Microbiology (2011), **157**, 1851–1875 DOI 10.1099/mic.0.049536-0

Review

Cytolethal distending toxin: a conserved bacterial genotoxin that blocks cell cycle progression, leading to apoptosis of a broad range of mammalian cell lineages

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Cytolethal distending toxin (CDT) is a heterotrimeric AB-type genotoxin produced by several clinically important Gram-negative mucocutaneous bacterial pathogens. Irrespective of the bacterial species of origin, CDT causes characteristic and irreversible cell cycle arrest and apoptosis in a broad range of cultured mammalian cell lineages. The active subunit CdtB has structural homology with the phosphodiesterase family of enzymes including mammalian DNase I, and alone is necessary and sufficient to account for cellular toxicity. Indeed, mammalian cells treated with CDT initiate a DNA damage response similar to that elicited by ionizing radiation-induced DNA double strand breaks resulting in cell cycle arrest and apoptosis. The mechanism of CDT-induced apoptosis remains incompletely understood, but appears to involve both p53-dependent and -independent pathways. While epithelial, endothelial and fibroblast cell lines respond to CDT by undergoing arrest of cell cycle progression resulting in nuclear and cytoplasmic distension that precedes apoptotic cell death, cells of haematopoietic origin display rapid apoptosis following a brief period of cell cycle arrest. In this review, the ecology of pathogens producing CDT, the molecular biology of bacterial CDT and the molecular mechanisms of CDT-induced cytotoxicity are critically appraised. Understanding the contribution of a broadly conserved bacterial genotoxin that blocks progression of the mammalian cell cycle, ultimately causing cell death, should assist with elucidating disease mechanisms for these important pathogens.

Introduction

Johnson and Lior's seminal observations in the 1980s identified a novel heat-labile toxin in culture filtrates obtained from certain *Escherichia coli*, *Shigella dysenteriae* and *Campylobacter jejuni* strains which caused distinctive and progressive cytoplasmic and nuclear enlargement of cultured mammalian cells, so called cytolethal distending toxin (CDT), and uncovered a novel paradigm amongst bacterial toxins and virulence mechanisms (Johnson & Lior, 1987, 1988a, b). It was not until many years later that Scott & Kaper (1994) identified the genes encoding CDT in *E. coli*, which set the stage for fundamental investigations into the ecology, biochemistry and molecular mechanisms of cellular toxicity associated with this novel bacterial toxin (Table 1). While a secreted protein cytotoxin was identified among *Haemophilus* (*Haem.*) *ducreyi* clinical isolates in the early 1990s by Purvén & Lagergård (1992), it was not until the late 1990s that this cytotoxin was conclusively shown to be encoded by a *cdt* gene cluster with homology to the previously identified *E. coli* genes (Cope *et al.*, 1997). This discovery extended the range of niches where CDT-producing bacteria are found to include mucocutaneous surfaces of the genital tract in addition to the intestinal tract. At that time, Pérès *et al.* (1997) first reported that the mechanism of mammalian cell intoxication by *E. coli* CDT involved arrest of the cell cycle at the G2/M phase. Soon after, these observations were extended to CDT produced by *Aggregatibacter* (formerly *Actinobacillus*) *actinomycetemcomitans* (Sugai *et al.*, 1998). Finally, Gelfanova *et al.* (1999) demonstrated that the underlying mechanism of CDT-induced cell death involved apoptosis.

The next milestone in CDT research came with the independent demonstration of structural conservation of amino acid residues and functional homology of CDTs from *E. coli* and *C. jejuni* to mammalian DNase I enzyme

Table 1. Major milestones in the CDT discovery time line

Discovery	Bacterium	Reference
Biological activity	*Shigella dysenteriae*	Johnson & Lior (1987)
	E. coli	Johnson & Lior (1988a)
	C. jejuni	Johnson & Lior (1988b)
	Haem. ducreyi	Purvén & Lagergård (1992)
Gene sequence	*E. coli*	Scott & Kaper (1994)
Cell cycle arrest	*E. coli*	Pérès *et al.* (1997)
	A. actinomycetemcomitans	Sugai *et al.* (1998)
Apoptosis	*Haem. ducreyi*	Gelfanova *et al.* (1999)
Nuclease activity	*C. jejuni*	Lara-Tejero & Galán (2000)
	E. coli	Elwell & Dreyfus (2000)
Crystal structure	*Haem. ducreyi*	Nešić *et al.* (2004)
Cell surface receptor	*E. coli*	Carette *et al.* (2009)

and correlation with DNA double strand breaks (DSBs) as the molecular basis of CDT-mediated mammalian cell cycle arrest (Elwell & Dreyfus, 2000; Lara-Tejero & Galán, 2000). With the understanding that CDT holotoxin consists of a heterotrimeric complex of CdtA, CdtB and CdtC subunits and reconstitution of the CDT holotoxin complex with individually expressed recombinant subunits, Lara-Tejero & Galán (2001) later proposed an AB toxin molecular model for CDT-induced cellular toxicity (as described below and by Lara-Tejero & Galán, 2001). This model has since been validated on the basis of high resolution crystal structure analysis of CDT from *Haem. ducreyi* and *A. actinomycetemcomitans* (Nešić *et al.*, 2004; Yamada *et al.*, 2006). The identification of a cell membrane protein requirement for CDT binding to a myeloid leukaemia cell line recently linked cell surface binding of the toxin to DNA damage (Carette *et al.*, 2009). However, considering that CdtA and CdtC subunits have structural features consistent with lectin binding domains, the functional significance of a cell membrane protein as a component of the CDT receptor molecule remains to be clarified.

Currently, CDT is the only member of the bacterial AB toxins that exhibits DNase activity and, irrespective of the bacterial species of origin, exerts genotoxic damage by causing DSBs leading to irreversible cell cycle arrest and apoptosis in a broad range of mammalian cell lineages (Alouf, 2006; Dassanayake *et al.*, 2005a; Frisan *et al.*, 2003; Gelfanova *et al.*, 1999; Hickey *et al.*, 2005; Hontz *et al.*, 2006a, b; Nešić *et al.*, 2004; Scott & Kaper, 1994; Whitehouse *et al.*, 1998; Yamada *et al.*, 2006; Young *et al.*, 2000b). The genes encoding CDT have now been found in more than two dozen bacterial species belonging to the *Gamma* and *Epsilon* classes of *Proteobacteria*. Many of these Gram-negative bacteria are clinically important mucocutaneous pathogens of humans and animals that are responsible for major food- and water-borne bacterial illnesses worldwide (Table 2). It is expected that additional members of the classes *Gamma-* and *Epsilonproteobacteria* will be found to harbour CDT in the future.

The biological activity of CDT is highly dependent on cellular targets; however, haematopoietic cells are more susceptible, by several orders of magnitude, than all other cell types, suggesting a potential immunomodulatory role of CDT in the pathogenesis of diseases caused by CDT-producing bacterial pathogens. In this review, we highlight the ecology of pathogens producing CDT, outline the molecular biology of bacterial CDT, including current structural features and biological properties of the toxin, and critically address the molecular mechanisms of CDT-induced cellular toxicity. The respective roles of ATM and p53 in CDT-induced DNA damage response (DDR) leading to arrest of the cell cycle and ultimately cell death by apoptosis are reviewed in detail.

CDT genes and encoded proteins

Distribution of CDT genes amongst bacteria

Currently, bacteria that harbour the CDT gene and display biological activity are restricted to the gamma and epsilon classes in the phylum *Proteobacteria* (Table 2). Within the *Gammaproteobacteria*, one subset belongs to the family *Pasteurellaceae* and includes *Haem. ducreyi*, the cause of a sexually transmitted disease known as chancroid (Abeck *et al.*, 1997), *Haemophilus parasuis*, a commensal organism of the upper respiratory tract associated with septicaemia in pigs (Yue *et al.*, 2009), and *A. actinomycetemcomitans*, a common contributing agent to chronic periodontitis and localized aggressive periodontitis (Henderson *et al.*, 2002). A second subset of *Gammaproteobacteria* belongs to the *Enterobacteriaceae* family and collectively these organisms are responsible for intestinal and urinary tract infections that can lead to systemic spread. All members of the *Epsilonproteobacteria* belong to the order *Campylobacterales* and include several species of *Campylobacter* and enterohepatic *Helicobacter* species (EHS) which are primarily associated with enterocolitis; however, some species cause bacteraemia/septicaemia, hepatitis and reproductive losses in humans and animals (Dassanayake *et al.*, 2005a, b; Ge *et al.*, 2008; Johnson & Lior, 1987; Liyanage *et al.*, 2010). It is noteworthy that, in addition to mammalian bacterial pathogens, a monophyletic group of bacteriophage-encoded CdtB orthologues has been found amongst facultative endosymbionts of sap-feeding insects (Degnan & Moran, 2008). Thus, the ecology of CDT-harbouring bacteria is restricted to certain Gram-negative bacteria that primarily occupy mucocutaneous niches where persistent colonization can either occur as a commensal or result in localized or disseminated infections and diseases in a broad range of mammalian hosts.

Table 2. Gram-negative mucocutaneous bacteria harbouring CDT, their respective colonization niches and associated clinical diseases in human and animal hosts

EPEC, Enteropathogenic *E. coli*; ExPEC, extraintestinal pathogenic *E. coli*; APEC, avian pathogenic *E. coli*; UTI, urinary tract infection; NTEC, necrotoxigenic *E. coli*; EHEC, enterohaemorrhagic *E. coli*; STEC, shiga toxin-producing *E. coli*; NHP, non-human primates; HUS, haemolytic uraemic syndrome.

Bacterium	CDT*	Host	Niche	Associated disease	Reference
Class *Gammaproteobacteria*					
Family *Pasteurellaceae*					
Haemophilus species					
Haem. ducreyi	HducCDT	Human	External genitalia	Chancroid	Purvén & Lagergård (1992), Cortes-Bratti *et al.* (1999)
Haem. parasuis	HparCDT	Pig	Upper respiratory mucosa	Septicaemia	Yue *et al.* (2009)
Aggregatibacter (formerly *Actinobacillus*) species					
A. actinomycetemcomitans	AactCDT	Human	Periodontal pocket/ gingival sulcus/ dental plaque	Periodontitis	Sugai *et al.* (1998), Shenker *et al.* (1999)
Family *Enterobacteriaceae*					
E. coli†					
EPEC/ExPEC	EcolCdtB-I	Human	Intestinal mucosa	Enterocolitis/ septicaemia	Asakura *et al.* (2007a), Pickett & Whitehouse (1999), Scott & Kaper (1994), Tóth *et al.* (2003)
	EcolCdtB-I	Human	Urogenital mucosa	UTI	Tóth *et al.* (2003)
APEC	EcolCdtB-I	Chicken	Intestinal mucosa	Septicaemia	Johnson *et al.* (2007)
EPEC	EcolCdtB-II	Human	Intestinal mucosa	Enterocolitis	Pickett *et al.* (1994), Pickett & Whitehouse (1999)
EPEC/ExPEC	EcolCdtB-III	Human	Intestinal mucosa	Enterocolitis	Bielaszewska *et al.* (2004), Pérès *et al.* (1997), Tóth *et al.* (2003)
NTEC	EcolCdtB-III	Cattle	Intestinal mucosa	Enterocolitis/ septicaemia	Johnson *et al.* (2010), Pérès *et al.* (1997), Pickett & Whitehouse (1999)
EPEC/ExPEC	EcolCdtB-IV	Human	Intestinal mucosa	Enterocolitis/ septicaemia	Tóth *et al.* (2003, 2009)
NTEC	EcolCdtB-IV	Human	Urogenital mucosa	UTI	Tóth *et al.* (2003)
	EcolCdtB-IV	Pig	Intestinal mucosa	Enterocolitis/ septicaemia	Tóth *et al.* (2003, 2009)
ExPEC	EcolCdtB-IV	Chicken	Intestinal mucosa	Septicaemia	Tóth *et al.* (2003, 2009)
EHEC/STEC	EcolCdtB-V	Human	Intestinal mucosa	Enterocolitis/HUS	Bielaszewska *et al.* (2004)
Shigella species					
Shig. boydii serotype 13 (*Escherichia albertii*)	SboyCDT	Human	Intestinal mucosa	Dysentery	Hyma *et al.* (2005), Johnson & Lior (1987)
Shig. dysenteriae	SdysCDT	Human	Intestinal mucosa	Dysentery	Johnson & Lior (1987), Okuda *et al.* (1997)
Salmonella species	SdysCDT				
S. enterica serotype Typhi	StypCdtB	Human	Intestinal mucosa	Typhoid fever	Haghjoo & Galán (2004)
Class *Epsilonproteobacteria*					
Family *Campylobacteriaceae*					
Campylobacter species					
C. jejuni	CjejCDT	Human	Intestinal mucosa	Enterocolitis	Fouts *et al.* (2005), Johnson & Lior (1988b), Young *et al.* (2007)
	CjejCDT	NHP	Intestinal mucosa	Enterocolitis	Fouts *et al.* (2005), Johnson & Lior (1988b), Young *et al.* (2007)
	CjejCDT	Cattle, sheep, pig	Intestinal mucosa	Enterocolitis	Bang *et al.* (2003), Inglis *et al.* (2005)
	CjejCDT	Sheep	Intestinal mucosa	Reproductive loss	Sahin *et al.* (2008)
	CjejCDT	Dog, cat, ferret	Intestinal mucosa	Enterocolitis	Fox *et al.* (1987), Young & Mansfield (2005)
	CjejCDT	Chicken	Intestinal mucosa	Commensal	Young *et al.* (2007)

Table 2. cont.

Bacterium	CDT*	Host	Niche	Associated disease	Reference
C. coli	CcolCDT	Human	Intestinal mucosa	Enterocolitis	Fouts *et al.* (2005), Pickett *et al.* (1996)
	CcolCDT	NHP	Intestinal mucosa	Enterocolitis	Dassanayake *et al.* (2005b)
	CcolCDT	Cattle, pig	Intestinal mucosa	Commensal	Bang *et al.* (2003)
	CcolCDT	Sheep, chicken	Intestinal mucosa	Commensal	Garrity *et al.* (2005)
C. upsaliensis	CupsCDT	Human	Intestinal mucosa	Enterocolitis/ bacteraemia	Fouts *et al.* (2005), Mooney *et al.* (2001), Pickett *et al.* (1996)
	CupsCDT	Pig, dog, cat, chicken	Intestinal mucosa	Commensal	Garrity *et al.* (2005)
C. hyointestinalis	ChyoCDT	Human	Intestinal mucosa	Enterocolitis	Edmonds *et al.* (1987)
	ChyoCDT	Cattle	Intestinal mucosa	Commensal	Inglis *et al.* (2005)
	ChyoCDT	Pig	Intestinal mucosa	Enterocolitis	Gebhart *et al.* (1983), Pickett *et al.* (1996)
C. lari	ClarCDT	Human	Intestinal mucosa	Enterocolitis	Fouts *et al.* (2005), Pickett *et al.* (1996), Shigematsu *et al.* (2006)
C. fetus subsp. *fetus*	CfetCDT	Human	Intestinal mucosa	Enterocolitis/ bacteraemia	Garrity *et al.* (2005), Johnson & Lior (1988b), Pickett *et al.* (1996)
	CfetCDT	Cattle	Intestinal mucosa	Enterocolitis	Ohya *et al.* (1993)
	CfetCDT	Cattle, sheep	Urogenital mucosa	Reproductive loss	Garrity *et al.* (2005)
C. fetus subsp. *venerealis*	CvenCDT	Human	Intestinal mucosa	Enterocolitis	Asakura *et al.* (2008), Moolhuijzen *et al.* (2009)
	CvenCDT	Cattle	Urogenital mucosa	Reproductive loss	Garrity *et al.* (2005), Moolhuijzen *et al.* (2009)
Family *Helicobacteriaceae*					
Enterohepatic Helicobacter species					
Hel. hepaticus	HhepCDT	Laboratory mice	Intestinal mucosa	Enterocolitis/ hepatitis	Young *et al.* (2000b)
Hel. bilis	HbilCDT	Laboratory mice	Intestinal/Biliary mucosa	Typhlocolitis/ hepatitis	Fox *et al.* (2004b), Fox (2007)
	HbilCDT	Dog	Intestinal mucosa	Commensal	Hänninen *et al.* (2005), Kostia *et al.* (2003)
Hel. mastomyrinus	HmasCDT	Laboratory mice	Intestinal mucosa	Proctitis	Shen *et al.* (2005)
	HmasCDT	Mastomys	Liver	Hepatitis	Shen *et al.* (2005)
Hel. cinaedi	HcinCDT	Human	Intestinal mucosa	Septicaemia	Taylor *et al.* (2003)
	HcinCDT	NHP	Intestinal mucosa/ liver	Colitis/hepatitis	Fernandez *et al.* (2002), Fox *et al.* (2001)
	HcinCDT	Laboratory mice	Intestinal mucosa	Typhlocolitis	Shen *et al.* (2009)
Hel. canis	HcanCDT	Human	Intestinal mucosa	Bacteraemia	Leemann *et al.* (2006)
	HcanCDT	Dog	Intestinal mucosa/ liver	Enterocolitis/ hepatitis	Fox *et al.* (1996)
Hel. pullorum	HpulCDT	Human	Intestinal mucosa	Enteritis	Ceelen *et al.* (2006), Young *et al.* (2000a)
	HpulCDT	Chicken	Intestinal mucosa	Enteritis/hepatitis	Ceelen *et al.* (2006), Young *et al.* (2000a)
	HpulCDT	Laboratory mice	Intestinal mucosa	Commensal	Boutin *et al.* (2010)
Hel. winghamensis	HwinCDT	Human	Intestinal mucosa	Enteritis	Melito *et al.* (2001)

*CDT designation according to a modification of the nomenclature proposed by Cortes-Bratti *et al.* (2001a). Only the genes encoding CDT have been described in *Haem. parasuis* and *C. fetus* subsp. *venerealis*; the biological activity of CDT has not been determined in these bacteria.
†Variants of CdtB identified amongst pathotypes of *E. coli* are designated EcolCdtB-I to -V.

Nomenclature of CDT proteins

To simplify the designation of CDT produced by various bacteria, Cortes-Bratti *et al.* (2001a) proposed a nomenclature system which has been widely adopted by the scientific community. Since then, the list of bacteria that harbour CDT has expanded significantly (Table 2). Consequently, the original system consisting of the capitalized first letter of the genus followed by a single

lower case species letter initial is no longer adequate, and some bacterial species now have overlapping designations; for example, Hp for *Haemophilus parasuis* and *Helicobacter pullorum* or Hc for *Helicobacter cinaedi* and *Helicobacter canis*. Therefore, we propose a modification of the previous nomenclature system in which the first three letters of the bacterial species in lower cases are placed after the capitalized first letter of the bacterial genus, followed by 'CDT' (Table 2).

CDT operon

The CDT holotoxin consists of a heterotrimeric complex of three subunits designated CdtA, CdtB and CdtC with corresponding approximate molecular masses of 23–30, 28–29 and 19–21 kDa according to bacterial host species (Haghjoo & Galán, 2004; Hu & Stebbins, 2006). In nearly all bacteria, CdtA, CdtB and CdtC subunits are encoded by adjacent or slightly overlapping *cdtA*, *cdtB* and *cdtC* genes which together form a constitutively expressed operon on the chromosome (Fig. 1). *E. coli* is unique amongst CDT-producing bacteria in that at least five divergent variants of CdtB have been found within this single species so far, which also differentially segregate according to *E. coli* pathotypes (Table 2; Janka *et al.*, 2003). Since it is presently unknown whether or not the genes encoding the CdtA and CdtC subunits display sequence divergence similar to their corresponding CdtB variants, and until this can be resolved, we propose to designate these toxins as EcolCdtB-I to -V. Similar to other CDT-producing bacteria, EcolCdtB-I, -II, -IV and -V are encoded by chromosomal gene loci (Asakura *et al.*, 2007a; Johnson *et al.*, 2007; Oswald *et al.*, 1994; Pérès *et al.*, 1997; Tóth *et al.*, 2009). However, analyses of sequences upstream and downstream of operons encoding EcolCdtB-I and -IV variants revealed lambdoid prophage genes (Asakura *et al.*, 2007a; Tóth *et al.*, 2009), whereas homologues of bacteriophages P2 and lambda are found in *E. coli* strain O157 : H$^-$ 493/89 harbouring EcolCdtB-V (Janka *et al.*, 2003). These gene arrangements are reminiscent of bacteriophage-encoded CdtB orthologues found amongst facultative endosymbionts of sap-feeding insects in which acquisition of *cdtB* has been attributed to horizontal gene transfer (HGT; Degnan & Moran, 2008). Indeed, carriage of the CDT operon by certain enteropathogenic *E. coli* with EcolCdtB-I on a lysogenic phage background mediates HGT among related species, and confers enhanced bacterial toxicity during phage induction (Asakura *et al.*, 2007a; Johnson *et al.*, 2007; Oswald *et al.*, 1994; Pérès *et al.*, 1997; Tóth *et al.*, 2009). Also unique to *E. coli* is the location of the operon encoding EcolCdtB-III which is found on a large conjugative plasmid called pVir (Johnson *et al.*, 2010). Finally, limited evidence to suggest that the *cdt* gene cluster of *A. actinomycetemcomitans* might have been part of a genomic island has been proposed (Doungudomdacha *et al.*, 2007).

A notable exception to the heterotrimeric CDT model is *Salmonella enterica* serotype Typhi (*S.* Typhi) in which the genes encoding CdtA and CdtC are missing (Haghjoo & Galán, 2004). In keeping with our proposed CDT nomenclature, and since only the CdtB subunit is present in *S.* Typhi, the toxin should be designated StypCdtB. The *cdtB* gene of *S.* Typhi is located in a region of the chromosome with features consistent with a pathogenicity islet acquired by HGT and delineated by insertion sequences and a transposase gene (Haghjoo & Galán, 2004). Although the significance of these various CDT gene arrangements is incompletely understood, it is likely that they represent mechanisms of HGT which together might play a role in expanding the range of pathogenic bacteria that can establish persistent colonization and potentially cause infection and disease.

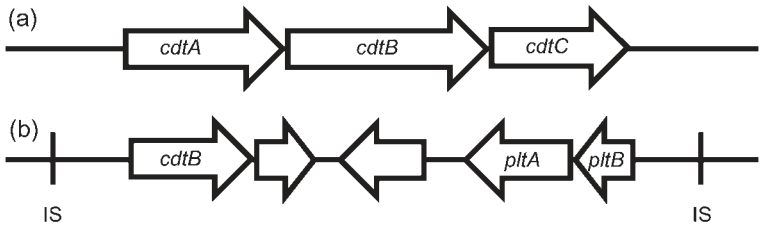

Fig. 1. Organization of the CDT gene locus. (a) In nearly all CDT-producing bacteria, the CDT gene cluster consists of three adjacent or slightly overlapping genes *cdtA*, *cdtB* and *cdtC*, encoding the corresponding CdtA, CdtB and CdtC protein subunits and is located on the chromosome, except for EcolCdtB-III in which the CDT gene cluster is found on a large conjugative plasmid called pVir. (b) In *S.* Typhi, the genes encoding CdtA and CdtC are missing; instead the StypCdtB protein subunit is encoded by the *cdtB* gene located upstream of *pltA* and *pltB* genes which encode PltA and PltB, respectively, homologues of the pertussis toxin ADP-ribosylating 'A' subunit and one of the five components of its heteropentameric 'B' subunit, respectively. Together, these are located on a pathogenicity islet delineated by insertion sequences (IS).

CDT protein structure and function

On the basis of structural and functional characteristics of reconstituted recombinant heterotrimeric CDT subunits, Lara-Tejero & Galán (2001) proposed an AB toxin molecular model for CDT-induced cellular toxicity (Lara-Tejero & Galán, 2001). Accordingly, the catalytically active 'A' subunit is attributable to the CdtB subunit, whereas the CdtA and CdtC subunits, which together display a high degree of sequence diversity, are assembled as a heterodimeric binding 'B' subunit mediating target host cell membrane specificity and cellular uptake of CdtB effector protein (Hu *et al.*, 2006; Hu & Stebbins, 2006; Nešić *et al.*, 2004). In *S.* Typhi, which lacks *cdtA* and *cdtC*, the holotoxin is instead composed of a single 'B' subunit and two 'A' subunits. The *cdtB* gene which encodes StypCdtB is located on a pathogenicity islet upstream of *pltA* and *pltB* genes, respectively, encoding pertussis-like toxin A and B (PltA and PltB) which are homologues of the pertussis toxin ADP-ribosyltransferase 'A' subunit and one of the five components of its heteropentameric 'B' subunit, respectively (Song *et al.*, 2010; Spanò *et al.*, 2008). PltA and PltB form a heterotrimeric complex with StypCdtB, where StypCdtB and PltA act as the 'A' or active toxic subunit, while PltB mediates the binding specificity or 'B' subunit function essential for toxin delivery and cellular toxicity (Song *et al.*, 2010; Spanò *et al.*, 2008). The holototoxin which displays both nuclease and ADP-ribosyltransferase activities has been referred to as 'typhoid toxin' (Song *et al.*, 2010; Spanò *et al.*, 2008).

Consistent with the notion that CdtB is the active subunit, comparative *in silico* structural analysis of predicted *cdtB*-encoded amino acid sequences of human and animal bacterial pathogens revealed conserved endonuclease/exonuclease/phosphatase super family domain with approximately 25–40 % sequence identity with phosphodiesterase enzymes including mammalian DNase I (Elwell & Dreyfus, 2000; Lara-Tejero & Galán, 2000). Despite the limited overall sequence homology with DNase I, *cdtB*-encoded catalytic, DNA- and divalent cation-binding residues are highly conserved among the various CDT-producing bacterial pathogens, suggesting a critical role for nuclease activity in host–pathogen interaction (Fig. 2; Elwell & Dreyfus, 2000; Hu & Stebbins, 2006; Lara-Tejero & Galán, 2000; Nešić *et al.*, 2004).

The catalytic residues corresponding to mammalian DNase I-H134 (HducCdtB-H160), DNase I-D212 (HducCdtB-D238) and DNase I-H252 (HducCdtB-H274) are conserved among all bacterial CdtBs (Nešić *et al.*, 2004). Moreover, mutagenesis analysis of EcolCdtB-II-E86 residue and comparative sequence analysis confirmed the high degree of conservation of the corresponding DNase I-E78 catalytic residue amongst all CdtB (Elwell & Dreyfus, 2000). Conversely, those residues corresponding to divalent cation binding DNase I-E39 (CjejCdtB-E60), DNase I-D168 (CjejCdtB-D185) and DNase I-D251 (CjejCdtB-D260) as well as those corresponding to critical DNA-binding DNase I-R111 (HducCdtB-R144) and DNase I-N170 (HducCdtB-N201) are highly conserved among all bacterial CdtB (Nešić *et al.*, 2004; Pickett & Lee, 2005). One additional DNA-binding DNase I-R117 (HducCdtB-R41) residue, which is present in all CdtBs, has also been confirmed to be present in both AactCdtB and HducCdtB by mutational analysis (Nešić *et al.*, 2004; Shenker *et al.*, 2007). Differences in DNA-binding residue conservation between bacterial CdtB and mammalian DNase I probably account for reduced *in vitro* nuclease activities found with AactCdtB, EcolCdtB-II and HhepCdtB (Dassanayake *et al.*, 2005a; Elwell & Dreyfus, 2000; Mao & DiRienzo, 2002). Additionally, differences in nuclease and cytotoxic activities of AactCdtB from clinical isolates have been attributed to a single amino acid substitution at position H281 (a residue not known to be associated with catalysis, DNA binding or divalent cation binding; Nishikubo *et al.*, 2006).

More recently, high resolution crystallographic analysis of reconstituted heterotrimeric HducCDT holotoxin from individually expressed recombinant subunits (2.0 Å resolution; Nešić *et al.*, 2004) or complete AactCDT operon expressed as a holotoxin in *E. coli* (2.4 Å resolution; Yamada *et al.*, 2006) and an EcolCdtB-II subunit (1.73 Å resolution; Hontz *et al.*, 2006b) have provided further support to earlier observations and confirmed that CdtB closely resembles mammalian DNase I. Comparative protein modelling revealed a high degree of key structural features conservation, namely the canonical four-layered fold of the mammalian DNase I family, the deeply grooved aromatic patch and the active site of CdtB from diverse bacterial species including CjejCDT, HhepCDT, EcolCDT-I, EcolCDT-II, EcolCDT-III, AactCDT and HducCDT (Hu *et al.*, 2006). While the heterotrimeric holotoxin forms a ternary complex between CdtB subunit and ricin-like lectin domains within CdtA and CdtB (Nešić *et al.*, 2004), the lectin domains form a deeply grooved, highly aromatic patch which is critical for cellular toxicity, presumably through binding to target cell surface biomolecules (Nešić *et al.*, 2004). Interaction between the N-terminal 13 amino acids of CdtC with the active nuclease site of CdtB suggests an auto-inhibitory function. Accordingly, the CdtB nuclease would be active only after release from CdtC and target cell entry.

Although the residues required for nuclease activity of CdtB are highly conserved, the overall amino acid sequence of CdtB varies among bacteria. While StypCdtB together with HducCdtB and AactCdtB form a distinct cluster, the CdtB produced by EHS and *Campylobacter* species forms a separate cluster which is distinct from CdtB produced by members of the *E. coli*/*Shigella* group (Degnan & Moran, 2008). Interestingly, a similar clustering pattern is seen when the amino acid sequences of CdtA and CdtC are compared between different bacterial pathogens (Eshraghi *et al.*, 2010). The high degree of nucleotide and amino acid sequence homologies of CDT produced by *Haem. ducreyi* and *A. actinomycetemcomitans* as well as those produced by EHS and *Campylobacter* species suggests differential infection niches that may correlate with bacterial adaptation to

Fig. 2. Comparative amino acid sequence of bacterial CdtB and mammalian DNase I. Sequence alignment was done using CLUSTALW_2. Numbers at the bottom correspond to amino acid positions of bovine or human DNase I. Residues required for enzymic activity [catalytic (red), divalent cation binding (blue) and DNA binding (green)] in human DNase I are indicated in bold. The residues predicted to be required for CdtB nuclease activity and which have been confirmed by site-directed mutagenesis are highlighted in yellow. The numbers in parentheses represent the number of intervening amino acid residues that are omitted (excluding gaps).

persistent colonization in specific hosts. Alternatively, the possibility that these structural differences are indicative of variable bacterial target cell binding specificities and intracellular nuclease subunit signalling pathways remains to be determined.

Production of CDT

The prevalence of the CDT gene and biological activity varies among clinical isolates within individual bacterial species known to harbour CDT-producing members (Table 3). Assessment of clinical *E. coli* isolates for determination of EcolCdtB gene prevalence prior to current descriptions of the existence of variants I–V probably underestimated the percentage of strains harbouring the *cdt* gene operon (Ansaruzzaman *et al.*, 2000; Clark *et al.*, 2002; Janka *et al.*, 2003; Johnson & Stell, 2000; Okeke *et al.*, 2000; Tóth *et al.*, 2003). While the prevalence of the CDT gene and activity among clinical isolates of CDT-producing species of the families *Pasteurellaceae*, *Campylobacteriaceae* and *Helicobacteriaceae* is generally greater than 85 %, a consistent finding with members of the *Enterobacteriacea* family has been less than 14 % prevalence. Considering the specificity of PCR screening, the possibility that additional variants could account for these discrepancies cannot be ruled out completely. Nevertheless, a notable exception among *E. coli* are clinical isolates of sorbitol fermenting enterohaemorrhagic *E. coli* (EHEC) O157 : H^-, in which 87 % have been found to harbour a *cdt* gene cluster (Janka *et al.*, 2003). The near absence of a *cdt* gene cluster in most EHEC O157 : H7 strains and all *E. coli* O55 : H7/H^- strains examined together with the presence of phage flanking regions in EHEC O157 : H^- strain 493/89 *cdt* led Janka *et al.* (2003) to propose that a mobile element containing the *cdt* gene cluster might have been acquired after this lineage diverged from EHEC O157 : H7.

In addition to varying distribution among clinical isolates, the biological activity within individual bacterial species is variously found either in culture supernatant (and thus is presumably actively secreted), associated with bacterial cells or both (Table 4). Moreover, the activity of CDT recovered from these different fractions can range from relatively high, intermediate, low to absent, and this may be relevant to disease pathogenesis for individual bacterial pathogens. Similar to other virulence factors, the possibility that these alterations in CDT production are attributable to high numbers of passages on laboratory media and artificial selection of strains that are less virulent cannot be ruled out completely.

The early studies of Johnson & Lior (1988a) assessed CjejCDT in culture supernatant; however, assessment of cell-associated CDT among clinical *C. jejuni* isolates revealed relatively high biological activities in most strains with few strains exhibiting comparatively lower levels of activity (Pickett *et al.*, 1996). Strains of *C. jejuni* that harbour the *cdt* gene cluster but lack CDT biological activity have also been found (Abuoun *et al.*, 2005; Dassanayake *et al.*, 2005b). Two types of mutations within the *cdt* gene operon that can account for a lack of CDT activity have been found in these strains; a deletion across

Table 3. Prevalence of CDT gene and activity amongst clinical isolates of different bacteria

ND, Not done.

CDT	No. positive/no. tested (%)		Reference
	Gene*	Activity	
HducCDT	ND	6/10 (60)	Purvén & Lagergård (1992)
	11/12 (91.6)	11/12 (91.6)	Cope *et al.* (1997)
	82/100 (82)	89/100 (89)	Ahmed *et al.* (2001)
AactCDT	43/50 (86)	43/50 (86)	Ahmed *et al.* (2001)
	34/40 (85)	39/40 (97.5)	Fabris *et al.* (2002)
	40/45 (88.8)	40/45 (88.8)	Yamano *et al.* (2003)
EcolCdtB†	17/430 (5)‡	17/430 (5)	Bielaszewska *et al.* (2004)§
	14/202 (6.9)‡	14/202 (6.9)	Orth *et al.* (2006)‖
	35/362 (7.9)‡	ND	Hinenoya *et al.* (2009)¶
	14/100 (14)‡	14/100 (14)	Bielaszewska *et al.* (2009)#
SboyCDT	ND	1/19 (5.2)	Johnson & Lior (1987)
SdysCDT	ND	4/12 (33.3)	Johnson & Lior (1987)
CjejCDT	20/20 (100)‡	20/20 (100)	Pickett *et al.* (1996)
	10/11 (90)**	20/21 (95.2)	Pickett *et al.* (1996)
	16/16 (100)‡	16/16 (100)	Dassanayake *et al.* (2005b)
	27/27 (100)	10/11 (90.9)	Asakura *et al.* (2007b)
CcolCDT	12/12 (100)‡	0/12 (0)	Pickett *et al.* (1996)
	16/16 (100)‡	0/16 (0)	Dassanayake *et al.* (2005b)
	19/19 (100)	0/10 (0)	Asakura *et al.* (2007b)
CupsCDT	ND	5/5 (100)	Mooney *et al.* (2001)
CfetCDT	ND	25/26 (100)	Ohya *et al.* (1993)
	20/20 (100)	10/10 (100)	Asakura *et al.* (2007b)
HcinCDT	11/11 (100)‡	11/11 (100)	Taylor *et al.* (2003)

*Tested for *cdtA*, *cdtB* and *cdtC* by PCR.
†Recent reports that tested for the presence of all currently known variants (I–V) of EcolCdtB were included.
‡Tested only for *cdtB* by PCR.
§Non-O157 STEC: three CdtB-III and 14 CdtB-V were tested.
‖STEC: seven CdtB-III and seven CdtB-IV were tested.
¶Twenty-one CdtB-1, three CdtB-II, four CdtB-III, three CdtB-IV and four CdtB-V were tested.
#STEC O91: all strains were CdtB-V.
**Tested only for *cdtB* by Southern blot.

cdtA and *cdtB*, and a non-synonymous mutation in the *cdtB* gene (Abuoun *et al.*, 2005). In the closely related species *Campylobacter coli*, the *cdt* genes encoding each of the three CDT subunits are consistently present, and although this species lacks biological activity, the underlying significance of maintaining a *cdt* gene operon has not been investigated (Asakura *et al.*, 2007b; Dassanayake *et al.*, 2005b; Pickett *et al.*, 1996). Clinical isolates which lack *cdt* genes and/or CDT activity have also been found amongst *Haem. ducreyi*, *A. actinomycetemcomitans*, *E. coli*, *Shigella* species and EHS (Abuoun *et al.*, 2005; Ahmed *et al.*, 2001; Asakura *et al.*, 2007b; Dassanayake *et al.*, 2005b; Fabris *et al.*, 2002; Hyma *et al.*, 2005; Janka *et al.*, 2003; Johnson & Lior, 1987; Solnick & Schauer, 2001; Yamano *et al.*, 2003).

Information concerning the molecular mechanism of bacterial synthesis and secretion of CDT is limited (Deng *et al.*, 2001; Ueno *et al.*, 2006). A difference of approximately 2 kDa between the observed and calculated molecular masses of EcolCdtB-II subunit has been attributed to cleavage of a putative N-terminal signal peptide sequence involved in secretion across the inner membrane by a general export pathway (Dreyfus, 2003). Consistent with these observations, Ueno *et al.* (2006) demonstrated the presence of a lipid-binding consensus motif (lipobox) and lipoprotein cleavage site in AactCdtA signal peptide. In addition to AactCdtA, putative lipobox motifs are also present in HducCdtA, CjejCdtA, HhepCdtA, EcolCdtA-I, -II and –III, and EcolCdtC-I, -II and -III (Ueno *et al.*, 2006; R. N. Jinadasa & G. E. Duhamel, unpublished observations). While the periplasmic CDT holotoxin consists of the uncleaved AactCdtA, AactCdtB and AactCdtC subunits, the secreted holotoxin complex in culture supernatant has cleaved AactCdtA (Ueno *et al.*, 2006). A similar post-translational cleavage of HducCdtA probably accounts for the two forms with approximate molecular masses of 23 and 17 kDa seen with recombinant HducCdtA (Frisk *et al.*, 2001).

Table 4. Production of CDT by bacterial pathogens

ND, Not done. Low or high is indicated relative to the other bacterial location. Strains of *Campylobacter* species *Haem. ducreyi*, *A. actinomycetemcomitans*, *E. coli*, *Shigella* species and EHS that lack a full complement of CDT genes and/or activity have been described elsewhere (Abuoun *et al.*, 2005; Ahmed *et al.*, 2001; Asakura *et al.*, 2007b; Dassanayake *et al.*, 2005b; Fabris *et al.*, 2002; Hyma *et al.*, 2005; Janka *et al.*, 2003; Johnson & Lior, 1987; Solnick & Schauer, 2001; Yamano *et al.*, 2003).

CDT	Bacterial location		Reference
	Secreted*	Cell-associated†	
HducCDT	Present	Present	Purvén & Lagergård (1992), Ahmed *et al.* (2001), Cope *et al.* (1997), Gelfanova *et al.* (1999)
AactCDT	Present	Present	Ahmed *et al.* (2001), Fabris *et al.* (2002), Sugai *et al.* (1998), Yamano *et al.* (2003)
EcolCdtB-I	High	High	Tóth *et al.* (2003)
EcolCdtB-II	Present	Present	Pickett *et al.* (1994)
EcolCdtB-III	Present	Present	Tóth *et al.* (2003)
EcolCdtB-IV	Absent	Present	Tóth *et al.* (2003)
EcolCdtB-V	Present	ND	Bielaszewska *et al.* (2005), Janka *et al.* (2003)
SboyCDT	Present	Present	Hyma *et al.* (2005), Johnson & Lior (1987)
SdysCDT	Present	ND	Johnson & Lior (1987)
CjejCDT	High	High	Dassanayake *et al.* (2005b), Pickett *et al.* (1996)
CcolCDT	Absent	Absent	Asakura *et al.* (2007b), Dassanayake *et al.* (2005b), Pickett *et al.* (1996)
CupsCDT	Present	High	Mooney *et al.* (2001), Pickett *et al.* (1996)
ChyoCDT	ND	High	Pickett *et al.* (1996)
ClarCDT	ND	Low	Pickett *et al.* (1996)
CfetCDT	Present	High	Asakura *et al.* (2007b), Ohya *et al.* (1993), Pickett *et al.* (1996)
EHS	ND	Present	Chien *et al.* (2000), Hänninen *et al.* (2005), Kostia *et al.* (2003), Shen *et al.* (2005), Taylor *et al.* (2003)

*CDT activity in unconcentrated culture supernatant.
†CDT activity in bacterial lysate or enriched outer membrane preparation.

Cell-associated CjejCDT is found primarily in the periplasmic space of *C. jejuni*, whereas culture supernatant contains primarily CjejCDT tightly associated with outer membrane vesicles (Lindmark *et al.*, 2009). In the context of intestinal infection, packaging and release of CDT into outer membrane vesicles may serve a protective function against enzymic digestion, thus allowing uptake of intact protein toxin by host absorptive enterocytes. Further studies on the mechanism of CDT production and release by bacterial pathogens should provide important insights for the design of therapeutic modalities aimed at inhibiting CDT production or neutralizing CDT activity during infection.

Molecular mechanisms of cellular toxicity

Cellular entry of CdtB

A causal relationship between the requirement for heterotrimeric CdtABC holotoxin and cellular toxicity is well established for AactCDT, CjejCDT, EcolCdtB-I and -II and HducCDT (Akifusa *et al.*, 2001; Lara-Tejero & Galán, 2001; Pickett *et al.*, 1994, 1996; Purvén *et al.*, 1997; Scott & Kaper, 1994; Shenker *et al.*, 2005). In these pathogens, CdtA and CdtC subunits bind host cell membrane lipid raft microdomains before internalization of CdtB (Fig. 3; Boesze-Battaglia *et al.*, 2006, 2009; Carette *et al.*, 2009; Cortes-Bratti *et al.*, 2000; Lara-Tejero & Galán, 2001; Nešić

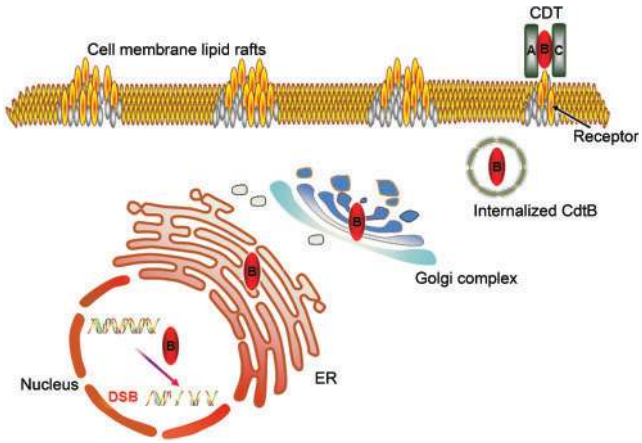

Fig. 3. CDT uptake and intracellular transport in mammalian cells. Binding of the 'B' subunit consisting of CdtA and CdtC protein subunits of CDT heterotrimeric holotoxin to a cell membrane receptor located within lipid rafts is followed by rapid endocytosis of the 'A' active CdtB protein subunit in a clathrin-coated pit and retrograde transport via the Golgi complex and endoplasmic reticulum (ER) before nuclear localization signal-mediated active transport to the nucleus and DNA double strand breaks (DSB).

et al., 2004; Shenker *et al.*, 2005). The integrity of lipid membrane raft microdomains is critical for binding of AactCDT to human T-cell leukaemia Jurkat cells, presumably involving binding of host cell membrane cholesterol by a putative AactCdtC subunit cholesterol recognition/interaction amino acid consensus domain (Boesze-Battaglia *et al.*, 2009). As predicted, pretreatment of cells with CdtA–CdtC complex protects against intoxication by CdtABC holotoxin, but not by CdtB alone, again demonstrating that the internalization of CdtB is critical for toxicity (Deng & Hansen, 2003).

Structural analysis of CdtA and CdtC has revealed the presence of ricin-like lectin domains, suggesting that an interaction with host cell membrane carbohydrate molecules might mediate CDT binding (Eshraghi *et al.*, 2010; Hu *et al.*, 2006; Nešić *et al.*, 2004). This is consistent with observations by McSweeney & Dreyfus (2005) demonstrating a critical role for cell surface *N*-linked glycoprotein playing a critical role in binding of EcolCdtA and EcolCdtC to cultured human epithelioid cervical carcinoma HeLa cells. Because fucose-specific lectins could block EcolCDT-II-mediated cell cycle arrest and CdtA and CdtC subunits could bind immobilized fucose, a sugar moiety containing fucose has been suggested as a likely component of the EcolCDT-II host cell receptor (McSweeney & Dreyfus, 2005). These findings are consistent with reduced binding of single amino acid AactCdtA subunit mutants to fucose-containing glycoprotein and correlation with reduced cytotoxicity of corresponding reconstituted AactCDT holotoxin for cultured Chinese hamster ovary (CHO) cells (Cao *et al.*, 2005). However, this is diffult to reconcile with data suggesting that GM3 glycosphingolipid is the CDT host cell membrane receptor for intoxication of human monocytic U937 cells by AactCDT unless target cell membrane receptors vary between cells of different lineages (Mise *et al.*, 2005). More recent studies examining the susceptibility of target cell lines expressing a wide range of surface membrane biomolecules to CDT representing each of the three distinct CdtA–CdtC sequence clusters described earlier suggest that a cell surface polypeptide component rather than glycoconjugates is the receptor that is most likely to be responsible for CDT–host cell specificity (Eshraghi *et al.*, 2010). These findings are consistent with a requirement for a putative G protein-coupled transmembrane protein, designated TMEM181, which localizes to membrane lipid rafts in induction of EcolCdtB-I cytotoxicity by a novel loss-of-function haploid genetic screen using a highly sensitive myeloid leukaemia cell line (Carette *et al.*, 2009). However, the possibility that TMEM181 is part of a complex that constitutes a functional receptor, that it plays a role in trafficking of a receptor–CdtB complex or that it represents a ligand unique to EcolCdtB-I cannot be ruled out. Nevertheless, the demonstration that the expression level of TMEM181 is rate limiting for intoxication of cell lines with differential sensitivity to CDT provides a molecular basis to explain the variable susceptibilities of cells from different lineages to intoxication. These apparent discrepancies between previously reported specificities might indicate the lack of a requirement for a shared molecular receptor among CDT produced by different bacterial pathogens. In support of this hypothesis is the observed differential receptor specificity of AactCDT/HducCDT, EcolCdtB-III and CjejCDT when compared against a battery of cell receptor molecular targets (Eshraghi *et al.*, 2010). Since CDT-producing bacteria occupy different mucocutaneous niches and display diverse pathogenetic mechanisms, variable cell receptor requirement might indicate adaptation to the specificities of host cell targets.

Following receptor-mediated internalization in clathrin-coated pits, CdtB undergoes retrograde transport to the endoplasmic reticulum (ER) via the Golgi complex (Fig. 3; Cortes-Bratti *et al.*, 2000; Guerra *et al.*, 2005). The lack of protease inactivation or serum neutralization of HducCdtB and HducCdtC within minutes of cell surface binding suggests that conformational changes of cell surface-bound toxin are followed by rapid internalization. Consistent with this observation, irreversible inhibition of CHO cell proliferation occurs within 2 min of exposure to EcolCdtB-II (Aragon *et al.*, 1997). Pharmacological, chemical or genetic disruption of clathrin-mediated endocytic pathways as well as Golgi complex transport abolish CDT cytotoxicity (Cortes-Bratti *et al.*, 2000). Once taken up by mammalian cells, the catalytic subunit CdtB localizes to the nucleus presumably via alternate nuclear localization signals (NLSs; Lara-Tejero & Galán, 2000; McSweeney & Dreyfus, 2004; Nishikubo *et al.*, 2003). An N-terminal NLS has been proposed for AactCdtB, while two C-terminal NLSs have been found in EcolCdtB-II (McSweeney & Dreyfus, 2004; Nishikubo *et al.*, 2003). On the basis of highly conserved N-terminal amino acid sequences corresponding to the putative NLS of AactCdtB among all known bacterial CdtB orthologues, a modular structure consisting of an N-terminal domain responsible for nuclear transport and a C-terminal DNase-like domain capable of exerting DSBs has been proposed (McSweeney & Dreyfus, 2004; Nishikubo *et al.*, 2003). Unlike the 'A' subunit of other AB toxins which generally translocates from the ER directly into the cell cytosol by a process of ER-associated degradation (ERAD), translocation of HducCdtB is ERAD-independent with the toxin subunit moving directly from the ER lumen to the nucleoplasm without unfolding (Guerra *et al.*, 2009).

In *S.* Typhi, bacterial internalization into a *Salmonella*-containing vacuole of host cells leads to concurrent expression of StypCdtB, PltA and PltB which assemble into functional holotoxin complexes that are packaged into transport vesicles destined for extracellular secretion and intoxication of infected (autocrine) and uninfected neighbouring (paracrine) host cells (Fig. 4; Spanò *et al.*, 2008). The absence of CDT receptor on infected host cells has been proposed as a protective mechanism against cytotoxicity allowing intracellular survival of *S.* Typhi, and thus persistence in infected host tissues (Spanò *et al.*, 2008).

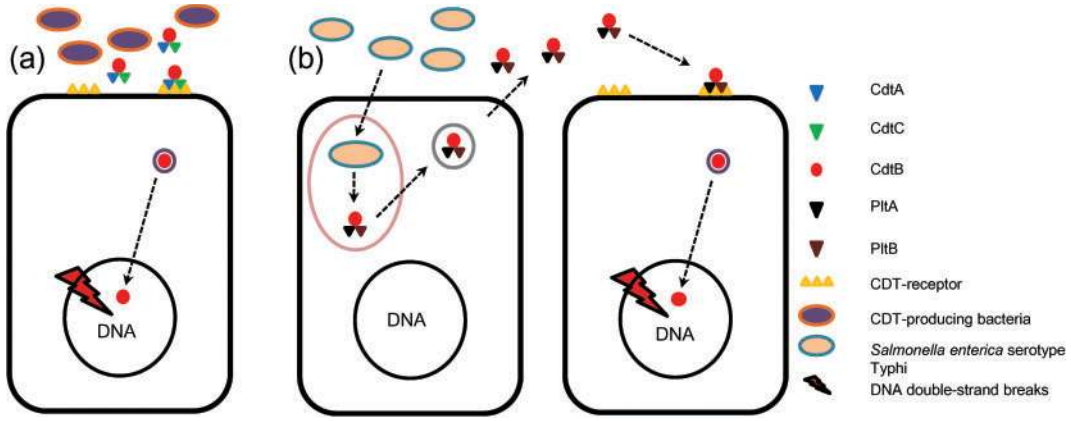

Fig. 4. Interaction of CDT with mammalian cells. (a) In nearly all CDT-producing bacteria, binding of CdtA and CdtC protein subunits of functional holotoxin complex or 'B' subunit to the host cell membrane receptor is followed by internalization of CdtB protein subunit or active 'A' subunit by clathrin-coated-pit-mediated endocytosis, nuclear translocation and host cell DNA damage. (b) In *S.* Typhi, intracellular uptake of bacteria into a *Salmonella*-containing vacuole is followed by concurrent expression of StypCdtB, PltA and PltB protein subunits, secretion and assembly into functional holotoxin complexes that are packaged into transport vesicles destined for extracellular secretion. Binding of PltB protein subunits or the 'B' subunit of functional holotoxin complex to the infected (autocrine) and to uninfected neighbouring (paracrine) host cell membrane receptors is followed by internalization of StypCdtB and PltA protein subunit or active 'A' subunit, nuclear translocation and DNA damage.

Nuclease and phosphatase activities of CdtB

The structural features of CdtB that predict a nuclease function are in agreement with demonstration of bacterial plasmid DNA digestion by recombinant EcolCdtB-II (Elwell & Dreyfus, 2000), AactCDT (Cao *et al.*, 2005; Nishikubo *et al.*, 2006) and HhepCdtB (Dassanayake *et al.*, 2005a), and also with DNA DSBs in mammalian cells intoxicated with CjejCdtB (Lara-Tejero & Galán, 2000) or HducCDT (Frisan *et al.*, 2003). These observations have been extended to nuclear DNA fragmentation by transient expression, cellular transfection or nuclear microinjection of mammalian cells with CdtB alone (Frisan *et al.*, 2003; Lara-Tejero & Galán, 2000). Finally, rescue of a mouse B cell line from AactCDT-induced cytotoxic death with the DNA endonuclease inhibitor aurintricarboxylic acid further confirms the endonuclease-mediated DNA damage requirement for CdtB-mediated cellular toxicity (Ohguchi *et al.*, 1998).

While it is well-established that cellular toxicity is mediated by the nuclease function of CdtB, other studies suggest that phosphatidylinositol-3,4,5-triphosphate (PIP_3) phosphatase activity plays a role in cellular toxicity (Shenker *et al.*, 2007). This is based in part on *in silico* comparative analysis of predicted amino acid sequences suggesting that several phosphatases involved in cell cycle regulation and signal transduction, including inositol phosphatases, contain a protein fold similar to Mg^{2+}-dependent endonucleases including mammalian DNase I and bacterial CDT (Dlakić, 2000). Demonstration of PIP_3 phosphatase activity is more clearly seen when using human leukaemia T-cell lines with constitutively elevated PIP_3 levels because of mutations in SHIP1 and/or PTEN (Shenker *et al.*, 2007). Therefore, an alternative mechanism of cellular toxicity might result from depletion of cell membrane PIP_3 and suppression of the protein kinase B (PKB)/Akt signalling pathway (Shenker *et al.*, 2007). However, when compared with site-specific phosphatase-defective CdtB mutants or specific PIP_3 phosphatase inhibitors, the nuclease activity of AactCdtB alone was sufficient for induction of cell cycle arrest and apoptosis of a proliferating human monocytic U937 cell line (Rabin *et al.*, 2009). Similarly, cell cycle arrest and death of haploid *Saccharomyces* (*Sacc.*) *cerevisiae* yeast strains requires CdtB DNase I-like catalytic residues and nuclear localization (Matangkasombut *et al.*, 2010). Since yeasts lack PIP_3, CdtB-induced DSB alone is sufficient to account for toxicity in this model (Matangkasombut *et al.*, 2010). Taken together, these data suggest that the predominant mechanism of cellular toxicity varies according to target cell lineage with nuclease activity and DSB as the most prevalent mechanism, and PIP_3 phosphatase activity plays an accessory role in certain situations.

CDT-induced DNA repair response and cell cycle arrest

Mammalian cell cycle regulation is responsible for the proper maintenance of several physiological processes, including the maintenance of intact mucosal epithelial barriers and clonal expansion of lymphocyte subsets during adaptive immune response, both of which constitute important defence mechanisms against colonization and infection of mucocutaneous interfaces by bacterial pathogens (Oswald *et al.*, 2005). Therefore, CDT-producing bacteria might have evolved a specialized mechanism to

disrupt key mammalian cell functions in order to establish persistent colonization and cause disease in certain niches (Nougayrède *et al.*, 2005; Oswald *et al.*, 2005). On the basis of their ability to modulate the cell cycle and cause cell death, CDT has been classified as an inhibitory cyclomodulin (Nougayrède *et al.*, 2005; Oswald *et al.*, 2005). In fact, CDT was the first bacterial toxin shown to cause cell cycle arrest in mammalian cells (Tóth *et al.*, 2009).

Cell cycle fidelity is maintained by redundant DNA damage checkpoint mechanisms, which are evolutionarily conserved signalling pathways that validate the integrity and accuracy of DNA replication at each phase of cell division (Bartek & Lukas, 2007; Jackson & Bartek, 2009). Activation of DNA damage checkpoints results in cell cycle arrest so that DNA can be repaired or, if damage is severe, progress to programmed cell death by apoptosis (Bartek & Lukas, 2007; Hoeijmakers, 2001). The DNA damage checkpoint network comprises upstream DNA damage sensors, signal transducers and downstream effectors (Bartek & Lukas, 2007). Specific pathways preferentially sense and respond to distinct types of DNA damage and initiate dedicated protective outputs (Hoeijmakers, 2001). Because DSBs are among the most severe DNA lesions, repair mechanisms involving error free homologous recombination and error-prone non-homologous end joining mechanisms will act synergistically to repair DSBs (Ciccia & Elledge, 2010). It is currently believed that CDT-induced DSB triggers a DDR similar to that caused by ionizing radiation (IR)-induced DSB and mediated by the phosphoinositide 3-kinase-related kinase ataxia telangiectasia mutated (ATM), encoded by the gene mutated in the rare autosomal recessive genetic disorder ataxia telangiectasia (AT; Fig. 5; Bartek & Lukas, 2007; Cortes-Bratti *et al.*, 2001b; Derheimer & Kastan, 2010; Jackson & Bartek, 2009).

The ATM-dependent IR-induced DDR involves both induction of cell cycle arrest and initiation of DNA repair (Derheimer & Kastan, 2010). Major components of the ATM-dependent DNA damage signalling pathway include (i) the multifunctional MRN protein complex consisting of Mre11, Rad50 and Nbs1, (ii) histone H2AX, (iii) the cell cycle checkpoint regulator protein kinase Chk2 and (iv) the transcription factor p53 (Bartek & Lukas, 2007; Derheimer & Kastan, 2010; Jackson & Bartek, 2009). Initial DSBs are recognized by the MRN complex, which recruits ATM to the damage site (Bakkenist & Kastan, 2003; Bartek & Lukas, 2007; Hoeijmakers, 2001; Lavin & Kozlov, 2007; Lavin, 2008). The serine-threonine protein kinase ATM, which normally exists as inactive dimers, dissociates and is activated by autophosphorylation in the presence of DSBs. Activated ATM subsequently phosphorylates a large array of substrates, including histone H2AX, Chk2 and p53 (Matsuoka *et al.*, 2007). Although the precise events surrounding ATM activation are incompletely understood, recent evidence suggests that ATM and MRN complex act in harmony to sense DSBs (Derheimer & Kastan, 2010). Activated MRN complex and phosphorylated H2AX (γH2AX) initiate and amplify the DNA repair process by

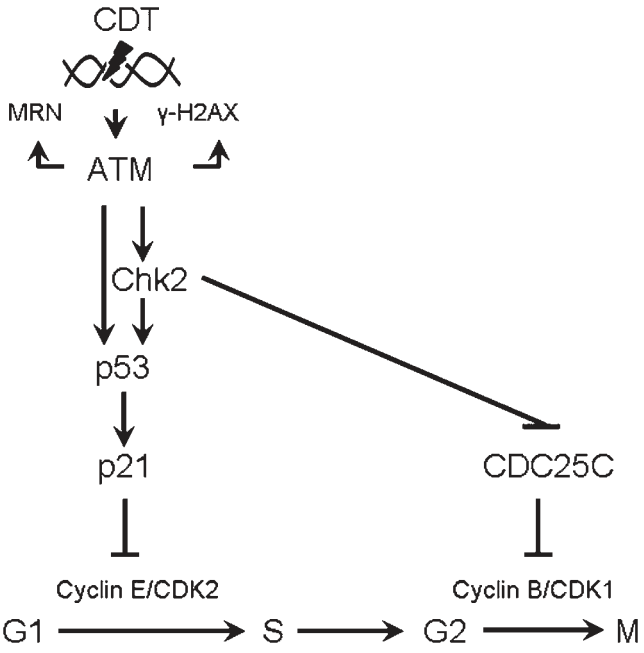

Fig. 5. Pathways of CDT-induced cell cycle arrest in mammalian cells. Following CDT-induced DNA double strand breaks, the ATM-mediated DNA damage response is initiated with subsequent activation of the multifunctional protein complex consisting of Mre11, Rad50 and Nbs1 (MRN), histone H2AX, the cell cycle regulator checkpoint kinase 2 (Chk2) and the transcription factor p53. Downstream p53-induced activation of p21 and Chk2-mediated inactivation of cell division cycle 25 (CDC25) C phosphatase blocks cell cycle progression by inhibition of CDK–cyclin complexes and thus prevents entry into mitosis while promoting DNA repair.

stabilizing the DNA lesion and providing a platform for the binding of other DNA repair proteins (Derheimer & Kastan, 2010). Activated Chk2 reduces cyclin-dependent kinase (CDK) activity by several mechanisms including activation of the transcription factor p53 and subsequent induction of cyclin inhibitor p21 (Jackson & Bartek, 2009). CDKs are key regulators of the cell cycle and the inhibition of CDKs can result in arrest of the cell cycle progression at the G1/S, intra-S and G2/M checkpoints, allowing time for DNA repair before proceeding with replication or mitosis (Jackson & Bartek, 2009). Additionally, other ATM substrates have key roles in affecting cell cycle checkpoint function, such as Smc1 for intra-S phase, as reviewed by Derheimer & Kastan (2010).

The main evidence in support of CDT-induced DDR is based on demonstration of MRN and γH2AX activation shortly after exposure of susceptible cells to CDT. A large percentage of HeLa cells display γH2AX foci 2 h after HducCDT exposure, and nearly all cells are positive for γH2AX foci within 6–8 h post-exposure (Li *et al.*, 2002). Both HducCDT and IR induce similar levels of Mre11 foci in HeLa cells and primary human dendritic cells (Li *et al.*,

2002). Nuclear microinjection of purified recombinant HducCdtB into HeLa cells induces Mre11 foci to the same extent as HducCDT holotoxin-treated cells within 1 h, suggesting that the active subunit CdtB is sufficient and necessary to elicit DDR (Li *et al.*, 2002). Increased Rad50 foci and γH2AX are also seen in primary human fibroblasts treated with CjejCDT (Hassane *et al.*, 2003), in primary and established human endothelial cells treated with EcolCdtB-V (Bielaszewska *et al.*, 2005) and cultured human intestinal epithelial H407 cells exposed to HhepCDT (Liyanage *et al.*, 2010). As expected, γH2AX is readily detected in immortalized wild-type human B lymphocyte lines after HducCDT exposure, but it is absent in ATM-defective leukaemic B cell lines obtained from AT patients (Li *et al.*, 2002). Analysis of diploid *Sacc. cerevisiae* yeast strains has provided additional evidence in support of CDT-induced DDR. *Sacc. cerevisiae* strains that lack Mre11, Rad50 or Xrs2, the budding yeast repair proteins equivalent to the MRN complex of mammalian cells, or other proteins required for DSB repair such as Rad51 or Rad55, are exquisitely sensitive to CjejCdtB, suggesting a critical role for HR in repair of CDT-induced DNA damage (Kitagawa *et al.*, 2007).

Other evidence in support of a CDT-induced DDR is based on demonstration of cell cycle checkpoint activation, which further confirms data obtained by monitoring DNA damage signalling. Human HL fibroblast and larynx carcinoma HEp-2 cell lines display similar cell cycle checkpoint response kinetics consisting of p53, Chk2 and Cdk1 phosphorylation and upregulation of p21 and p27, following exposure to IR or HducCDT (Cortes-Bratti *et al.*, 2001b). In common with IR, rat fibroblast cell lines exposed to HhepCDT exhibit c-Myc and ATM-dependent activation of DNA damage checkpoint responses (Guerra *et al.*, 2010). Even though this is not a genotoxin-specific phenomenon, formation of actin stress fibres through the ATM-dependent activation of small GTPase RhoA is seen in HeLa cells following HducCDT-induced DSBs (Frisan *et al.*, 2003). Formation of actin stress fibres is also seen in Hep-2 cells exposed to HpulCDT and CHO cells exposed to EcolCDT-II (Aragon *et al.*, 1997; Ceelen *et al.*, 2006). More recently, the formation of actin stress fibres in HeLa cells exposed to HducCDT was shown to result from the activation of nuclear RhoA-specific guanine nucleotide exchange factor (GEF) Net1 (Guerra *et al.*, 2008).

Currently, CDT is known to cause ATM-dependent cell cycle arrest at the G2/M and G1/S transitions, although definitive molecular analysis could further strengthen these observations. The CDT-mediated G2/M arrest is a result, at least in part, of the activation of Chk2 by activated ATM upon sensing DSBs. Activated Chk2 phosphorylates and inactivates cell division cycle 25 (CDC25) C phosphatase. The resulting accumulation of phosphorylated cyclin B–CDK 1 complex prevents mitotic entry (Ge *et al.*, 2008; Smith & Bayles, 2006). The mechanism for CDT-mediated G1/S arrest is thought to be p53-dependent. Activated ATM phosphorylates p53, and the resulting upregulation of p21 inhibits cyclin E–CDK2, which blocks S-phase entry (Ge *et al.*, 2008; Smith & Bayles, 2006). However, p53-independent upregulation of p21 following CDT treatment has been reported (Smith & Bayles, 2006). Although there is limited evidence supporting CDT-mediated intra-S-phase checkpoint activation in yeast, the potential contribution of this checkpoint has yet to be investigated in mammalian systems (Matangkasombut *et al.*, 2010).

Limitations in our understanding of the molecular mechanisms of CDT-induced cell cycle arrest lie in the techniques that are commonly employed to address this question. With the exception of a few instances indicating accumulation of phosphorylated Cdk1 (Cdc2) prior to cell cycle arrest, CDT-induced arrest of the cell cycle relies primarily on demonstration of altered patterns of DNA staining with propidium iodide and analysis by flor-escence-activated cell sorting (Bielaszewska *et al.*, 2005; Comayras *et al.*, 1997; Cortes-Bratti *et al.*, 2001b; Pérès *et al.*, 1997). At least two G2/M checkpoints exist in mammalian cells: an ATM-dependent transient checkpoint which activates in G2 phase cells immediately after DNA damage and a prolonged ATM-independent checkpoint which is activated several hours after damage and reflects accumulation of cells in the G2 phase that were initially damaged during S-phase (Weiss *et al.*, 2003; Xu *et al.*, 2002). Because CDT might induce arrest at multiple stages of the cell cycle, measurement of DNA content alone cannot distinguish between these two checkpoints, highlighting the need to delineate the mechanism of CDT-induced cell cycle arrest in greater detail.

CDT-mediated apoptosis

Apoptosis is a physiological mechanism of cell death present in multicellular organisms for the controlled elimination of unwanted cells. Similar to creating a block in the cell cycle, bacterial toxins can induce apoptosis of host cells to facilitate colonization, persistent infection and chronic disease by hampering healing (Fig. 6; Nougayrède *et al.*, 2005; Ohara *et al.*, 2011; Oswald *et al.*, 2005). Diverse factors and stimuli can initiate signalling pathways leading to apoptotic cell death by two major mechanisms; the extrinsic pathway involving death receptor activation and the intrinsic (or mitochondrial) pathway that can be activated by several stimuli (environmental toxicants, drugs and toxins) that provoke cell stress or damage (Taylor *et al.*, 2008). The extrinsic pathway is activated by the binding of extracellular death ligands (for example FasL and tumour necrosis factor α) to transmembrane death receptors (for example FAS), while the intrinsic pathway is activated by stimuli that alter the mitochondrial outer membrane permeability (Ow *et al.*, 2008). In some cases, the extrinsic pathway can also be activated by certain DNA-damaging drugs (Roos & Kaina, 2006). The activation of the intrinsic pathway depends on the activation of one or more members of the BH3-only protein family including Bax, Bid, Puma and Noxa (Taylor *et al.*, 2008). Accumulation of activated BH3-only proteins beyond a critical level

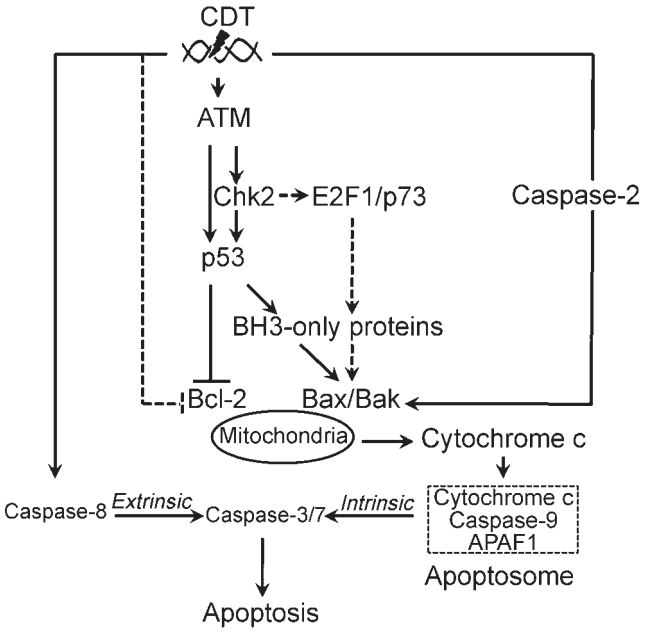

Fig. 6. Pathways of CDT-mediated apoptosis in mammalian cells. Following CDT-induced DNA double strand breaks, the ATM-mediated DNA damage response is activated, which (directly or through Chk2) phosphorylates and activates p53 leading to activation of the intrinsic apoptotic pathway. Accumulation of fully phosphorylated active p53 overrides anti-apoptotic Bcl-2 while promoting the formation of pro-apoptotic Bcl-2-associated X (Bax) and Bcl-2 homologous antagonist killer (Bak) protein oligomers on the mitochondrial outer membrane by transcriptional upregulation of pro-apoptotic BH3-only proteins. These interactions result in the release of cytochrome *c* from mitochondria and activation of caspase-9 within the apoptosome. Activated caspase-9 in turn activates the executioner caspases-3 and -7 leading to apoptotic cell death. Activation of the extrinsic apoptotic pathway following DSB through caspase-8 has been suggested in human B lymphoblastoid JY and monocytic 28SC cell lines (Hickey *et al.*, 2005; Shenker *et al.*, 2001). In a p53-mutated background, the intrinsic apoptotic pathway may be activated by either caspase-2-mediated Bax/Bak translocation, Chk2 causing E2F1/p73-mediated transcriptional upregulation of pro-apoptotic BH3-only proteins and leading to Bax/Bak mitochondrial translocation, or degradation of Bcl-2 (putative interactions are drawn as dotted lines).

overrides the inhibitory effect of anti-apoptotic B-cell lymphoma-2 (BCL-2) family proteins (such as Bcl-2 itself) and promotes the formation of pro-apoptotic BCL-2 family protein oligomers of BCL-2-associated X (Bax) and Bcl-2 homologous antagonist killer (Bak) on mitochondrial outer membrane (Ow *et al.*, 2008; Taylor *et al.*, 2008). Accumulation of the Bax–Bak complex results in the release of mitochondrial intermembrane proteins, including cytochrome *c* into the cytosol (Ow *et al.*, 2008; Taylor *et al.*, 2008). A feature in common to both of these pathways is the activation of caspases that are involved in early signalling events as well as in the final proteolysis phase of cell death (Creagh *et al.*, 2003). Stimulation of the extrinsic pathway leads to activation of caspase-8 that can process and activate downstream caspase-3 and caspase-7 (Taylor *et al.*, 2008). Stimulation of the intrinsic pathway involves multiple sequential alterations including signalling that results in release of cytochrome *c* from mitochondria and formation of the apoptosome (Ow *et al.*, 2008). This structure facilitates cytochrome *c*–APAF-1-dependent activation of caspase-9, which in turn activates caspase-3 leading to the execution phase of cell death by apoptosis (Creagh *et al.*, 2003; Taylor *et al.*, 2008).

The status of p53 is a key factor that determines the sensitivity of eukaryotic cells to apoptosis, and this is particularly critical for genotoxin-induced apoptosis where cells with the wild-type p53 genotype display greater sensitivity than cells with a p53-null genotype (Gudkov & Komarova, 2003; Roos & Kaina, 2006). In p53 wild-type cells, DNA damage leads to accumulation of fully phosphorylated active p53, and transcriptional activation of the pro-apoptotic p53 targets BH3-only proteins including Bax, Bid, Puma and Noxa; however, in p53-null cells apoptosis sensitivity can be mediated through several alternative mechanisms, including the degradation of anti-apoptotic Bcl-2, and Chk1/Chk2-E2F1/p73-mediated upregulation of pro-apoptotic p53 targets BH3-only proteins (Fig. 6; Gudkov & Komarova, 2003; Roos & Kaina, 2006). The p53-independent mechanism is somewhat less influential for facilitating apoptosis than direct activation of Bax by p53, and thus p53-null cells are generally more resistant to genotoxin-induced apoptosis (Roos & Kaina, 2006). Additionally, sensitivity to toxicant or drug-induced apoptosis varies considerably in different cell lines, and this is attributed to differences in the expression level of the anti-apoptotic protein Bcl-2 which has a protective function for mitochondrial integrity (Zamzami *et al.*, 1996). High Bcl-2 expression in certain lymphoid tumour cell lines is associated with resistance to chemotherapeutic drug- and bacterial toxin-induced apoptosis (Bloom *et al.*, 2006; O'Brien *et al.*, 2001). Significant resistance to bacterial toxin-induced apoptosis was demonstrated in the B-cell lymphoma EW36 cell line model that expresses a high Bcl-2 level (Bloom *et al.*, 2006; O'Brien *et al.*, 2001). Thus, the level of Bcl-2 expression can play an important role in target cell sensitivity to a wide variety of agents including bacterial toxins. This may be particularly true for toxins such as CDT that can induce a DNA-damage pathway involving mitochondria-mediated regulation by Bcl-2 family proteins.

The mechanisms involved in CDT-induced apoptosis are incompletely understood and currently thought to be dependent on the particular type of target cell (Belibasakis *et al.*, 2004; Dreyfus, 2003). Based on the observations of early DNA fragmentation and activation of DDR in several p53 wild-type and p53-defective cell lines, it is now clear that apoptosis is a downstream event of CdtB-induced DDR, which can be mediated through both p53-dependent and -independent pathways (Cortes-Bratti *et al.*, 2001b;

Frisan *et al.*, 2003; Liyanage *et al.*, 2010; Ohara *et al.*, 2004). Similar to other genotoxins, cells with wild-type p53 are more sensitive to CDT-induced apoptosis than p53-deficient cells. For example, in human leukaemia T-cell lines, caspases are activated earlier in p53 wild-type MOLT-4 cells (highly CDT sensitive) than they are in p53-mutated Jurkat cells (Cheng & Haas, 1990; Ohara *et al.*, 2004).

The CDT produced by several bacteria can induce apoptosis in a broad range of cell types including proliferating and non-proliferating primary cells of fibroblastic (primary human fibroblasts; Wising *et al.*, 2005), endothelial (human umbilical vein endothelial cells, Bielaszewska *et al.*, 2005), haematopoietic origin [human peripheral blood mononuclear cells (HPBMC), Shenker *et al.*, 2001; HPBMC, $CD4^+$ T cells and $CD14^+$ monocytes, Wising *et al.*, 2005], and established cell lines of epithelial [H407, Liyanage *et al.*, 2010; HeLa, Mooney *et al.*, 2001; immortalized human gingival keratinocytes (HIGK), Alaoui-El-Azher *et al.*, 2010; Ca9-22, Yamamoto *et al.*, 2004; HEp-2, HeLa and HaCaT, Wising *et al.*, 2005], endothelial (human EA.hy 926 and human brain microvascular endothelial cells, Bielaszewska *et al.*, 2005), and haematopoietic (MOLT-4 and Jurkat, Ohara *et al.*, 2004; 28SC, Hickey *et al.*, 2005; THP-1, Wising *et al.*, 2005) lineages (reviewed by Smith & Bayles, 2006). Similarly, caspase pathway activation following CDT intoxication has been documented in cultured epithelial (H407, Liyanage *et al.*, 2010; HeLa, Wising *et al.*, 2005; Ca9-22, Yamamoto *et al.*, 2004; HIGK, Alaoui-El-Azher *et al.*, 2010), endothelial (EA.hy 926, Bielaszewska *et al.*, 2005) and haematopoietic (MOLT-4 and Jurkat, Ohara *et al.*, 2004; 28SC, Hickey *et al.*, 2005; HPBMC, Shenker *et al.*, 2001; THP-1 and $CD4^+$ T cells, Wising *et al.*, 2005) lineages.

Recently, we reported that activation of the intrinsic (mitochondrial) apoptotic pathway follows DDR activation by HhepCDT treatment of human intestinal epithelial H407 cells (Liyanage *et al.*, 2010). Sequential upregulation of Bax and downregulation of Bcl-2 led to cytochrome *c* release and the subsequent activation of caspase-9 within 5 h of exposure to HhepCDT (Liyanage *et al.*, 2010). While the activation of caspase-9 peaked at 12 h, caspase-3 was not activated until 24 h after HhepCDT exposure, and the activity of caspase-8 did not change (Liyanage *et al.*, 2010). Based on caspase activation patterns in this and other cell lines, and together with other experimental evidence, the major mechanism for CDT-induced apoptosis appears to be through the ATM-dependent intrinsic pathway (Fig. 6; Alaoui-El-Azher *et al.*, 2010; Bielaszewska *et al.*, 2005; Hickey *et al.*, 2005; Ohara *et al.*, 2004; Shenker *et al.*, 2001; Yamamoto *et al.*, 2004). In support of this hypothesis is a significant reduction of CDT-mediated apoptosis in human B-cell lines with overexpressed Bcl-2, supporting the suggestion of an intrinsic pathway-dependent mechanism of apoptosis activation by CDT (Ohguchi *et al.*, 1998; Shenker *et al.*, 2001). However, elevation of caspase-2 activity following AactCDT treatment of p53-mutated Jurkat cells suggests a direct caspase-2-mediated Bax/Bak translocation as the mechanism of intrinsic apoptotic pathway activation (Ohara *et al.*, 2004). Although previous studies showed complete inhibition of AactCDT-induced apoptosis of human T-cell leukaemia Jurkat and MOLT-4 cell lines by the general caspase inhibitor z-VAD-fmk at 16 h (Ohara *et al.*, 2004), failure of this inhibitor to completely rescue AactCDT-induced cell death at 24–48 h suggested a caspase-independent (late) cell death pathway (Ohara *et al.*, 2008). This apparent late cell death in approximately 35 % of treated cells has been attributed to intracellular accumulation of reactive oxygen species (Ohara *et al.*, 2008). However, overexpression of Bcl-2 completely inhibited late cell death, suggesting that this pathway is also mediated through alterations in mitochondrial membrane permeability (Ohara *et al.*, 2008). Finally, simultaneous activation of both intrinsic and extrinsic apoptotic pathways has also been suggested with human B lymphoblastoid JY and monocytic 28SC cell lines (Hickey *et al.*, 2005; Shenker *et al.*, 2001).

The role of ATM in CDT-mediated apoptosis is incompletely characterized. There is limited evidence suggesting ATM wild-type SN-B1 and JAC-B2 cells (Epstein–Barr virus-transformed B lymphocytes from healthy donors) are more susceptible to HducCDT-induced apoptosis compared with ATM-defective (lymphoblastoid cell lines from AT patients; Cortes-Bratti *et al.*, 2001b). Based on caspase activation patterns, pharmacological inhibition of ATM or siRNA knock down of Chk2, it was recently shown that AactCDT-induced apoptosis of HIGK is mediated through the ATM-dependent DDR pathway (Alaoui-El-Azher *et al.*, 2010).

CDT cellular specificity and susceptibility

It has been proposed that the effects of CDT are cell-type-dependent (Belibasakis *et al.*, 2004; Dreyfus, 2003). Irrespective of the bacterial source of CDT and before characteristic nuclear and cytoplasmic distension are seen, cultured cell lines of epithelial and endothelial origins primarily arrest in the G2/M phase (Elwell *et al.*, 2001; Lara-Tejero & Galán, 2001; Smith & Bayles, 2006; Whitehouse *et al.*, 1998; Wising *et al.*, 2005; Young *et al.*, 2000b), whereas cells of fibroblastic origin arrest both in the G1/S and G2/M phases of the cell cycle (Belibasakis *et al.*, 2004; Cortes-Bratti *et al.*, 2001b; Hassane *et al.*, 2003; Smith & Bayles, 2006; Wising *et al.*, 2005). In contrast, cell lines of haematopoietic lineage including lymphocytes, monocytes, macrophages and dendritic cells, not only are several orders of magnitude more susceptible to CDT (pg versus μg as described below and in Fig. 7) but also display rapid apoptosis after a transient arrest of the cell cycle (Belibasakis *et al.*, 2004; Cortes-Bratti *et al.*, 2001b; Hassane *et al.*, 2003; Smith & Bayles, 2006).

The lowest effective dose of recombinant CDT that can intoxicate cell lines of haematopoietic lineage varies between 10 and 50 pg ml^{-1} compared with 1 and 5 μg ml^{-1} for other cell types (Shenker *et al.*, 2007). More than

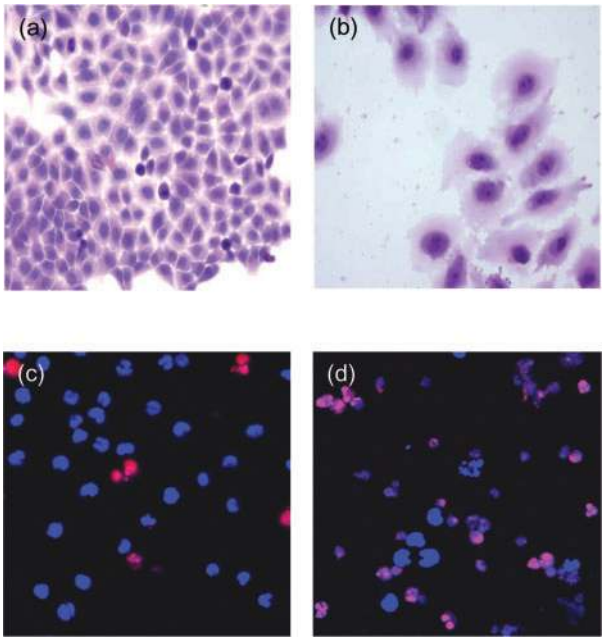

Fig. 7. Comparison of CDT-mediated cytotoxicity in human epithelial cervical carcinoma HeLa (ATCC CCL-2) and T-cell leukaemia MOLT-4 (ATCC CRL-1582) cell lines. Reconstituted recombinant CjejCDT was incubated with HeLa cells before fixing and staining. (a, b) Light photomicrographs (60× original magnification) of formalin-fixed and haematoxylin and eosin stained HeLa cells incubated with control medium (DMEM) (a) or DMEM containing 25 μg CjejCDT ml^{-1} (b) for 72 h. Note the marked nuclear and cytoplasmic enlargement of CjejCDT-treated cells compared with normal control cells. (c, d) Confocal laser scanning photomicrographs (40× original magnification) of un-fixed Hoechst 33342 and propidium iodide double stained MOLT-4 cells incubated with control medium (RPMI) (c) or RPMI containing 100 ng CjejCDT ml^{-1} (d) for 24 h. More than 85 % of the cells treated with CjejCDT (d) displayed early apoptotic changes characterized by condensed and fragmented chromatin (blue) or late apoptotic changes consisting of similar nuclear changes and cytoplasmic uptake of propidium iodide (pink). Less than 5 % of dead (red) cells are present in the control untreated culture.

90 % of either human Jurkat T cells or THP-1 monocytic cells undergo apoptosis within 24–48 h after treatment with 100 ng HducCDT ml^{-1}, while only 30 % of HeLa or HaCaT epithelial cells or primary human fibroblasts are intoxicated under the same conditions (Wising *et al.*, 2005). Based on their extreme susceptibility, lymphocytes have been proposed as the *in vivo* target of CDT, suggesting that immunomodulation is responsible for persistent bacterial colonization (Ge *et al.*, 2005; Pratt *et al.*, 2006; Shenker *et al.*, 2007). Although CDT is a broad range genotoxin (Ge *et al.*, 2005; Pratt *et al.*, 2006; Shenker *et al.*, 2007), it remains to be shown whether this apparent cell type specificity is in part attributable to inherent differences in cell surface receptor binding of CdtA and CdtC to host cell membrane based on: (i) host cell surface biomolecule chemical composition or density, (ii) intrinsic differences in CdtB uptake and nuclear translocation or (iii) variable target cell lineage DDR competence (Carette *et al.*, 2009; Eshraghi *et al.*, 2010). However, other factors that can determine the outcome of CDT interactions with susceptible cells, including differential receptor intrinsic affinity and also amino acid sequence divergence of CdtA and CdtC binding subunits encoded by different bacteria, need further detailed analysis (Carette *et al.*, 2009; Eshraghi *et al.*, 2010).

The mutational status of individual cell lines is another critical factor that can determine activation of specific checkpoint and apoptotic pathways, and thus the stage of cell cycle arrest and kinetics of progression to apoptosis in response to genotoxic injury. As described earlier, two of the most important mediators of CDT-induced DDR are ATM and p53, both of which play critical roles in determining the stage of cell cycle arrest and pathways and efficiency of apoptosis execution. ATM is required for the initiation of G1/S, intra-S and G2/M checkpoint arrest (Derheimer & Kastan, 2010; Xu *et al.*, 2002). However, there are at least two distinct G2/M checkpoints, and only the rapid-transient checkpoint is ATM-dependent (Xu *et al.*, 2002). Most established cell lines are tumour derived and frequently contain mutations in tumour suppressors, including *p53* (Cheng & Haas, 1990). Similar to ATM, p53 is required for the initiation and maintenance of G1/S checkpoint and can play a role in arrest at the G2/M stage of the cell cycle (Giono & Manfredi, 2006). As described earlier, *p53* wild-type cells are more susceptible to apoptosis compared with *p53* defective cells (Roos & Kaina, 2006).

Since the cellular responses to CDT-induced damage are very similar to IR-induced DDR, the inherent differential tissue susceptibility to IR-induced DNA damage may also be involved in the pathogenesis of diseases caused by CDT-producing bacterial pathogens (Gudkov & Komarova, 2003; Smith & Bayles, 2006). Rapidly proliferating cells including haematopoietic and intestinal epithelial cells are the most radiosensitive cell types, while non-proliferating cells of the nervous, respiratory, urinary, endocrine, musculoskeletal and mesenchymal tissues are relatively radioresistant (Gudkov & Komarova, 2003). The rate of cell division does not always correlate with cellular radiosensitivity, as extremely radiosensitive adult thymus, spleen and bone marrow stem cells consist mostly of quiescent cells (Gudkov & Komarova, 2003). However, the expression levels of p53 and several p53-responsive pro-apoptotic genes including *bax* and *fas*/*apo1* correlate with tissue radiosensitivity, where highly radiosensitive tissues have higher expression levels of *p53* and *p53*-responsive pro-apoptotic genes (Gudkov & Komarova, 2003). As a general rule, haematopoietic cells undergo rapid apoptosis following IR exposure, whereas fibroblasts undergo permanent growth arrest and epithelial cells, with the exception of the intestinal epithelium which undergo rapid p53-mediated apoptosis, exhibit reversible arrest, while

p53-deficient cells from all lineages tend to display a brief arrest (Gudkov & Komarova, 2003).

In this context it is interesting to note that the CDT-induced G1/S cell cycle arrest in fibroblasts has been observed exclusively in primary cell lines, which are likely to have wild-type p53, and thus retain an intact G1/S checkpoint (Hassane *et al.*, 2003). In contrast, G2/M cell cycle arrest does not depend on p53 for initiation, and therefore it is possible for established p53-deficient cell lines to retain a functional G2/M checkpoint (Giono & Manfredi, 2006). Indeed, the G2/M cell cycle arrest is the predominant type of cell cycle arrest associated with CDT toxicity (Smith & Bayles, 2006). At least in T-cell leukaemia cell lines, susceptibility to CDT-induced apoptosis is dependent on the p53 status of the cell line, as p53 wild-type cells are more susceptible to apoptosis compared with p53-deficient cells (Ohara *et al.*, 2004).

The differential tissue susceptibility to IR mirrors the cell type susceptibility to CDT (haematopoietic lineage hypersusceptibility, growth arrest in epithelial, endothelial or fibroblast lineages and CDT resistance in p53-defective cells from all lineages). To our knowledge, epithelial cell lines primarily show G2/M phase cell cycle arrest and a slow progression to apoptosis which can take several hours to days after CDT treatment. Investigating the mechanism of CDT intoxication in primary intestinal epithelial cells should provide additional mechanistic insights into the pathogenesis of disease caused by CDT-producing bacterial pathogens.

It is conceivable that the CDT-induced DDR might be distinct from that induced by IR, yet some of the pathways may overlap. The ability of a cell line to generate DDR is also an important consideration which determines genotoxin susceptibility, particularly for agents that cause DSBs (Roos & Kaina, 2006). While DSBs can be repaired by either relatively error free homologous recombination (HR) or error-prone non-homologous end joining (NHEJ), the latter may lead to chromosomal rearrangements particularly in cells in which this mechanism is preferentially utilized, such as cells in G1 phase of the cell cycle (i.e. resting fibroblasts, hepatocytes) or in lymphocytes undergoing V(D)J recombination-associated DSB repair during clonal selection (Callén *et al.*, 2007; Roos & Kaina, 2006). In HR-defective cells or cells that preferentially repair DSBs by NHEJ, the resulting chromosomal rearrangements can lead to apoptosis (Roos & Kaina, 2006). At least in *Sacc. cerevisiae* yeast, two types of mutations display hyper-sensitivity to AactCdtB: strains defective in sensing DNA strand breaks and strains with defective HR repair (Matangkasombut *et al.*, 2010). Since yeasts use HR as their primary means of DSB repair, the data suggest that cells defective in DSB repair are more sensitive to CdtB.

Role of CDT in disease

Naturally occurring infections with *Haem. ducreyi* and *A. actinomycetemcomitans* can elicit serum IgG antibodies to individual CdtABC protein subunits indicating development of a host adaptive immune response to toxin expressed during infection (Ando *et al.*, 2010; Mbwana *et al.*, 2003; Xynogala *et al.*, 2009). It has been suggested that the absence of AactCdtC-specific IgG in sera of patients with localized aggressive periodontitis compared with those with generalized aggressive periodontitis might be attributable to differences either in CdtC expression by different strains or in the extent or duration of these infections in an individual patient (Ando *et al.*, 2010). Serum antibodies that can neutralize the biological activity of CDT have been demonstrated in patients with chancroid (anti-HducCDT; Mbwana *et al.*, 2003) and periodontitis (anti-AactCDT; Ando *et al.*, 2010; Xynogala *et al.*, 2009), and in patients who had recovered from campylobacteriosis (anti-CjejCDT; Abuoun *et al.*, 2005). Moreover, development of high levels of CDT-neutralizing IgG in sera and genital tissues of mice immunized with HducCDT toxoid compared with native toxin suggests a potential vaccine application for CDT toxoid in protection against chancroid (Lundqvist *et al.*, 2010). However, it remains to be determined whether serum IgG antibodies to individual CDT subunits can (i) prevent acute infection, (ii) contribute to recovery from active infection or (iii) protect against reinfection.

Aside from demonstrating an adaptive humoral immune response to CDT following natural infection or immunization, nearly all studies on the role of CDT in disease have focused on *in vitro* models of eukaryotic cell genotoxicity, and direct experimental evidence demonstrating the role of CDT in disease of human and animal hosts is limited (Ge *et al.*, 2005; Smith & Bayles, 2006; Stevens *et al.*, 1999). Failure to demonstrate differences in skin colonization and lesion development among human volunteers and rabbits inoculated with wild-type *Haem. ducreyi* compared with an isogenic strain with an inactivated *cdtC* gene suggests that CDT is not required for cutaneous infection with this pathogen (Stevens *et al.*, 1999; Young *et al.*, 2001). While *cdtA*-, *cdtB*- and *cdtC*-negative mutant strains were no longer cytotoxic for cultured HeLa cells *in vitro*, a difference in the onset and degree of cutaneous changes elicited by mutant strains compared with the corresponding wild-type *Haem. ducreyi* parent strain in a rabbit model of chancroid could not be demonstrated, further suggesting that CDT does not contribute to the early stage of skin infection in this model (Lewis *et al.*, 2001). Although a dose-dependent inflammatory response was seen following intradermal inoculation of rabbits with reconstituted recombinant HducCDT holotoxin, but not with individual subunits, incomplete characterization of the cellular infiltrate and mechanism of inflammation hinder interpretation of these observations (Wising *et al.*, 2002). Similarly, intradermal inoculation of rabbits with an avirulent *Haem. ducreyi* strain together with 10 μg of reconstituted recombinant HducCDT holotoxin resulted in development of larger and ulcerated skin lesions when compared with the avirulent strain alone (Wising *et al.*,

2005). Considering that HducCDT is highly toxic to a variety of mammalian cells *in vitro*, it is conceivable that local cellular damage was responsible for the dermal response seen with purified toxin in the rabbit model. In the context of sexually transmitted disease associated with *Haem. ducreyi*, HducCDT might play a role in persistent infection and delayed healing which together can promote further disease transmission rather than initiation of mucocutaneous infection.

Preliminary *in vivo* evidence suggesting that CDT contributes to mucosal epithelial cell damage and altered healing response, which extends the earlier observations of Wising *et al.* (2002) in the rabbit model of chancroid ulcer, has recently been reported in a gingival rat model of AactCDT (Ohara *et al.*, 2011). In this model, oral sulcular epithelial cell degeneration and sloughing accompanied by local arrest of epithelial cell regenerative response was found within 3 days after *in situ* inoculation of oral gingival sulci of rats with purified parent AactCDT holotoxin expressed in *E. coli*, but not with AactCDT holotoxin reconstituted with a mutated CdtB DNase I-like H274A catalytic site (Ohara *et al.*, 2011).

Since CDT is produced by several intestinal bacterial pathogens, early studies examined the role of purified CDT and mutant strains in the pathogenesis of diarrhoeal disease. Development of profuse watery diarrhoea accompanied with intestinal fluid accumulation and colonic epithelial cell damage within 12 h after intragastric inoculation of conventional suckling mice with purified SdysCDT expressed in *E. coli* suggested a role for CDT in diarrhoeal disease associated with *Shigella dysenteriae* infection (Okuda *et al.*, 1997). These observations were further supported by demonstration of impaired translocation of *C. jejuni* CDT mutant compared with the isogenic wild-type parent strain across the intestinal epithelial barrier of adult SCID mice at 2 h, but not at 6 or 24 h after intragastric inoculation with 10^9 c.f.u. bacteria (Purdy *et al.*, 2000). Although the data suggest a potential role for CDT in direct intestinal epithelial cell damage and disease, intragastric administration of massive doses of toxin or bacteria, respectively, in immature and immunocompromised hosts might not be representative of the natural disease. Therefore, it is still unclear whether CDT contributes to intestinal epithelial barrier dysfunction and systemic spread of CDT-producing bacteria.

Because HhepCDT is the only known virulence factor found in *Helicobacter* (*Hel.*) *hepaticus*, a host-adapted pathogen of mice (Suerbaum *et al.*, 2003), laboratory mice have been used as a model to uncover pathogenetic mechanisms associated with CDT in infection and disease. Studies in laboratory mice, mostly with *Hel. hepaticus* (Ge *et al.*, 2005; Pratt *et al.*, 2006; Young *et al.*, 2004), but also with *Hel. cinaedi* (Shen *et al.*, 2009) and *C. jejuni* (Fox *et al.*, 2004a) and others (reviewed by Ge *et al.*, 2008) suggest that CDT potentially contributes to bacterial virulence. On the basis of a more rapid intestinal clearance of *Hel. hepaticus* CdtB-negative mutants compared with the wild-type parent strain in orally inoculated conventional and IL-$10^{-/-}$ deficient mice, a role for HhepCDT in resistance against host defence mechanisms has been suggested (Ge *et al.*, 2005; Pratt *et al.*, 2006). The lack of serum IgG1 and significantly lower IgG2c responses to *Hel. hepaticus* in IL-$10^{-/-}$ mice infected with a *Hel. hepaticus* CdtB-negative mutant compared with mice infected with wild-type *Hel. hepaticus* up to 8 months post-inoculation suggest an immunomodulatory role for HhepCDT in this model (Pratt *et al.*, 2006). In these studies, IL-$10^{-/-}$ mice that recovered from infection with the CdtB-negative mutant strain were partially protected against subsequent challenge with either the mutant or wild-type *Hel. hepaticus* strains (Pratt *et al.*, 2006). However, because protection of mice recovered from infection with wild-type *Hel. hepaticus* was not evaluated, it is unknown whether partial protection is a function of a lack of exposure to CDT or a characteristic of *Hel. hepaticus* infection in IL-$10^{-/-}$ mice. In other studies using highly susceptible A/JCr male mice, the prevalence and level of caecal colonization by a *Hel. hepaticus* CdtB-negative mutant were reduced compared with the wild-type parent strain at 4 and 10 months post-inoculation, whereas hepatic colonization levels were similar for both strains at 4 months, but reduced at 10 months post-inoculation in mice inoculated with the mutant strain (Ge *et al.*, 2007). Either strain produced similar degrees of hepatic inflammation early after infection, but only mice infected with the wild-type strain progressed to develop dysplastic changes by 10 months post-inoculation (Ge *et al.*, 2007). Taken together, these studies demonstrate a role for HhepCDT in modulating host adaptive immune response so that persistent intestinal colonization leads to systemic translocation of bacteria and localization to the liver where chronic infection can result in development of hepatocellular carcinoma. More recently, infection of immunodeficient mice engrafted with human haematopoietic stem and progenitor cells with an *S.* Typhi mutant strain lacking PltB suggested a role for typhoid toxin in persistent infection (Song *et al.*, 2010). However, none of the mice developed clinical disease in this model. Future studies with laboratory mice should uncover more precisely the stage in pathogenesis of disease and the specific cellular targets of CDT that are responsible for persistent infection and development of disease.

High concentrations of all three CjejCDT subunits are present in detergent extracts of purified outer membrane preparations of *C. jejuni*, suggesting that it is primarily membrane-associated (Hickey *et al.*, 2000; Lindmark *et al.*, 2009). Incubation of *C. jejuni* in the presence of 25 mM or 0.1 % bile acid sodium deoxycholate, a concentration physiologically relevant to the intestinal lumen, releases the membrane-associated CdtA, CdtB and CdtC subunits into the culture supernatant (Hickey *et al.*, 2005). Since CjejCDT holotoxin can elicit established human intestinal epithelial cell lines to produce CXCL8, a potent pro-inflammatory chemokine responsible for recruitment of

polymorphonuclear neutrophils (PMNs) in the intestinal mucosa, a role for CDT in initiation of host innate defence has been suggested (Hickey *et al.*, 2000; Konkel *et al.*, 2001; Murphy *et al.*, 2011; Young *et al.*, 2007). However, since PMNs are expected to eliminate *C. jejuni*, the benefit of eliciting a pro-inflammatory response seems less desirable if bacterial infection is prevented. Given that massive translocation of PMNs across the intestinal epithelium, as seen in the initial stages of campylobacteriosis, results in increased intestinal permeability and leakage of extracellular fluid into the gut lumen (Blikslager *et al.*, 2007; Dasti *et al.*, 2010), alterations in the gut microenvironment might indirectly promote local expansion of *C. jejuni*, thus allowing increased shedding of bacteria in faeces, contamination of the environment and further spread of the disease to susceptible hosts.

Development of a host adaptive immune response to an individual CDT protein subunit is seen following spontaneous infection and disease caused by CDT-producing *A. actinomycetemcomitans*, *C. jejuni* and *Haem. ducreyi*. However, experimental infections with *Hel. hepaticus* in a laboratory mouse model clearly show that CDT can modulate both the level and isotype antibody response of the host and establish persistent infection. Future studies should define which bacterial clearance mechanism is specifically targeted by CDT and its relationship to host immune response modulation.

Conclusions and future directions

Since the discovery of CDT in 1987, our understanding of the ecology of CDT-producing bacterial species has greatly expanded and the molecular biology of CDT-induced cellular genotoxicity has improved significantly. However, the natural history and contribution of CDT produced by individual bacterial pathogens in the context of mucocutaneous colonization, initiation and persistence of infection and disease in their respective host niches remain incompletely understood. Of paramount importance is the production of CDT by all major food- and water-borne pathogens, including several pathotypes of *E. coli* and species of *Campylobacter*, *Shigella* and *Salmonella*. Greater than 85 % prevalence of the CDT gene among clinical isolates of CDT-producing species of the families *Pasteurellaceae*, *Campylobacteriaceae* and *Helicobacteriaceae* suggests a critical role for this toxin in the pathogenesis of diseases caused by members of these families. In contrast, less than 14 % prevalence of CDT gene carriage together with evidence of HGT in clinical isolates of the enterobacteriaceae implies a conditional requirement for maintenance of this toxin in certain members of this family. Further studies on the ecology of CDT are clearly needed in order to clarify the significance of this toxin in different bacterial pathogens affecting a range of host species, including human beings.

Currently, CDT is the only member of a novel class of AB-type bacterial toxins that displays nuclease activity and translocates to the nucleus of a broad range of mammalian cell lineages where it exerts genotoxic damage resulting in cell cycle arrest and apoptosis. Further studies are needed in order to more precisely establish the relative importance of nuclease and phosphatase activities in disease pathogenesis. While the role of CDT-producing bacterial pathogens in orchestrating host adaptive immune response leading to persistent infection, inflammation and chronic disease such as periodontitis, chancroid, enterocolitis and hepatitis is relatively well documented, the underlying mechanisms, particularly in the context of infection and chronic disease associated with persistence and delayed healing, are incompletely understood. Given the availability of genetically engineered mouse models, determining the cellular targets of CDT in relation to specific stages of the disease process and host immune response modulation has the potential to expand our understanding of the role of this toxin in host–pathogen interactions. CDT is the only known virulence factor produced by *Hel. hepaticus* and *Helicobacter bilis*, and chronically infected mice develop inflammation-associated hepatic and colon cancer (Ericsson *et al.*, 2010; Fox *et al.*, 2011). Given the association of these and other CDT-producing bacterial pathogens with cancer and the ability of CDT to cause DNA damage in a wide range of mammalian cell lineages, a largely unexplored and provocative area of future research will be to determine whether or not CDT-producing bacteria can act as co-factors in promoting cancer development in their respective host niches. We hope that this review will generate interest among our colleagues and stimulate the next generation of microbiologists to tackle this evolving field of CDT-mediated microbial pathogenesis.

Acknowledgements

The authors would like to thank members of the Weiss and Duhamel laboratories for helpful discussions, and specifically Dr Gabriel Balmus for assistance with preparation of figures. This paper was partially supported by funds provided by the National Institutes of Health, National Institute of Allergy and Infectious Diseases R21 Grant 1AI085332 (to G. E. D.), the Qatar Foundation (Qatar National Research Fund Grant NPRP 08-465-4-009 to G. E. D.) and the United States Department of Agriculture, Cooperative State Research, Education, and Extension Service, Collaborative Research Program (to G. E. D. and R. S. W.).

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