. & Ahmadian, M. R. (2004). Always look on the bright site – what does it mean? EMBO Rep 5, 1130–1136.

Eggerer, M., Giesemann, T., Jank, T., Satchell, K. J. & Aktories, K. (2007). Auto-catalytic cleavage of Clostridium difficile toxins A and B depends on a cysteine protease activity. J Biol Chem 282, 25314–25321.

Etienne-Manneville, S. & Hall, A. (2002). Rho GTPases in cell biology. Nature 420, 629–635.

Faust, C., Ye, B. & Song, K.-P. (1998). The enzymatic domain of Clostridium difficile toxin A is located within its N-terminal region. Biochem Biophys Res Commun 251, 100–105.

Florin, I. & Thelestam, M. (1983). Internalization of Clostridium difficile cyto
toxin into cultured human lung fibroblasts. Biochim Biophys Acta 763, 383–392.

Frisch, C., Gerhard, R., Aktories, K., Hofmann, F. & Just, I. (2003). The complete receptor-binding domain of Clostridium difficile toxin A is required for endocytosis. Biochem Biophys Res Commun 300, 706–711.

Geyer, M., Wilde, C., Selzer, J., Aktories, K. & Kälbitzer, H. R. (2003). Glucosylation of Ras by Clostridium sordellii lethal toxin: consequences for the effector loop conformations observed by NMR spectroscopy. Biochemistry 42, 11951–11959.

Giesemann, T., Jank, T., Gerhard, R., Maier, E., Just, I., Benz, R. & Aktories, K. (2006). Cholesterol-dependent pore formation of Clostridium difficile toxin A. J Biol Chem 281, 10808–10815.

Greco, A., Ho, J. G., Lin, S. J., Palcic, M. M., Rupnik, M. & Ng, K. K. (2006). Carbohydrate recognition by Clostridium difficile toxin A. Nat Struct Mol Biol 13, 460–461.

Hammond, G. A. & Johnson, J. L. (1995). The toxigenic element of Clostridium difficile strain VPI 10463. Microb Pathog 19, 203–213.

Ho, J. G., Greco, A., Rupnik, M. & Ng, K. K. (2005). Crystal structure of receptor-binding C-terminal repeats from Clostridium difficile toxin A. Proc Natl Acad Sci U S A 102, 18373–18378.

Hofmann, F., Busch, C., Prepens, U., Just, I. & Aktories, K. (1997). Localization of the glucosyltransferase activity of Clostridium difficile toxin B to the N-terminal part of the holotoxin. J Biol Chem 272, 11074–11078.

Hundsberger, T., Braun, V., Weidmann, M., Leukel, P., Sauerborn, M. & von Eichel-Streiber, C. (1997). Transcription analysis of the genes tcdA-E of the pathogenicity locus of Clostridium difficile. Eur J Biochem 244, 735–742.

Jank, T., Reinert, D. J., Giesemann, T., Schulz, G. E. & Aktories, K. (2005). Change of the donor substrate specificity of Clostridium difficile toxin B by site-directed mutagenesis. J Biol Chem 280, 37833–37838.

Jank, T., Giesemann, T. & Aktories, K. (2007a). Clostridium difficile glucosyltransferase toxin B – essential amino acids for substrate-binding. J Biol Chem 282, 35222–35331.

Jank, T., Giesemann, T. & Aktories, K. (2007b). Rho-glucosylating Clostridium difficile toxins A and B: new insights into structure and function. Glycobiology 17, 15R–22R.

Just, I. & Gerhard, R. (2004). Large clostridial cytotoxins. Rev Physiol Biochem Pharmacol 152, 23–47.

Just, I., Selzer, J., Wilm, M., von Eichel-Streiber, C., Mann, M. & Aktories, K. (1995). Glucosylation of Rho proteins by Clostridium difficile toxin B. Nature 375, 500–503.

Krivan, H. C., Clark, G. F., Smith, D. F. & Wilkins, T. D. (1986). Cell surface binding site for Clostridium difficile enterotoxin: evidence for a glycoconjugate containing the sequence Galβ1-3Galβ1-4GlcNAc. Infect Immun 53, 573–581.

Larsen, R. D., Rivera-Marrero, C. A., Ernst, L. K., Cummings, R. D. & Lowe, J. B. (1990). Frameshift and nonsense mutations in a human genomic sequence homologous to a murine UDP-Galβ1-3Galβ1-4GlcNAc α(1,3)-galactosyltransferase cDNA. J Biol Chem 265, 7055–7061.

Mani, N. & Dupuy, B. (2001). Regulation of toxin synthesis in Clostridium difficile by an alternative RNA polymerase sigma factor. Proc Natl Acad Sci U S A 98, 5844–5849.

McDonald, L. C., Killgore, A., Thompson, A., Owens, R. C., Jr, Kazakova, S. V., Sambol, S. P., Johnson, S. & Gerding, D. N. (2005). An epidemic, toxin gene-variant strain of Clostridium difficile. N Engl J Med 353, 2433–2441.

Pfeifer, G., Schirmer, J., Leemhuis, J., Busch, C., Meyer, D. K., Aktories, K. & Barth, H. (2003). Cellular uptake of Clostridium difficile toxin B: translocation of the N-terminal catalytic domain into the cytosol of eukaryotic cells. J Biol Chem 278, 44535–44541.

Popoff, M. R., Rubin, E. J., Gill, D. M. & Boquet, P. (1988). Actin-specific ADP-ribosyltransferase produced by a Clostridium difficile strain. Infect Immun 56, 2299–2306.

Pothoulakis, C., Gilbert, R. J., Cladaras, C., Castagliuolo, I., Semenza, G., Hitti, Y., Montcrief, J. S., Linevsky, J., Kelly, C. P. & other authors (1996). Rabbit sucrose-isomaltase contains a functional intestinal receptor for Clostridium difficile toxin A. J Clin Invest 98, 641–649.

Qa’Dan, M., Spyres, L. M. & Ballard, J. D. (2000). Ph-Induced conformational changes in Clostridium difficile toxin B. Infect Immun 68, 2470–2474.

Reineke, J., Tenzer, S., Rupnik, M., Koschinski, A., Hasselmayer, O., Schrattenholz, A., Schild, H. & von Eichel-Streiber, C. (2007).
Autocatalytic cleavage of *Clostridium difficile* toxin B. *Nature* 446, 415–419.

Reinert, D. J., Jank, T., Aktories, K. & Schulz, G. E. (2005). Structural basis for the function of *Clostridium difficile* toxin B. *J Mol Biol* 351, 973–981.

Rifkin, G. D., Fekety, F. R., Silva, J. & Sack, R. B. (1977). Antibiotic-induced colitis. Implication of a toxin neutralised by *Clostridium sordellii* antitoxin. *Lancet* 310, 1103–1106.

Rolfe, R. D. & Song, W. (1993). Purification of a functional receptor for *Clostridium difficile* toxin A from intestinal brush border membranes of infant hamsters. *Clin Infect Dis* 16, S219–227.

Rupnik, M., Braun, V., Soehn, F., Janc, M., Hofstetter, M., Laufenberg-Feldmann, R. & von Eichel-Streiber, C. (1997). Characterization of polymorphisms in the toxin A and B genes of *Clostridium difficile*. *FEMS Microbiol Lett* 148, 197–202.

Rupnik, M., Avesani, V., Janc, M., von Eichel-Streiber, C. & Delmé, M. (1998). A novel toxinotyping scheme and correlation of toxinotypes with serogroups of *Clostridium difficile* isolates. *J Clin Microbiol* 36, 2240–2247.

Rupnik, M., Pabst, S., Rupnik, M., von Eichel-Streiber, C., Urlaub, H. & Soling, H. D. (2005). Characterization of the cleavage site and function of resulting cleavage fragments after limited proteolysis of *Clostridium difficile* toxin B (TcdB) by host cells. *Microbiology* 151, 199–208.

Sandvig, K., Spilsberg, B., Lauvrak, S. U., Torgersen, M. L., Iversen, T. G. & van Deurs, B. (2004). Pathways followed by protein toxins into cells. *Int J Med Microbiol* 293, 483–490.

Sehr, P., Joseph, G., Genth, H., Just, I., Pick, E. & Aktories, K. (1998). Glucosylation and ADP-ribosylation of Rho proteins – effects on nucleotide binding, GTPase activity, and effector-coupling. *Biochemistry* 37, 5296–5304.

Selzer, J., Hofmann, F., Rex, G., Wilm, M., Mann, M., Just, I. & Aktories, K. (1996). *Clostridium novyi* z-toxin-catalyzed incorporation of GlcNAc into Rho subfamily proteins. *J Biol Chem* 271, 25173–25177.

Sheahan, K.-L., Cordero, C. L. & Fullner Satchell, K. J. (2007). Autoprocessing of the *Vibrio cholerae* RTX toxin by the cysteine protease domain. *EMBO J* 26, 2552–2561.

Shears, S. B. (2001). Assessing the omnipotence of inositol hexakis-phosphate. *Cell Signal* 13, 151–158.

Sullivan, N. M., Pellett, S. & Wilkins, T. D. (1982). Purification and characterization of toxins A and B of *Clostridium difficile*. *Infect Immun* 35, 1032–1040.

Torres, J. F. (1991). Purification and characterisation of toxin B from a strain of *Clostridium difficile* that does not produce toxin A. *J Med Microbiol* 35, 40–44.

Tucker, K. D. & Wilkins, T. D. (1991). Toxin A of *Clostridium difficile* binds to the human carbohydrate antigens I, X, and Y. *Infect Immun* 59, 73–78.

Vetter, I. R., Hofmann, F., Wohlgemuth, S., Herrmann, C. & Just, I. (2000). Structural consequences of mono-glucosylation of Ha-Ras by *Clostridium sordellii* lethal toxin. *J Mol Biol* 301, 1091–1095.

von Eichel-Streiber, C. (1992). A dual model for the architecture of *Clostridium difficile* toxins A and B. In *Bacterial Protein Toxins*, pp. 113–122. Edited by B. Witholt. Stuttgart, Jena, New York: Fischer.

von Eichel-Streiber, C., Laufenberg-Feldmann, R., Sartingen, S., Schulze, J. & Sauernborn, M. (1992). Comparative sequence analysis of the *Clostridium difficile* toxins A and B. *Mol Gen Genet* 233, 260–268.

von Eichel-Streiber, C., Boquet, P., Sauernborn, M. & Thelestam, M. (1996). Large clostridial cytotoxins – a family of glycosyltransferases modifying small GTP-binding proteins. *Trends Microbiol* 4, 375–382.

Voth, D. E. & Ballard, J. D. (2005). *Clostridium difficile* toxins: mechanism of action and role in disease. *Clin Microbiol Rev* 18, 247–263.

Warny, M., Pepin, J., Fang, A., Killgore, G., Thompson, A., Brazier, J., Frost, E. & McDonald, L. C. (2005). Toxin production by an emerging strain of *Clostridium difficile* associated with outbreaks of severe disease in North America and Europe. *Lancet* 366, 1079–1084.

Wren, B. W. (1991). A family of clostridial and streptococcal ligand-binding proteins with conserved C-terminal repeat sequences. *Mol Microbiol* 5, 797–803.