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# TOXIN PRODUCTION IN *CLOSTRIDIUM DIFFICILE*

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Dedicated to two of the most delightful persons I know,

JOHANNA & SEBASTIAN

## ABSTRACT

*Clostridium difficile* is a Gram-positive anaerobic spore-forming intestinal bacterium and a potential human pathogen. It causing symptoms ranging from mild *C. difficile* associated diarrhea (CDAD) to severe inflammation of the colon including pseudomembranous colitis and fulminant colitis. Over 300 000 and about 10 000 CDAD episodes are diagnosed annually in the USA and Sweden, respectively. Onset of CDAD is typically associated with antibiotic therapy. These drugs may disrupt the colonization resistance of the colon microflora and allow for overgrowth of *C. difficile*. Pathogenic strains of *C. difficile* usually produce two toxins, A and B. These main virulence factors cause severe tissue damage due to their ability to both disrupt the assembly of the cytoskeleton in enterocytes and to induce inflammation in the colon.

In this study several novel factors influencing toxin production in *C. difficile* were identified and further investigated. A specific subset of amino acids and particularly cysteine was found to suppress the induction of the toxin yields in *C. difficile* by 100 to 1000-fold. Glucose also lowered toxin synthesis and furthermore, reduced the uptake and metabolism of certain amino acids. When toxin synthesis was already initiated amino acids stopped toxin synthesis much more efficiently than glucose, indicating that amino acids are more directly sensed and linked to *C. difficile* toxin expression, whereas glucose has a more indirect effect. The induction of toxin synthesis in media lacking a carbohydrate source and with limiting levels of key amino acids was preceded by the expression of several alternative metabolic pathways involved in energy production as shown by proteomic analysis. When these pathways apparently were no longer able to supply energy sufficient for growth, toxin synthesis was induced. Examples of candidate regulatory proteins sensing metabolic stress (ATP/GTP and amino acid levels) are CodY, LepA, Obg and Tex. The *C. difficile* toxins were also found to be temperature regulated with maximum expression at 37°C. This effect was dependent on the auto-regulated alternative sigma factor TcdD. Toxins were released by an export mechanism and not, as previously postulated, through lysis of the bacteria. During high toxin production and export a TolC-like protein accumulated in the growth medium. This putative pore-forming protein could represent a novel protein secretion machinery in Gram-positive bacteria and be involved in toxin release by *C. difficile*.

## LIST OF ARTICLES

This thesis is based on the following papers, which will be referred to by their roman numerals.

I. Karlsson S, Burman LG, Åkerlund T. Suppression of toxin production in *Clostridium difficile* VPI 10463 by amino acids. Microbiology. 1999; 145: 1683-1693.

II. Karlsson S, Lindberg A, Norin E, Burman LG, Åkerlund T. Toxins, butyric acid, and other short-chain fatty acids are coordinately expressed and down-regulated by cysteine in *Clostridium difficile*. Infection and Immunity. 2000; 68: 5881-5888.

III. Mukherjee K, Karlsson S, Burman LG, Åkerlund T. Proteins released during high toxin production in *Clostridium difficile*. Microbiology. 2002; 148: 2245-2253.

IV. Karlsson S, Dupuy B, Mukherjee K, Norin E, Burman LG, Åkerlund T. Expression of *Clostridium difficile* toxins A and B and their sigma factor TcdD is controlled by temperature. Infection and Immunity. 2003; 71: 1784-1793.

V. Karlsson S, Burman LG, Åkerlund T. Proteome study of *Clostridium difficile* during high and low toxin synthesis. Manuscript.

# TABLES OF CONTENTS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| AAD | Antibiotic associated diarrhea |
| BDM | Basal defined medium |
| BHI | Brain heart infusion medium |
| bp | Base pair |
| CDAD | *Clostridium difficile* associated diarrhea/disease |
| CDM | Complete defined medium |
| CDT | *Clostridium difficile* binary toxin |
| EIA | Enzyme immunoassay |
| Gal | Galactose |
| GalNAc | N-Acetyl Galactosamine |
| GAP | GTPase activating protein |
| GDI | Guanine nucleotide dissociating inhibitor |
| GEF | Guanine nucleotide exchange factor |
| GDP | Guanosine diphosphate |
| GI | Gastrointestinal |
| GTP | Guanosine triphosphate |
| Ig | Immunoglobulin |
| LCT | Large clostridal cytotoxins |
| MDM | Minimal defined medium |
| PAGE | Polyacrylamide gel electrophoresis |
| PAI | Pathogenicity island |
| PaLoc | Pathogenicity locus |
| PMC | Pseudomembranous colitis |
| PY | Peptone yeast medium |
| PYG | Peptone yeast glucose medium |
| SCFA | Short-chain fatty acids |
| SDM | Supplemented defined medium |
| SDS | Sodium dodecyl sulfate |
| SLP | Surface layer protein |
| TcdA | *Clostridium difficile* toxin A |
| TcdB | *Clostridium difficile* toxin B |
| UDP | Uridine diphosphate |
| 2-D | Two-dimensional |

# INTRODUCTION

## The clostridia

The genus *Clostridium* is a phylogenetically heterogeneous group of bacteria that share some fundamental characteristics including rod-shape, obligate anaerobic metabolism, Gram-positive cell wall and endospore formation. Another trait that is highly developed among the clostridia is the production of many diverse exotoxins, and clostridia produce more exotoxins than any other bacterial genus (Hatheway, 1990). The heterogeneity of the clostridia is clearly seen when the GC ratios of the chromosomal DNA are compared. The group members range in GC content from 24% to 55% (Cato *et al.,* 1986). Approximately 80 bacterial species belonging to the clostridia have been described and these are ubiquitously found in the environment, soil, water, sewage, and as part of the microbial flora of the gastro intestinal (GI) tract of man and animals (Cato *et al.,* 1986). Circa 30 *Clostridium spp.* are recognized as being pathogenic to animals and/or man and about one third of these are causing disease in humans, Table 1 (Murray *et al.,* 2002). Three key features, closely linked to what defines the genus, are crucial to all clostridial infections; (i) potential to survive harsh environmental conditions by the ability to form spores, (ii) rapid growth in oxygen-deprived environments due to its anaerobic metabolism, (iii) cytotoxicity or invasiveness mediated by the production of various toxins.

| | |
|---|---|
| *C. difficile* | *C. difficile* associated diarrhea<br>Pseudomembranous colitis |
| *C. perfringens* | Food poisoning/ Gas gangrene/<br>Soft tissue infection/ Enteritis<br>necroticans/ Septicemia |
| *C. tetani* | Tetanus |
| *C. botulinum* | Botulism |
| *C. septicum* | Gas gangrene/ Septicemia |
| *C. tertium* | Opportunistic infections |
| *C. barati* | Botulism |
| *C. butyricum* | Botulism |
| *C. histolyticum* | Gas gangrene |
| *C. novyi* | Gas gangrene |
| *C. sordellii* | Bacteraemia/ Gas gangrene |

Table 1. The major human pathogenic clostridia.

## *Clostridium difficile*

*Clostridium difficile* was first described in 1935 as *Bacillus difficilis,* the name originating from the obvious shape of the bacterium and the difficulty to isolate and grow it in the laboratory (Hall & O'Tool, 1935). *C. difficile* was isolated from feces of a newborn child without clinical symptoms. However culture filtrates from the same isolate injected into animals lead to death of the animals raising concerns already from the start that the bacterium could be a pathogen. Few additional studies were carried out and *C. difficile* drew little medical or other scientific attention during the next 40 years. In the 1970ies the correlation between the administration of the antibiotic clindamycin and the occurrence of severe pseudomembranous colitis (PMC) was observed (Tedesco *et al.,* 1974a; Tedesco *et al.,* 1974b). In the late 70's several case reports showed cytotoxic activity in stools from patients with antibiotic induced colitis (Larsson *et al.,* 1977). The observed cytotoxity could be neutralized by anti-toxin raised against *C. sordellii* but the feces were culture negative for this organism (Rifkin *et al.,* 1977). Bartlett and colleagues were the first to show that *C. difficile* was the etiological agent of PMC (Bartlett *et al.,* 1978). Today we know that the neutralizing effect of *C. sordellii* anti-toxin is due to the fact that *C. sordellii* and *C. difficile* produce toxins belonging to the same protein family and this enables the antitoxin to cross-react with both types of toxins.

# The human GI tract

The absorption and secretion of fluids and nutrients is central to the function of the human intestines. The GI tract is comprised of four main segments: namely, the oesophagus, stomach, small intestine and large intestine. The small intestine is further divided into three regions: the duodenum, jejunum and the ileum. The surface area of the small intestine wall is increased enormously by a folded structure from where projections of small villi occur. The cells that make up the surface is in addition to this covered with microvilli resulting in an enormous increase of the surface area of the small intestines. This allows for the efficient uptake of nutrients. The large intestine is divided into the caecum, ascending colon, transverse colon, descending colon, sigmoid colon, rectum and anus. The term large bowel and colon are used as a collective terms for the lower GI tract that includes the large intestine from the caecum to the rectum

(Vander *et al.*, 1994). The movement of electrolytes and other solutes is dependent on active transport mechanisms, which control fluid transport throughout the GI tract. $Cl^-$ is the predominant electrolyte that drives fluid secretion and is accompanied by the movement of $Na^+$ and water. The malfunctioning of these mechanisms produces symptoms ranging from constipation to diarrhea.

From the stomach to the anus the luminal surface of the GI tract is covered by a single layer of epithelial cells the majority of these being enterocytes or mucus producing goblet cells. Other specialized cells are also present in the GI tract such as M-cells (antigen uptake), various enteroendocrine cells (hormone production) and Paneth cells (production of animicrobial substances). Below the epithelium cells is a layer of connective tissue, the lamina propria, through which small blood vessels, nerve fibers and lymphatic ducts passes. Below the lamina propria, a thin layer of smooth muscles, the muscularis mucosae, is present. The epithelium, the lamina propria and the muscularis mucosae are commonly referred to as the mucosa. In the mucosa the gut-associated lymphoid tissue (GALT) is located. The GALT includes Peyer's patches, which are focal aggregates of lymphoid tissue. Each Peyer's patch contains follicles of B-cells, T-cells and dendritic cells and is covered with follicle-associated epithelium cells and M-cells (Debard *et al.*, 1999). The GALT also includes macrophages, lymphocytes, plasma cells and other immune cells. Below the mucosa we find the sub-mucosa. This connective tissue layer contains a network of nerve cells and blood and lymphatic vessels. The next layer of the GI wall, the muscularis externa, is made up of a series of circular and longitudinal muscles in close contact with the enteric nerve system. The muscularis externa is responsible for the peristaltic movements of the GI tract. Finally, surrounding all these different layers is a thin layer of connective tissue called the serosa (Vander *et al.*, 1994).

## Importance of the indigenous GI tract micro flora

The GI tract of man contains 1 to 2 kg bacteria, and as often quoted, their number exceeding that of the eukaryotic body cells by 10-fold (Moore *et al.*, 1974). This bacterial ecosystem within the human body, the microbiota, is of great importance for both normal and patho-physiological processes and the number of species present may exceed 500, although exact numbers remain to be defined, due in part to the fact that <30% of microorganisms are non-cultivable with current microbiologic methods

(Nord *et al.*, 1984, Mai *et al.*, 2004). The concentration of bacteria steadily increases from the stomach (almost zero between meals), via the duodenum, jejunum, ileum finally to the colon. In the lower part of the colon the bacterial concentration reaches an impressive level of $10^{11}$-$10^{12}$ microorganisms per g feces (Savage, 1981). The bacterial composition of the microbiota is specific to each part of the GI tract, and studies indicate that in a healthy individual the anaerobic species outnumber the aerobic ones by at least 10-fold and that each individual has unique gut microbial community that is relative unchanged over time (McCartney *et al.,* 1996). The most abundant bacteria isolated from the colon are members of the bifidobacteria, *Lactobacillus* and *Bacteroides* (Evaldson *et al.,* 1982). Quantification of the bacterial composition in human feces by using different methods of oligonucleotide probe hybridization, that also can detect bacteria that are viable but difficult to cultivate, have shown that bacteroides-prevotella group of bacteria is abundant and that also several groups of clostridia (sub-groups leptum and coccoides) are present in high numbers (Sghir *et al.,* 2000). In these studies the proportion of *Bifidobacteria* and *Lactobacillus* was less than 2% of the total GI flora.

Humans depend on the microflora for the acquisition of vitamins (K and B), degradation of mucin (Hill *et al.,* 1997), the conversion of cholesterol to coprostanol, and the degradation of fibers (high molecular weight carbohydrates) to short-chain fatty acids (SCFA) (Simon & Gorbach, 1986). Butyrate arising from microbial fermentation is important for the energy metabolism and normal development of colonic epithelial cells (Pryde *et al.,* 2002). A role of the colonic flora also in the homeostasis of the amino acid lysine has been proposed (Metges, 2000).

The microbiota, together with normal anatomic and physiological functions of the GI tract such as proper production of gastric hydrochloric acid, secretory immunoglobulins, intact mucosa and peristaltic movements, also play an important role in the prevention of colonization of the bowel by potentially pathogenic bacteria, a function often referred to as colonization resistance (Rolfe, 1996). The microbial part of the colonization resistance is mediated by the competition for essential nutrients and epithelial attachments sites, the production of specific anti-microbial substances and a cross talk with the immune system (Mai & Morris, 2004). Several studies have highlighted the regulatory role of the commensals for the proper development of a functional immune system as well as for the important regulatory

effect on the GALT through out life (Hooper & Gordon, 2000; MacDonald & Petterson, 2000). With all this important functions mediated and controlled by the bacteria within us it is not surprising that antibiotic therapy that may disrupt both the total number and the composition of the microbiota often results in different GI tract symptoms, especially antibiotic associated diarrhea.

## Antibiotic associated diarrhea (AAD)

The most potent inducers of AAD are antibiotics with a poor absorption or high biliary excretion that results in the accumulation of high titers in the feces such as the broad spectrum β-lactams (i.e. ampicillin and amoxicillin), clindamycin and cephalosporins (McCarty *et al.,* 1993; McFarland *et al.,* 1995; Winston *et al.,* 1991). AAD is in most cases not associated with an infection but caused by a shift in the osmotic balance in the intestine. The reduced microbiota is unable to adequately degrade unabsorbed carbohydrates in the feces (Rao *et al.,* 1988). The intact polysaccharides accumulated in the feces bind water resulting in osmotic diarrhea. Except for the diarrhea few additional symptoms are present and no colonic inflammation is induced. This benign form of AAD is for obvious reasons not transmissible between persons and the symptoms normally stop soon after antibiotic therapy is ended.

## *Clostridium difficile* associated diarrhea

In 25-50 % of AAD cases disruption of the colonic flora has also allowed overgrowth of *C. difficile* and onset of infection due to this organism (McFarland, 1998). The bacteria have typically been introduced from the environment as a spore or were already present in the GI tract as a constrained member of the present flora. Up to 15% of the population and 90-100% of newborns may be are healthy carriers of *C. difficile,* (i.e. culture positive without showing symptoms of disease) (Kato *et al.*, 2001). The bacterium itself is not invasive and its pathological manifestations are exclusively related to the production of toxins that results in an inflammation of the colonic mucosa (colitis) with an accompanying *Clostridium difficile* associated diarrhea (CDAD). Many *C. difficile* strains are naturally tolerant to certain antibiotics, notably clindamycin and third generation cephalosporins and the incidence and

severity of CDAD is usually influenced of the use of such clinical agents (Johnson *et al.,* 1999; Sambol *et al.,* 2001). CDAD may range from a mild diarrhea with few additional symptoms to severe forms with over 10 stools per day, including bloody stools, accompanied by several additional signs such as elevated body temperature, cramps, abdominal pain and rised level of leucocytes and nitric oxide in the feces (Enocksson *et al*., 2004; Wilcox, 2003). The first treatment, if possible, is to stop the administration of the inducing antibiotic allowing the normal flora to reestablish. This is often sufficient in the mild form of CDAD, but in the more severe infections specific antibiotic therapy directed towards *C. difficile* is given, metronidazole being the drug of choice followed by oral vancomycin and fusidic acid. Other causes of CDAD less common than antibiotics are other agents or interventions that disturb the GI flora such as surgery, nasogastric tube feeding, endoscopy, cytotoxic agents, laxatives and enemas (McFarland, 1998).

## Pseudomembranous colitis

In approximately 3-5 % of CDAD cases the infection progresses further resulting in a more severe form of inflammation called pseudomembranous colitis (PMC). The term originates from the whitish yellow visible plaques (pseudomembranes) covering parts of the inner colonic wall. These areas are composed of necrotic colonic cells and invading leucocytes. The correlation between *C. difficile* and PMC is 95-99% (McFarland *et al.,* 1995). In other words, *C. difficile* is the sole cause of PMC. PMC patients are generally not able to recover without antibiotic therapy directed towards *C. difficile*.

## Fulminant colitis and peritonitis

Fulminant colitis is the most severe outcome of a *C. difficile* infection and develops in circa 1-3% of the CDAD patients (Dallal *et al.,* 2002; Rubin *et al.,* 1995). Characteristic symptoms clearly indicates a systemic inflammation including high fever, hypotension and elevated white blood cell counts in combination with several patho-physiological changes in the GI tract, such as PMC, toxic megacolon, prolonged ileus and risk of perforation of the colonic wall and life threatening intra-

abdominal infection (peritonitis) (Dallal *et al.,* 2002). Fulminant colitis often needs prompt surgical action by removal of the colon (colectomy) but with unsatisfactory results including mortality rates as high as 38%-80% (Klipfel *et al.,* 2000; Synnott *et al.,* 1998).

## Hospital epidemiology of CDAD

Soon after *C. difficile* was shown to be the etiological agent of PMC in 1978, the organism was found to be associated also with at least one third of AAC cases with symptoms ranging from mild to severe diarrhea, mainly in hospitalized patients (see above). During the last two decades CDAD has become a rapidly increasing problem worldwide and currently *C. difficile* is responsible for an estimated health care costs of 1 billion US dollar annually in the US (Kyne *et al*., 2002). In a nation-wide study the incidence in Sweden, the number of CDAD cases was greater than all diagnosed domestic cases of diarrhea caused by classical bacterial and parasitic agents taken together (*Salmonella, Shigella*, entero-toxigenic and other diarrhea causing *E. coli, Campylobacter, Yersinia*, amoeba, *Giardia*) (Karlström *et al.,* 1998). Furthermore, 28% of the cases were not associated with current or recent hospital stay and thus were, apparently community acquired.

The main cause of the dramatic rise of the CDAD problem is thought to be the introduction of new antibiotics having an antibacterial spectrum and pharmacokinetic properties enabling profound disruption of the patients´ colonic flora except for the spores of *C. difficile.* In addition, the major group of antibiotics currently used in hospitals, the cephalosporins, have little or no impact on vegetative *C. difficile,* increasing the likelihood of overgrowth of the organism in the patients´ large bowel during or following antibiotic therapy. An additional risk factor for the emergence of CDAD is the excretion of large numbers of *C. difficile* spores by patients with diarrhea followed by transmission of the organism both between patients and from the hospital environment where the *C. difficile* spores can be found ubiquitously and survive for months to years (Kim *et al.,* 1981).

In a majority of studies using typing of the *C. difficile* isolates, CDAD has been found to be due to transmission of certain strains of the organism within hospitals. For

example, using the current reference method for typing of *C. difficile*, PCR-ribotyping, over 50% of isolates from UK CDAD patients nation-wide belonged to one type (UK1) compared to only 5% of community associated cases (Brazier, 2001). Apparently, this strain has spread within and between most UK hospitals. However, recent studies of *C. difficile* isolates from patients in Swedish hospitals indicated that a majority of the patients apparently fell ill due to their endogenous strain (PCR-ribotype) of the organism, whereas only about 17% and 27% had strains isolated also from other patients in the same hospital ward within 2 or 12 months (Norén *et al.*, 2004). Apparently, the quality of hospital hygiene may influence hospital transmission of *C. difficile* and thus, the size of the local CDAD problem.

## Susceptibility of the host to *C. difficile* infection

One of the typical characteristics of *C. diffilce* infection is the age related susceptibility with high colonization frequency among infants and the elderly (Hall & O'Tool, 1935; Holst *et al.,* 1981). The poor developed intestinal flora of infants in combination with an initial high exposure of *C. difficile* spores from hospital environments explains the high colonization rates among infants. Interestingly, the lack of symptoms in infants even if high levels of *C. difficile* toxins are present in the feces is still not fully understood. Absence of a functional toxin receptor in the immature mucosa, or its masking by a thick mucus layer has been proposed as explanation of this lack of susceptibility to the *C. difficile* toxins (Lyerly *et al.,* 1988). Furthermore, the secretory component of sIgA, received from the mother s breast milk has been shown to bind and inactivate toxin A (Dallas & Rolfe, 1998).

Several factors act synergistically in making the elderly especially vulnerable to *C. difficile* infection, including a generally poor colonization resistance due to an age-related reduction of the total level of anaerobes and especially the *Bifidobacteria* in their microbiota (Hopkins *et al.,* 2001; Hopkins *et al.,* 2002). In addition, elderly are high-level consumers of antibiotics with the accompanying risk of further disrupting the already low intrinsic colonization resistance. With increased age patients are hospitalized more frequent and for longer periods of time resulting in exposure to high doses of different spores of *C. difficile* strains from the hospital environment and thus, higher, colonization rates. During life we acquire protective antibodies against *C. difficile* toxins, both systemic IgG and intestinal secretory IgA (Johnson *et al.,* 1992).

As we grow old these titers are lowed with the consequence that the effect of *C. difficile* toxins is more pronounced in the elderly resulting in higher attack rate and worse symptoms (Kelly *et al.,* 1996; Kyne *et al.,* 2000; Kyne *et al.,* 2001). Finally, the nutritional state of the old are often poor a factor likely to affect both the colonization and the production of toxins from *C. difficile* (Mahe *et al*., 1987; Ward & Young, 1997).

## Recurrence of CDAD

Approximately 20% (10%-50%) of CDAD patients develop one or several recurrences (Joyce & Burns, 2002; Kyne & Kelly *et al.,* 2001). The recurrence can be either from endogenous persistence of *C. difficile* or its spores (relapse) or by the acquisition of a new strain from the environment (reinfection) and the general view has been that there is a 1:1 ratio between these two types of recurrences (Wilcox *et al*., 1998). However, recent *C. difficile* data from Sweden show that 90% of these are relapses, again supporting that hygienic precautions can reduced the risk of reinfection (Svenungsson *et al.,* 2003). Patients who have relapsed once are at an even higher risk for further relapses, and the relapse rate in patients who have suffered two ore more previous relapses is as high as 65 % (McFarland *et al.,* 1994). Relapse probably occurs due to a combination *C. difficile* spores not affected by antibiotic therapy and poor reestablishment of the protective flora, and in reinfection a high dose of infecting spores from the environment and a high intrinsic susceptibility of the host to *C. difficile* infection (Barbut *et al.,* 2000; Kyne & Kelly, 2001).

## Antibiotic therapy of *C. difficile* infections

In one out of four CDAD cases withdrawal of the causative agent is possible and followed by resolution of the infection. In the remaining cases *C. difficile* specific antibiotic therapy is necessary. Vancomycin and metronidazole are the mostly used drugs and have been shown to yield a similar cure rate (93-98%) and relapse rate (6-16%) (Teasley *et al.,* 1983). Vancomycin given orally is not absorbed and yields high

faecal concentrations (>1000mg/l). Oral metronidazole is normally well absorbed but in CDAD patients the excretion of metronidazole via the inflamed colonic mucosa and via biliary secretion results in therapeutic concentrations in the feces (Bolton & Culshaw 1986). Metronidazole may also be given intravenously also with high cure rates. Due to the occurrence of vancomycin resistant enteroccocci (VRE) and recently also vancomycin resistant *Staphyloccocus aureus* (VRSA), vancomycin has become second line therapy (Poduval *et al.,* 2000). Thus, metronidazole is currently the drug of choice for CDAD in both the EU and the US.

## Non-antibiotic therapy of *C. difficile* infection

Several alternative non-antibiotic therapies have been used to treat *C. difficile* infection and especially to reduce the major problem of relapse. Probiotic strategies including e.g. the non-pathogenic yeast *Saccharomyces boulardii* (McFarland *et al.,* 1994, Surawicz *et al.,* 2000), *Lactobacillus GG* (Gorbach *et al.,* 1987), *Bifidobacteria* (Nord *et al.,* 1997), *Bacteroides spp.* (Tvede *et al.,* 1989) are examples of this. The combination of a high dose of vancomycin (2g/day) together with *S. boulardii* has shown to reduced the risk of relapse from 45% to 26% (Surawicz *et al.,* 2000). The protective effect of *S. boulardii* is at least in part mediated by a 54 kDa protease with a specific activity against both the *C. difficile* toxins and toxin receptors (Czerucka *et al.,* 2002). Administration of non-toxigenic strains of *C. difficile* (Merrigan *et al.,* 2003, Seal *et al.,* 1987) and enemas containing diluted feces from healthy volunteers reduced the risk of developing CDAD and the risk of relapse (Schwan *et al.,* 1983). Toxin binding compounds (Heerze *et al.,* 1994) and passive (Beales, 2002; Salcedo *et al.,* 1997) or active immunization (Aboudola *et al.,* 2003; Kotloff *et al.,* 2001) represent new alternatives for therapy and prophylaxis of CDAD.

## *C. difficile* virulence factors

One definition of a microbial virulence factor is that the loss of that component or function specifically impairs the degree of pathogenicity (virulence) of the microorganism but does not affect the general viability. Example of typical bacterial virulence factors are, toxins, tissue degrading enzymes, adhesins, invasins and

capsules. Most pathogens have evolved a number of different virulence factors working synergistically in order to enable a successive colonization and occasionally infection. For *C. difficile* several virulence factors has been proposed.

**Toxins A and B**

The two major virulence factors in *C. diffilcile* are the toxins A (308 kDa) and B (270 kDa). The toxins share a high level of homology both at the amino acid level (62% similarity) as well as in their general structure and enzymatic function, probably as a result of gene duplication. Toxin A and B are the prototype members of a protein family named Large Clostridial Cytotoxins (LCT) (Just *et al.,* 1995a; Just *et al.,* 1995b) also including the lethal and hemorrhagic toxin from *C. sordellii* (Genth *et al.,* 1996; Just *et al.,* 1996; Popoff *et al* 1996) and α-toxin from *C. novyi* (Selzer *et al*., 1996) Table 2.

| Organism | Toxin name | Mw (kDa) | Co-substrate |
|---|---|---|---|
| *C. difficile* | toxin A | 308 | UDP-glucose |
| *C. difficile* | toxin B | 270 | UDP-glucose |
| *C. sordellii* | lethal toxin | 271 | UDP-glucose |
| *C. sordellii* | hemoragic toxin | 300 | UDP-glucose |
| *C. novyi* | α-toxin | 250 | UDP-N-acetyl-glucosamine |

Table 2. Members of the family of Large Clostridial Cytotoxins (LCT).

The LCTs are large single-chain proteins with three separate functional domains predicted to reflect particular events in the intoxication process of eukaryotic cells. The carboxy-terminal part is composed of repetitive micro-domains enabling receptor recognition and binding, the central part is characterized by a stretch of hydrophobic amino acids and has a putative role in translocation the toxins across the cell membrane, whereas the amino-terminal harbors the enzymatic (toxic) function (von Eichel-Streiber *et al.,* 1996). As the name indicates LCTs are typical of clostridia, but interestingly some strains of *Escherichia coli* belonging to the groups of enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) of the serotype O157:H7 encode a large (366 kDa) toxin shares significant homology with

the LCT that in the amino-terminal part (Burland *et al.,* 1998; Klapproth *et al.,* 1996). These proteins are called lymphostatins as they have shown to block the proliferation and cytokine expression of human lymphocytes without inducing apoptosis or otherwise killing the cells. These effects were not associated by changes in the epithelial cell cytoskeleton, suggesting a different mechanism of action than the LCT (see below) (Klapproth *et al.,* 2000). Elucidation of the mechanism of action of lymphostatin awaits further investigation.

Toxin A is a potent enterotoxin that stimulates fluid secretion and inflammation when administered into the animal intestine (Mitchell *et al.,* 1986) and shows hemagglutinating activity against rabbit erythrocytes (Krivan *et al.,* 1986). Toxin A causes extensive damage to the epithelial cells of the GI tract. The villus tips of the epithelium are initially disrupted followed by the destruction of the brush border membrane. The mucosa is finally denuded with massive infiltration of neutrophils resulting in loss of normal functions of the epithelium (Triadafilopoulos *et al.,* 1987).

Toxin B is often referred as a cytotoxin. The relevance of toxin B in the outcome of CDAD has been less verified than toxin A. The reason is that *in vitro* studies have shown that, although toxin B is approximately 1000- fold more potent as a cytotoxin *in vitro* than toxin A, the administration of toxin B to the GI tract in several different animal models results in little or no inflammation (Mitchel *et al.,* 1986; Triadafilopoulos *et al.,* 1987). However the last years have shown several cases of CDAD and PMC were the isolated *C. difficile* strains were toxin A negative, toxin B positive ($A^-B^+$) (Johnson *et al.,* 2001; Limaye *et al.,* 2000; Toyokawa *et al.,* 2003). A, study using human intestinal xenografts in an immunodeficient mice model showed that toxin B alone, indeed is capable of inducing an inflammatory response in the human GI tract (Savidge *et al.,* 2003). Toxin B was also in a recent study shown to cause a massive internalization of the apical located $Na^+/H^+$ exchanger NHE3 central to the absorption of salt and water across the intestinal epithelium (Hayashi *et al.*, 2004). This effect may very well contribute to the pathological role of this toxin. Taken together, these results suggests that toxin B functions as an enterotoxin in man, thus making the designations enterotoxin and cytotoxin less relevant. Thus, the importance of toxin B for the outcome of CDAD and PMC is apparently more important than previously appreciated. The absence of effect of toxin B in the *in vivo* models suggests a lack of functional receptor for toxin B in the animals tested. A

significance of both toxins is further supported by the fact that a majority of clinically important strains of *C. difficile* produce both toxins and that toxins A and B have a synergistic pathological effect on the intestine (Libby *et al.,* 1982; Lyerly *et al.,* 1988).

**Pathogenicity locus (PaLoc) of *C. difficile***

Many virulence factors are physically clustered on the bacterial chromosome in regions referred to as pathogenicity islands (PAIs) (Hacker & Kaper, 2000). PAIs comprise large regions of DNA (10-200 kbp), present only in the pathogenic strains or species of the bacteria. PAIs often have a markedly different GC% ratio and a different codon usage than the rest of the genome indicating that PAIs represent DNA imported by horizontal transfer. In addition, PAIs frequently contains regulatory elements such as two-component response regulators or alternative sigma factors and are often flanked by small directly repeated sequences supporting their horizontal transfer (Hacker & Kaper, 2000).

Toxin A and B in *C. difficile* were in 1995 shown to be part of a 19.6 kbp PAI including three additional reading frames (Hammond & Johnson, 1995). This chromosomal fragment from the strain VPI 10463 was later named the pathogenicity locus (PaLoc) and its five open reading frames were designed *tcdA-E* according to their size. The organization of the PaLoc is shown in Figure 1.

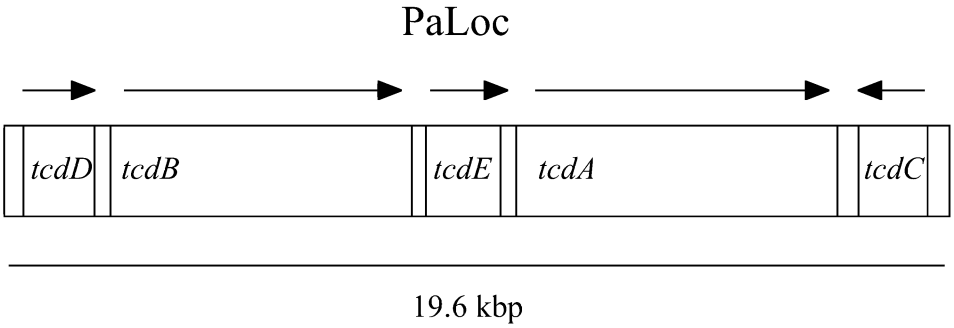


Figure 1. *C. difficile* VPI 10463 Pathogenicity Locus (PaLoc), arrows indicate direction of transcription.

Non-toxigenic strains of *C. difficile* contain a 115 bp sequence flanked by two insertion sequences at the site where the PaLoc is inserted (Braun *et al.,* 1996). Several variants of the *C. difficile* PaLoc have been identified including, deletions, insertions and different restriction patterns. As a result, *C. difficile* can currently be divided into twenty toxinotypes (I to XX) (Rupnik *et al.,* 1998, Rupnik *et al.,* 2003). The PaLoc structure of VPI 10463 is denoted toxinotype 0. Despite this high variability within the PaLoc all clinically important strains of *C. difficile* express a variant of either or both toxin A and toxin B, emphasizing the fact that the main virulence factors of *C. difficile* indeed are the two toxins. Transcriptional studies of the PaLoc showed that the five genes are transcribed coordinately and in response to the phase of growth, and indicated that the toxins A and B are transcribed as part of a large polycistronic mRNA species (17.5 kbp) (Hammond *et al.,* 1997; Hundsberger *et al.,* 1997). Other studies have shown that the toxin genes are transcribed also individually from their own promoters (Dupuy & Sonenshein, 1998; Moncrief *et al.,* 1997).

The transcription of the first four genes in PaLoc, *tcdDBEA*, peaks during stationary phase and correlates with the synthesis of toxin A and B (Hundsberger *et al*., 1997). Transcription of the last gene, *tcdC*, is high in early exponential growth phase and declines as growth continues and is transcribed in opposite direction of *tcdDBEA* (Hundsberger *et al*., 1997) This suggested that *tcdC* could be a negative regulator. *C. difficile* strain 8864, the so far sole member of the toxinotype X, is extremely cytotoxic and has a truncated *tcdC* gene (Boriello *et al.,* 1992; Lyerly *et al.,* 1992). This indirectly supports that TcdC is a negative regulator but little additional data have been published to verify a negative role of *tcdC* in *C. difficile* toxin synthesis.

The ORF denoted *tcdE* is located between the toxin genes *tcdB* and *tcdA*. Expression of *tcdE* in *Escherichia coli* caused bacterial death and *tcdE* displays structural features similar to a class of bacteriophage proteins called holins (Tan *et al.,* 2001). Holins are cytolytic proteins that cause lysis of bacterial hosts. The release of the two large toxins from the bacterium is still not well understood but as both toxins lacks signal peptide the function of TcdE has been proposed to facilitate the release of the toxins A and B to the extracellular environment.

The first gene in the PaLoc, *tcdD*, is located immediately upstream of the toxin B gene and encodes a small protein, 22kDa, that was shown to function as a positive factor for the expression of the promoters of both toxins A and B (Moncrief *et al.,* 1997). When *tcdD* was present in trans the expression of reporter gene constructs carrying the promoters of either toxin A or B were induced by 500- and 800- fold, respectively in *E. coli*. TcdD has a helix-turn-helix DNA motif and sequence similarity to other clostridial proteins that act as positive regulators of toxin expression, such as TetR in *C. tetani*, BotR in *C. botulinum* and UviA in *Clostridium perfringens* (Garnier & Cole, 1988; Marvaud *et al.,* 1998a; Marvaud *et al.,* 1998b). The positive impact on toxin production by TcdD has been shown to be due to its role as an alternative sigma factor enabling the RNA polymerase to transcribe *tcdA* and *tcdB* (Mani & Dupuy, 2001). Furthermore, *tcdD* is also positively auto-regulated and have two putative promoters (P1 and P2) located upstream of *tcdD* (Mani *et al.,* 2002). The –35 regions of these promoters show a high homology to each other and to the promoters of *tcdA* and *tcdB* whereas their –10 regions are are divergent and resembles more the sigma factor 70 promoters active during vegetative growth (Mani *et al.,* 2002). Therefore, it has been postulated that the –10 region of the *tcdD* promoter mediates a basal low-level transcription during early growth and that the –35 promoter is responsible for the auto-regulation of TcdD and thus, induction of the toxins during the late log phase (Mani *et al.,* 2002).

**Binary toxin**

Some strains of *C. difficile* encode an additional chromosomal toxin, not belonging to the LCT family, the binary actin-specific ADP-ribosyltransferase (Braun *et al.,* 2000; Popoff *et al.,* 1988). The *C. difficile* binary toxin (CDT) is composed of an enzymatic (CDTa) and a binding component (CDTb) and shows high homology of both activity and structure to the *C. perfringens* ι-toxin (Popoff *et al.,* 1988). CDAD strains belonging to the predominant PCR-ribotypes are binary toxin negative and only 6% of toxigenic *C. difficile* isolates produce also CDT (Stubbs *et al.,* 2000). Binary toxin has a toxic effect on cells in culture but its role in CDAD is not yet well understood (Perelle *et al.,* 1997). A recent study addressed the correlation of toxin A and toxin B negative strains expressing CDT ($A^-B^-CDT^+$) and CDAD. Out of 402 $A^-B^-$ strains 8 were found to produce CDT (2%) and of these only two were isolated from patients with diarrhea

(Geric *et al.,* 2003). The authors concluded that any synergistic effect of producing both toxin A and B and the binary toxin apparently is of minor clinical importance.

### Additional virulence factors

Several other virulence factors in *C. difficile* have been proposed including proteolytic enzymes (Poilane *et al.,* 1998), capsule (Davies *et al.,* 1990), the cell wall protein Cwp66 (Waligora *et al.,* 2001), flagellin related proteins FliC and FliD (Tasteyre *et al.*, 2001), the heat shock protein/chaperon GroEL (Hennequin *et al.,* 2001), the fibronectin binding protein Fbp68 (Hennequin *et al.,* 2003) and surface-exposed proteinaceous layer proteins (SLPs) (Cerquetti *et al.,* 2000). *C. difficile* is unusual in expressing two SLPs, a high and a low molecular weight variant. The binding of bacterial SLPs to enterocytes or extracellular matrix (ECM) is regarded important in the colonization of the gut by bacteria representing both the normal resident flora and pathogens and among the candidate colonizing factors of *C. difficile* a lot of focus has been on the SLPs. The high molecular weight subunit of the *C. difficile* SLP is well conserved among strains and has been demonstrated to bind specifically to the brush borders of the enterocytes mediated by a so far not identified ligand and to ECM ligands via collagen I, thrombospondin, and laminin (Calabi *et al.,* 2002). The low molecular SLP shows greater strain variation than the high molecular variant and may reflect the need for *C. difficile* to avoid immune recognition by surface antigen variation (Calabi *et al.*, 2001).

## Receptors and cellular uptake of toxins A and B

To reach their substrates in the cytosol the catalytically active domain of toxins A and B must cross the cellular membrane. It is generally accepted that both toxin A and B bind via their repetitive carboxy-terminal sequence to specific receptors on the colocytes enabling receptor mediated endocytosis (Barth *et al.,* 2001; Florin & Thelestam, 1983; Henriques *et al.,* 1987; von Eichel-Streiber *et al.,* 1991). However, the relevant receptor(s) on human colonic cells has not yet been identified. The carboxy-terminal part of toxin A has been shown to bind to the sugar structure Galα1-

3Galβ1-4GlcNAc, present on the brush border membranes on hamsters and on the red blood cells in rabbits (Krivan *et al.,* 1986). Moreover, toxin A binds to the human blood antigens Lewis I, X and Y (Tucker & Wilkins, 1991). These data suggest that the minimal consensus structure for a toxin A receptor is Galβ1-4GlcNac. Recent studies have supported that a fully intact carboxy-terminal part of toxin A is required for successful endocytosis (Frisch *et al.,* 2003). No potential receptor structures for toxin B have so far been found. That toxin B indeed binds via a receptor is supported by the fact that toxin B binds better to some cell lines than others and that the binding kinetics can be saturated (Chaves-Olarte *et al.,* 1997). The potent cytotoxic effect of toxin B on a broad range of eucaryotic cells suggests that its receptor(s) is almost ubiquitous.

Bacterial toxins that act on intracellular targets can reach the host cell cytosol by two different pathways. In one of these the toxin, including cholera toxin, shiga toxin and *Pseudomonas aeruginosa* exotoxin A, is internalized after binding to specific receptors to be transported to the endoplasmatic reticulum (ER) by a retrograde pathway where the translocation through the ER-membrane out to the cytosol takes place (Chaudhary *et al.,* 1990; Majoul *et al.,* 1998; Sandvig *et al.,* 1992). The other route for toxin uptake is to enter the cytosol by escaping the low pH compartment of the endosome. The prototype bacterial toxins using this uptake are diphtheria toxin, anthrax toxin, *C. botulinum* C2 toxin and *C. perfringens* binary ι-toxin (Barth *et al.,* 2000; Blocker *et al.,* 2001; Madshus *et al.,* 1991; Singh *et al.,* 1996). Also *C. difficile* toxin A and B depends on the acidic environment in the endosome for entry into the cytosol (Florin & Thelestam, 1986; Henriques *et al.,* 1987). Two members of the LCTs, *C. difficile* toxin B and *C. sordellii* lethal toxin, have been shown to undergo conformational changes during low pH conditions that results in pore formation that is thought to mediate the transfer of the toxin from the endosome to the cytosol (Barth *et al.,* 2001). Whether the whole toxin or only its enzymatic amino-terminal part is delivered to the cytosol has not been fully clarified. Both toxin A and B can intoxicate cells upon microinjection, indicating that no proteolytic processing of the toxins is needed per se in order to enable cytotoxicity (Müller *et al.,* 1992). However, the microinjected toxins had a somewhat lower toxicity per mol than toxins taken up by the normal route. For toxin B it was further shown that the carboxy-terminal and middle parts of the toxin remains in the endosome and that only the amino-terminal enzymatic domain was translocated from the endosome to the cytosol (Pfeifer *et al.,*

2003). Both these observations support the concept that the toxins are intracellular processed.

## Toxin targets, the Rho subfamily of small GTPase proteins

The main targets of toxins A and B are members of the Rho subfamily of small GTPase proteins. These proteins functions as master regulators of the integrity of the actin cytoskeleton in eukaryotic cells but also play important roles in diverse functions such as phospholipid metabolism, intracellular vesicle trafficking, smooth muscle contraction, cell cycle progression, cell transformation, apoptosis and transcriptional activation (Genth *et al.,* 1999; Symons & Settleman, 2001). Both toxins use UDP-glucose as a co-substate and transfer the glucose moiety to a specific threonine residue of the different GTPase iso-forms, Rho, Rac and Cdc42 (Just *et al.,* 1995a; Just *et al.,* 1995b). Moreover, Toxin A modifies also the Rap iso-form belonging to the Ras subfamily of small GTPases proteins (Chaves-Olarte *et al.,* 1997). Rho governs stress fibers involved in the regulation of cell shape, Rac controls the formation of sheet like ruffles such as lammelipodia and Cdc42 is involved in the regulation of thin spiky protrusions such as microspikes/filopodia (Symons & Settleman, 2001). Members of the Rho subfamily of small GTPase function as molecular switches. The proteins are activated by binding GTP and deactivated by de-phosporylation of GTP to GDP. The proteins are activated by various growth factors and cytokines, i.e. by bradykinin, TNF-α and IL-1 for Cdc42 (Kozma *et al.,* 1995; Puls *et al.,* 1999) and can in addition regulate each others activities via intracellular cross-talk (Matozaki *et al.,* 2000).

In their active form, Rho, Rac and Cdc42 have affinity for Ser/Thr kinases (e.g. Rho kinase) for downstream signaling. Three classes of accessory proteins also tightly regulate the activities of the Rho-subfamily proteins. These accessory proteins control the guanine nucleotide binding state of Rho, Rac and Cdc42 include; Guanine nucleotide Exchange Factors (GEFs), GTPase activating proteins (GAPs) and Guanine nucleotide Dissociating Inhibitor (GDIs).

The GDP/GTP cycle of the Rho subfamily of small GTPase proteins is further accompanied by intracellular translocation of an inactive GDP-bound cytosolic form to an activated GTP-bound form located in the cell membrane. The proper stimuli activate GEF so that GEF binds to the Rho protein and catalyses the release of the bound GDP. In addition, GEF stabilizes the nucleotide free protein and promotes binding of GTP to Rho whereby the affinity between Rho and GEF is lowered. GEF dissociates leaving the activated Rho located in the cell membrane. Binding of GTP induces a conformational change that allows the different iso-forms to bind to their various effector proteins. The Rho protein has an intrinsic GTPase activity and spontaneously hydrolyses GTP to GDP, an activity further enhanced by GAP. The inactive Rho protein dissociates from the effector protein and is released from the membrane and trapped in the cytosol by binding to GDI. In addition to sequester the Rho protein in the cytosol, GDI is also involved in the correct sub-cellular addressing of the GEF-induced activated Rho to the cell membrane (Genth *et al.,* 1999). The result of these complex regulatory pathways is, among others, that a proper organization of the cells cytoskeleton is maintained. The regulatory cycle of members of the Rho subfamily of small GTPase proteins is outlined in Figure 2.

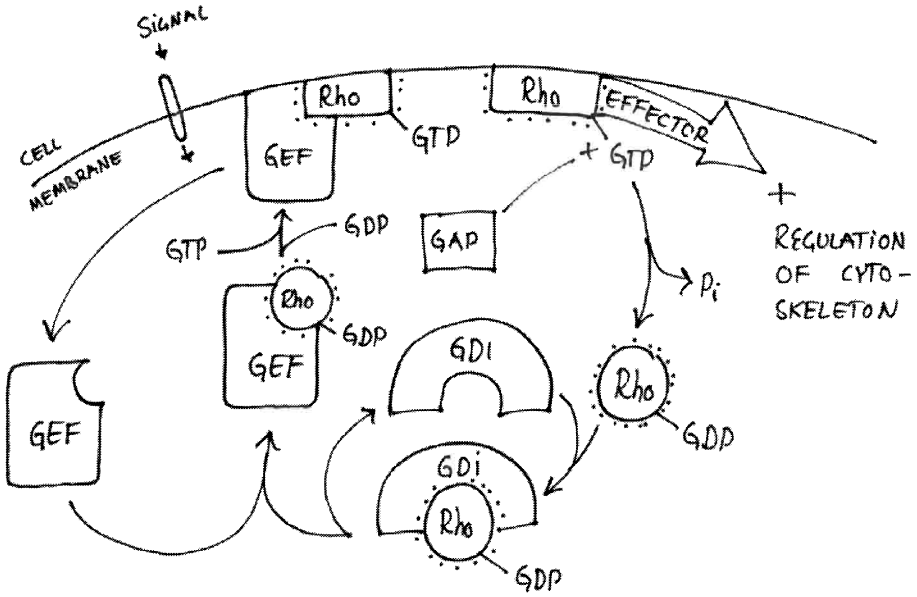


Figure 2. The regulatory cycle of the Rho subfamily of small GTPase proteins.

The mono-glucosylation of the members of the Rho subfamily of small GTPase proteins catalyzed by *C. difficile* toxins A and B occurs at the amino acids Thr 37 and Thr 35 for Rho and Rac/Cdc42, respectively, and completely blocks the interaction with the corresponding effector proteins (Just *et al.,* 1995a; Just *et al.,* 1995b). In addition, the glucosylation prevents the GAP induced hydrolysis of GTP to GDP (Sehr *et al.,* 1998). The outcome of the glucosylation is that the corresponding GTP-binding protein is trapped in the cytoplasmic cell membrane in an inactive GTP-bound state, resulting in a loss of control of the actin cytoskeleton, Figure 3.

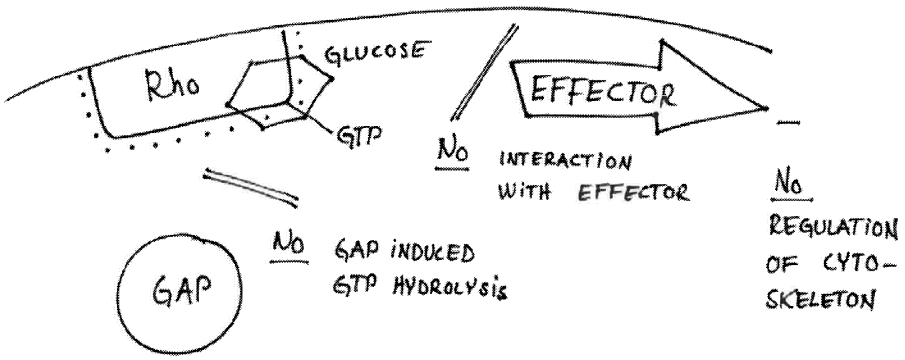


Figure 3. Effects of *C. difficil*e toxin catalyzed glucosylation of the Rho subfamily of small GTPase proteins.

## *C. difficile* toxins A and B and inflammation

Both toxins A and B have been shown to induce apoptosis in human intestinal cells (Fiorentini *et al.,* 1988; Mahida *et al.,* 1996). However, the toxin-mediated inflammation leading to CDAD is not only dependent on the direct cytotoxic effect mediated by the induction of apoptosis or by the loss of a functional regulation of the actin cytoskeleton leading to the destruction of epithelial villi, cell detaching from the ECM and opening of tight junctions (Nusrat *et al.,* 2001; Pothoulakis, 2000). The second important mechanism involves the activation of the enteric neurons, immune cells and inflammatory cells in the intestinal mucosa by the *C. difficile* toxins resulting in the pronounced local infiltration of neutrophils, a characteristic typical of the pseudomembranes observed in PMC. The inflammatory process is caused by the toxin-induced production of proinflammatory mediators such as leukotrien $B_4$, prostaglandin $E_2$ and TNF-α and IL-8 from the enterocytes (Alcantara *et al.,* 2001; Branka *et al.,* 1997; Triadafilopoulos *et al.,* 1989). The toxins also directly activate monocytes to realease IL-1β, TNF-α and IL-6 (Flegel *et al.,* 1991; Linevsky *et al.,* 1997). Several observations indicate that the induction of these mediators is independent of the Rho-glucosylation. For example, within 5 to 15 minutes after exposure to toxin A more than 80% of the cellular ATP levels dropped in Chinese hamster ovary cells, whereas cell rounding and Rho glucosylation did notcommence until often 15 to 30 minutes. Toxin

A also caused reduction of mitochondrial membrane potential and a 2 to 3-fold increase in reactive oxygen radicals (He *at al.,* 2000). Studies of human colocytes have confirmed the Rho-independent early role for mitochondria-derived oxygen radicals in the stimulation of IL-8 release by toxin A, an activation involving a nuclear translocation of NF-κB before release of IL-8 (He *et al.,* 2002). Recent studies indicate that also IFN-γ plays an important role in toxin A induced enteritis in mice (Ishida *et al*., 2004).

Once the proinflammatory factors are produced they activate primary afferent nerves in the mucosa that in turn produce neurotransmitters such as substance P (SP) and the calcitonin gene-related peptide (CGRP) (Castagliuolo *et al.,* 1997; Keates *et al.,* 1998). The importance of the enteric nerve system for the inflammatory response caused by *C. difficile* is supported by animal experiments. Rats pretreated with capsaisin, which prevents the effect of afferent neurons of the gut, showed virtually no response to administration of toxin (Castaglioulo *et al.,* 1994). The produced SP and CGRP trigger enteroendocrine N-cells in the epithelium to release neurotensin (NT), a potent compound inducing mast cell activation (Castagliuolo *et al.,* 1999). The de-granulation of mast cells results in vasodilatation, neutrophil infiltration accompanied by release of even more proinflammatory mediators (Holtzer *et al.,* 1992; Wershil *et al.,* 1998). SP can directly promote homing of neutrophils and auto-induce enteric nerve cells to release more SP (Mantyh *et al.,* 1996). Toxin A can also directly activate enteric nerves (Xia *et al*., 2000) and be a chemo-attractant for neutrophils (Kelly *et al.,* 1994). A summary of the inflammatory actions of the *C. difficile* toxins is outlined in Figure 4.

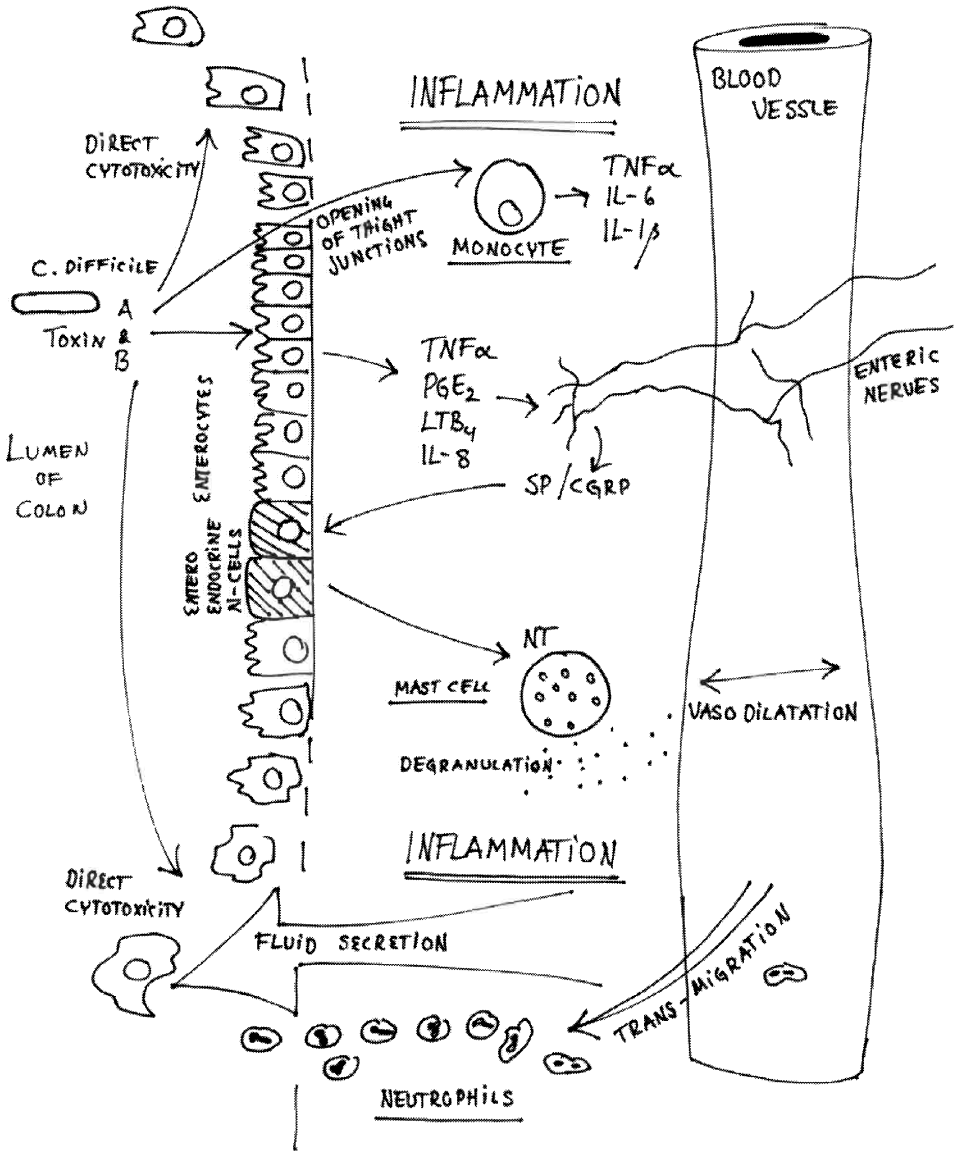


Figure 4. The inflammatory processes induced by *C. difficile* toxins A and B.

# THE PRESENT STUDY

## Aims

The aim of this thesis was to identify environmental factors that affect the toxin production in the human bacterial pathogen *Clostridium difficile* and to clarify the mode of action of these factors. A better understanding of the regulation of its toxins may lead to novel approaches to prophylaxis and therapy of CDAD.

# Results

## Paper I

### Suppression of toxin production in *Clostridium difficile* VPI 10463 by amino acids

**Karlsson S**, Burman LG, Åkerlund T. Microbiology 1999; 145: 1683-1693.

Aims: To better understand the regulation of toxin synthesis in the high-level toxin producing strain *C. difficile* VPI 10463 by investigating how growth rate, growth phase, buffering and composition of the growth media with respect to biotin, glucose and amino acids influenced toxin production.

Results: During growth in the defined media BDM, SDM, CDM or in the complex medium PYG the growth rate of *C. difficile* VPI 10463 ranged from 0.4 to 1.6 cell doublings per hour. The intracellular toxin level as measured as units (U) of toxin A + B per μg cellular protein was $\leq$ 1 in all media. These results indicated that that the growth rate per se did not have any major influence on toxin expression. Limiting the levels of the vitamin biotin, a co-factor involved in carboxylation reactions, resulted in a marked decrease of the growth rate of *C. difficile*. The intracellular toxin level dramatically increased during biotin starvation and reached levels as high as 100 U per μg protein.

The published data on the impact of addition of fermentable carbohydrates on toxin synthesis to *C. difficile* cultures were conflicting. Glucose had been shown to either up- or down-regulate toxin production when added to a rich growth medium (Dupuy & Sonenshein, 1998; Osgood *et al.,* 1993). We confirmed that *C. difficile* toxin production in PY medium was suppressed when glucose was added. However, glucose had opposite effects in minimal defined medium. Our hypothesis to explain the different results obtained in defined and complex media was that *C. difficile* was carrying out a mixed fermentation of both carbohydrates and amino acids and that the levels of certain amino acids in glucose limited PY medium became low enough to induce a stress response, including toxin production. Furthermore, we speculated that in the presence of glucose as preferred fuel these amino acids were spared and toxin production was not induced. In defined media the levels of these key amino acids would be in such

excess that this stress response never occurred. This hypothesis was supported as the induction of toxin synthesis was prevented in glucose-limited PY medium by a blend of nine amino acids (C, G, I, L, M, P, T, V and W) present in the defined medium SDM. The addition of other amino acids (A, D, F, H, K, R, S and Y) did not prevent toxin induction in PY showing that the expression of toxins was linked to limitation of only a specific subset of amino acids, Figure 5.

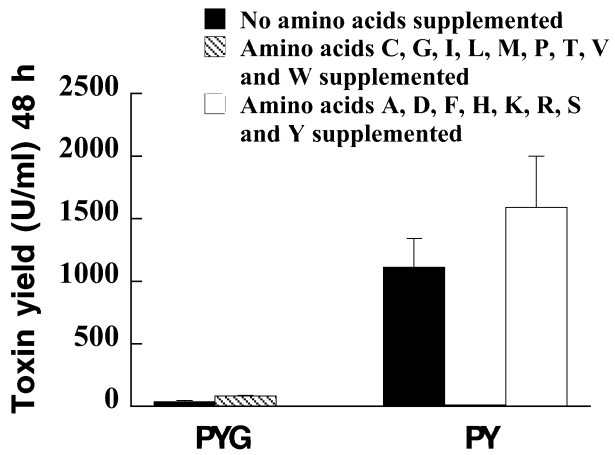


Figure 5. In glucose-supplemented peptone yeast medium (PYG) the yield of *C. difficile* toxins was low regardless of addition of extra amino acids. The lack of fermentable glucose in PY medium resulted in elevated toxin synthesis. This induction was blocked by the addition of a specific subset of amino acids whereas a blend of other amino acids had no such impact.

Nutritional shifts of *C. difficile* cultures (up or down) with respect to amino acids concentrations markedly altered the growth rate but were not associated with marked changes of toxin synthesis. This indicated that amino acid-mediated regulation of toxin expression was not part of a stringent response. The lowered toxin synthesis in PYG medium correlated with a drop in pH but phosphate buffering of PY and PYG resulted in neutral pH in both media after 48 h of growth without restoring toxin production in PYG cultures. Thus pH per se did not govern toxin expression. A bicarbonate buffer did enhance toxin production in both media but again only the glucose-limited PY culture responded with lowered toxin expression upon the addition of the amino acids C, G, I, L, M, P, T, V and W. A first 2-D gel analysis showed that high toxin production correlated with induction of several cytosolic proteins.

## Paper II

### Toxins, butyric acid, and other short-chain fatty acids are coordinately expressed and down-regulated by cysteine in *Clostridium difficile.*

**Karlsson S**, Lindberg A, Norin E, Burman LG, Åkerlund T. Infection and Immunity 2000; 68: 5881-5888.

Aims: To test if the toxin suppressing effect of the nine amino acids identified in paper I was a general response and thus, independent on the type of growth medium and *C. difficile* strain. We also wanted to evaluate if any specific amino acid(s) was more potent than the others in preventing toxin induction and if so, to see whether analogues and derivatives of this amino acid had a similar activity. Finally, we wanted to further identify the metabolic pathways in *C. difficile* that apparently were co-regulated with its toxins.

Results: Reduced toxin expression due to the specific amino acid mixture from paper I was seen also for all other strains than VPI 10463 tested and in other rich media than PY (i.e. BHI). Thus, the toxin down-regulating impact of certain amino acids was neither medium nor strain-dependent. A screen of individual amino acids as well as different combinations showed that cysteine followed by proline were most potent in suppressing toxin synthesis. The remaining seven amino acids (G, I, L, M, T, V and W) were not able to reduce toxin expression when supplied one by one but showed a synergistic effect and down-regulated toxin synthesis when added together, Figure 6.

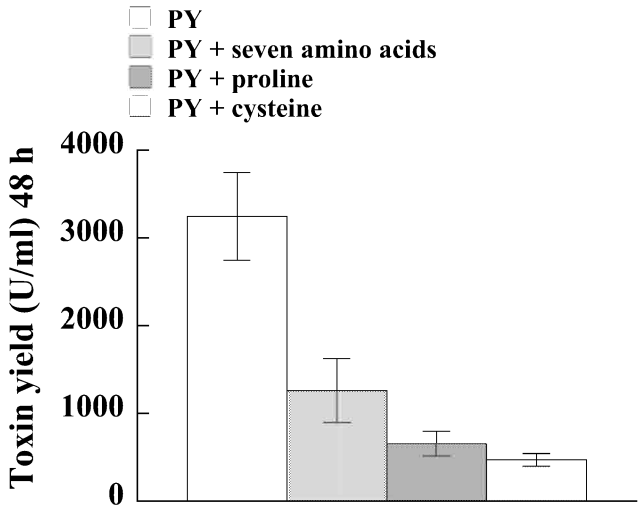


Figure 6. Impact of supplementing a blend of the amino acids G, I, L, M, T, V and W and the amino acids P and C on toxin yields from *C. difficile* VPI 10463 grown 48h in PY medium.

The impact of cysteine on toxin yields was further tested for 28 clinical isolates of *C. difficile* and in all isolates toxin production was suppressed. For the isolate showing the highest toxin level, the reduction by 10 mM cysteine was more than 100-fold, Figure 7.

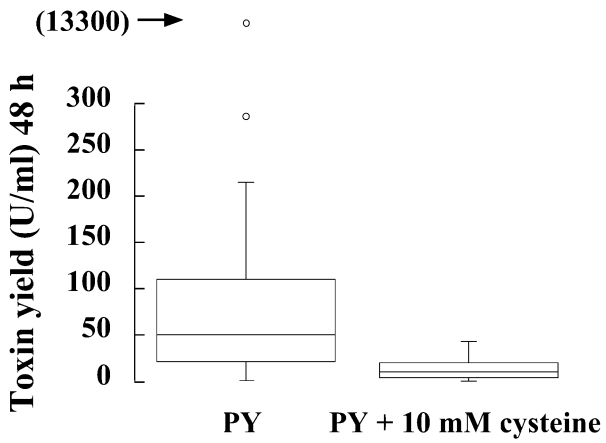


Figure 7. Toxin yields from 28 clinical isolates of *C. difficile* after 48h growth in PY medium with or without cysteine added.

As cysteine influences the reduction/oxidation potential ($E_h$) of the medium we addressed the question wheter the change in the environmental $E_h$ could play a major role in the cysteine-dependent down-regulation of the toxins. The redox active compound thioglycolate did not alter toxin expression while cysteine derivatives such as glutathion (a tripeptide containing cysteine), acetylcysteine and cystine (the redoxe inactive dimer of cysteine) had an impact similar to that of cysteine. These data showed that that toxin expression in *C. difficile* was not regulated by $E_h$ per se but by molecules having a cysteine moiety. The effect of L *vs* D forms of cysteine and cystine was also investigated. For both compounds the L form was over 10-fold more potent than the D form again arguing for that the suppressing effect on toxin production was not due to their ability to change the environmental $E_h$ but depended on another property of cysteine and related molecules.

Initial studies of the pattern of *C. difficile* proteins by 2-D PAGE analyses during growth in the absence or presence of cysteine showed that several proteins were down-regulated together with the toxins upon addition of cysteine. After N-terminal amino acid sequencing and database searches, four of these were identified as putative metabolic enzymes showing homology to 4-hydroxyphenylacetate-3-hydroxylase, formate tetrahydrofolate dehydrogenase, indolepyruvate ferredoxin oxidoreductase, and 3-hydroxybutyryl-CoA dehydrogenase, respectively. The corresponding genes and operon structures indicated that specific metabolic pathways involved in generation of ATP during production of butyric acid were down-regulated during low toxin production. To verify this, we analyzed the short-chain fatty acid (SCFA) fermentation products accumulating in the medium during high and low toxin synthesis. This pattern was complex but generally showed that growth in a cysteine-rich medium and thus resulting in low toxin production, gave low levels of SCFA and *vice versa*. In particular, the SCFA butyric acid was found in large amounts during high toxin production.

In addition, butyric acid elevated toxin production when added to cultures, while addition of butanol, the end product of butyric acid metabolism during so-called solventogenesis, suppressed toxin production. It was concluded that toxin expression is linked to specific metabolic processes including amino acid metabolism and ATP generation by butyric acid formation.

## Paper III

### Proteins released during high toxin production in *Clostridium difficile.*

Mukherjee K, **Karlsson S**, Burman LG, Åkerlund T. Microbiology 2002; 148: 2245-2253.

Aims: Toxins A and B from *C. difficile* are among the largest bacterial toxins recognized however the mechanism of their release from the bacterial cell is not known. The toxins start to accumulate in the growth medium upon entry into stationary phase. As both toxins lack a signal peptide, one hypothesis has been that they are released by bacterial lysis during sporulation (Kamiya *et al.,* 1992). We here studied the kinetics of toxin export and identified additional proteins co-released with the toxins in order to increase the understanding of the mechanism of toxin export.

Results: During growth in PY medium we observed an accumulation of toxins A and B in the growth medium without signs of concomitant bacterial lysis or sporulation. The relative intracellular level of toxin A and B was 3:1 whereas the corresponding extra cellular ratio was 1:1. This indicated that the release of toxins from *C. difficile* is not due to a non-specific mechanism (e.g. lysis) but rather by a more defined mode of export yet to be understood.

By 2-D SDS PAGE we further characterized the repertoire of exported *C. difficile* proteins during high and low toxin production. The set of released proteins were quite limited further arguing against cell lysis as export mechanism.

A 47 kDa protein was found to be exported with similar kinetics as the toxins. N-terminal amino acid sequencing of this protein indicated that it was processed and showed a homology to TolC, a protein involved in outer membrane pore formation and export of toxins in Gram-negative bacteria. The ORF of this protein was located in an operon with genes encoding homologues to the proteins HlyD and AcrB/D/F secretion family. In Gram-negative bacteria these proteins represents specific inner membrane fusion proteins that TolC docks to resulting in the formation of a channel connecting

the interior and exterior of the bacterium. These *C. difficile* proteins may represent a novel type of protein secretion apparatus in Gram-positive bacteria.

Another *C. difficile* protein found in the medium together with the toxins was identified as a homologue to XkdK, a protein present in the prophage PBSX of *Bacillus subtilis*. Similar to the toxins of *C. difficile*, XkdK lacks a N-terminal signal sequence and the *B. subtilis* XkdK is exported via phage-specific holins.

The major extracellular *C. difficile* proteins were shed surface layer proteins (SLP), which depend on typical *sec*-dependent leader (signal) peptides for their export. Searches of the *C. difficile* genome revealed that the SLP operon was complex and included its own *secA* gene and on active plus some ten silent SLP genes with similar architecture. N-terminal sequencing and PCR analysis revealed a high degree of sequence variability in the SLP gene *slpA* among different *C. difficile* strains. These results indicated that the SlpA can be involved in *C. difficile* antigenic variation.

## Paper IV

### Expression of *Clostridium difficile* toxins A and B and their sigma factor TcdD is controlled by temperature.

**Karlsson S**, Dupuy B, Mukherjee, K, Norin E, Burman LG, Åkerlund T. Infection and Immunity 2003; 71, 1784-1793.

Aims: To clarify the mechanism of temperature-dependent regulation of *C. difficile* toxins.

Results: We first found that the expression of *C. difficile* toxins A and B protein was regulated by temperature with maximum levels at 37°C and very low levels at 22°C and 42°C both in complex and defined media. Control experiments showed that low levels were not due to instability of the toxin or poor bacterial growth.

RNase protection assays showed that the both *C. difficile* toxins were induced at the level of transcription upon a temperature shift from ambient temperature to 37°C but not to 42°C Figure 8.

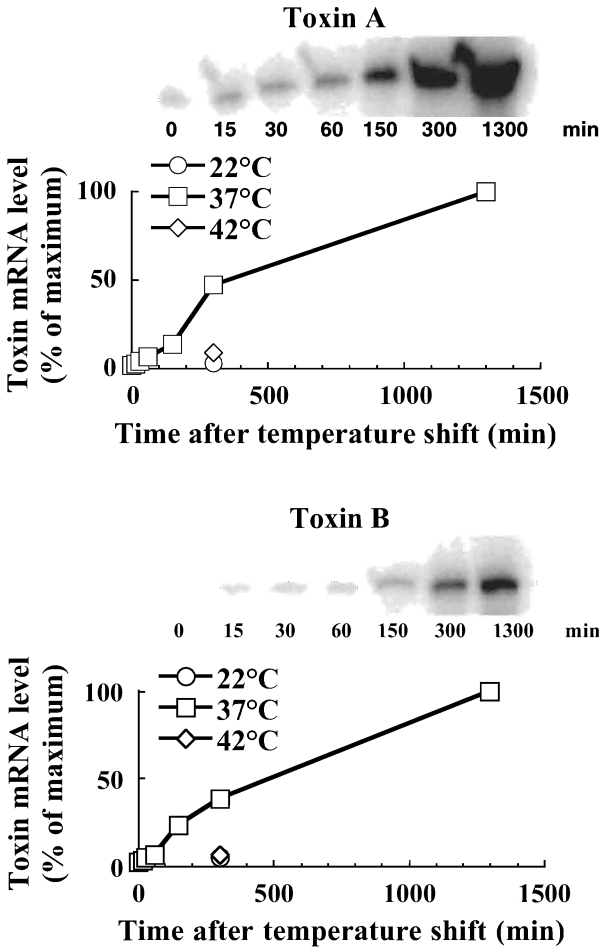


Figure 8. Expression of *C. difficile* toxin A and toxin B mRNA after a temperature shift from 22°C to 37°C or 42°C. The amount of transcripts at 37°C is shown in full, whereas the toxin mRNA levels in 22°C and 42°C cultures are indicated only at 300 min.

The fact that temperature influenced toxin expression at the mRNA level indicated that the *C. difficile* toxin specific sigma factor TcdD could play an important role in this regulation. Using a *C. perfringens* model system with the reporter gene *gusA*

fused to the promoters of either the toxin A or toxin B genes or *tcdD* we found that the presence of *tcdD* in trans enabled up-regulation of both toxin A and B Figure 9. In addition, *tcdD* was found to be positively auto-regulated specifically at 37°C. The presence of glucose in these experiments blocked the induction of toxins and TcdD at 37°C suggesting that similar regulatory networks were present in *C. difficile* and *C. perfringens*.

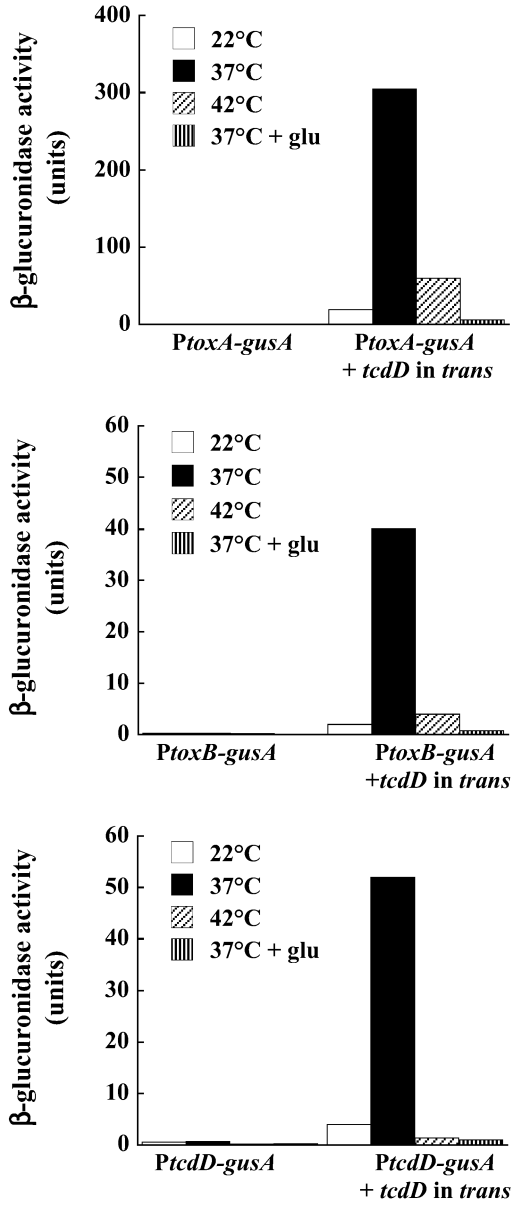

Figure 9. Expression of the reporter gene *gusA* linked to the promoters of the toxin A and B genes or to that of *tcdD* in the absence or presence of the toxin specific alternative sigma factor gene *tcdD* in trans. The experiments were carried out at 22°C, 37°C and 42°C in PY with and without glucose added.

Additional proteins showing a temperature dependent regulation similar to that of the toxins were identified. The results supported our earlier findings that growth conditions supporting toxin production are linked to expression of enzymes in metabolic pathways yielding butyric acid as main metabolic end product. Butyric acid was accordingly found by gas chromatography to be produced more in 37°C cultures than at the other growth temperatures.

## Paper V

## Proteome study of *Clostridium difficile* during high and low toxin synthesis.

**Karlsson S**, Burman LG, Åkerlund T. Manuscript.

Aims: To monitor the kinetics of amino acid uptake from the growth media $PY_0$, $PY_{10}$ and PYG by *C. difficile* VPI 10463. To follow changes of the proteome during the growth curve in these media in order to better characterize the metabolic changes associated with induction and repression of toxin synthesis. As only TcdD so far has been shown to be directly involved in the regulation of toxin expression this study also aimed at identifying additional proteins having a role in the toxin regulation.

Results: Addition of the toxin suppressing amino acids (i.e. cystein) rapidly turned off the accelerating toxin production in late log phase *C. difficile* cultures whereas the addition of glucose did not. The presence of glucose in the medium caused a general reduction of the uptake of amino acids from the environment. This was especially apparent for the branched-chained amino acids isoleucine, leucine and valine but also for cysteine and proline. The level of cysteine and proline was reduced to basal levels already at an $OD_{600}$ of 0.8 in all media. Taken together, these results supported that

amino acids had a more direct role in modulating toxin synthesis in *C. difficile* compared to glucose and that glucose slowed down the utilization of free amino acids in the culture obviously replacing these as fuel. In addition, proline and cysteine were preferred amino acids for fueling purposes apparently reflecting the fact that these amino acids are superior for stopping toxin synthesis.

By analyzing triplicate sets of 2-D PAGE visualizing major *C. difficile* proteins expressed during different parts the growth curve in various media using the software PDQest, we observed 347 proteins that were co-regulated with the toxins. Of these, 64 were successfully identified by MALDI-TOF MS. A majority of these proteins were up-regulated during growth in $PY_0$ and thus positively correlated with toxin expression. In agreement with previous studies, enzymes involved in generation of ATP during producton of butyric acid were induced. For example, enzymes involved in the pathway seen in *Clostridium acetobutylicum* during the acidogenic part of the biphasic metabolism of solventogenesis as well as enzymes similar to those responsible for the mixed fermentation of succinate and ethanol in *C. kluveri* leading to butyric acid as end product were induced. Fermentation of proline was specifically indicated by the induction of the two proteins proline isomerase and D-proline reductase. These enzymes are important in the coupled oxidation and reduction fermentation of specific pairs of amino acids, an unusual type of fermentation unique to clostridia and called the Stickland reaction. The use of amino acids as fuel in $PY_0$ cultures was also suggested by the induction of several other enzymes involved in amino acid fermentation (i.e. R-phenyllactate/2-hydroxyglutaryl-CoA dehydrates, indolepyruvate ferredoxine oxidoreductase and shikimate 5-dehydrogenase). During growth in $PY_0$ *C. difficile* is possibly also carrying out anaerobic respiration in order to generate ATP. Some of the enzymes induced indicated that the Wood-Ljungdahl pathway for autotrophic $CO_2$ fixation was taking place. In this reaction $CO_2$ is reduced to CO and in the following steps acetyl-CoA is produced that in turn generates ATP by substrate level phosphorylation and further via the acidogenic pathway. Taken together, during growth in $PY_0$ induction of the *C. difficile* toxins was preceded by induction of many different ATP generating pathways, apparently representing final attempts to generate sufficient energy. This emphased the association between lack of fuel and toxin expression.

During growth in the presence of cysteine or glucose the many enzymatic systems resulting in extra energy production observed in $PY_0$ were strongly down-regulated showing that by having cysteine or glucose as fuel the cells were no longer subjected to starvation or expressing the toxins. The proteomic data also showed that the $F_0F_1$ ATPsynthase was specifically induced during growth in $PY_{10}$ implying that in the presence of high levels of cysteine *C. difficile* could build up a proton motive force across the cell membrane to be used for energy production (ATP generation). During growth in PYG several glycolytic enzymes were up-regulated as expected.

A *C. difficile* homologue to the toxin expression (Tex) accessory protein, involved in the regulation of pertussis toxin in *Bordetella pertussis* was found to be induced during growth conditions where toxin production was low. This protein could be an important negative factor for toxin expression working to balance the inducer TcdD. the level of GTP-sensing proteins such as Obg and LepA were also influenced by the presence of cysteine and glucose. Obg and LepA belong to a large GTP-binding/regulatory family of proteins characterized by micromolar binding constants for GTP/GDP, low GTPase activity and rapid dissociation constants for either GTP or GDP. This indicated that the regulation of *C. difficile* toxins is dependent on factors sensing the GTP pool. The explicit role of Obg in bacteria is not well known but it is associated with the ribosome and essential for growth and plays important role in the phosphorelay and thus for the initiation of sporulation in *B. subtilis.* Also the role of LepA is unclear. LepA is a GTPase exclusively found in the bacterial lineage and shows homology to several elongation factors. The role of LepA is thus likely to be similar to that of Obg, i.e. interacting and modulating the activity of the ribosome.

# Discussion

*C. difficile* is currently the major cause of nosocomial diarrhea in the western world and usually related to antibiotic therapy. In a country like Sweden, having a highly developed food and water hygiene, diarrheal episodes caused by *C. difficile* outnumber those due to the classical etiological agents taken together (Karlström *et al.*, 1998). Only in the US the number of CDAD cases is estimated to be at least 300 000 per year (Kyne *et al.*, 2002) and the corresponding number in Sweden is thought to be around 10 000. Clostridia are notorious for having a lifestyle dependent of the fermentation of amino acids (Barker, 1981). For example, *C. tetani* has the capability of degrading many amino acids but lack genes for sugar utilization (Brüggemann *et al.*, 2003) and *C. sticklandii* is the model organism for a specific type of fermentation to generate ATP by the coupled oxidation and reduction of specific pairs of amino acids (Gottschalk, 1986). We have in this study found that temperature and the availability of certain amino acids in the environment and the metabolism of these are crucial to the regulation of the expression of the major virulence factors, toxins A and B in *C. difficile.* In the following, the current state of knowledge of the regulation of these toxins will be discussed.

## Down-regulation of toxins by glucose and specific amino acids

The presence of rapidly fermentable carbohydrates (e.g. glucose) in a complex growth medium prevents the long-term induction of toxin, e.g. in over-night cultures, as shown here **(paper I)** and by others (Dupuy & Sonenshein, 1998). This dramatic reduction in toxin production was however medium dependent and was not observed in defined medium **(paper I).** A possible reason for this discrepancy is that the defined medium had higher levels of certain amino acids replacing glucose as fuel and thus, kept toxin expression at a low level. This hypothesis was supported by the fact that not all amino acids were alike in suppressing toxin induction (**paper I, II**). The two most potent amino acids that down-regulated toxin production were cysteine and proline (**paper II**). The importance of amino acids was further shown when the levels of free amino acids in *C. difficile* culture supernatants were analyzed. Here it was apparent that cysteine and proline together with the branched-chained amino

acids, isoleucine, leucine and valine, were rapidly taken up by *C. difficile* (**paper V**). Adding toxin-suppressing amino acids or glucose at the end of the logarithmic growth phase of *C. difficile* cultures in $PY_0$, i.e. under low cysteine and glucose conditions and thus predisposing the cells to high toxin production in stationary phase, showed that the effect of amino acids and glucose differed in one important aspect. While the addition of amino acids rapidly stopped the otherwise accelerating toxin production glucose did not **(paper V)**. This indicated that the crucial amino acids are sensed and down regulate the toxin instantly whereas, the less dramatic suppression by glucose was dependend on slower or more complex cellular processes, such as the fermentation of glucose followed by reconstitution of the energy level in the cell. Its impact on toxin expression could be due to that glucose slowed the utilization of crucial amino acids by *C. difficile* thus preventing these to reach critically low levels that induced toxin synthesis **(paper V)**.

In several bacteria the preferred sources of nitrogen are ammonium and glutamine/glutamate (Fisher, 1999), and some bacteria measure the general level of nitrogen in the environment by sensing the intracellular pool of glutamine (Ikeda *et al*., 1996). The nitrogen metabolism in the model organism of Gram-positive bacteria, *B. subtilis,* is controlled by at least three global nitrogen regulatory proteins TnrA, GlnR and CodY. As judged by its impact on growth rate and uptake kinetics, the preferred amino acid in *B. subtilis* is glutamine followed by arginine (Atkinson & Fisher, 1991). In our experiments *C. difficile* did not show any preference for glutamine, glutamate or arginine during growth, neither did we see any induction of metabolic pathways involved in the synthesis of these amino acids during high or low toxin production **(paper V)**. In addition, no homologues to the GlnR or TnrA genes were found on the *C. difficile* chromosome. These results indicated that *C. difficile* differs markedly from *B. subtilis* with respect to nitrogen metabolism and further supported that the glutamine/glutamate levels and GlnR and TnrA has no role in the amino acid dependent regulation of the *C. difficile* toxins.

The third important regulator in nitrogen metabolism CodY is present in many Gram-positive bacteria, including all sequenced clostridia. CodY is a pleiotropic transcriptional repressor that responds to the level of GTP and branched-chained amino acids in the cell (Guédon *et al*., 2001). The *C. difficile* CodY homologue has a predicted pI and molecular mass of 5.3 and 29 kDa. During our proteomic studies of

*C. difficile* we did not detect a protein in this region of our 2D-gels that differed in expression depending on the growth phase or toxin production. In *B. subtilis* the expression of CodY does not change to a large extent during the growth curve but the activity of the protein does (Ratnayake-Lecamwasam *et al.,* 2001). Thus, our results do not exclude that the activity of CodY in *C. difficile* is dramatically altered depending on growth conditions or that CodY is important in the regulation of toxin expression. Examples of proteins and cellular functions known to be regulated by CodY in *B. subtilis* are dipeptide permeases, histidine degradation, isoleucine-leucine, and -valine biosynthesis, acetyl-CoA synthase, motility, competence and sporulation (Fisher *et al*., 1999; Guédon *et al.,* 2001; Ratnayake-Lecamwasam *et al.*, 2001). The presence of a CodY homologue and the observed preference for amino acids including isoleucine, leucine and valine makes CodY an interesting candidate for coupling generation of energy, amino acid levels and toxin production in *C. difficile*. In addition, the induction of *C. difficile* acetyl-CoA synthase during high toxin production in cysteine-limited media could indicate that a similar regulation of this enzyme by CodY is occurring in *B. subtilis* and *C. difficile* **(paper V)**.

The importance of sensing the change in the cellular level of GTP was in our studies further supported by the different expression of the proteins Obg and LepA **(paper V)**. The precise role of Obg in bacteria is not well known but it is essential for growth and plays an important role in the phosphorelay and thus, the initiation of sporulation in *B. subtilis* (Kok *et al.,* 1994). In our study, Obg was induced in the presence of cysteine and thus negatively correlated with toxin expression. In *B. subtilis* Obg is is associated with the ribosome protein L13 and necessary for activation of the general stress sigma factor B (Scott *et al.,* 2000). The three-dimensional structure of the *B. subtilis* Obg indicated that the GDP binding pocket of the protein also could bind ppGpp, crucial to the so-called stringent response, suggesting a more complex regulatory role of Obg than previously appreciated (Buglino *et al.,* 2002). In a number of bacterial systems the RelA protein (stringent factor) senses the increased binding of uncharged tRNA molecules (due to limited levels of free amino acids) to the ribosomal A-site and converts GTP to the highly phosphorylated compounds ppGpp and pppGpp (Chatterji & Ojha, 2001). The elevated levels of (p)ppGpp have been suggested to directly alter the promoter recognition of the RNA polymerase and reduce the expression of genes having growth-related functions, e.g. encoding ribosomal proteins or involved in DNA and cell wall synthesis. These events elicited

by amino acid starvation are known as the stringent response. However, markers typical of the stringent response were found neither in $PY_0$ cultures and as expected, nor in $PY_{10}$ cultures. Furthermore, metabolic down shifts with respect to amino acid concentration did not induce toxin production **(paper I)** and thus, our data do not suggest that toxin expression in *C. difficile* is under stringent control. A possible role of Obg in *C. difficile* toxin regulation needs to be further investigated.

LepA was induced during high cysteine levels. Little is known about the function of this protein except that it is a GTP binding protein and strictly found in the bacterial lineage of organisms (Caldon *et al.,* 2001). As it shows homology to several elongation factors (EF-G, EF-Tu and IF2), the functional role of LepA is likely to be similar to that of Obg, i.e. interacting and modulating the activity of the ribosome (Caldon *et al.,* 2001). Like CodY, LepA may be regulating gene expression in response to the levels of GTP and amino acids (i.e. cysteine) in the cell.

So far, only one protein directly influencing the expression of the *C. difficile* toxins has been found, the alternative sigma factor TcdD (Mani & Dupuy, 2001; Mani *et al.*, 2002). The apparent positive auto regulation of TcdD indicates that the ability of this protein to induce itself and thus, also toxin synthesis, is repressed during the logarithmic phase to be restored during entry into the stationary phase. In a model system where TcdD was encoded by a plasmid construct in *C. perfringens* the addition of glucose suppressed the expression of *tcdD* similar to the effect seen on toxins in *C. difficile* (**paper IV**). This indicated that similar regulatory mechanisms modulated the activity of TcdD in these two systems. A protein suggested to negatively influence toxin expression in *C. difficile* is TcdC, encoded by the last ORF in the *C. difficile* PaLoc (Hundsberger *et al*., 1997). As *C. perfringens* does not have a TcdC homologue any importance of TcdC in down regulating TcdD activity was not supported in these experiments. In the event CodY (see above) has a role in modulating toxin expression in *C. difficile* this could also be the case in the *C. perfringens* model-system as the CodY protein of these bacteria share 58% amino acid similarity. Another interesting candidate regulator of toxin synthesis in *C. difficile* is the Tex homologe. In *B. pertussis* Tex is involved in the regulation of the pertussis toxin expression (Fuchs *et al*., 1996**)** and we observed a reduction of the Tex levels in *C. difficile* during high toxin production indicating that Tex could be a suppressor of toxin synthesis.

Further studies on the functions of the *C. difficile* proteins CodY, Obg, LepA and Tex are likely to be fruitful for the understanding on how toxin A and B are regulated with respect to energy generation and GTP and amino acid levels in the cell.

We found that up-regulation of the toxin was preceded by induction of several specific alternative energy producing pathways in *C. difficile* apparently in response to a state were energy and the levels of free amino acids are low **(paper V).** This could be interpreted such that the toxins are produced by *C. difficile* as a "last resort" response in order both to access nutrients by colonic tissue degradation in the current host and inducing diarrhea to get transmitted to other hosts. Examples of such alternative pathways were some involved in fermentation of amino acids, and the autotrophic synthesis of acetyl-CoA by anaerobe respiration of $CO_2$ (Wood-Ljungdahl pathway), and two pathways involved in the mixed fermentation of succinate and ethanol. A connection between $CO_2$ assimilation and toxin synthesis was observed also when we increased the levels of bicarbonate in the medium **(paper I)**.

Several of the metabolic pathways induced prior to high toxin production had butyric acid as major end product**.** This compound was also shown to accumulate in the medium and in addition, to stimulate toxin production per se when exogenously added to *C. difficile* **(paper II)**. One of the operons induced during high toxin production included the genes for 3-hydroxybutyryl-CoA dehydrogenase [3-hbd], acetyl-CoA acetyltransferase and butyryl-CoA dehydrogenase. Operon structure similar to that of the *C. difficile* 3-hbd operon is present in several Gram-positive bacteria. However, the metabolic function of these seems to differ. In *C. difficile*, induction of the 3-hdb operon paralleled that of an operon including succinate semialdehyde dehydrogenase indicating energy generation also by the simultaneous fermentation of succinate and ethanol as present in *C. kluyveri* (Gottschalk, 1986). In *C. acetobutylicum* a related 3-hbd operon is present and part of the biphasic metabolism of acidogenesis/solventogensesis during the fermentation of glucose (Dürre *et al*., 1995). Solventogenesis in *C. acetobutylicum* is a process where acidic fermentation products (i.e. acetate and butyrate) are reinternalized and oxidized to acetone and butanol. This shift generates a delay in cell death due to the prevention of further increase in the toxic acidic end-products and allows for the differentiation of the organism into a dormant endospore. A proteomic study of proteins specifically

induced during solventogenesis in *C. acetobutylicum* did however not reveal any proteins similar to those found by us during elevated toxin synthesis (Schaffer *et al.*, 2002; **paper V**), neither did we see any correlation between up-regulation of toxin production and sporulation in *C. difficile*. Yet another example of a different mode of regulation of a 3-hbd operon is found in *B. subtilis.* Here, the operon is under a $\sigma^E$ dependent regulation and thus, only expressed during the intermediate stages of sporulation just after the formation of a mother cell and a pre-spore (Bryan *et al.*, 1996). In *B. subtilis* this operon is subjected to glucose dependent catabolite repression and is likely to be involved in fine-tuning the metabolic state of the mother cell in response to carbon source availability during endospore formation.

We found that exogenous butyric acid induced toxin synthesis in *C. difficile*, whilst adding butanol to cultures had the opposite effect (**paper II**). As high levels of butyric acid will be converted to butanol at the expense of ATP and excess butanol can be converted to butyric acid during generation of ATP these seemingly odd observations in fact supported the connection between scarcity of energy and toxin induction.

Like glucose, the presence of cysteine in the *C. difficile* culture medium prevented both the induction of the alternative metabolic pathways and the toxin synthesis. The rapid uptake of most cysteine (approximately 10 mM) during the logarithmic growth phase indicated that cysteine was used in fueling pathways. Whereas the energy production from glucose is easy to understand (glycolytic fermentation) it is not clear how enough energy was generated from cysteine alone to prevent induction of the alternative metabolic pathways as the state of knowledge of how cysteine is metabolised in Gram-positive bacteria, and especially clostridia, is poor. One possibility alternative is that cysteine was decomposed to alanine by desulfurases and this process is coupled to the transfer of protons across the cell membrane. This additional proton motive force could then be used for synthesis of ATP. The accumulation of alanine in the culture supernatant and the induction of a $F_0F_1$ ATPsynthases specifically in $PY_{10}$ cultures indicated that this mode of ATP generation from cysteine could be present in *C. difficile* **(paper V).** Future studies of this pathway in *C. difficile* and the coupling to toxin expression are needed.

## Up-regulation of toxins by biotin limitation

Limiting the level of biotin in defined media is a potent ways to induce high toxin yields in *C. difficile in vitro* (Yamakawa *et al.*, 1996; **paper I**). Biotin is a water-soluble vitamin that plays important roles in carboxylation reactions in the cell including carbohydrate, lipid and amino acid metabolism (McMahon, 2002).

During toxin synthesis induced by biotin starvation in *C. difficile* the enzyme phosphoribosylformylglycinamidine (FGAM) synthetase is up-regulated (Maegawa *et al.*, 2002). The FGAM synthetase is catalyzing the fourth step in the *de novo* synthesis of purines and is dependent on the presence of glutamine to function. The biotin effect can be bypassed by adding glutamine (Yamakawa *et al.*, 1998). A proposed mechanism of the biotin effect is that biotin limitation results in a low level glutamine and that this prevents the proper synthesis of purines resulting in block of DNA synthesis, generation of ATP/GTP and growth (Maegawa *et al.*, 2002). The dramatic decline of growth of *C. difficile* during biotin starvation and the observation that the bacteria tended to lyse already at low OD made comparisons of the *C. difficile* proteome during growth in this defined media and complex medium difficult. But it was obvious that the protein expression patterns differed markedly between these two growth styles **(paper I)**. Interestingly, also during elevated toxin expression in *C. difficile* growing in complex media there was indications that the pathway for *de novo* purine synthesis was induced, as the enzyme catalyzing the ninth step in this pathway, 5-phosphoribosyl-4-carboxamide-5-aminoamidazole (AICAR) transformylase was slightly up-regulated **(paper V)**.

Man lacks the ability to synthesize biotin and thus depends on receiving this vitamin from various food sources. In a healthy individual fecal excretion of biotin exceeds the diary intake indicating that gut flora also play a role in providing biotin (Combs, 1992). Studies indicate that malnourished patients with inflammatory bowel disease have significantly lower serum biotin levels than healthy controls (Abad-Lacruz *et al.*, 1988). Whether the combination of a poor general nutritional status and a large bowel flora reduced by antibiotic therapy could result in such low biotin levels in the feces that toxin synthesis in *C. difficile* is induced and biotin limitation thus, represents a true risk factor for CDAD, is not known.

## Temperature dependence of toxin expression

Temperature is one of the key environmental signals that pathogenic bacteria exploit in order to successfully express virulence factors at the proper time and location (Konkel & Tilly, 2000). Prokaryotes use several different ways to sense changes in temperature and to translate these in to adequate cellular responses. Temperature can change the supercoiling of DNA often in combination with temperature-dependent alterations of DNA-binding proteins resulting in the silencing of the promoter of the gene to be regulated. An example of such a response in virulence is the transcriptional regulator VirF of Shigella. At low temperatures *virF* is repressed by the binding of H-NS to its super-coiled promoter (Tobe *et al.*, 1993). Also mRNA can directly respond to temperature by forming hairpin structures preventing gene expression and allowing translation only at specific temperature intervals. Examples of this type of regulation is found in *Yersinia pestis* were the mRNA of the transcriptional regulator LcrF responsible for inducing several virulence-related proteins forms stem-loop structures at 25°C that prevents the Shine-Dalgarno sequence from being exposed (Hoe & Goguen, 1993). Also the change in viscosity of the cell membrane may be used to monitor temperature. In *B. subtilis* the two-component system DesK/DesR is involved in regulating the enzymes that modify the saturation of the membrane phospholipids. It is believed that the change of membrane viscosity due to temperature activates the membrane embedded sensory kinase DesK resulting in activation the regulator DesR (Aguilar *et al.*, 2001). The phosporylated DesR then regulates the activity of lipid desaturases. Finally and not surprisingly, proteins can directly respond to temperatures. This type of regulation can either be more general as in the case of H-NS where its overall DNA binding ability is altered by temperature or more specific as the *Salmonella typhimurium* protein TlpA. TlpA binds specifically to its own promoter at low temperatures thereby preventing transcription (Hurme *et al.*, 1997).

No temperature regulation of virulence factors corresponding to the transfer to a temperature representing a mammalian host was known in clostridia until we found that toxins in *C. difficile* were expressed with maximum at 37°C **(paper IV)**. The temperature effect occurred at the level of transcription and was at a higher regulatory level than the nutritional factors known to influence toxin production (i.e. low levels of biotin, cysteine or glucose, elevated levels of butyric acid) **(paper IV)**. This

indicated that temperature is working by controlling the activity of a fundamental factor in toxin expression. The positive auto-regulation of TcdD was here found to be optimal at 37°C **(paper IV)** indicating that temperature dependent expression of toxin A and B in *C. difficile* indeed was mediated by TcdD, the alternative sigma factor controlling PaLoc expression.

TcdD shows similarity to other regulatory proteins in other clostridia such as the BotR, TetR and UviA in *C. botulinum*, *C. tetani* and *C. perfringens*, respectively. All these proteins share similarity with the extracellular function (ECF) sigma factors (Mani *et al.*, 2002). The ECF sigma factors are typically bound and inactivated by an anti-sigma factor often located in the cytoplasmic membrane. Upon adequate environmental stimuli, including temperature (Burger *et al.*, 2000), the anti-sigma factor releases the ECF sigma factor to the cytosol (Hughes & Mathee, 1998). The role of a putative anti-sigma factor for BotR, TetR, UviA or TcdD is however speculative as no cognate anti-sigma factor have been isolated for any of these proteins. The possibility that temperature affects the secondary structure of the TcdD transcript and thus, only allows for efficient translation at certain temperatures is unlikely as the actual level of TcdD mRNA was found to increase upon a temperature shift from 22°C to 37°C. Maybe TcdD senses temperature directly or that the curvature of the *tcdD* promoter is altered by temperature mediating efficient transcription only at 37°C. The specific nature of how *tcdD* is regulated by temperature needs further studies.

## Export of toxins

As mentioned, the *C. difficile* toxins are induced in late logarithmic phase and first accumulate in the cytoplasm followed by release of over 50% of the toxin pool during early stationary phase **(paper III)**. The mechanism of export of toxins A and B by *C. difficile* is however poorly understood. A majority of bacterial proteins to be secreted are transported across the cytoplasmic membrane by the general mechanism, the Sec pathway. The Sec pathway is composed of a proteinaceous channel comprising SecY, Sec E, SecG and inserted into the membrane and a translocation motor consisting of the protein SecA (Mori *et al.,* 2001). Typical Sec-dependent proteins have a signal peptide in their N-terminal part that directs the newly synthesized proteins to the Sec

machinery for further transport across the cell membrane. The translocation of proteins by the Sec machinery is mediated by a thread-like mechanism where the protein is transported through the pore in an unfolded state (Mori *et al*., 2001). The *C. difficile* toxin A and B lack typical signal peptides and their large size (270 and 308 kDa) suggests that exporting these proteins by a thread-like mechanism, delivering them into the supernatant in a denatured form, would represent an especially difficult task and most likely result in the export of nonfunctional proteins. The alternative way would to be exported fully folded. One example of such a transport system is the Tat (twin-arginine translocation) pathway (van Dijl *et al*., 2002). Apart from the fact that it makes use of completely different cellular components the Tat pathway is distinguishable from Sec pathway by two additional critical features. First, it specifically recognizes signal peptides with an Arg-Arg-motif. Second, Tat pathways are capable of transporting tightly folded proteins and even multimeric enzyme complexes. Thus, a main function of Tat pathways seems to be the translocation of proteins that fold too rapidly, or too tightly, to allow their translocation via the Sec pathway. However, no apparent Arg-Arg motif is present in the toxins and thus, the Tat pathway is unlikely to be involved in the export of the *C. difficile* toxins.

Early studies showed that the extracellular toxin levels in *C. difficile* cultures were influenced by various harsh treatments such as elevated temperature, certain antibiotics and major changes of the oxidation-reduction potential of the growth medium (Onderdonk *et al.,* 1979). The kinetics of these responses indicated that this effect was influencing the release rather than the actual synthesis of the toxins and thus, suggested that the toxins are delivered by a specific export mechanism. A correlation between of toxin export and sporulation in *C. difficile* has been also been suggested (Kamiya *et al*., 1992). In this scenario the lysis of the mother cell would release the preformed toxins in a process similar to that of the enterotoxin in *C. perfringens* (Rood, 1998). In our studies we did not find any correlation between appearance of spores and toxin production in either laboratory strains or clinical isolates of *C. difficile*. In addition, by proteomic analysis we did not find that bulk proteins were released together with the toxins showing that lysis and toxin release are two separate processes (**paper V).** Instead, we found that during high toxin export a 47 kDa protein with homology to the outer membrane protein TolC typically found in Gram-negative bacteria accumulated in the supernatant (**paper III**). Three TolC monomers embedded into the outer membrane form a pore with an inner diameter of

35Å (Lewis, 2000). The TolC of Gram-negative bacteria docks to specific membrane fusion proteins located in the inner membrane and this event mediates a conformational change in TolC that allows passage of various compounds from the cytosol to the exterior. In *E. coli* examples of such membrane fusion proteins are HlyB and AcrB and EmrB (Lewis, 2000). Interestingly, we found that the *C. difficile* TolC homologue was part of a putative operon also encoding Hly and Acr homologues (**paper III**). We speculate that this Hly/Acr/TolC operon may represent a novel Gram-positive secretion apparatus playing an important role in toxin secretion in *C. difficile.*

## Proposed model for toxin regulation in *C. difficile*

*C. difficile* senses the levels of specific amino acids and the general energy level of the cell and when limiting levels of these components occur several alternative energy producing pathways are induced. When these pathways become insufficient for maintaining growth and the cell is facing a prolonged period of low levels of amino acids and ATP/GTP, toxin synthesis is initiated as one final survival strategy (the other being sporulation). Examples of, negative regulatory proteins sensing the ATP/GTP and amino acids are CodY, LepA, Obg and Tex. After lifting their repression of the *tcdD* promoter, TcdD rapidly accumulates due to its auto-regulation and out-competes other sigma factors for RNA polymerase. This allows for a massive expression of toxins.

The presence of glucose slows the uptake and fermentation of key regulatory amino acids (cysteine, proline, isoleucine, leucine and valine) and prevents the induction of toxin production. This is supported by the fact that after *C. difficile* has fermented on amino acids and thus, lowered the levels of these in its environment, glucose is less efficient than these amino acids in preventing the induction of toxin synthesis. Amino acids are thus sensed more directly than glucose. Cysteine is the most effective amino acid suppressing toxin induction. This amino acid is rapidly utilized and during growth in cysteine rich medium the ATPsynthase was specifically up-regulated indicating that this type of metabolism can generate ATP, perhaps by building up the proton motive force. Several of the alternative energy producing pathways induced prior to toxin production results in the accumulation of butyric acid in the

environment. High level of butyric acid is itself a factor triggering toxin expression. Thus, the down-regulation of these pathways by amino acids or glucose is also indirectly suppressing toxin production by reducing the butyric acid levels.

The mechanism of induction of toxin synthesis by biotin limitation remains obscure. Insufficient levels of biotin impair several important metabolic pathways of the cells, notably those involving carboxylation reactions. It is possible that the biotin effect is linked with disruption of purine synthesis and prevention of an effective $CO_2$ assimilation thereby limiting the ATP/GTP pool and energy status of the cell and inducing toxin synthesis.

TcdD synthesis, crucial to toxin production occurs via a positive auto-regulatory loop that was temperature dependent with maximum expression of TcdD at 37°C. At 22°C or 42°C no TcdD or toxin production was present regardless of the nutritional status of the growth media. Several of the metabolic enzymes induced during low levels of cysteine ($PY_0$ medium) and thus, high toxin production were also found to be temperature regulated similar to TcdD and the toxins. This suggested that TcdD is involved also in the regulation of these pathways or that a more global regulator affecting the expression of these is inhibited during low temperature. A dependence on cysteine and temperature for toxin expression similar to that in *C. difficile* is also found in *B. pertussis*. The regulation of toxin production in this organism depends on the regulatory protein Tex. A similar protein was here found to be present in *C. difficile* and suppressed at high toxin production. It is possible that Tex is a key regulatory protein in both the general change of protein expression due to altered temperature and involved in the regulation of the *C. difficile* toxins A and B.

## Concluding remark, novel therapy and prophylaxis of CDAD

As current therapy of CDAD depends on additional antibiotics and is accompanied by a risk of recurrences of at least 20% one main goal of this work was do find novel ways to prevent and treat CDAD. Relapse probably occurs due to a combination of remaining *C. difficile* spores not affected by antibiotic therapy and poor reestablishment of the protective large bowel flora. The results presented in this thesis show that certain amino acids are effective in down-regulating toxin synthesis in *C.*

*difficile*. As the administration of amino acids to the colon is expected not to cause any major disturbance of the GI flora we propose that the local delivery of specific toxin down-regulating amino acids may become a novel and ecological strategy for therapy and prophylaxis of CDAD.

We have in preliminary (unpublished) *ex vivo* experiments shown that effective down-regulation of toxin synthesis in *C. difficile* by cysteine also takes place in a fecal milieu. To feces from CDAD patients and healthy volunteers (inoculated with *C. difficile* to obtain toxin production) we added 10 to 30 mM cysteine. This resulted in dramatically lowered toxin levels compared to controls where cysteine was omitted. For example, the toxin levels after 24 h of incubation was more than 100-fold lower if 30 mM cysteine was present. During these experiments the viable counts of *C. difficile* was lowered approximately 5-fold. No major alterations of the major bacterial genera in the colon such as *Lactobacillus*, *Bifidobacteria* or *Bacteroides* were observed. These experiments showed that also in a complex environment mimicking the human colon cysteine efficiently and specifically targeted *C. difficile* toxin production. In these experiments we found another putative risk factor for CDAD, differing between healthy individuals namely, the capacity of feces per se to degrade the produced *C. difficile* toxins (unpublished). In feces from CDAD patients incubated *ex vivo* exposed to cysteine, toxin levels were lowered to almost zero within 2h (unpublished). We ascribe the result to the synergistic effect of cysteine stopping toxin synthesis by *C. difficile* and the rapid degradation of preexisting toxins by proteases in the feces. When preparations with high levels of *C. difficile* toxin were mixed with feces from healthy human volunteers the toxin half-life ranged from 25-60 minutes apparently due to degradation by normal digestive enzymes such as trypsin and chymotrypsin. In additional experiments different strains or clinical isolates of *C. difficile* were inoculated into fecal suspensions from healthy donors and screened for their ability to support toxin production we could consistently see that each individual feces typically supported either high or low levels of toxin production. Apparently, in addition to a presence of *C. difficile* the specific composition of the patient's feces (e.g. nutrients, bacterial flora, proteolytic enzymes) is likely to affect the attack rate of CDAD and disease outcome.

Studies of the intestinal tract of several types of mammals showed that glucose concentrations in the feces were below 0.4 mM in the colon (Ferraris *et al.,* 1990).

The probable reasons for this low level is that glucose is the most important carbohydrate in human food and thus, rapidly absorbed in the GI tract and in addition, that any glucose remaining in the gut lumen is the preferred sugar for intestinal bacteria. The uptake of free amino acids in humans is maximum in the mid-lower jejunum and is near complete as the feces leaves the ileum and enters the colon (Bos *et al.,* 2002). This indicates that in order for amino acids or glucose to be used for suppression of *C. difficile* toxin synthesis of *in vivo* these compounds must be delivered locally, i.e. into the colon. The general preference of enteric bacteria for glucose as fuel indicates that glucose would be short lived in the large bowl lumen. In addition, our studies showed that an already induced toxin synthesis in *C. difficile* does not stop upon the addition of glucose. In contrast, cysteine was both rapidly acting and remained so despite a presence of the fecal flora. I therefore suggest that specific amino acids (i) may serve as novel therapeutic agents to down-regulate toxin production in *C. difficile*, if (ii) administered locally to the colon by a capsule that is dissolved specifically at this location of the human GI tract. I think that this strategy of combining inhibition of *C. difficile* toxin synthesis by e.g. cysteine without further disturbing the colonic microbiota together with the intrinsic capacity of feces to degrade *C. difficile* toxins can be used clinically to prevent or limit the pathological effects of toxinogenic strains of *C. difficile* in the colon of patients receiving antibiotic therapy.

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