Antibiotics and *Clostridium difficile*

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CONTENTS

REFERENCE LIST

APPENDICES

DECLARATION

The author performed all the investigations presented in this thesis unless otherwise stated.

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ABSTRACT

Clostridium difficile is an important cause of antibiotic-associated diarrhoea in the developed world. Antibiotics, especially broad-spectrum agents, predispose the patient to *C. difficile* disease through depletion of the protective bowel flora and antibiotics have also been shown to affect the toxin production of *C. difficile*.

The aims of this thesis were to investigate the antibiotic susceptibility patterns of *C. difficile* with relation to the S-type of the isolates over a period of 18 months. The relationship of sub-MIC antibiotics and the growth and toxin production of *C. difficile* had not been extensively studied and so this relationship was investigated using ELISA for toxin A, RT-PCR for the transcription profile and analysis of total cell protein using 2D gel electrophoresis and MALDI-TOF mass spectrometry.

Detailed growth curves were performed on strains NCTC 11223, the sequenced strain 630 and an endemic isolate 338a. Toxin A was shown to be produced upon entry to stationary phase in agreement with other studies. OD_{600} was found to be a good predictor of growth phase and allowed this measurement to be used for subsequent experiments.

MICs were performed on 186 random isolates of *C. difficile* collected during an 18-month epidemiological study to investigate the patterns of sensitivity to six different antibiotics. No evidence of resistance was seen to the two treatment antibiotics and all strains were resistant to cefoxitin (MICs 64-256μg/ml), the antibiotic used in most selective media. Most strains (98.9%) had intermediate resistance or were resistant to ceftriaxone. The MIC_{50} and MIC_{90} of the strains to amoxycillin and clindamycin were very close (8 and 16 for amoxycillin and 16 for clindamycin) but the range of MICs was great. Clindamycin resistance was common with 67% of strains resistant (MICs of ≥ 8μg/ml), 25% with intermediate resistance (MIC ≥4μg/ml) and only 8% sensitive (MICs of ≤2μg/ml). Twelve isolates from six different patients had very high resistance to clindamycin with MICs ≥128μg/ml. Multiple isolates from the same patient, taken at different times, showed changes in susceptibility patterns over time. The only major change in susceptibility over the time period was in clindamycin resistance; some strains appearing to become more resistant while others became less resistant. No differences were apparent in the MIC_{50} and MIC_{90} of the different S-types of *C. difficile* identified, although some S-types were present

in very small numbers. No links between antibiotics prescribed and susceptibility patterns were found.

Three strains (NCTC 11223, strain 630 and endemic isolate 338a) were cultured in sub-lethal concentrations of the six antibiotics (1/2, 1/4 and 1/8 of the MIC) over 104 hours and growth and toxin A measured three times a day. The effects varied between strain and antibiotic. The most common effect on the growth of the strains was to increase the initial lag period by ca. 4h compared to the antibiotic-free controls though the clindamycin resistant strain NCTC 11223, (MIC ≥512μg/ml) showed no lag whatsoever in comparison to the controls when grown in this antibiotic. The most common effect on toxin A production was in the onset of toxin elaboration. Normally toxin began to appear in low levels in early stationary phase before accumulating to high levels by the start of decline. In the presence of sub-MIC antibiotics this onset appeared before that of the antibiotic-free controls. This effect was seen with metronidazole, amoxycillin and clindamycin, rarely with vancomycin and never with cefoxitin. Results suggest a very complex relationship between the effects of growth and toxin production, which is strain dependent.

RT-PCR was used to analyse transcripts (toxins, *groEL*, *tcdC*, *tcdC* and 16S RNA) with and without the presence of sub-MIC antibiotics. There was a major problem with DNA contamination which was eventually solved but very little RNA was extracted using the Qiagen kit. Throughout this work only transcripts from the 16S RNA genes were clearly seen though the sensitivity was improved by using the RT Sensiscript enzyme.

The protein profile of strain 630 in the presence and absence of ceftriaxone was studied using proteomics. 2D gels, MALDI-TOF analysis and a *C. difficile* MASCOT database were utilised to identify proteins from total cell extracts of strain 630. No differences were found between the protein profiles with and without ceftriaxone but 40 spots were picked from the gels for further identification. The *C. difficile* S-layer proteins were identified along with GroEL and GroES, acetyl Co-A dehydrogenase, NADH oxidase and proteins from the electron transport system. This work has provided essential information on successful procedures for proteomic analysis in *C. difficile* and the MASCOT database will be invaluable for further studies.

PUBLICATIONS

Drummond LJ, McCoubrey J, Smith DGE, Starr JM and Poxton IR (2003). Antibiotic sensitivity patterns in *Clostridium difficile* in geriatric in-patients over an 18-month period. *Journal of Medical Microbiology 52*: 259-263.

Drummond LJ, Smith DGE and Poxton IR (2003). Effects of sub-MIC concentrations of antibiotics on growth of and toxin production by *Clostridium difficile*. *Journal of Medical Microbiology 52*: 1033-1038.

List of Abbreviations

1D	1 dimensional
2D	2 dimensional
AAD	Antibiotic-Associated Diarrhoea
ABC	ATP Binding Cassette
AIM	Anaerobic Investigation Medium
AP-PCR	Arbitrarily-primed PCR
ARU	Anaerobe Reference Unit
ATP	Adenine triphosphate
BHI	Brain Heart Infusion broth
bp	base pair
CDAD	*Clostridium difficile* – Associated Diarrhoea
CDD	*Clostridium difficile* Disease
CDT	*Clostridium difficile* Toxin
CHCA	α-Cyano-4-hydroxycinnamic acid
CMB	Cooked Meat Broth medium
CCEY	Cycloserine-Cefoxitin Egg-Yolk agar
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
EDTA	Ethylenediamine-tetraacetic acid
ELISA	Enzyme-linked Immunosorbent Assay
FAA	Fastidious Anaerobe Agar
GTP	Guanidine triphosphate
IAA	Ioadacetamide

IgA	Immunoglobulin A
IgG	Immunoglobulin γ
IM	Intra-muscular
IPG	Immobilised pH Gradient
IV	Intra-venous
kDa	kilodaltons
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization – Time of Flight
MIC	Minimum Inhibitory Concentration
MLS	Macrolide, Lincosamide and Streptogramin B resistance determinant
MPRL	Microbial Pathogenicity Research Laboratory
mRNA	Messenger ribonucleic acid
MW	Molecular weight
NCCLS	National Committee for Clinical Laboratory Standards
NCTC	National Collection for Type Cultures
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PaLoc	Pathogenicity locus
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
PMC	Pseudomembranous colitis
PPY	Proteose peptone yeast extract medium
ORF	Open reading frame
RAPD	randomly amplified polymorphic DNA

REA	Restriction enzyme analysis
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RT-PCR	Reverse Trancriptase – Polymerase Chain Reaction
SDS	Sodium dodecyl sulphate
sIgA	Secretory immunoglobulin A
tRNA	Transfer RNA

INTRODUCTION

1.1 History and description of *Clostridium difficile*

Clostridium difficile is a Gram-positive, spore-forming bacillus. It forms sub-terminal spores and its dimensions are ca. 1μm by 3μm. It is an obligate anaerobe and is the most common cause of nosocomial antibiotic-associated diarrhoea (AAD). It was first encountered in 1935 as a commensal in the faeces of healthy infants (Hall & O'Toole, 1935) and received its name because in the beginning it was slow and difficult to grow. In 1969 it was reported in a study by Hammarstrom and colleagues to be involved in the formation of anti-colon autoantibodies in germ-free rats. Rats monocontaminated with *C. difficile* developed antibodies to a colon polysaccharide likely resulting from a break in tolerance caused by administration of the bacterium. It emerged as a problem in the late 1970s when it became implicated as the cause of AAD and pseudomembranous colitis (PMC) after broad-spectrum antibiotic use (Tedesco *et al.*, 1974). The frequent association of AAD with the antibiotic clindamycin gave rise to the term clindamycin-associated diarrhoea. In 1978, *C. difficile* was identified as the source of a cytotoxin found in the stools of patients with PMC (Bartlett *et al.*, 1978; Larson *et al.*, 1978) which led to increased interest in the organism. Since then *C. difficile* disease (CDD) has emerged as a major problem in hospitals and long-term care facilities with an increase in patient morbidity and mortality. Acquisition of *C. difficile* often leads to extended hospital stays while the patient recovers, which results in increased costs on the hospital. One estimate is that in an average sized hospital 100 cases of *C. difficile* infection can be

expected each year with an extra annual cost of £400,000 and 2100 lost bed days (Spencer 1998a). The cost of treatment per individual patient may reach four thousand pounds (Shek *et al.*, 2000). The length of stay and the associated costs due to *C. difficile* disease in the United States was estimated at over $1.1 billion per year by Kyne *et al.* (2002).

1.2 *C. difficile* disease

C. difficile is involved in a spectrum of disease ranging from mild, self-limiting diarrhoea through severe diarrhoea to PMC with toxic megacolon or perforation (Borriello, 1998).

The most common form of CDD is a mild, antibiotic-associated illness characterised by watery diarrhoea containing mucus but no blood and the absence of systemic symptoms. This self-limiting form of the disease generally resolves itself upon cessation of the antibiotic regime. It is thought that 20% of mild diarrhoea associated with antibiotics is caused by *C. difficile* (Kelly & LaMont, 1998).

Asymptomatic carriage of *C. difficile* is extremely common within hospitals and long-term care facilities. Asymptomatic carriers and contaminated surfaces appear to act as a reservoir for *C. difficile*. Epidemiological studies within hospitals indicate a high level of carriage, symptomatic and asymptomatic, in the patient population. McCoubrey *et al.* (2003) found a carriage rate of 30% within two geriatric wards and McFarlane *et al.*, (1989) found a similar figure of 26% within a general medical ward.

C. difficile also causes a more serious type of AAD in the form of colitis without pseudomembrane formation. This is characterised by abdominal pain, malaise,

anorexia, nausea and watery diarrhoea. The patients may also present with dehydration and a mild fever.

Pseudomembrane formation is a characteristic manifestation of full-blown *C. difficile* colitis. The pseudomembranes appear as yellowish plaques, 2-10mm in diameter, and are formed from mucin and fibrin which interlace the polymorphonuclear leukocytes present as part of the inflammatory response (Borriello, 1990). *C. difficile* is the aetiological agent of PMC with the organism being cultured in 95-100% of cases.

Fulminant colitis is the most serious of all forms of CDD and occurs in only 3% of cases (Kelly & LaMont, 1998). Serious complications such as perforation of the colon and death occur more often with this form of CDD than all the others. Patients with fulminant colitis may suffer severe abdominal pain, high fever, diarrhoea, chills and severe leukocytosis (up to 40000 white blood cells/μl) (Kelly & LaMont, 1998).

Table 1.1 below shows the incidence of *C. difficile* within groups of patients. This table has been adapted from the review by Kelly and LaMont (1998).

Table 1.1 Incidence of *C. difficile* in different populations

Subject population	***C. difficile* positive**
Pseudomembranous colitis	95-100%
Antibiotic-associated diarrhoea	10-30%
Hospital in-patients	20%
Healthy adults	0-3%
Healthy neonates and infants	25-80%

As mentioned previously *C. difficile* is the aetiological agent of PMC which is a serious form of CDD. Various studies, including one carried out in our lab, have

shown a carriage rate of ca. 15-30% in hospital in-patients. This is unsurprising as these patients are in contact with contaminated surfaces and both symptomatic and asymptomatic carriers of the organism. It has been estimated that 10-30% of AAD is caused by *C. difficile*. Studies on healthy adults within the population estimate a carriage rate of 0-3% but this rises to 4.2-15.3% in one study of Japanese individuals (Kato *et al.*, 2001). The reasons for this are unclear but dietary factors are likely to affect the composition of the bowel flora.

1.3 Risk factors for *C. difficile* disease

The main risk factors for developing *C. difficile* disease are: (i) use of broad-spectrum antibiotic agents to disrupt the colonic microflora; (ii) age and immune status of the host; (iii) presence of the host in an environment where spores are prevalent; (iv) virulence of the infecting strain (Borriello, 1998). Other risk factors include the presence and severity of underlying disease, anti-ulcer medications, nasogastric tubes and the administration of multiple antibiotics (Bignardi, 1998). The role of antibiotics in CDD will be discussed at length later in this Introduction due to its importance as a risk factor. The prevalence of CDD in elderly patients is interesting and it is likely that this relates to the state of their immune system compared to healthy adults. Decreasing levels of antibodies due to increasing age appears to be involved in this general susceptibility. These elderly patients often have underlying disease which further affects the immune system. There is also some evidence that they have a less protective normal flora which allows *C. difficile* entry into this niche (Hopkins *et al.*, 2002). The last risk factor, mentioned above, is due to the existence of non-toxigenic strains which are not associated with disease and the fact that some toxigenic strains appear to be more prevalent and possibly more

virulent than others (Wilcox & Fawley, 2000). Strains with very different levels of toxin production have been described including VPI 10463 which is regarded as extremely virulent in the hamster model and produces high levels of both toxins. The three strains used in this study produce different levels of toxin A (see Chapter 3) which would naturally affect the ability of these strains to cause disease due to the fact that CDD is mediated by release of the toxins into the colon. There are now over 20 toxinotypes (Rupnik *et al.*, 2003), which may reflect very different levels of production including the loss of one or two toxins. Any difference in their ability to cause disease is not well characterised.

The course of the disease can be summarised by Figure 1.1 adapted from Kelly and LaMont (1998).

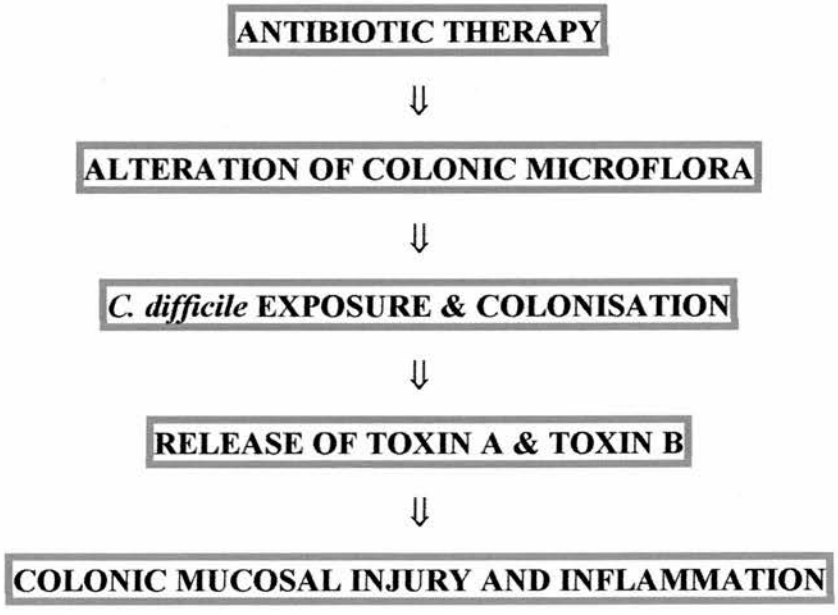

Figure 1.1 Pathogenesis of *C. difficile* diarrhoea and colitis.
This flow diagram shows the progression of the disease caused by toxigenic strains of *C. difficile*.

This figure illustrates the most important stages of the process of setting up CDD. Antibiotic therapy leads to the depletion of the protective healthy flora and allows

acquisition and overgrowth of *C. difficile*. Once *C. difficile* is established the two toxins can be released to cause mucosal injury and disease.

1.4 Gut flora and colonisation resistance

When broad-spectrum antibiotics are administered to patients their action seriously depletes the normal gut flora. Subsequently the numbers of viable bacteria in the colon decrease to such an extent that the factors that prevent *C. difficile* growth (as yet not clearly defined) are removed (Larson & Welch, 1993). "Colonisation resistance" is no longer apparent. If *C. difficile* is present it can survive this onslaught due to its resistance, natural and acquired, to many of these antibiotics and the survival capacity of spores. If the organism is sensitive to the antibiotic, as is the case with amoxycillin, it can survive in the gut through the formation of spores. Once the antibiotic levels drop the spores germinate and cause disease. *C. difficile* can now proliferate, either from endogenous spores or an exogenous source. As *C. difficile* causes disease when the normal flora of the colon is disrupted, antibiotics become the most important and easily alterable risk factor. Starr *et al.* (1997) have shown that by limiting the use of cefotaxime, one of the most common precipitating antibiotics, the incidence of *C. difficile* disease was drastically reduced even in patients not receiving cefotaxime. So, altering the use of cefotaxime not only removed a major risk to the patients but also built up a form of host immunity (seen also in measles epidemiology) which prevented the spread of *C. difficile*. The risk of cefotaxime therapy (and that of its active metabolite desacetylcefotaxime) has been elegantly shown recently through the use of a triple-stage chemostat model of the human gut (Freeman *et al.*, 2003). A stable mixture of *C. difficile* and the normal gut flora were analysed before and after the addition of cefotaxime and its metabolite. As expected

decreases in bacterial counts (notably bifidobacteria and bacteriodes), proliferation of *C. difficile* and subsequent increases in cytotoxin levels were observed.

The normal colonic flora of healthy adults and infants has been shown by Borriello and Barclay (1986) to be capable of preventing colonisation by *C. difficile*. This phenomenon is dependent on the presence of viable organisms; faecal filtrates are not inhibitory to *C. difficile* growth (Borriello & Barclay, 1986; Larson & Welch, 1993). The faecal flora of infants was shown to be inhibitory to *C. difficile* growth by Yamamoto-Osaki *et al.* (1994) in continuous culture. Addition of a mixture of amino acids depleted from the medium resulted in an increase in *C. difficile* numbers. They suggested that depletion of amino acids from the medium (used by the normal flora) may be responsible for the inhibition of *C. difficile* rather than any inhibitors produced by the flora. There are many theories in regard to the actual mechanism by which colonisation resistance operates. Perhaps the most accepted is that the gut flora of the healthy colon out-competes *C. difficile* for nutrients, including amino acids, and space.

The bacteria responsible for this inhibition of *C. difficile* colonisation have not been fully characterised but candidates include the lactic acid bacteria *Lactobacillus* spp., *Bifidobacterium* spp. and the most common inhabitant of the colon, *Bacteriodes* spp. (Rolfe *et al.*, 1981; Rolfe, 2000). The importance of the lactic acid bacteria is apparent in the flora differences between breast-fed and bottle-fed infants. Borriello and Barclay (1986) found the faeces of breast-fed infants to be significantly more inhibitory to *C. difficile* growth than the faeces of bottle-fed infants. Duffy *et al.* (1999) states that feeding infants breast milk leads to the proliferation of lactic-acid bacteria whereas feeding them formula leads to a proliferation of coliforms. Breast

milk contains unique factors, *N*-acetylglucosamine-containing oligosaccharides, which stimulate the growth of these lactic-acid bacteria (Duffy *et al.*, 1999). An early study by Cooperstock *et al.* (1983) found that infants who were fed on formula milk were four times more likely (62 versus 16%) to be colonised by *C. difficile* than breast-fed infants. Human breast milk, in addition to its role in the proliferation of lactic acid bacteria, contains secretory IgA (sIgA) which has been shown to inhibit toxin A binding to hamster epithelium (Dallas & Rolfe, 1998). The secretory component found in breast milk also has an inhibitory effect on toxin A binding (Dallas & Rolfe, 1998). The fact that breast-fed faeces is significantly more inhibitory to *C. difficile* highlights the possible role of the lactic acid bacteria in colonisation resistance. However, many in-vitro studies using the lactic-acid bacteria have shown them to be less inhibitory than the more complex mix of bacteria found in healthy adult faeces (Fuller, 1991).

It is extremely important that the factors and organisms contributing to colonisation resistance be elucidated to give us a greater understanding of the disease and its outcome. It is also important to find out which bacterial species or products have an inhibitory effect on *C. difficile* to further the field of pre- and probiotics as an alternative treatment for *C. difficile* diarrhoea and colitis. Probiotics are already used to treat *C. difficile* disease utilising organisms such as *Saccharomyces boulardii* and *Lactobacillus* GG with varying degrees of success. Alternatives to the treatment antibiotics vancomycin and metronidazole are required due to the high incidence of recurrence of symptoms (5-24%) once therapy stops (Wilcox *et al.*, 1998).

1.5 Toxins A and B description and mode of action

C. difficile causes damage through its two high molecular weight exotoxins which are some of the biggest bacterial toxins known. Toxin A is an enterotoxin with a predicted molecular weight of 308kDa and toxin B is a cytotoxin with a predicted molecular weight of 270kDa (Dupuy & Sonenshein, 1998). Toxin A also has cytotoxic properties but toxin B exceeds this cytotoxicity by an estimated factor of 10-1000 (100-1000 - Hundsberger *et al.*, 1997; 10 – Riegler *et al.*, 1995). The toxins damage the colonic mucosa by glycosylating small GTP-binding proteins from the Rho subfamily (Dillon *et al.*, 1995; Just *et al.*, 1995a; Just *et al.*, 1995b). The carboxy terminal domain of toxin A binds to carbohydrate moieties on the surface of the mucosal epithelium. It is predicted that after the tight junctions are disrupted on the damaged cell, the toxin B receptor becomes exposed leading to both toxins being endocytosed via coated pits (Karlsson *et al.*, 1999). They then are free to glycosylate GTPases at Threonine 37 rendering them inactive. This leads to the depolymerisation of actin filaments, disruption of the cytoskeleton, cell rounding and cell death (Dillon *et al.*, 1995; Just *et al.*, 1995a; Just *et al.*, 1995b). Intoxicated cells show a retraction of cell processes and cell rounding caused by a loss of F-actin and an increase in G-actin. The breakdown of the actin cytoskeleton and the inability of the modified Rho proteins to regulate tight junction complexes leads to increased colonic permeability and subsequent watery diarrhoea (Nusrat *et al.*, 2001).

Toxin A has been shown to be a potent activator and chemoattractant for human leukocytes (Knoop *et al.*, 1993). In addition to its direct cytotoxic action toxin A also affects neurons in the enteric nervous system and induces them to release substance P (Castagliuolo *et al.*, 1997). Recruitment of neutrophils is then thought to

follow through substance P activation of mast cells. This recruitment leads to increased intestinal secretion and mucosal inflammation (Wershil *et al.*, 1998). Pseudomembranes are formed by sloughed epithelial cells, leukocytes and fibrin (Knoop *et al.*, 1993). The mucosal surface underneath this pseudomembrane contains petechial lesions and ulcerations of the mucosa consisting of epithelial necrosis and a marked leukocyte infiltration of the lamina propria (Knoop *et al.*, 1993).

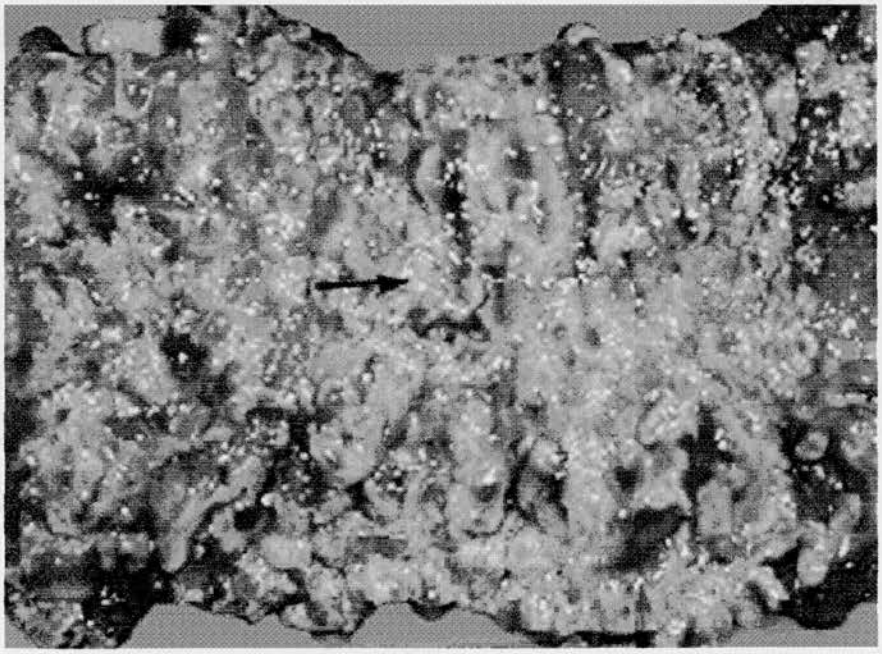

Figure 1.2 Pseudomembrane formation

This figure shows a section of colon affected by PMC. The arrow points to the pseudomembranous plaques which can measure up to 2cm in diameter. Taken from Sanders at http://radiology.uchc.edu/code/444.htm

Another interesting property of toxin A is its role in adherence. Borriello *et al.* (1988) showed poorly virulent or avirulent non-toxigenic *C. difficile* strains adhered less well than highly virulent *C. difficile*. Another experiment from this study showed that co-administration of toxin A with a non-toxigenic strain resulted in improved

adherence of that strain. It is clear that toxin A affects adhesion of *C. difficile* directly or mediated through the damage it causes to the mucosal epithelium.

1.6 The Pathogenicity Locus (PaLoc) or Toxigenic Element

The toxins are encoded on a chromosomal genetic unit of 19.6kb that is called the toxigenic element (Hammond *et al.*, 1995) or the Pathogenicity Locus (PaLoc) (Braun *et al.*, 1996). Figure 1.3 shows the layout of the element in diagrammatical form. This 19.6kb section on the *C. difficile* genome is present only in toxigenic strains; replaced in non-toxigenic strains by a section of 115 base pairs (Braun *et al.*, 1996; Cohen *et al.*, 2000b). This element is conserved even in strains of very different toxigenicity (Hammond *et al.*, 1997). In addition to the toxin genes this region contains three accessory genes (Hundsberger *et al.*, 1997). Outside the PaLoc there resides a few interesting sequences upstream and downstream which may be important in pathogenicity. Braun *et al.* (1996) showed that the PaLoc is integrated into one site on the chromosome in a unidirectional orientation. They also found the borders of the PaLoc to be almost perfectly conserved in all the toxigenic strains. This defines the PaLoc as a distinct genetic element.

Analysis of the PaLoc by Braun *et al.* (1996) did not identify any plasmid-, phage- or transposon-like elements. They also found no characteristic inverted repeats which are common at the borders of mobile genetic elements. Potential tRNA genes, which are often used as integration sites for mobile genetic elements, were also absent. From this it can be concluded that PaLoc by itself is not a mobile genetic element.

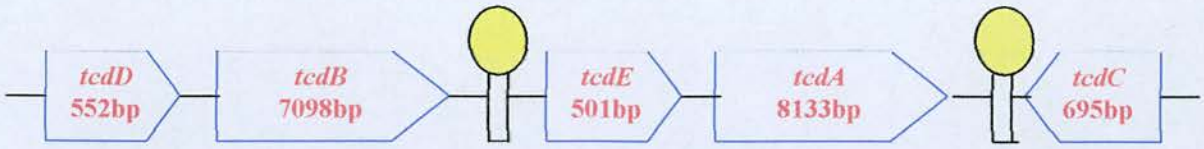

Figure 1.3 The Pathogenicity Locus of *C. difficile*.

Figure 1.3 shows the genes present in the PaLoc. The arrows represent the direction the genes are transcribed and included is their size in base pairs. The stem loop structures are also shown. The stem loop between *tcdA* and *tcdC* acts as a bidirectional transcription terminator. The alternative terminology is shown here in the order of the above figure. *txe1*(*tcdD*), *toxB*(*tcdB*), *txe2*(*tcdE*), *toxA*(*tcdA*), *txe3*(*tcdC*). Adapted from Braun *et al.* 1996.

1.6.1 The toxin genes

The toxin genes are located within 1.4kb of one another (Hammond & Johnson, 1995). Toxin A, the enterotoxin, is 309kDa in size and is coded for by *tcdA*. Toxin B, the cytotoxin, is 270kDa in size and is coded by *tcdB*. *tcdA* consists of 8133 base pairs and *tcdB* of 7098 base pairs. Sequencing by von Eichel Strieber *et al.* (1992) showed 48% identity between the two toxin genes and a further 15% similarity of amino acids. It was postulated that the genes had arisen by gene duplication. This theory is likely as the proteins also share a number of structural features. These include a putative nucleotide binding site, a central hydrophobic region, four conserved cysteines and a long series of repeating units at their carboxyl ends (putative receptor) (von Eichel Strieber *et al.*, 1992). The genes are consecutive and transcribed in the same direction (Hammond *et al.*, 1997).

1.6.2 The accessory genes

The accessory genes are *tcdC, tcdD and tcdE* and what is known about their functions is described below. Where a definitive function is unknown putative ones are proposed. As can be seen in Figure 1.3, *tcdD and E* are transcribed in the same

direction as the two toxin genes. *tcdC* is transcribed in the opposite direction. Below, the putative functions of these accessory proteins.

tcdC

The product of this gene is thought to be involved in a negative regulatory function primarily due to the fact that when it is transcribed, the other gene transcripts of the PaLoc are at low levels (Hundsberger *et al.*, 1997). *tcdC* is an open reading frame of 695 base pairs (bp) and its resulting protein, TcdC, is composed of polar amino acids which make it highly acidic. It also contains stretches of repetitive amino acids at various lengths. After a database search to find similarities between TcdC and other proteins, Hundsberger *et al.* (1997) found no match for TcdC. Spigaglia and Mastrantonio (2002) carried out a molecular analysis on the PaLoc of 51 strains of *C. difficile* to look for polymorphisms in the accessory proteins. Within the toxigenic strains 25% contained polymorphisms in the *tcdC* gene. No correlation between disease severity and variant TcdC strains was found though it is possible that changes in this protein would affect toxin production. For example, they found 1 allele with a nonsense mutation that reduced the TcdC protein from 232 to 61 amino acids. Lack of a functional protein may lead to abrogated repression of the toxin genes. This may be a partial explanation for the differences in virulence common between the strains of *C. difficile*.

tcdD

The *tcdD* gene (552bp) encodes TcdD, a protein of ~22kDa, which was postulated to be a positive regulator of the transcription of the toxin genes (Hundsberger *et al.*, 1997; Moncrief *et al.*, 1997). It is a protein of basic nature and has a helix-turn-helix motif. This is a typical attribute of DNA-binding bacterial response regulators

(Moncrief *et al.*, 1997). It also has an unusually high content of lysine residues within its C-terminal region (Moncrief *et al.*, 1997). Its function was further investigated by a database search by Moncrief *et al.* (1997) who found 27.2% sequence identity (41.3% similarity) with UviA, a putative positive regulator of bacteriocin production in *Clostridium perfringens*. It also had 21.8% sequence identity (41.9% similarity) with ORF-22 (open reading frame-22), a putative positive regulator of botulinum neurotoxin. When Moncrief *et al.* (1997) expressed *tcdD* in *E. coli* with the toxin genes they found that when it was expressed in *trans*, the expression of toxin A increased 500-fold and toxin B increased 800-fold. This confirmed their hypothesis that *tcdD* is involved in the positive regulation of toxin production. Recent discoveries by Mani and Dupuy (2001) and Mani *et al.* (2002) have shown it to be an alternative sigma factor that confers on *C. difficile* RNA polymerase the ability to recognise the *tcdA* and *tcdB* promoters. Three things suggested that TcdD was a sigma factor and not a positive regulator. The fact that it is unable to bind to the toxin promoters; that it interacts directly with the core of RNA polymerase and upon binding to RNA polymerase allows it to recognise the toxin promoters. TcdD also stimulates its own synthesis and responds to environmental stimuli. Toxin expression is affected by various environmental stimuli including growth phase, amino acids, antibiotics and the presence of a rapidly metabolised carbon source (see section 1.8). Karlsson *et al.* (2003) have also shown *tcdD* and the toxins to be controlled by temperature with low levels at 22°C and 42°C and maximum levels at 37°C. Mani *et al.* (2001) demonstrated TcdD production to be upregulated upon the entrance to stationary phase and repressed in

the presence of glucose; mirroring the effect on toxin production.

tcdE

tcdE is an open reading frame of 501bp intergenic to the two toxin genes. It is located 122bp downstream from the *tcdB* stop codon and 727bp upstream from the start codon of *tcdA* (Hammond and Johnson, 1995). The putative protein encoded is of 166 amino acids and has a predicted molecular weight of 19kDa. Predictions suggest the protein is membrane spanning, highly hydrophobic with a hydrophobic N-terminus and a highly charged C-terminus. Hundsberger *et al.* (1997) showed *tcdE* to be polycistronically transcribed along with *tcdD* and the toxin genes suggesting an important role for this protein in *C. difficile* toxin production or release. It was thought that a role in the release of toxin was possible due to the lack of any signal peptide on the toxins. Tan *et al.* (2001) tested this hypothesis and found evidence of holin function in TcdE. Holin proteins are cytolytic proteins produced by bacteriophages that cause lysis in the infected cell to facilitate release of the new phage. Transformation experiments using *Escherichia coli* showed that when *tcdE* was expressed it led to a decrease in culture turbidity which could be seen as lysis of the cells. Electron microscope pictures of *E. coli* expressing *tcdE* clearly showed disruption of the membrane illustrated by numerous membrane folds and a merging of the cytosol and periplasm in places. They failed to visualise or purify TcdE and so further studies are underway but the results achieved present a good case for the holin function of this protein.

1.6.3 Upstream of the PaLoc

3.2kb of the upstream nucleotide sequence and 5.2kb of the downstream were analysed in the paper by Braun *et al.* (1996). Upstream of the toxin B gene was *cdu2*,

cdu2', *cdu1* and *tcdD*. Another ORF was discovered (*cdu3*) but this was not examined any further as they only had the sequence for 238bp of the 3' end. All the ORFs were orientated in the same direction as the *tcdB* gene. Downstream of the toxin A gene, five ORFs were identified- *tcdC* and *cdd1-4*. All the ORFs were orientated in the opposite direction to *tcdA* apart from *cdd1*.

Figure 1.4 shows this region in diagrammatical form.

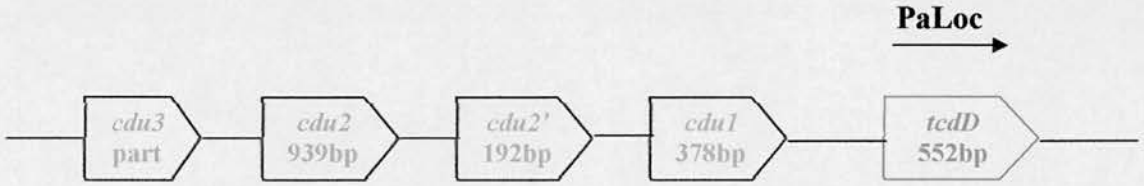

Figure 1.4 Upstream of the PaLoc

Figure 1.4 shows the region upstream of the PaLoc including *tcdD* of the PaLoc. The arrows show the direction the genes are transcribed. The gene sizes are given in base pairs. In non-toxigenic strains there is a stem loop structure present after *cdu1* that is likely the transcription terminator for this gene. In toxigenic strains this is where the PaLoc integrates. Adapted from Braun *et al.* 1996.

cdu1

cdu1 is a gene of 378 base pairs and the promoter is homologous to the consensus sequences of promoters described by Rood and Cole (1991) for Gram-positive bacteria (Braun *et al.*, 1996). Cdu1, the product of the *cdu1* gene, showed a high similarity to repressor proteins described for some Gram-positive bacteria. Cdu1 and three of these repressor proteins showed 50% similarity (13.1% identity) to one another (Braun *et al.*, 1996) with Cdu1 showing the most similarity to Pen1, a β-lactamase repressor of *Bacillus licheniformis*. Mec1, a methicillinase repressor, and Bla1, a β-lactamase repressor, from *Staphylococcus aureus*, were the other two. The proteins are especially similar in the DNA binding domain and their N-terminal

regions which contain basic lysine residues. These residues may facilitate a protein-DNA binding function. Due to the similarity of Cdu1 to these proteins, it is tempting to speculate that it has a DNA binding character and is involved in some form of as yet unexplained regulation.

cdu2 and cdu2'

cdu2 is a gene of 939 base pairs. Its promoter is also similar to that of Gram-positive bacteria. Cdu2 has 48% similarity to NapA, a Na^+/H^+ antiporter of *Enterococcus hirae*. NapA has 12 membrane-spanning domains and using the algorithm of Klein, Braun *et al.* (1996) were able to determine that Cdu2 had 11. When they searched *cdu2'*, an ORF of 192bp downstream of *cdu2*, they found an additional putative membrane-spanning domain. If these two proteins can functionally link then it would represent a functional protein of 376 amino acids similar to NapA which has 383. When Braun *et al.* (1996) fused these two amino acid sequences together it increased the relatedness between NapA and the fusion protein by 51%. Other similarities between the proteins include the hydrophobicity and the p*I*. Cdu2' has no promoter sequence and this suggests co-regulation of the two genes. All of these findings suggest that Cdu2 and Cdu2' form a functional protein that acts as a Na^+-transporter in *C. difficile*.

1.6.4 Downstream of the PaLoc

Figure 1.5 represents the downstream region of the PaLoc.

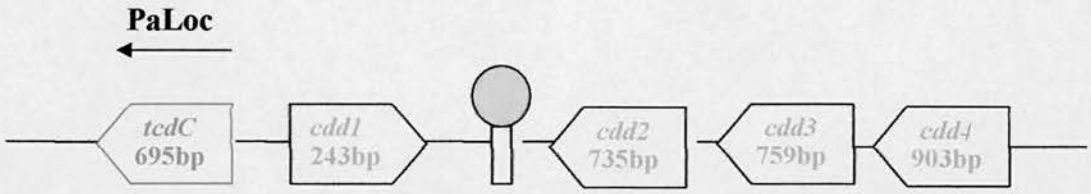

Figure 1.5 The downstream region of the PaLoc.

Figure 1.5 shows the genes downstream of the PaLoc including *tcdC*. A stem loop structure is also shown. The arrows represent the direction of transcription and the length of the genes in base pairs is given. Adapted from Braun *et al.* 1996.

cdd1

cdd1 is 243 base pairs in length. The predicted amino acid sequence of the protein showed no similarity to any others in the database at that time.

cdd2-4

These three ORFs are situated within a few base pairs of each other. Their lengths are 735, 759 and 903 base pairs respectively. The organisation of the genes suggests a functional linkage between them. No promoter could be found for the three genes but it is not unusual for promoters to be situated far from the ORF, perhaps further upstream from the sequence determined so far. Cdd4 was found to have 56.2% similarity (43.2% identity) to the putative permease (BcrA) of *Bacillus licheniformis* bacitracin-resistance ABC transporter (Braun *et al.*, 1996). BcrA is part of a family of ATP-binding proteins which have been found in a number of transport systems. Walker A and B, two motifs thought to form a nucleotide binding pocket, are highly conserved among these ATP-binding proteins and are also conserved in Cdd4. As

well as BcrA, the bacitracin-resistance ABC transporter contains two hydrophobic proteins (BcrB and C) which are presumed to form a diffusion channel. *cdd2* and *cdd3* both encode hydrophobic proteins which according to the algorithm of Klein predicted six membrane-spanning domains in each protein. The two proteins could form 12 such domains through the membrane to form a channel like those in other bacterial transport systems. Cdd2 has 38.9% similarity (19.7% identity) with BcrB and Cdd3 has 34.5% similarity (13.8% identity) with BcrC. These similarities are high for membrane-spanning domains as it is common for them to differ greatly in their primary structures (Braun *et al.*, 1996).

All these findings strongly suggest that *cdd2-4* encode an ABC transporter in *C. difficile*. ABC transporters have many different substrates and functions including the expulsion of antibiotics and the secretion of various virulence factors.

The role of these genes up- and downstream from the PaLoc and their potential role in virulence and pathogenicity may explain why the PaLoc is always integrated where it is.

1.7 Transcription and regulation of the PaLoc

Hundsberger *et al.* (1997), using a semi-quantitative reverse transcriptase PCR, showed that all five open reading frames within the PaLoc were transcribed. They also found growth-phase dependence in the transcription patterns of this element. During early exponential phase high *tcdC* transcripts were present and transcripts of the other genes were low. This situation was reversed in the late exponential and stationary phase with low levels of *tcdC* mRNA and high levels of the other four gene transcripts at this stage. This gave further credence to the thought that TcdC has some kind of negative regulatory function.

Hundsberger *et al.* (1997) and Hammond *et al.* (1997) both support the theory that the toxin genes are transcribed both polycistronically and monocistronically as they found that when the PaLoc was transcribed, a 17.5kb transcript that hybridised with probes for *tcdA, B, D* and *E* was present. Individual transcripts which hybridised with only one of the probes were also found. The 17.5kb transcript is thought to be processed to individual transcripts. The processing first makes the toxin A transcript available leaving behind an 8.1kb section which hybridises with B and D or B and E. Further processing then yields the individual mRNAs. This can be seen in Figure 1.6.

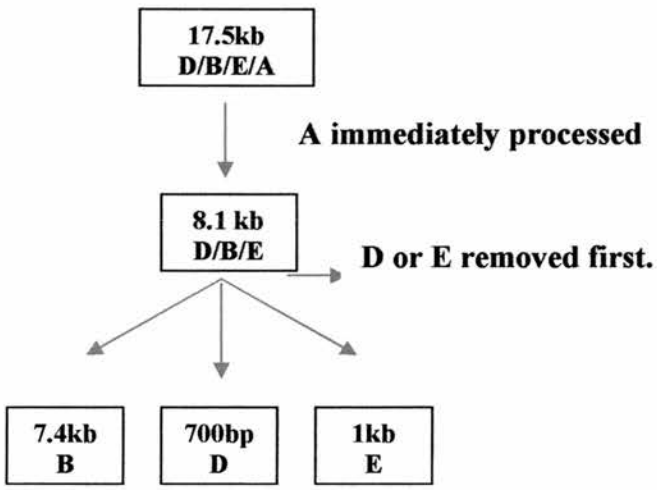

Figure 1.6 Processing of the 17.5kb transcript.

The 17.5kb transcript is produced and the toxin A mRNA is immediately processed to leave an 8.1kb transcript that hybridises with probes from *tcdD*, *tcdB* and *tcdE*. Further processing follows with D or E being removed from the transcript. Finally the individual transcripts of the genes are produced. Adapted from Hundsberger *et al.* 1997.

1.7.1 Promoters

Primer extension analysis by Hundsberger *et al.* (1997) suggested that in the late exponential phase (and the stationary phase) the two toxin genes are transcribed

using their own promoters. In the early exponential phase when high levels of *tcdC* transcripts are present, the 17.5kb read-through fragment is transcribed at low levels. Dupuy and Sonenshein (1998) agree as they found lower numbers of the large, 17.5kb fragment suggesting that it is not responsible for the majority of toxin transcripts. Primer extension by Hammond *et al.* (1997) showed a transcriptional initiation site 236bp upstream of the translational start codon for *tcdD*. This sequence contained promoter sequences TATTTT and TATGTC corresponding to the –10 and –35 regions. The sequence and the spacing compares well with a consensus clostridial promoter proposed by Young *et al.* (1989). Upstream of the promoter for the PaLoc (-40 to –60) is a region rich in AT and A stretches which are seen in many Gram-positive promoters. Several *E. coli* promoters contain these stretches which function as binding-sites for the α subunit of RNA polymerase. This region is likely to be the promoter for the 17.5kb transcript.

Hundsberger *et al.* (1997) attempted to locate the promoter sequences for the genes of the PaLoc in their 5' untranslated regions. All of the putative promoters for the genes of the PaLoc fit well with the promoters for other clostridial genes. One of their characteristics is long spacing between the transcription and translation initiation sites (Hundsberger *et al.*, 1997). Using primer extension analysis of *tcdA* and *B* to find their transcription initiation sites and their promoters showed that the proposed promoter sequences match well with the actual sequences. The –35 and –10 boxes of the predicted *tcdC,D* and *E* promoters match well with the actual sequences of the *tcdA* and *tcdB* promoters. This suggests that the proposed *tcdC, D* and *E* promoters will be relatively accurate. The predicted promoter of *tcdD* has an interesting perfect 26bp palindrome covering the –35 region. The promoters-

predicted or deduced- contain other features like Gram-positive promoters. They have A clusters at positions –41 to –45 and conserved nucleotides next to the –35 and –10 boxes. All the promoters apart from p*tcdC* have a –35 box identical to that of ultraviolet-inducible promoters found in the *C. perfringens* bacteriocin locus. This raises questions as to how the stationary phase genes (*tcdA,B,D,E*) are affected by environmental stress.

1.7.2 Transcription terminators

Von Eichel-Streiber *et al.* (1990), demonstrated the presence of a stem loop structure between *tcdB* and *tcdE* that they surmised may function as a transcription terminator. This however cannot be the case as the presence of readthrough transcripts between the genes have been found by many. A second stem loop structure is present between *tcdA* and *tcdC*. As there is no readthrough mRNA species between *tcdA* and *tcdC* using *tcdC* or *tcdA* promoters, the stem loop structure between these two genes possibly serves as a bi-directional transcription terminator (Hundsberger *et al.*, 1997).

In non-toxigenic strains the 19.6kb PaLoc is replaced by a region of 115bp (Braun *et al.*, 1996). This region displays no similarity to any sequence within the PaLoc or the database. It does not form an ORF but it contains a stem loop of 20bp and a loop of 12 unpaired bases located 12 bases downstream of *cdu1*. In non-toxigenic strains this loop may function as a transcription terminator of *cdu1*. In toxigenic strains this stem loop is lost which could lead to a lack of termination of *cdu1*. This may be important in the regulation of the PaLoc and result in a polar effect on *tcdD*, the alternative sigma factor of the toxin genes and the next ORF upstream of *cdu1*. The stem loop

may also act as a recognition site for the integration of the PaLoc element (Braun *et al.*, 1996).

1.8 Environmental effects on *C. difficile* and the PaLoc

Various studies over the years have sought to elucidate the conditions which have an effect on the production of the two toxins. This was done primarily to seek understanding of the disease process and the virulence of the organism as a whole. Many conditions have been shown to affect toxin production in *C. difficile*. These include growth phase which has already been mentioned, temperature, fermentable carbon sources, amino acids, biotin concentration and sub-inhibitory concentrations of antibiotics.

Growth phase

The growth phase effect on toxin production has already been discussed. Toxin first appears during late exponential and peaks in stationary phase with high levels present throughout decline. However, a study by Karlsson *et al.* (1999), using different defined and complex media, found no dramatic effect on the rate of toxin production at different growth rates and phases. Observations from the majority of groups disagree with these findings as toxin clearly starts to appear with the onset of stationary phase. The relevance of this finding *in vivo* is unclear, as the classical growth states exist less precisely.

Temperature

Onderdonk *et al.* (1979) showed that an increase in temperature from 37°C to 45°C resulted in a 100-1000-fold increase in toxin production. No change in the viable cell density or spore formation was seen with this increase. The stress of a temperature increase will activate the heat stress response and if the toxin promoters are sensitive

to such a response then it is likely that toxin production is affected. The onset of an inflammatory reaction to the administration of antibiotics, the initial factor in the disease, may result in an increase in temperature which could result in an in-vivo effect on toxin levels. Onderdonk *et al.* (1979) showed a small change in the caecal temperature of hamsters given antibiotics, which if it occurs in humans could be important in the progression of disease.

Oxidation-reduction potential

Onderdonk *et al.* (1979) changed the Eh of a continuous culture experiment from –360mV to +100mV. This caused a 10-100-fold increase in toxin production, measured by cytotoxicity assay. The effect occurred very quickly (4hrs) after the increase in Eh suggesting that it was due to a change in the release of the toxins from the cell. The Eh is important in the proton motive gradient and any changes would be expected to affect membrane transport and permeability.

Sub-inhibitory concentrations of antibiotics

There has been little work done in this area and the studies that exist often disagree. Onderdonk *et al.* (1979) added sub-inhibitory concentrations of vancomycin, penicillin and clindamycin to their continuous culture set-up and reported that vancomycin and penicillin, but not clindamycin increased the levels of toxin produced. Vancomycin and penicillin at 0.1μg/ml caused 100-1000-fold increases in toxin, measured by cytotoxicity assay. Clindamycin at 0.5μg/ml caused no increase in toxin levels. This agreed with the results of Barc *et al.* (1992) who found no increase in toxin production when they treated *C. difficile* with clindamycin. They did not use any other antibiotics in their study. The paper by Honda *et al.* (1983) did find an effect with sub-inhibitory concentrations of clindamycin. They found

clindamycin (at 1μg/ml) and cephaloridine (at 1.25μg/ml) to stimulate cytotoxin and enterotoxin production. Tetracycline had no effect in this study. As well as their undisputed effect on the protective bowel flora, antibiotics may cause an increase in temperature in the host intestine which may in turn affect toxin production of *C. difficile*. More work is needed in this area to clarify the effect of sub-inhibitory antibiotics on *C. difficile*.

Studies in other bacteria have shown various effects caused by sub-inhibitory concentrations of antibiotics. Braga *et al.* (2000) showed sub-MIC concentrations of cefodizime to decrease the virulence of *E. coli* by interfering with bacterial adhesion to human epithelial cells. This was thought to occur by interfering with the physio-chemical characteristics of the bacterial cell surface. A later study by Braga *et al.* (2001) found the adhesiveness of *S. aureus* to be affected through the inhibition of expression of adhesins on the cell surface. Dal Sasso *et al.* (2003) showed the adhesiveness of both *E. coli* and *S. aureus* to be decreased by sub-MIC concentrations of gemifloxacin. Levner *et al.* (1977) demonstrated enterotoxigenic *E. coli* or *Vibrio cholerae* grown in the presence of the antibiotic lincomycin, an inhibitor of protein synthesis, to produce elevated levels of heat-labile enterotoxin (*E. coli*) or choleragen (*V. cholerae*). The appearance of filamentous forms of bacteria have often been reported (Lorian, 1993; own observations) demonstrating the ultrastructure effects caused by sub-inhibitory concentrations of antibiotics. Coyle *et al.* (2003) showed that concentrations of clindamycin and linezolid, alone and in combination with penicillin, reduced the early release of streptococcal pyrogenic exotoxin A: a problem in severe *Streptococcus pyogenes* infection. Linezolid was also shown to decrease virulence factors in *S. aureus*, affecting α- and δ-haemolysin

and in *S. pyogenes*, streptolysin O and DNase (Gemmel & Ford, 2002). Sub-inhibitory concentrations of β-lactams and other cell wall antibiotics were shown by Nichterlein *et al.* (1996) to reduce the production of listeriolysin in *Listeria monocytogenes* in concentrations which did not affect growth and would therefore not induce morphological changes in the bacteria. Ohlsen *et al.* (1998) investigated the effects of sub-inhibitory concentrations of 31 antibiotics on the expression of the *S. aureus* alpha-toxin gene (*hla*) and found the β-lactams to strongly induce its expression and clindamycin to completely inhibit its expression.

Glucose concentration

Dupuy and Sonenshein (1998) found the presence of excess glucose in culture media to repress toxin expression and production though growth in this situation was enhanced as expected. Other fermentable carbon sources, such as fructose, had the same effect. Non-fermentable carbon sources, such as starch or sucrose, had no effect on toxin production or growth. This suggested that toxin production was controlled by catabolite repression. Karlsson *et al.* (1999) agreed with these results though they found the opposite effect in a defined medium. In the defined medium, glucose-starvation produced lower toxin levels instead of enhanced ones. This decreased the case for catabolite repression as a regulator of toxin production. Their explanation for this, if not catabolite repression, was that perhaps glucose starvation leads to consumption of amino acids and the resultant shortage of amino acids signals the induction of toxin synthesis. This suggested that starvation for, or metabolism of, certain amino acids rather than glucose is coupled to toxin production and regulation.

Biotin concentration

Yamakawa *et al.* (1996) studied the effect of biotin concentration on the production of toxins. Biotin is a vitamin which is essential for growth in *C. difficile* and other bacteria. They found that in a biotin-limited medium, bacterial growth was decreased as expected but toxin production was greatly increased. This increase in toxin levels was due to an increase in de-novo production of toxin rather than an increase in the release of toxin from the cell. This was found using data gathered from sonicated cell extracts and culture supernatant. Biotin is a prosthetic group in certain carboxylation-catalysing enzymes so perhaps this is important in the role it plays in toxin expression. These biotin-containing enzymes may produce metabolic intermediates that play a role in regulation of toxin production. This effect occurred in all toxigenic strains tested suggesting that it is a conserved phenomenon in these isolates. Biotin is produced by the natural flora of the intestine, and the disruption of these in antibiotic therapy may be important in setting up this effect and therefore the onset of disease. The successful use of bacteriotherapy in some cases may be due partly to the reinstatement of biotin in the intestine. Karlsson *et al.* (1999) also studied the effects of biotin-limitation on toxin expression and achieved results that agreed with Yamakawa *et al.* (1996). They postulated that this occurred because biotin is required for most CO_2-fixation reactions, for example the first step of fatty acid synthesis where acetyl-CoA is converted to malonyl-CoA by acetyl-CoA carboxylase. In *E. coli* fatty acid and protein synthesis are coupled and may be co-ordinately regulated. The toxin genes may be uncoupled from this regulation and are therefore able to be expressed. As well as the upregulation of toxin production during biotin-limitation, Karlsson *et al.* (1999) found several 22kDa proteins to be

upregulated. Although no mention of TcdD was made, this is the size of the putative positive regulator of toxin expression and is a possible candidate for the identity of this protein.

Follow-up experiments by Yamakawa *et al.* (1998) showed that adding biotin to biotin-limited media inhibited the enhanced toxin production originally caused by low levels of biotin. This is important as biotin therapy may be an option in the treatment of CDD. They also found that by adding asparagine, glutamic acid or glutamine at a concentration of 10mM they could achieve similar toxin-inhibiting properties as biotin. Lysine was also found to inhibit toxin production and it also inhibited growth of the organism.

Amino acid concentrations

In the Yamakawa *et al.* (1998) paper mentioned before, the inhibitory effects of asparagine, glutamic acid, glutamine and lysine were studied and found to be inhibitory to toxin production. Karlsson *et al.* (1999) found that addition of nine amino acids (cysteine, glycine, isoleucine, leucine, methionine, proline, threonine, tryptophan and valine) downregulated toxin production. The follow-up study (Karlsson *et al.*, 2000) showed seven of the amino acids to exhibit moderate suppression of toxins and proline and particularly cysteine to greatly affect toxin production. When Ikeda *et al.* (1998) increased the concentrations of histadine, methionine, valine, isoleucine, proline and leucine, toxin A and B production increased 6.9-fold and 32-fold respectively. An increase in isoleucine concentration produced the most marked effect. The effect of these amino acids, especially isoleucine, leads to the possibility that products in the metabolic pathways of these

amino acids may play a role in triggering the regulatory genes involved in toxin production.

Bicarbonate concentration

Karlsson *et al.* (1999) observed that elevating the bicarbonate concentration in the medium increased toxin levels by 10-fold. An explanation for this may be that this is likely to affect several biosynthetic reactions including biotin-dependent carboxylation. In some bacteria carboxylation is coupled to amino acid anabolism which in turn may be linked to toxin expression.

In all of these areas more work is needed to elucidate the role of media as different groups using different media produce different results. In 1992, Kamiya *et al.*, found a correlation between cytotoxin production and sporulation although in non-toxigenic strains the lack of the PaLoc does not mean that they cannot sporulate. It may be that the stresses that cause the organism to produce toxin also have an effect on sporulation. They both occur during the stationary phase so this is the most likely explanation. More work is needed to qualify the situations that affect the regulation of the PaLoc genes.

1.9 Other virulence factors

S-layer proteins

C. difficile has on its outer surface a crystalline array of two glycoproteins which make up the S-layer. They were first described by Kawata *et al.* (1984) who reported the presence of two proteins of differing molecular weights. These different sized proteins each form a lattice which superimpose on one another to form the complete

S-layer. The inner lattice is hexagonally arranged and is thicker than the outer, squarely arranged lattice (Cerquetti *et al.*, 2000).

Many Gram positive and Gram negative organisms possess an S-layer and they have many different functions. They can function as protective coats, barriers to antibiotics and other toxic substances and be involved in adhesion. In members of the Archaea they are often the only layer outwith the plasma membrane and are involved in cell shape and division (Sleytr & Beveridge, 1999).

The high MW protein of the S-layer ranges in size from 42-56kDa and there is much cross-reactivity between strains in this protein. The lower MW protein ranges in size from 36-45kDa and is more immunogenic and unrelated between different *C. difficile* strains (Cerquetti *et al.*, 1992). The proteins are easily stripped from the cell by different methods including urea, EDTA and guanidine hydrochloride. The guanidine hydrochloride method is used in our laboratory to type *C. difficile* using the MW of the proteins. The most common isolate found in Edinburgh and the UK has the designation 5236 which is the sizes in MW of the proteins when they are run on an SDS gel after extraction with guanidine hydrochloride.

Calabi *et al.* (2001) found the gene that encodes the S-layer proteins and discovered that they are both derived from one ORF (*slpA*) which undergoes post-translation modification to form two distinct proteins. The post-translational processing first involves the removal of a signal peptide followed by cleavage of the protein to form the two functional S-layer proteins. They are extremely abundant in terms of total cell protein and must confer an important function as they are so expensive for the cell to produce. The precursor protein SlpA contains a C-terminal highly conserved anchoring domain to attach the protein to the cell surface (Karjalainen *et al.*, 2001).

The most obvious suggestion for a role for S-layer proteins was in adherence to the gut mucosa due to the fact that they are the most abundant proteins on the surface of the cell. Calabi *et al.* (2002) demonstrated the S-layer proteins binding to the surface epithelium and subjacent lamina propria of the human and mouse digestive tract. They also discovered that *C. difficile* adhered to Hep-2 cells and that this adherence was inhibited with the addition of antibodies to the high MW protein. The use of purified recombinant S-layer proteins led to the discovery that the binding was mediated by the high MW protein. Potential ligands were collagen I, thrombospondin and vitronectin but no binding was seen with collagen IV, fibronectin or laminin.

These results demonstrate that the role of the S-layer in *C. difficile* is in adherence of the bacterium to the gut mucosal tissues and therefore plays an important part in the virulence of this organism.

Flagella

Flagella are important virulence factors in many species where their role in motility, adherence and invasion of the bacterium is an essential part of their ability to cause disease. Delmée *et al.* (1990) reported that *C. difficile* serogroups A1-12, G and K possess flagella on their surface. Figure 1.7 shows an electron micrograph of *C. difficile* and its flagella.

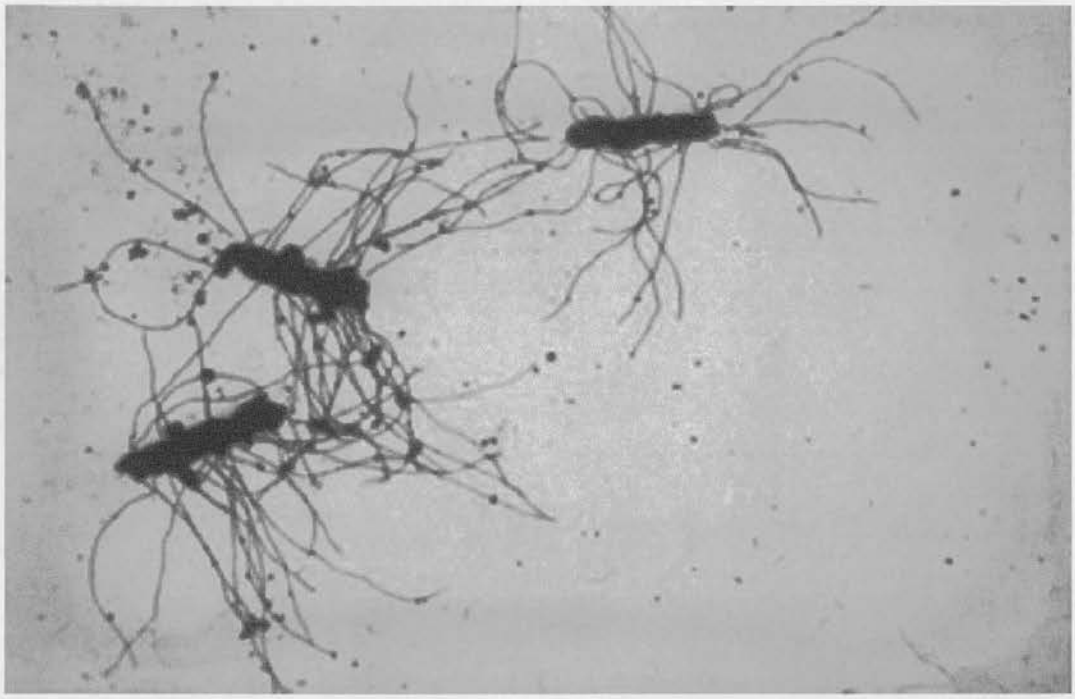

Figure 1.7 Flagella of *C. difficile*

This figure shows *C. difficile* and its flagella negatively stained with phosphotungstic acid. Picture courtesy of IR Poxton.

Almost all cases of PMC are caused by serogroups A, C or H. Serogroup A always possesses flagella, C never has them and H sometimes has them (Delmée *et al.*, 1990). The lack of flagella in other virulent serogroups seems to suggest that flagella are not an essential virulence factor and that adherence of *C. difficile* to the intestinal mucosa is not dependent on flagella. However, flagella do seem to be able to play some part in the adhesion of *C. difficile* to the mucosal tissue as Tasteyre *et al.* (2001) showed two flagellar proteins (FliC and FliD) and crude flagellar extracts to bind to in-vitro mouse caecal mucus. In a non-flagellated strain the association with the mouse cells was 10-fold lower than in the flagellated strain. Although obviously

not essential for the virulence or adhesion of *C. difficile* the possession of flagella may add to the virulence of a toxigenic *C. difficile* strain.

Fimbriae

Fimbriae are commonly involved in virulence as they can mediate adherence of the organism to its environment and undergo phase variation to avoid immune recognition. Borriello *et al.* (1988) investigated the presence of fimbriae in fifteen strains of *C. difficile* to attempt to elucidate the "colonisation factor" of this organism. Five out of the fifteen strains possessed fimbriae but there was no correlation between the presence or absence of these structures and the virulence (toxigenic status) of the isolates. It appears that fimbriae have no part in the virulence of *C. difficile*.

Hydrolytic enzymes

Seddon *et al.* (1990) investigated the presence of tissue degradative enzymes in *C. difficile* as they believed that they may contribute to the tissue damage seen in CDD. There was no direct correlation between toxigenic status, virulence or hydrolytic enzyme production but they found some level of hydrolytic enzyme activity in all strains investigated with some evidence for a higher level of activity in highly virulent strains. *In vivo*, these enzymes would facilitate the release of nutrients from the cells potentially adding to the fitness of *C. difficile*.

Capsule

Dailey *et al.* (1987) demonstrated the anti-phagocytic properties of *C. difficile* to polymorphonuclear leukocytes and it was suggested that the organism may possess a capsule. Capsules are well known virulence determinants and commonly impart anti-phagocytic and immune evasion properties on the bacterium. Davies and Borriello

(1990) tested 15 strains of *C. difficile* for the presence of a capsule. All 15 strains (9 toxigenic and 5 non-toxigenic) were shown to have one. As all 15 strains possessed a capsule it was decided that it had no correlation with the virulence of the isolate. The role of the capsule in disease is unclear as no non-capsulated mutants have been discovered or made to test it.

Other toxic factors

Some strains of *C. difficile* also possess an ADP-ribosylating toxin termed CDT (*C. difficile* toxin). This binary toxin is homologous to the iota toxin of *C. perfringens* and, the C2 toxin of *Clostridium botulinum* types C and D and *Clostridium spriroforme* toxin (Perelle *et al.*, 1997). All these toxins contain two independent protein chains that do not link either covalently or non-covalently. The binding component (MW of 43kDa in *C. difficile* CD196; Popoff *et al.*, 1988) associates with the cell surface and induces the internalisation of the enzymatic component (predicted MW 98kDa; Perelle *et al.*, 1997). The enzymatic component then catalyses the ADP-ribosylation of actin which leads to the disorganisation of the cytoskeleton. The gene for this component, *cdtA*, was cloned and sequenced by Perelle *et al.* (1997) from CD196 and showed 84.6% identity with the enzymatic component of the *C. perfringens* iota toxin. The binding component of CD196 was also sequenced and demonstrated 84.1% identity to the binding component of the iota toxin (Perelle *et al.*, 1997). The CDT toxin was first described by Popoff *et al.* (1988) during screening of various *C. difficile* isolates for ADP-ribosyltransferase activity. The strain CD196 was isolated from a 28-year-old woman who had developed CDD and PMC after amoxycillin treatment (Popoff *et al.*, 1988). Stubbs *et al.* (2000) tested 170 representative strains of *C. difficile* from the Anaerobe

Reference Unit (ARU), Cardiff, to look for the presence of the binary toxin. Fifty-nine of these strains contained the binary toxin, confirmed by immunoblotting and ADP-ribosyltransferase assay. It was calculated that 6.4% of *C. difficile* strains referred to the ARU contain the binary toxin. The role of the binary toxin in virulence is unclear, as the majority of virulent strains do not possess it. It is conceivable however, that this toxin could act synergistically with toxins A and B which may further exacerbate the cellular damage common in CDD to the detriment of the patient. Braun *et al.* (2000) analysed 17 equine *C. difficile* isolates, 41 canine isolates and 4 feline isolates for the presence of the *cdtA* gene. None of the feline or canine isolates contained CDT but 4 of the equine isolates did. They found no association between presence of the toxin and disease severity.

Another, less characterised toxin, has been described by Justus *et al.* (1982). This toxic substance altered motility and the electrical potential of the ligated rabbit intestine. No further reports of this toxin have surfaced.

1.10 Associated antibiotics

Risks of antibiotics

The close relationship between antibiotics and CDD has been the object of many studies over the years. The first antibiotic clearly associated with precipitating CDD was clindamycin in the late 1970s; to such a degree that it led to the term clindamycin-associated diarrhoea. Clindamycin is now considered such a risk factor that it is rarely used in the hospital environment because of its association with the disease. Antibiotics implicated most frequently with AAD include amoxycillin, co-amoxyclav, clindamycin and the cephalosporins (especially third generation) (Bignardi, 1998; Spencer, 1998b). These antibiotics have been shown to increase risk

of AAD by 10-70 times more than agents with narrow spectrums (Gorbach, 1999). One early study by Aronsson *et al.* (1985) found that 1st and 2nd generation cephalosporins (3rd generation cephalosporins were not yet licensed in Sweden) were implicated in CDD 40 times more than narrow-spectrum penicillins. Nelson *et al.* (1994), Golledge *et al.* (1989), Anand *et al.* (1994) and Cartmill *et al.* (1994) also found the extended-spectrum cephalosporins to be the most important risk factor for developing CDD.

The most problematic antibiotics in terms of precipitating CDD appear to greatly affect the large numbers of anaerobes, especially *Bacteroides* spp., in the gut. The 3rd generation cephalosporin ceftriaxone is largely excreted by the biliary tract and can therefore easily alter the flora of the bowel (Spencer, 1998b). Cefotaxime, another 3rd generation cephalosporin, and its metabolite desacetylcefotaxime act synergistically together against *Bacteroides fragilis* and other *Bacteroides* spp., important components of the healthy human flora (Spencer, 1998b).

In a prospective study by Starr *et al.* (2003) in two geriatric wards ceftriaxone was the only specific antibiotic to increase the risk of colonisation with *C. difficile*. Patients receiving other cephalosporins (not ceftriaxone) and non-cephalosporins were also more at risk of colonisation. For the conversion of culture-positive to toxin-positive patients receiving amoxycillin and cephalosporins (other than ceftriaxone) were most at risk. Antibiotics considered a risk factor for the overall conversion from culture-negative to toxin-positive were non-cephalosporins, amoxycillin and cephalosporins other than ceftriaxone.

Table 1.2 Antibiotics and risk of CDD

Frequency of Association with CDD	Antibiotic
Common	Clindamycin
	Cephalosporins – especially 3rd generation
	Amoxycillin
	Ampicillin
Less common	Chloramphenicol
	Erythromycin and other macrolides
	Penicillins e.g. anti-pseudomonal and ureido-
	Tetracyclines
	Trimethoprim sulphamethoxazole
	Quinolones
Rare	Bacitracin
	Parenteral aminoglycosides
	Parenteral metronidazole
	Parenteral vancomycin
	Rifampin
	Sulphonamides
	Teicoplanin

This table was adapted from Mylonakis *et al.* (2001) and Bignardi (1998) and shows the frequency with which different antibiotics are associated with precipitating CDD.

Ampicillin and amoxycillin are also commonly responsible for inducing CDD. Bartlett *et al.* (1981) found that ampicillin, clindamycin and cephalosporins accounted for 80% of the cases of CDD. Silva *et al.* (1984) found ampicillin to be the most important risk factor with it being implicated in 41 out of 130 cases. Antibiotics less frequently implicated in CDD are listed in Table 1.2 above. Anand *et al.* (1994), de Lalla *et al.* (1989), Tedesco *et al.* (1974) and Larson *et al.* (1977) all agree that anti-pseudomonal penicillins are less commonly responsible for inducing CDD. Anand *et al.* (1994) found the ureidopenicillins to have a low propensity to

predispose to CDD. They found that after the administration of 62000 doses of ticarcillin-clavulanate, there were no reported cases of CDD. The 4-fluoroquinolones are also rarely the causal agent of CDD despite large amounts of these agents being used in clinical practice. There is also a suggestion that ciprofloxacin is not bacteriocidal under anaerobic conditions which may mean that it is less disruptive to the flora of the bowel (Smith, 1988). Three reported cases of CDD apparently caused by ciprofloxacin were reported following episodes of salmonellosis which may have contributed or have been the predisposing factor (Spencer, 1998b). In another reported case the patient had received co-trimoxazole (common in inducing CDD) five weeks previous which may have predisposed for this episode (Spencer, 1998b). Golledge *et al.* (1992) investigated 213 patients receiving ciprofloxacin. Twenty-nine were being treated with ciprofloxacin for diarrhoea and 15 developed diarrhoea while on ciprofloxacin. None were culture positive for *C. difficile*.

Restrictive antibiotic policies

One cephalosporin, cefotaxime, was discovered in a study by Starr *et al.* (1997) to be an easily alterable risk factor. Cessation of cefotaxime use led to a marked decrease in the cases of CDD over and above the expected amount. It seemed that the restricted use of this antibiotic not only reduced number of cases of CDD but also protected patients not taking cefotaxime who would have picked up the organism while in the affected wards. It appeared that a reduction in cases of CDD led to reduced numbers of spores contaminating the environment which in turn protected other patients in a "herd immunity fashion", common in measles epidemiology. Reduction in the prescribing of third-generation cephalosporins resulted in a significant decrease in cases of CDD from 65 *C. difficile* positive patients per

100,000 to 20 patients per 100,000 in hospitals in Western Australia (Thomas *et al.*, 2002). McNulty *et al.* (1997) reduced their cefuroxime use in an elderly care unit by 90% and the cases of CDD dropped by a half and they have had small numbers of cases ever since. Ludlum *et al.* (1999) used a restrictive antibiotic policy in an elderly medicine department within a 900-bed teaching hospital in Cambridge. Restrictive use of injectable cephalosporins (usage down by 92%) resulted in a halving of the cases of CDD. The increase in costs was more than offset by the release of hospital beds previously taken up by patients with CDD.

1.11 Proteomics

The discipline of proteomics utilising 2D-gel electrophoresis has been around for decades but it has become more accessible due to the commercial availability of standardised equipment, precast gels and image analysis. Proteomics is used to analyse complex biological systems and is extremely adaptable and useful due to its global approach. Individual proteins expressed can be analysed and studied within the context of total protein produced by the cell. This allows patterns of expression e.g. co-ordinate expression to be studied, and any differences analysed between environmental conditions.

A typical proteomic set-up has three stages; sample preparation, separation of proteins (isoelectric point (IEP) and MW) and the identification of individual protein spots using mass spectrometry or N-terminal sequencing. The key to a good 2D gel is a complete, reproducible sample preparation. For example, if using total cell protein complete lysis of the cells is paramount to achieving the true picture of proteins produced. The next stage is the separation of proteins according to their IEP using gel strips containing an immobilised pH gradient. In the past this step was associated

with many problems as reproducibility of the pH gradient was extremely difficult with hand poured gels. These strips are now precast and are much more comparable even from different batches. In the 2nd dimension the proteins are run from the pH strip onto a SDS gel and separated according to their MW. Different environmental conditions can be compared to one another and spots of interest can be picked from the gel for further analysis.

Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry is the most common tool in identifying these proteins though N-terminal sequencing is also used for proteins that cannot be identified using MALDI-TOF. With MALDI-TOF spectrometry the proteins are trypsin digested and added to a suitable matrix (which confers a charge and allows them to "fly") before being processed through the machine. The peptides form a pattern of peaks that can then be compared to patterns from other proteins in a database. A percentage probability for the protein is given and a match may be found if this protein has been analysed before. One problem with 2D gel electrophoresis is that some proteins, especially hydrophobic and membrane proteins, do not separate or run well on these gels. Some estimates suggest that as little as 10% proteins can be visualised in this way. Low abundance and very large or very small proteins are also not seen. Even with silver staining low abundance proteins are very difficult to visualise.

Proteomic methods are especially useful in an organism for which detailed genomic studies are not yet available. Homology of the separated proteins with proteins from other species allows a prediction in the absence of a homologous sequence database.

1.12 Treatment of *C. difficile* diarrhoea

1.12.1 Antibiotic therapy and recurrences

The first step in the treatment of CDD is, where possible, the cessation of the offending antibiotic and supportive therapy with electrolyte and fluid replacement. Antiperistaltic drugs should, if possible, be stopped as they can worsen or hide the severity of disease. In patients who require antibiotics for treatment of a specific affliction a switch to lower risk antibiotics is preferred. This often leads to the natural recovery of the patient. In the case of frail or elderly patients treatment is often given immediately on emergence of symptoms to reduce the risk of complications. The two agents used in the treatment of CDD are vancomycin, and now more commonly, metronidazole. An oral course of either antibiotic for a period of 7-10 days is normal for uncomplicated CDD. Both antibiotics have similar performance in the treatment of CDD but metronidazole is the agent of choice because it's cheaper, has fewer side effects and is not implicated in the selection of glycopeptide-resistant enterococci.

Few reports of decreased susceptibility to these therapeutic agents have been reported (Barbut *et al.*, 1999; Peláez *et al.*, 2002; Johnson *et al.*, 2000; Brazier *et al.*, 2001). The majority of strains with reported decreased susceptibility to metronidazole have been non-toxigenic and are therefore considered clinically insignificant (Barbut *et al.*, 1999; Johnson *et al.*, 2000; Brazier *et al.*, 2001). A study by Peláez *et al.* (2002) found a resistance rate of 6.3% to metronidazole taking the breakpoint as 16μg/ml (there were no isolates with an MIC of 8μg/ml). A decreased susceptibility to vancomycin (MICs between 8-16μg/ml) was also found. No serotyping or ribotyping was carried out on these strains to give an idea of the clinical significance of these isolates though the clonal relatedness of the strains was

tested using Random Amplification of Polymorphic DNA (RAPD) and there was high levels of diversity between the isolates. This group did not investigate the toxin status of these strains which would be obviously important in relation to human disease. The Anaerobe Reference Laboratory in Cardiff reported in 2001 (Brazier *et al.*, 2001) the first UK isolate with a MIC to metronidazole of 16μg/ml. When typed this isolate was found to belong to PCR ribotype 010, a non-toxigenic group. Interestingly, three isolates from Paris tested by this group had similar MICs and were also non-toxigenic PCR ribotype 010s. Another study reporting decreased susceptibility to metronidazole in horses found 19% of equine isolates had MICs to metronidazole of 8-32μg/ml (Jang *et al.*, 1997) but these strains were not made available for confirmation suggesting they may have been falsely resistant (IR Poxton, personal communication). Barbut *et al.* (1999) found six isolates of *C. difficile* with MICs ranging from 8 - 32μg/ml to metronidazole though five out of these six were of the non-toxigenic serogroup D and therefore of little clinical importance. They found no evidence of decreased susceptibility to vancomycin.

The majority of patients respond well to treatment though there is a significant problem with recurrence of symptoms. It is estimated that up to 20% of patients suffer a recurrence of CDD after the cessation of the treatment antibiotic. The administration of another course of the treatment antibiotics often exacerbates the situation by further diminishing the bowel flora. Recurrences are thought to occur when the faecal levels of vancomycin/metronidazole fall (through the decrease in diarrhoea- levels are higher in liquid stools) which then allows the germination of spores in the gut and a further episode of CDD. One UK study showed that up to 37% of patients require an additional course of antibiotics due to symptom

recurrence (Wilcox *et al.*, 1996). These relapses may be due to a re-emergence of the initial *C. difficile* strain or the reinfection with another. A large study by Wilcox *et al.* (1998) showed that 56% of clinical recurrences are due to infections with different strains. Recurrences and re-infections are notoriously difficult to treat and have led to the search for alternative therapies for CDD. Other antibiotics have been examined namely bacitracin (Young *et al.*, 1985), fusidic acid (Cronberg *et al.*, 1984; Wenisch *et al.*, 1996) and teicoplanin (Wenisch *et al.*, 1996), with or without vancomycin or metronidazole but the trials were small and there was no significant benefit to the patient over the established regime.

1.12.2 Alternative treatments for CDD

Alternative therapies that have been explored for the treatment of CDD and recurrences include biotherapy in the form of probiotics or faecal enemas, immunotherapy and the use of soluble anionic polymers. Many studies and double blind clinical trials have focused on the use of probiotics to limit the severity and length of symptoms.

Probiotics and biotherapy

Saccharomyces boulardii has been extensively studied in relation to the prevention and treatment of CDD with mixed results. An early study in hamsters showed that administration of *S. boulardii* protected them against clindamycin-induced death (Toothaker & Elmer, 1984). One early human study looking at prevention of CDD showed that patients on antibiotics and receiving *S. boulardii* were less likely to develop diarrhoea. In patients who developed CDD however, diarrhoea was not significantly reduced (Surawicz *et al.*, 1989). A later study by the same authors followed 193 patients receiving β-lactams and administered, within 72 hours of

antibiotic therapy, *S. boulardii* or placebo to these patients (McFarland *et al.*, 1995). In the group receiving *S. boulardii*, AAD was significantly reduced (7.2% as opposed to 16.6%) in the placebo group. Another study, by Lewis *et al.* (1998), found no protective effect by *S. boulardii* in elderly patients; the patient group most associated with CDD. In relation to the treatment of CDD one large double-blind placebo-controlled trial by McFarland *et al.* (1994) using 124 patients, supplemented the standard therapy with or without *S. boulardii*. Administration of *S. boulardii* did not prevent recurrences of diarrhoea but patients receiving *S. boulardii* had fewer of these recurrent episodes than the placebo group. However, a follow-up study by the same group (Surawicz *et al.*, 2000) showed a beneficial effect only in combination with high-dose vancomycin therapy. *S. boulardii* has been shown to inhibit toxin A binding to rat ileum (Pothoulakis *et al.*, 1993). A follow-up study identified a protease released by *S. boulardii* as the cause of this inhibition and it was shown to inhibit toxins A and B in human colonic mucosa and prevented toxin A enteritis in rats (Castagliuolo *et al.*, 1999). This protease digests the toxin A molecule and its brush border membrane receptor. This appears to be partly responsible for the protective effect seen in some studies. *S. boulardii*-fed mice challenged with toxoid A demonstrated a 4.4 fold increase in intestinal anti-toxin a IgA. This effect on sIgA was also seen in the study by Qamar *et al.* (2001) using toxin A in mice to stimulate the immune response. This potential stimulatory effect of *S. boulardii* may also contribute to its protective effect. Clearly however, the efficacy of *S. boulardii* to prevent or treat CDD is still debated and the safety issues involved only complicate this further.

The use of *S. boulardii* in patients, both immunocompromised and immunocompetent, is a cause of much debate not least because some question the difference between *S. boulardii* and the invasive, virulent forms of *Saccharomyces cerevisiae*. McCullogh *et al.* (1998) were unable to distinguish between the strains genetically and found *S. boulardii* to be an asporogenous strain of *S. cerevisiae*. Two *S. boulardii* strains were tested in CD-1 and DBA/2N mouse models of systemic disease and showed intermediate virulence compared with virulent and avirulent strains of *S. cerevisiae*. Several reports of fungaemia reported in patients receiving *S. boulardii* further highlights the concerns of using this organism as a biotherapeutic agent.

Other probiotic organisms have been evaluated for the treatment or prevention of CDD including various *Lactobacillus* spp., *Bifidobacterium bifidum* and *Enterococcus faecium*. Many of these studies have failed to produce a statistically significant difference between administration of the probiotic or placebo and many of the promising studies were in very small groups (Alaverez-Olmos & Oberhelman, 2001).

An interesting study by Sambol *et al.* (2002) demonstrated that hamsters colonised with non-toxigenic isolates of *C. difficile* were protected from infection with toxigenic, disease-producing isolates. The problem of using this type of therapy will always be the difficulty in establishing the non-toxigenic strains in sufficient numbers to confer protection.

The administration of yoghurt or faecal enemas has been utilised to facilitate the recovery of patients with CDD. Bhaskarabhatla *et al.* (2001) reported the use of yoghurt containing *L. acidophilus* administered alongside antibiotics to reduce the

incidence of CDD with successful results. Patients ingesting yoghurt had significantly fewer cases of CDD compared to the control group. Bowden *et al.* (1981) used a faecal transplant from a healthy donor to treat 16 patients with PMC with no ill effects and good recovery. Persky *et al* reported in 2000 a successful treatment of CDD of lavage followed by administration of a donated stool to all segments of the colon. Synthetic faecal enemas, which are more acceptable to the patient, have been used with some success. Tvede and Rask-Madsen (1989) used a mixture of 10 faecal organisms and administered them after vancomycin therapy which led to a rapid loss of *C. difficile* and recovery of symptoms. Enemas are, however, very difficult and unpleasant to administer and receive and this undoubtedly reduces the likelihood of them being commonly used and accepted in the hospital environment.

Immunoglobulin and others

Animal studies have indicated that antibodies to the *C. difficile* toxins have a protective role in toxin neutralisation. In a study by Warny *et al.* (1994) the faecal IgA antitoxin A levels were significantly higher in patients who had had one episode of CDAD than in patients who had suffered repeated episodes whose titres were similar to the control group. Eight of the twenty-five non-immunocompromised patients who had CDAD for more than two weeks had faecal IgG levels significantly lower than in patients who had had CDAD for shorter periods. Accordingly, serum IgG and sIgA levels were lower in patients presenting with diarrhoea for more than two weeks or in those who suffered from relapses. Individuals who develop an efficient antibody response may be more likely to become asymptomatic carriers and the failure to produce this protective response appears to predispose to further

episodes of CDAD (Kyne *et al.*, 2000; Kyne *et al.*, 2001). Serum and mucosal antibodies specific to either or both of the *C. difficile* toxins are found in approximately two thirds of the healthy adult population, but the neutralising activity of the antibodies declines with an increasing age. This decline in activity could partly explain the higher incidence of CDD in elderly patients and the differences in antibody responses between individuals could account for the difference in severity of *C. difficile* disease including asymptomatic carriers. The correlation between higher antibody titres and self-limiting disease and production of neutralising antibodies coincides with the resolution of symptoms. High antibody responses in asymptomatic carriers indicate that the humoral immune response may confer protection and that a lower titre predisposes to severe disease (Kelly, 1996).

The apparent importance of antibody in CDD has led to number of small-scale studies looking at passive immunisation in addition to the standard treatment for CDD. Animal studies implied that the presence of toxin A specific antibodies confers protection from disease (Kim *et al.*, 1987; Libby *et al.*, 1982; Corthier *et al.*, 1991). Clindamycin-treated *C. difficile*-exposed hamsters were protected prophylactically with bovine colostral immunoglobulin in a study by Lyerly *et al.* (1991). Oral administration of this preparation also resulted in neutralising antitoxin activity in human faeces. Salcedo *et al.* (1997) reported the successful IV immunoglobulin treatment of two patients with severe PMC who were unresponsive to antibiotics. Four patients with recurrent CDAD were successfully treated with IV immunoglobulin which resulted in the recovery of the patients and no further episodes of CDD (Beales, 2002). Six children with chronic relapsing *C. difficile* colitis were found to have lower levels of anti-toxin A IgG than in healthy children

and adults (Leung *et al.*, 1991). Five of these children were administered IV IgG resulting in clinical resolution of symptoms. Salcedo *et al.* (1997) reported that many commercial immunoglobulin preparations contain significant levels of anti-*C. difficile* antibodies leading to the possibility of these being administered in cases of recurrent or severe CDD. The few success cases reported in the literature need to be expanded for the role of immunoglobulin to be elucidated but it may well be an treatment option in cases of non-respondance to the conventional treatment.

A formalin-inactivated *C. difficile* vaccine was investigated in hamsters (Torres *et al.*, 1995). Results suggested that optimal protection from death and diarrhoea caused by *C. difficile* was achieved with parenteral and mucosal administration of the vaccine. The safety and immunogenicity of a *C. difficile* toxin A and B vaccine was investigated by Kotloff *et al.* (2001). The vaccine was shown to be immunogenic with the best responses arising from the toxoids conjugated to alum rather than the response generated from soluble toxoids. The vaccine was well tolerated by the healthy volunteers with side effects mild and occasional. The vaccine is being further developed for prophylactic use. Ward *et al.* (1999a) investigated the immunogenicity of two toxin A fusion proteins in mice. Parts of the toxin A molecule were bound to an N-terminal polyhistidine tag or to the non-toxic binding domain of tetanus toxin. Significant levels of serum anti-toxin A antibodies were produced and were further increased and mucosal antibodies seen with the addition of a mucosal adjuvant in the form of *E. coli* heat-labile toxin (LT). Coadministration of toxin A-tetanus vaccine with a reduced toxicity LT adjuvant generated significant serum and mucosal antibodies with neutralising activity. Ward *et al.* (1999b) also investigated an attenuated (aromatic mutant) *Salmonella enterica* serovar Typhimurium vaccine

which expressed a toxin A non-toxic domain. Anti-toxin A serum and mucosal antibodies were increased and the serum from immunised mice was found to neutralise toxin A cytotoxicity.

The use of a soluble anionic polymer (GT160-246) was developed and tested for neutralisation properties against toxins A and B (Kurtz *et al.*, 2001). The results were very promising with protection of *C. difficile*-infected hamsters but during a phase 2 clinical trial the research was abandoned (personal communication, I.R. Poxton).

1.13 Typing of *C. difficile*

Over the years many methods have been used to distinguish between *C. difficile* strains. Before the advent or availability of genomic techniques typing schemes were based on phenotypic characteristics. Resistance patterns (Burdon *et al.*, 1982), combined use of plasmid analysis, immunoelectrophoresis of extracellular antigens and antibiograms (Wust *et al.*, 1982) and bacteriocin and bacteriophage analysis (Sell *et al.*, 1983) were some of the early attempts to distinguish between strains. Other methods included immunochemical analysis of EDTA extracts (Poxton *et al.*, 1984) and PAGE separation of [^{35}S]methionine incorporated cellular proteins (Tabaqchali *et al.*, 1984). The realisation of serotyping by Delmée *et al.* (1985) set the gold standard for typing of *C. difficile* over the next few years with its eventual ability to distinguish between 19 serogroups. A successful method developed in the Department of Medical Microbiology, Edinburgh University, involves analysing the surface layer proteins of *C. difficile* and is quick and extremely reproducible. The S-layer proteins are stripped from the cell by guanidine hydrochloride and separated on an SDS-polyacrylamide gel. The MW of the proteins are used to distinguish between types. The UK endemic isolate possesses S-layer proteins of MW 52kDa and 36kDa

and is given the designation 5236 (McCoubrey *et al.*, 2001). This typing system correlates well to PCR ribotyping (McCoubrey *et al.*, 2002).

Molecular methods based on the genome of the isolate are generally regarded as superior to phenotypic typing methods as they are not subject to change from growth medium or phase variation (not applicable in S-typing as S-layer proteins are stable). Restriction endonuclease analysis (REA) uses restriction enzymes to cut the entire genome into fragments which are then separated on a gel to produce a fingerprint. This method is highly discriminatory and reproducible but it is also extremely labour-intensive and technically demanding. Restriction fragment length polymorphism (RFLP) is another genomic method that initially starts with restriction enzyme digestion. The fragments are separated on a gel, southern blotted and hybridised with specific probes to analyse polymorphisms of the restriction sites. This method is less labour- and time-intensive but it is also less discriminatory (Brazier, 2001). Pulsed field gel electrophoresis (PFGE) is considered by many to the most optimum typing method for *C. difficile*. Chromosomal digests are prepared with an infrequent cutting enzyme such as *Sma*I, *Ksp*I, *Sac*II or *Nru*I which produce 10-20 fragments which represents the majority of the genome (Cohen *et al.*, 2000a). The technique is very sensitive but there are some important drawbacks to using this typing method. The major drawback to this technique is the problem with DNA degradation which renders many isolates untypeable. The untypeable strains were proven to be of PCR ribotype 001/serogroup G, the endemic UK isolate (Fawley & Wilcox, 2002). The technique is also cumbersome (with the need for agarose plugs etc.), time-consuming and expensive (especially for the equipment). So although this

method is extremely discriminatory and reproducible the time and cost constraints mean that it is used predominantly in typing studies.

The remaining methods utilise PCR to distinguish between isolates. Arbitrarily primed PCR (AP-PCR) and random amplified PCR (RAPD) both allow the detection of polymorphisms using primers with no known homology to the target genome. AP-PCR uses a single oligonucleotide primer and RAPD usually uses two short (ca. 10 nucleotides) primers (Brazier, 2001). AP-PCR correlates well with other methods, is rapid, easy and reproducible. It is also cost-effective but there are some drawbacks. The PCR reaction is extremely sensitive to changes in reagents, machines or conditions, which makes it very difficult to compare results between different laboratories (Brazier, 2001). RAPD is very cost effective, easy, quick and reproducible and is commonly used because of this. PCR ribotyping uses specific primers to the 16S-23S spacer region in *C. difficile*. This region is highly heterogenous in its sequence and in the number of copies the cell carries. The PCR is very stable and is not dependent on the amount of material present. This method is routinely used in the Anaerobe Reference Unit (ARU) in Cardiff and is easy, reproducible and cost-effective (Brazier, 2001). This method correlates well to other typing schemes and is considered the best method for the routine typing of *C. difficile*. A typing scheme developed by Rupnik *et al.* (1998) distinguishes between toxigenic *C. difficile* by the polymorphisms within the PaLoc. To date 20 toxinotypes have been found and it is a relatively simple and effective technique. The method correlates well to serotyping, PFGE and excellent correlation to PCR ribotyping (Rupnik *et al.*, 2001).

There remains difficulties in the typing of *C. difficile* as there is no unified approach to typing nor is there considerable consensus between laboratories.

AIMS

- To study the growth of *C. difficile* with respect to viable, total and spore counts and their relationship to OD_{600} with the view to using OD as a growth phase predictor.
- To investigate the relationship of growth phase and toxin production using a semi-quantitative toxin A ELISA.
- To study the MICs of six different antibiotics in randomly chosen clinical isolates and to utilise the available patient and strain data to look for resistance trends.
- To use sub-inhibitory concentrations of six different antibiotics in modified growth curves to investigate the effect on growth and toxin A production in three *C. difficile* isolates.
- To analyse mRNA transcripts (*tcdA*, *tcdB*, *tcdC*, *tcdD*, *groEL* and 16S RNA) in sub-MIC and control growth curves to see if they mirror the effect seen in toxin production.
- To develop a protocol for the proteomic study of *C. difficile* incorporating sample preparation, 2D analysis, MALDI-TOF analysis of peptides and the utilisation of a new *C. difficile* MASCOT database. To use this validated protocol to study the proteomic profile of *C. difficile* 630 in the presence and absence of the third generation cephalosporin ceftriaxone.

CHAPTER 2 MATERIALS AND METHODS

2.1 Growth curves

2.1.1 Growth curves set-up

The growth curves were performed in Anaerobic Investigation Medium (AIM) over a period of five days. Preliminary work had shown that overnight cultures (ca. 18 hrs) yielded good growth with few spores and would be a suitable inoculum when diluted. To pinpoint the onset of stationary phase readings were taken every four hours for 32 hours. Once this was established the important time points could be determined and the sampling times modified.

2.1.2 *C. difficile* strains

Three strains of *C. difficile* were chosen for this work. The reference strain NCTC 11223 (National Centre for Type Cultures) was chosen as it is commonly used in research and because it is easily available. The genome of *C. difficile* 630 has recently been sequenced and so this strain was a natural choice. During a recent epidemiological study of geriatric patients in the Royal Victoria Hospital (Edinburgh), an endemic strain emerged accounting for 78% of all *C. difficile* collected (McCoubrey, 2002). The number given to this strain was 338a. Approximately 30µl (a standard loopful) was added to 3ml of pre-reduced AIM from the stock culture in cooked meat broth (CMB; see Appendix 1 for recipes) and incubated overnight at 37°C in the anaerobic cabinet. This yielded approximately 10^8 bacteria/ml. Purity of the cultures was checked by Gram stain and retrospectively checked by incubation anaerobically and aerobically for 48 hours on Columbia blood agar. The total bacteria count was calculated using a 0.1mm Thoma counting chamber. Once the number of cells in the starter culture had been deduced the culture

was diluted to give approximately 5 X 10^4 cells/ml. Adding 1ml of this to the 99ml of AIM gave an inoculum of approximately 500 cells/ml. Later experiments used a larger inoculation to ensure minimal loss of cells during transfer.

2.1.3 Viable counts

Viable counts were carried out using the Miles and Misra technique. Samples were diluted ten-fold as necessary and 5 X 20µl drops were plated onto pre-reduced Columbia blood agar. These were incubated anaerobically for 48 hours.

2.1.4 Total counts

Total counts were carried out using a 0.1mm Thoma counting chamber. They were either counted immediately or fixed in 0.2% formaldehyde for later.

2.1.5 Spore counts

The samples for the spore counts were from the same dilution set as was used for the viable counts. The samples were placed in an 80°C waterbath for 20 minutes to kill off any vegetative cells. Five drops of 20µl were added to a Columbia blood agar plate and incubated for five days anaerobically before being counted.

2.1.6 Optical density (600nm)

Optical density was measured using 1ml of sample at 600nm against a media blank.

2.1.7 Toxin testing with the Techlab™ *C. difficile* toxin A kit

The 1ml sample used to measure the OD was spun down at 13000g for two minutes to remove the bacteria. This was frozen at -20°C until required. The supernatants collected over the week were thawed and 100µl diluted 1 in 2 in the buffer provided. A volume of 100µl of this used in the assay according to the manufacturers instructions. To allow for any degradation of toxin over time in the freezer, the

samples were assayed two weeks after the experiment had finished. This ensured that this effect never became a major factor. As with all ELISAs one can only truly compare the results within one plate so each experiment was carried out on one ELISA plate.

2.2 Minimum inhibitory concentrations (MICs)

(Using the National Committee for Clinical Laboratory Standards (1997). Methods for antimicrobial susceptibility testing of anaerobic bacteria.)

MICs were carried out using the agar dilution protocol in the NCCLS guidelines for Antimicrobial Susceptibility Testing of Anaerobes (NCCLS 1997). The isolates were sub-cultured from spores in cooked meat broth into pre-reduced (80% N_2, 10% H_2 and 10% CO_2 at 37°C) thioglycollate medium (Sigma T-9032) enriched with 5μg/ml haemin, 1μg/ml vitamin K_1 and 1mg/ml $NaHCO_3$ and incubated overnight anaerobically at 37°C. This yielded approximately 1 x 10^8 bacteria/ml. Purity of the cultures was checked by Gram stain and retrospectively checked by incubation anaerobically and aerobically for 48 hours on Columbia blood agar (Oxoid CM331 with 5% horse blood). The cultures (1-2μl) were spotted onto Brucella Agar (Oxoid CM169) supplemented with 5% defibrinated sheep blood, 5μg/ml haemin and 1μg/ml vitamin K_1 using a multi-point inoculator.

The concentrations used in the study were vancomycin (Sigma V2002, 8-0.125μg/ml), metronidazole (Sigma M1547, 8-0.125μg/ml), amoxycillin (Sigma A8523, 64-1μg/ml), clindamycin (Sigma C5269, 128-2μg/ml), cefoxitin (Sigma C4786, 256-8μg/ml) and ceftriaxone (Sigma C5793, 256-8μg/ml). For each plate used, controls were added and as an additional control 3 or 4 strains which had

already been tested. In total 1/3 of the strains in the study were tested two or three times.

MIC_{50} and MIC_{90} were calculated by pasting the MICs for all the strains into an Excel spreadsheet and sorted into ascending order using the sort function. The MIC_{50} was taken as the MIC of the strain that was halfway (50%) between the lowest and highest value. Similarly the MIC_{90} was taken as the MIC that would inhibit 90% of the strains tested.

2.3 S-layer typing

2.3.1 Growth of *C. difficile* for S-layer extraction

Starter cultures of *C. difficile* were set-up using 1ml of pre-reduced AIM. A loopful (ca. 30µl) from a CMB stock culture was added to the media and incubated anaerobically overnight at 37°C. The following day purity was checked by wet and Gram film; and retrospectively by plating anaerobically and aerobically on Columbia blood agar. This starter culture was then used to inoculate 4ml of pre-reduced Proteose Peptone Yeast medium (PPY) which was incubated overnight at 37°C.

2.3.2 Guanidine hydrochloride extraction of S-layer proteins

The purity of the cultures was checked as before and the starter culture plates checked for classic *C. difficile* colony morphology and smell. The cultures were centrifuged at 3000rpm for 20 minutes and the pellets resuspended in 4ml of PBS. This was centrifuged for 20 minutes at 3000rpm and the pellets again resuspended in 4ml PBS. The PBS wash was repeated once more and the pellet well drained. The pellet was resuspended in 0.3ml of 5M guanidine hydrochloride and transferred to an eppendorf and shaken at room temperature for 2 hours. This was microcentrifuged

for 2 minutes at 13000rpm and the supernatant collected. The microcentrifugation was repeated and the supernatant transferred to a clean eppendorf and stored at -20°C.

2.3.3 Visualisation of extracted S-layer proteins (SDS-PAGE)

The S-layer extracted proteins were dialysed overnight against water to remove any excess salt in the dialysis membrane (MW cut-off 10000 kDa). The S-layer protein extracts (ca. 1mg/ml) were added to an equal volume of double strength sample buffer and the proteins denatured in a 100°C boiling bath for 3 minutes. Forty µl of each mixture was run on a 10% gel using buffers described by Laemmli (1970), and the technique described by Hancock and Poxton (1988). A MW marker (Invitrogen Mark 12) was added according to the manufacturers instructions. See Appendix 1 for all buffers used.

2.3.4 Coomassie staining of the S-layer gels

The SDS gels were stained using Coomassie blue as described by Hancock and Poxton (1988). Details of the stains are given in Appendix 1.

2.4 Chelex DNA extraction

The DNA from the clindamycin-resistant strains was extracted using the procedure described by de Lamballarie *et al.* (1992). A starter culture of *C. difficile* was plated onto pre-reduced Fastidious Anaerobe Agar (FAA) and incubated anaerobically overnight. Ten big colonies from the FAA plates were emulsified into 100µl of a 5% chelex suspension. This was boiled for 10 minutes and then spun down for 2 minutes at 13000rpm. The supernatant was removed and frozen (-20°C) in small aliquots for future use.

2.5 Polymerase Chain Reaction (PCR) for *ermB*

The primers and conditions were taken from the paper by Farrow *et al.* (2001). Primers 6604 and 4120 have the following sequence;

6604 5'-TAA GAG TGT GTT GAT AGT GC-3'

4120 5'-TCA ATA GAC GTT ACC TGT TTA C-3'

The reaction mix was as follows;

Buffer (Roche)	10µl
dNTPs (1.25mM)	4µl of stock
Primers (10pmol)	2µl of working solution
Taq (Invitrogen, Cat no. 18038-026)	0.5µl (2.5 units)
Template DNA	10µl
Pyogen-free H_2O	to 100µl

The temperatures for the PCR were;

1 cycle	95°C	3 min
1 cycle	70°C	1 min
30 cycles	95°C	1 min
30 cycles	50°C	2 min
30 cycles	72°C	3 min
1 cycle	50°C	2 min
1 cycle	72°C	5min

HOLD AT 4°C

2.6 Visualisation of PCR products

Ten µl of the PCR product containing 10% gel loading buffer (Sigma G-2526) was run on a 0.8% agarose (Sigma A-9539) gel containing 1µl of ethidium bromide. The buffer used was TAE (Appendix 1).

2.7 Sub-Inhibitory Growth Curves

2.7.1 Set-up

Three strains were used in this work. The reference strain 11223, the sequenced strain 630 and a locally endemic strain termed 338a. Strains were grown from spores stored in cooked meat broth: Anaerobic Investigation Medium (AIM) with cooked meat particles (Brown *et al.*, 1996). A loopful (ca.30µl) was added to 3ml of pre-reduced AIM and incubated anaerobically overnight (80% H_2, 10% N_2, 10% CO_2 at 37°C). Appropriate purity checks were carried out on the starter cultures before use and they were then used to inoculate 30ml of AIM containing 1/2, 1/4 and 1/8 of the MIC to the particular antibiotic. The inoculum was 10^6 bacteria/ml and 0.3ml of a 10^8/ml starter culture (AIM overnight) was diluted 1/100 when added to the medium. The MICs garnered from Chapter 4 were used as a guideline. Antibiotics were prepared in sterile distilled water as 100X solutions with reference to the highest concentration required. Doubling dilutions were made in sterile distilled water and one volume of antibiotic was added to 100 volumes of broth.

2.7.2 Sampling

Each experiment was sampled 3 times a day for five days. In case of errors on the day two concentrations up and down from the desired concentrations were added and then discarded retrospectively (after 24-48 hours). A sample of 1ml was removed at each time point and the OD read at 600nm and the supernatant (13000g for 2 minutes) stored at -20°C for later toxin analysis.

2.7.3 Toxin analysis

Toxin A levels were assayed by ELISA with a ToxA kit (Techlab, Virginia, USA) according to the manufacturer's instructions. Prior to assay, 100µl of each sample was diluted in 100µl of the buffer provided, and 100µl of this was used in the assay. The plates were read at a dual wavelength of 450/620nm. The maximum OD value of the assay was 3.0 up to which OD was linear in respect to control toxin concentration. No further dilutions of supernates were made. Results (OD values) were plotted against time to evaluate when toxin was being elaborated and to show differences between antibiotic-free and antibiotic-containing cultures.

2.8 Effects of antibiotics on the secreted protein profile of *C. difficile*

A starter culture of strains 11223, 338a and 630 were set-up in 3ml of pre-reduced defined medium (DM; Karasawa *et al.*, 1995; see Appendix 1). This was incubated anaerobically overnight at 37°C and used to inoculate 30ml of DM with or without antibiotics. The same set-up used in the sub-MIC growth was followed here. This was to keep things as much the same as possible. Two important points in the growth curve were decided upon; late log and early stationary phase. Two ODs representing these points were used to determine when the experiment could be sampled. An OD_{600} of 0.6 was found through preliminary work to represent late log whereas an absorbance of 0.75 was used to represent early stationary phase. A volume of 5ml was removed at each point and the bacteria removed through centrifugation. The sample was then dialysed against distilled water (MW cut-off of 10,000Da) for 2 days to remove all the components of the medium. This was an important step as the medium not only contained salts but amino acids which would react in the protein

assay. Removing the amino acids would allow the accurate concentration of protein produced by the *C. difficile* to be deduced. After 2 days a protein assay was performed and the sample freeze-dried to concentrate the protein. From the assay values one could work out the amount of protein required to run on a SDS-PAGE. This sample was visualised on a 10% gel and stained with silver. Differences between antibiotic-free controls and antibiotic-containing experiments could be made.

2.9 RNA extractions

(Rneasy handbook, Qiagen (2001))

2.9.1 Preparation for RNA extractions

(Protect Bacteria Reagent Handbook, Qiagen (2001))

Samples for extraction were prepared using the Protect Bacteria reagent (Cat. no. 76506) from Qiagen. This allows the pellet to be stored at –70°C for up to 1 month prior to the extraction step. One part of the Protect bacteria reagent was added to 2 parts of the culture. This was centrifuged at 10000rpm for 2 minutes to remove the supernatant. The pellet could then be stored at -70°C until required (for up to 1 month).

2.9.2 RNeasy extraction

The RNeasy mini kit (Cat. no. 74104) was used. The protocol in the RNeasy handbook for RNA extraction from bacteria was followed. The pellets were thawed on ice and RNase-free reagents and equipment were used at all times. The procedure was carried out in an RNA clean room.

2.9.3 DNA digestion

The RNeasy handbook states that DNA is effectively removed by the column with no need for a DNA removal step. If required a DNA digestion can be carried out on the column as part of the RNA extraction process (RNase-free DNase Set (Cat. no. 79254)). This proved inadequate and an alternative method was provided by Qiagen. The volume of RNA eluted from the column (30-50µl) was made up to 80µl with RNase-free water. To this 10µl of buffer RD1 was added along with 2µl of the DNase enzyme. This was incubated for 20 minutes at room temperature and then 10µl of 140mM EDTA was added to inactivate the DNase. Incubation in a 65°C waterbath for 5 minutes removed the EDTA and the sample was now ready for use. The RNA was quantified using spectrophotometry at 260nm.

2.9.4 Reverse Transcriptase PCR

The Qiagen Omniscript RT-PCR kit was used (Cat. no. 205111). The protocol found in the RNeasy Handbook was followed. The Qiagen Sensiscript (Cat. no. 205211) was also used; again according to the handbook instructions.

2.10 PCR for *tcdA*, *tcdB* and 16S RNA

Five microlitres of the cDNA generated from the RT reaction were used in the PCR. The primers for toxins A and B were taken from the papers by Tang *et al.* (1994) and Gumerlock *et al.* (1993). The primers for the 16S RNA region were designed using the 16S sequence of strain 630.

toxin A 602bp

YT28 5' GCA TGA TAA GGC AAC ACA GTG G 3'

YT29 5' GAG TAA GTT CCT CCT GCT CCA TCA A 3'

toxin B	399bp
YT18	5' GTG TAA CCT ACT TTC ATA ACA CCA G 3'
YT17	5' GGT GGA GCT TCA ATT GGA GAG 3'
16S RNA	250bp
16S for	5' GGC TAG CGT TAT CCG GAT TTA CTG 3'
16S rev	5' ATC TAA TCC TGT TTG CTC CCC ACG 3'

The reaction mix was as follows;

Buffer (Invitrogen)	5µl
dNTPs (1.25mM)	2µl of stock
Primers (10pmol)	1µl of working solution
$MgCl_2$ (Invitrogen)	3µl
Taq (Invitrogen, Cat no. 18038-026)	0.2µl (1 unit)
Template DNA	5µl
Pyogen-free H_2O	to 50µl

The temperatures for the PCR were;

1 cycle	94°C	4 min
40 cycles	94°C	45 sec
40 cycles	55°C	30 sec
40 cycles	72°C	45 sec
1 cycle	72°C	10 min

HOLD AT 4°C.

2.11 PCR for *tcdC*, *tcdD* and *groEL*

The primers for *tcdC* and *tcdD* were taken from the paper by Braun *et al.* (1996). The primers for *groEL* were taken from the paper by Hennequin *et al.* (2001).

tcdC 345bp

***tcdC* for** 5' GCA CCT CAT CAC CAT CTT CAA 3'

***tcdC* rev** 5' TGA AGA CCA TGA GGA GGT CAT 3'

tcdD 300bp

***tcdD* for** 5' AAA AGC GAT GCT ATT ATA GTC AAA 3'

***tcdD* rev** 5' CCT TAT TAA CAG CTT GTC TAG AT 3'

groEL 350bp

***groEL* for** 5' GCT GAA GAT GTA GAA GGT GAA G 3'

***groEL* rev** 5' TAC AAC AGC TAC TCC TCC AGC 3'

The reaction mix from section 2.10 was used and the temperature cycles are shown as follows;

1 cycle	94°C	4 min
40 cycles	94°C	45 sec
40 cycles	53°C	30 sec
40 cycles	72°C	45 sec
1 cycle	72°C	10 min

HOLD AT 4°C.

This is the same as section 2.10 except for the annealing temperature. The melting point of the primers for *groEL* was lower than the other primer sets so it was necessary to drop this temperature. This set of PCR conditions also worked for the other 5 primer sets and so from this point on these temperatures were used for all cDNA PCRs.

2.12 Visualisation of PCR products

Ten µl of the PCR product containing 10% gel loading buffer (Sigma G-2526) was run on a 2% agarose (Sigma A-9539) gel containing 1µl of ethidium bromide. TAE buffer was used.

2.13 2D gel electrophoresis

2.13.1 Sample Preparation

Strain 630 was grown from spores overnight in AIM to produce a starter culture. Purity was checked as before. This starter culture was used to inoculate the appropriate volume of AIM to produce the cells for the 2D work. This was then grown overnight under anaerobic conditions as before. For the experiments containing sub-MIC antibiotics the AIM contained the concentration of the antibiotic correlating to 1/2, 1/4 and 1/8 the MIC. To ensure the correct concentrations were achieved, 2 concentrations up and down from the predicted MIC were included and extras discarded retrospectively. The overnight culture was centrifuged for 2 minutes at 13000g using a microcentrifuge. The cells were kept on ice at all times and washed three times in ice-cold PBS to remove media components and salts. To each pellet 200µl of lysis buffer (see Appendix 1) was added and the sample vortexed for 30 minutes at room temperature. Three rounds of 10-second tip sonication followed (50% output) to ensure complete lysis of the cells. The sample was then processed through the Amersham Clean-up Kit (Amersham Biosciences 80-6484-51). This precipitates the proteins and removes the salts. The resulting pellet was then rehydrated directly into rehydration buffer (see Appendix 1) The sample was then ready for addition onto the appropriate IPG strip. If protein quantification was required the pellet resulting from the clean-up kit could be rehydrated into lysis

buffer and samples removed (typically 2 X 10µl) for analysis. Upon quantification the appropriate volume of protein in lysis buffer could then be added to rehydration buffer (+ 10µl of the appropriate IPG buffer and 10µl of 280mg/ml dithiothreitol (DTT)) and subsequently used in the 2D gel experiment.

2.13.2 1st Dimension

The samples could be treated in two ways for the separation of the proteins in the 1st dimension. They could be added straight to the coffin and placed directly into the IPGPhor for ca. 24h. This included an overnight rehydration of the strip before the voltage was stepped up for the actual separation. The alternative method was to apply the sample and rehydrate the IPG strip overnight in a reswelling tray (no temperature control or voltage). The strip was then transferred to a coffin the next morning and run throughout the day. Both methods were used throughout this work. The IPG strips used in this work were 7cm pH 3-10 linear (17-6001-11), 13cm pH 3-10 non-linear (17-6001-15) and 18cm pH 3-10 non-linear (17-1235-01) all from Amersham Biosciences. Once the 1st dimension separation was complete the gel strip was frozen (-70°C) in a suitable container (petri dish, falcon tube, universal) until required for the 2nd dimension.

2.13.3 2nd Dimension

The IPG strips were prepared for the 2nd dimension separation by immersion in SDS equilibration buffer (see Appendix 1). SDS equilibration buffer plus DTT (200mg DTT per 20ml) was added to the strips and they were shaken for 15 minutes. This was then replaced with SDS equilibration buffer plus IAA (800mg per 20ml) for a further 15 minutes. The buffer was decanted and the strips carefully blotted to remove excess liquid. The strips were placed on a 12.5% gel (250 × 110 × 0.5,

Amersham Biosciences 80-1261-01) or a 12-14% gel (245 × 180 × 0.5, Amersham Biosciences 17-1236-01) and run on a MultiPhor II system with buffer strips (Amersham Biosciences 17-1342-01) and Mark 12 MW marker (10µl on a paper square). The proteins were run out of the strip for 30 minutes at 600V / 20mA / 40W. The strips and the MW marker paper was then removed and the cathode buffer strip moved to where they had been. The gel was then run for ca. 90 minutes at 600V / 40mA / 40W until the dye front had just disappeared under the anode buffer strip. The gel was then removed to a suitable container for staining.

2.13.4 Colloidal Coomassie Blue

The gel was placed in the fixative solution (40% methanol, 10% acetic acid) for 90 minutes. This was decanted and 400ml of colloidal coomassie blue (Genomic solutions 80-0216 or 80-0017) and left overnight. Several washes of destain (25% methanol) were then added to achieve a clear picture for scanning and analysis. See Appendix 1 for the recipes.

2.14 MALDI-TOF Mass Spectrometry

2.14.1 Trypsin Digestion of 2D Protein Spots

Protein spots of interest were excised from the gel and cut into small pieces of ca. 1-2mm in diameter and placed into a clean, sterile eppendorf. The spots were covered with 100mM ammonium bicarbonate/50% acetonitrile and left for 15 min at room temperature on a vortex. The supernatant was carefully removed and the previous step was repeated at least three times until the stain was completely removed from the gel pieces. After the stain was gone they were covered in 100% acetonitrile for 10 min to dehydrate. The supernatant was removed and the pieces left to dry at room temperature for 20 min to remove the excess acetonitrile. The gel pieces were then

covered with 10µl of sequencing grade modified trypsin (Promega V5111) in 25mM ammonium bicarbonate and left at room temperature for 15 min. If the gel pieces had rehydrated to above the solution level then 25mM ammonium bicarbonate was added to recover the pieces. They were left in a 37°C incubator (not a waterbath) for at least 16 hours.

2.14.2 MALDI-TOF

CHCA was resuspended in 400µl of 50% acetonitrile/0.1% TFA and 0.5µl of this was used with 0.5µl of the trypsin digest in the mass spectrometer.

2.15 *C. difficile* MASCOT database

The sequence of strain 630 was kindly donated by the Sanger Centre for the development of a MASCOT database. The MASCOT database predicts open reading frames and then predicts the products of these open reading frames. The peptide fragments were used to search the database to find a match for the proteins garnered from the 2D gels.

CHAPTER 3 Growth curves of *Clostridium difficile*

AIMS

1. To obtain accurate growth curves for *C. difficile* with respect to viable, total, spore counts and optical density (OD_{600}).
2. To analyse the relationship between cell counts (viable, total and spore) and optical density with the view to using only OD_{600} in future work.
3. To investigate the production of toxin in relation to growth phase.

It was necessary at the beginning of this project to establish accurate growth curves for *C. difficile*. The relationship of cell counts (viable, total) to OD_{600} was crucial in investigating the use of OD_{600} as a measure of growth phase. Elucidating the pattern of toxin production under these conditions was essential for the work to be carried out in the rest of this thesis. Toxins A and B were known to be produced after ca. 24h of culture but accurate timings using this medium were not available. This was essential for future work as well as fundamental for understanding growth phase and toxin production.

RESULTS

3.1 Growth curves set-up and strains

The growth curves were carried out in Anaerobic Investigation Medium (AIM) over a period of five days. Preliminary work had shown that overnight cultures (ca. 18h) yielded good growth with few spores. To pinpoint the onset of stationary phase

readings were taken every 4h for 32h. Once this was established the important time points could be determined and the sampling times modified in future experiments.

Three strains of *C. difficile* were chosen for this work. The reference strain NCTC 11223 (National Centre for Type Cultures) was chosen as it is commonly used in research and because it is easily available. The genome of *C. difficile* 630 has recently been fully sequenced and so this strain was included. During a recent epidemiological study of geriatric patients in the Royal Victoria Hospital (Edinburgh), an endemic strain (S-type 5236) emerged accounting for 78% of all *C. difficile* isolates collected (McCoubrey, 2002). A representative of this group, termed 338a, was chosen for use in the growth curves study. All three strains produce both toxin A and toxin B.

3.2 Growth curves set 1

This first set of growth curves was carried out every 4h for the first 36h. This was important to catch the onset of stationary phase. Strains 338a and 630 were used.

3.2.1 Growth of *C. difficile*

Figure 3.1 shows the OD_{600}, viable, total and spore counts for strains 338a and 630. Toxin production will be discussed for these growth curves with Figure 3.2. In both strains the late log/early stationary phase boundary occurred between 12 and 14h. Stationary phase lasted for ca. 16h before the cells went into decline. Cell concentrations calculated by viable and total counts correlated well to one another as can be seen in Figure 3.1. The OD readings gave a good measure of growth phase except when the cells were in log phase. The OD_{600} values only become readable at a concentration of ca. 10^6 cells/ml. This leads to a short delay of ca. 4h where the phase of the culture is indeterminable and appears at a earlier stage than the viable

and total counts until the cultures reach stationary phase. After this point OD is a good indicator for the growth phase of the cultures. The levels of the OD values and viable counts drop off into decline unlike the total counts, which remain high due to the counting of both viable and ghost cells. During this first set of growth curves spore counts (see Materials and Methods for protocol) were measured using the same dilutions as used for the viable counts. This was a mistake as this led to the low number of spores being missed at the lower time points when few spores were expected. Due to this error the number of spores could only be counted once they reached ca. 10^4 cells/ml. From this data however, it is apparent that strain 338a produced greater numbers of spores than strain 630. In strain 338a the spore numbers peaked at ca. 5 X 10^6/ml with strain 630 peaking at ca. 10^5/ml.

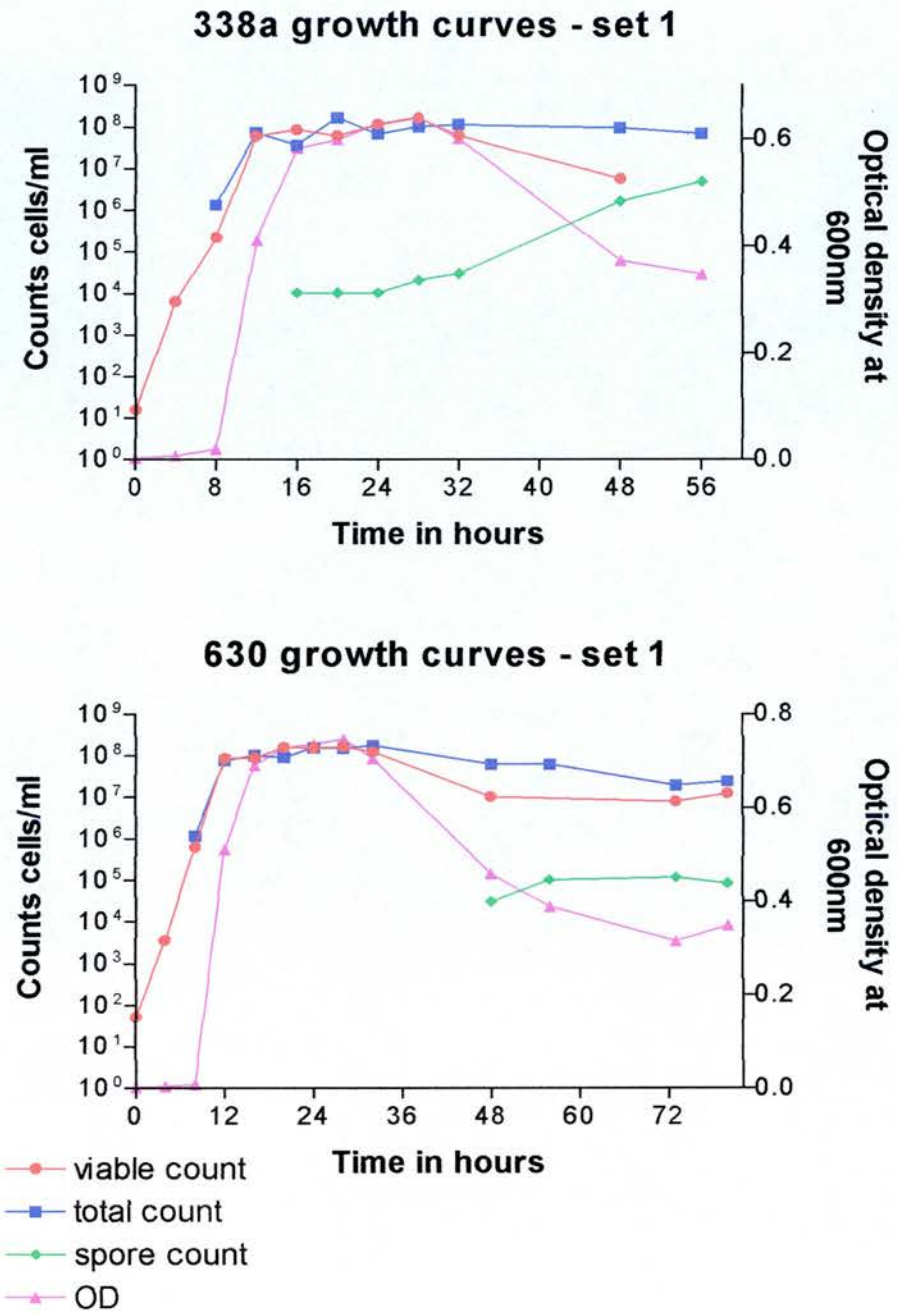

Figure 3.1 **Growth curves**

This figure shows the growth of the two strains. This was measured using viable and total counts and optical density (OD). The spore counts were also measured and are shown here.

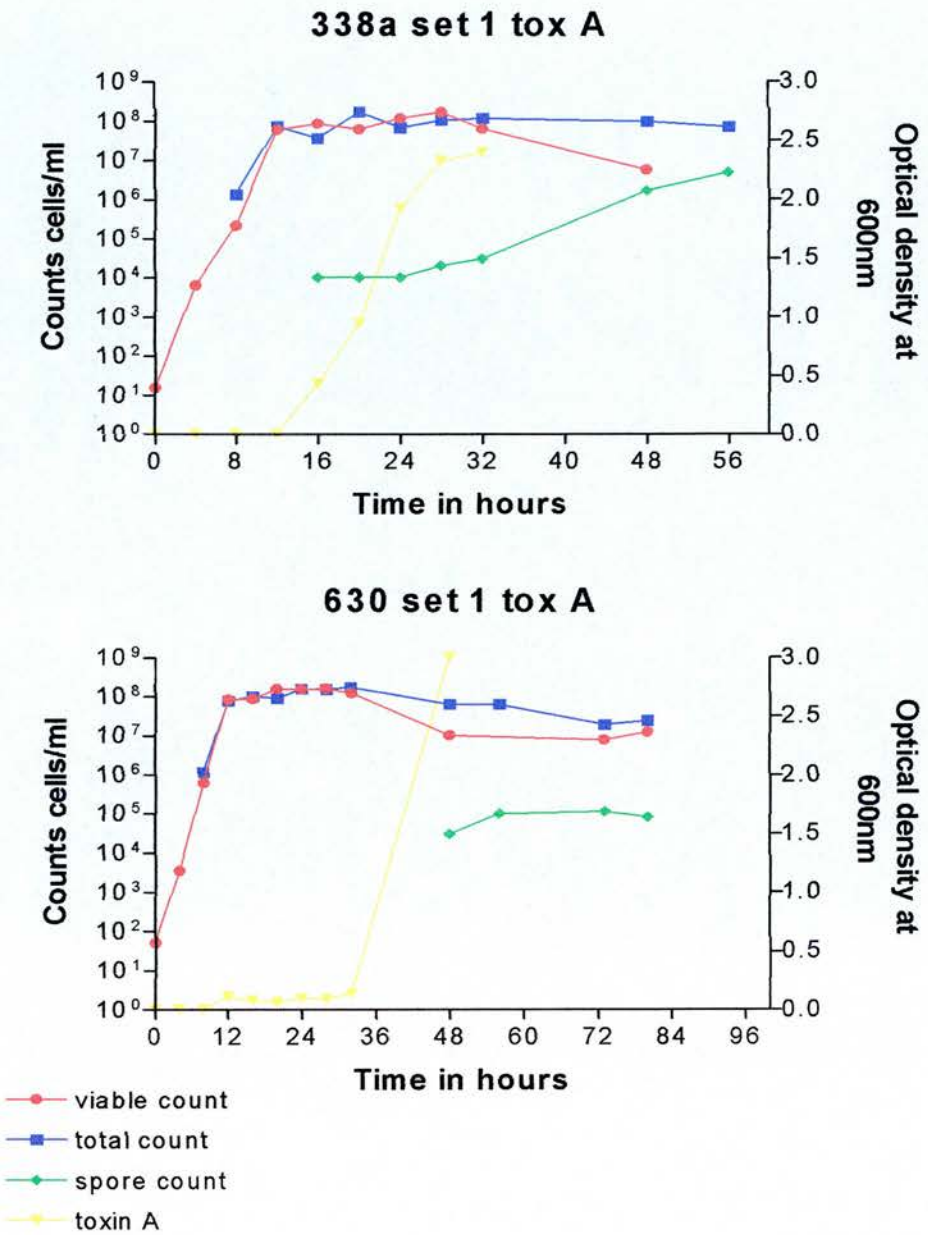

Figure 3.2 Growth curve including toxin production

This graph shows the same growth curve as Figure 3.1 but without OD and including toxin A production.

3.2.2 Growth and toxin A production

Figure 3.2 shows the viable, total and spore counts and the toxin production of strains 338a and 630. As is clear from the graphs, strain 338a began to produce reasonable levels of toxin A before strain 630. By 16h measurable toxin was clearly seen in this strain. Slight increases in toxin levels were seen in strain 630 but by 32h very little toxin A had been produced. By 48h, the next time point measured, greater levels of toxin A were present. A common feature of toxin production in these strains was that sometime between 32h and 48h toxin A levels reached and exceeded the limits of the assay.

3.3 Growth curves sets 2 and 3

The growth curves were repeated twice more with a few modifications. The inoculum used was 10000 cells/ml instead of 100 cells/ml to decrease error and the long early-log phase period seen with a lower inoculum. All three strains were used on these occasions and based on the data obtained from the previous experiment the sampling times were modified. No samples were taken between 14h and 25h as this period had been shown to represent the bulk of stationary phase and missing out these time points was deemed possible. The long early-log phase period seen in the previous experiment was decreased slightly and this was taken into consideration when deciding on sampling times. Error bars, although they contain too few data, were included to give an idea of the variation between the two experiments.

3.3.1 Growth of *C. difficile*

Figure 3.3 again shows the relationship between viable and total counts and OD_{600}. As in Figure 3.1 optical density is accurate for predicting growth phase, except for the apparent delay of ca. 4h where the phase cannot be determined until the culture

reaches a density of 10^6 cells/ml. As in the previous set viable and total counts correlate well especially during log and stationary phase. Once the cultures go into decline it becomes more difficult to get accurate total counts due to the presence of debris and ghost cells which are difficult to count. The OD values again mirror the viable counts into decline which will correlate with the lysing of cells and the production of spores. The difference in sporulation between the strains is less marked than in the previous set. In the first set of growth curves strain 338a produced ca. 10^6 spores/ml and strain 630 ca. 10^5/ml. The second set of growth curves show all 3 strains producing ca. 10^5 spores/ml.

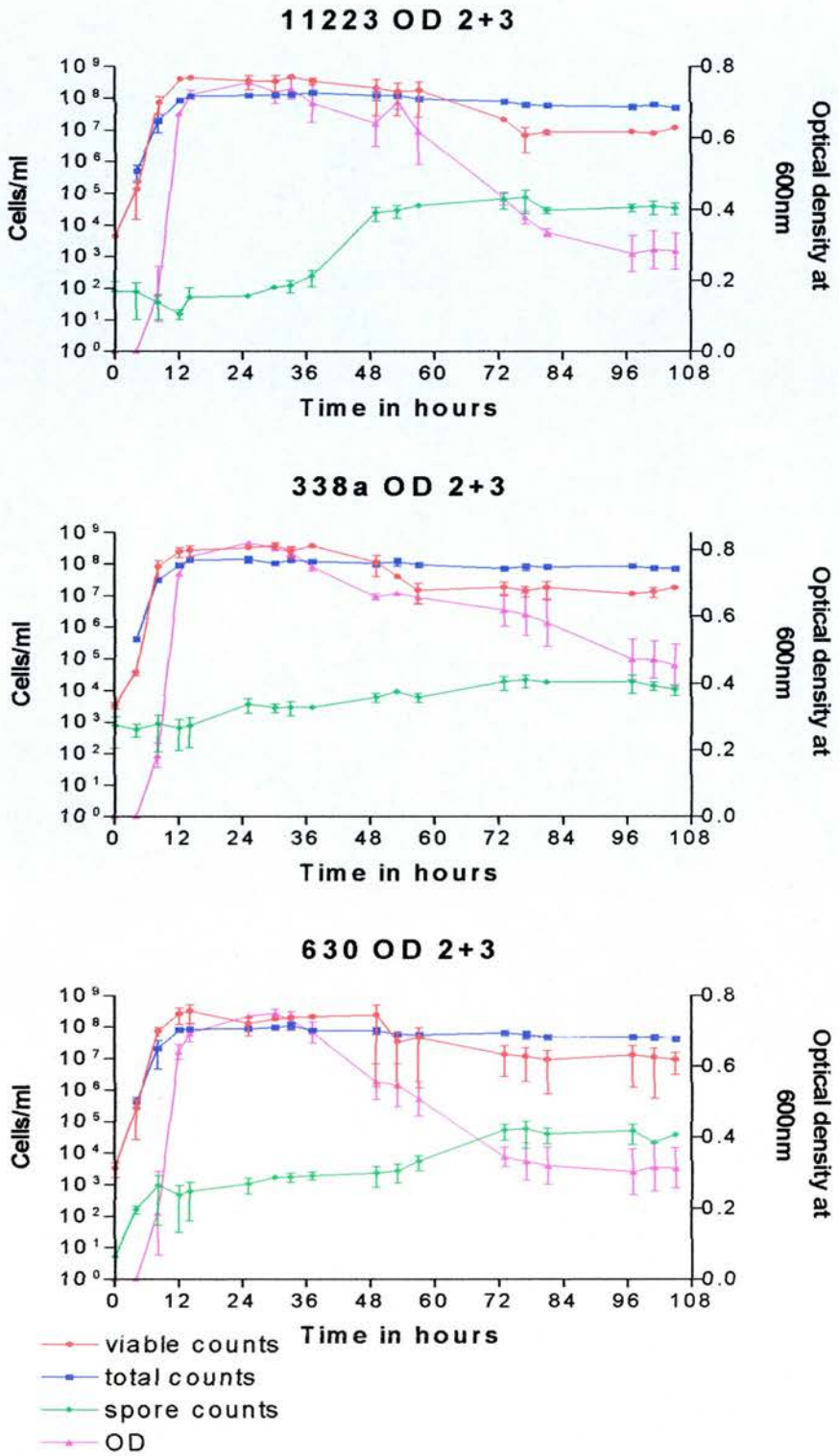

Figure 3.3 **Growth curves set 2 and 3 without toxin A**

These graphs show OD, viable, spore and total counts.

3.3.2 Growth and toxin A production

Figure 3.4 shows the growth and toxin production of the three strains for two experiments. Whereas the growth profiles of the strains varies little, toxin production between the strains is different. The most striking difference is the levels of toxin produced. Strain 11223 toxin levels rarely exceed the limits of the assay but strains 338a and 630 commonly exceed it by ca. 48h. The time toxin is first produced also differs between strains. Strain 338a produces toxin first with it appearing at 12h and rising sharply from then on. Toxin A in strain 630 appears at ca. 24h and in strain 11223 at ca. 36h.

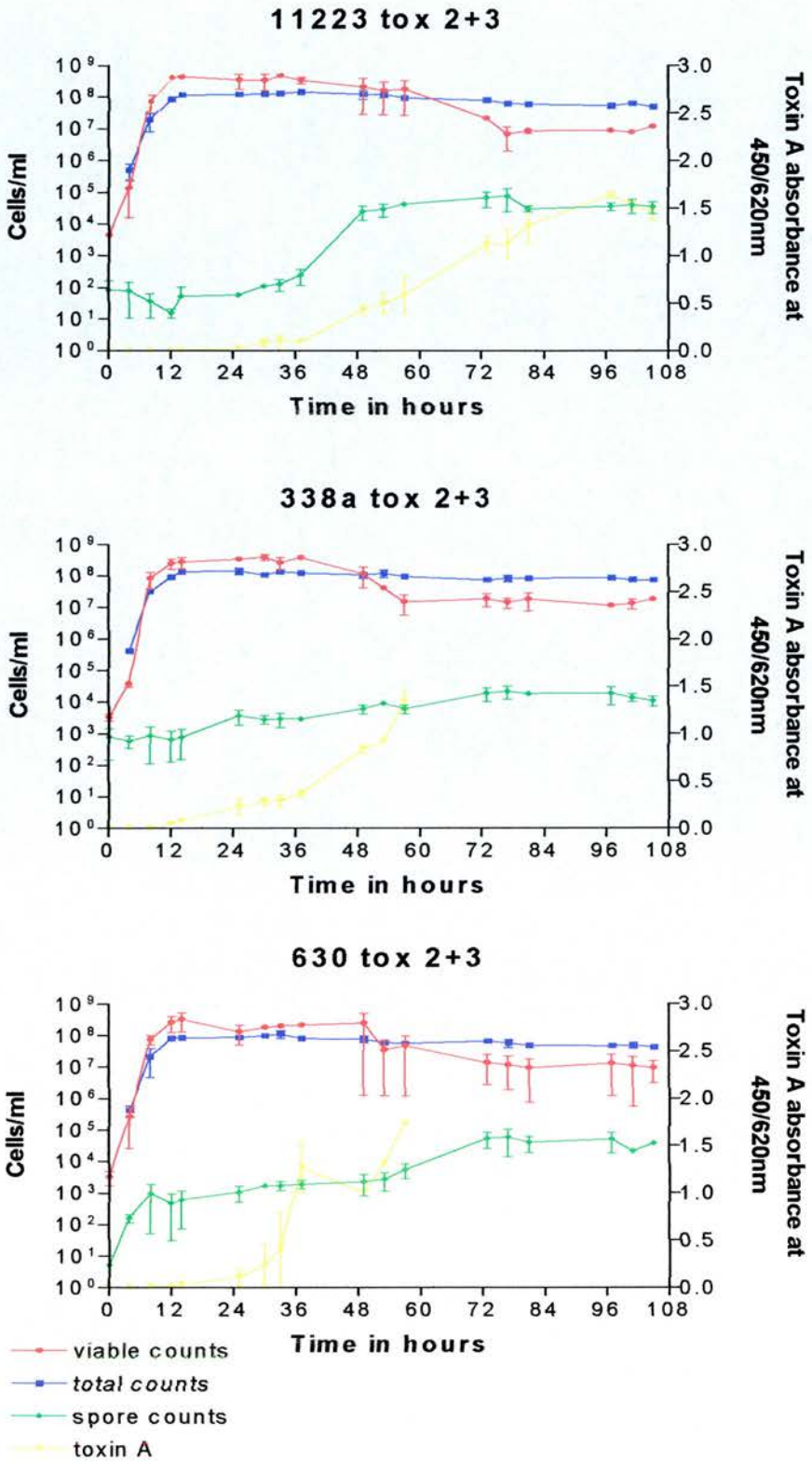

Figure 3.4 **Growth curves set 2 and 3 with toxin A**

These graphs show OD, viable and counts with toxin A levels.

3.4 Discussion

These experiments were essential in clarifying the growth curves in *C. difficile*. The relationship between toxin production and growth phase is extremely important and it was necessary to know at what point toxin was being released. The data shown (as well as the preliminary data not shown) highlight the fact that growth pattern of the three strains are similar. They all enter stationary phase by 12-16h and the cell/ml concentration peaks at ca. 10^8/ml. All three go into decline at ca. 36h with the viable cell count and OD levels falling while the total count remains high, picking up ghost cells and viable bacteria. The initial set of growth curves showed strain 338a to produce more spores (ca. 5 X 10^6/ml) than strain 630 (ca. 10^5/ml). This fitted in well with the knowledge that strain 338a is endemic in hospitals and so would unsurprisingly produce high numbers of spores to facilitate its survival. However, during the next two sets of growth curves strain 338a produced the same number of spores as 11223 and 630 (ca. 10^5/ml). Before this work was undertaken it was thought that strain 11223 produced few spores. This work has shown this not to be the case. If 11223 had produced fewer spores then it would have been interesting as this would have fit in with the theory of Kamiya *et al.* (1992) concerning a link between sporulation and toxin production. Addition of a sporulation inhibitor to a *C. difficile* culture resulted in a decrease in cytotoxin production as well as the expected decrease in sporulation. This suggested that the two were closely related. However, it is not an exclusive relationship as non-sporulating strains still produce toxins though it may suggest that they are under the same sort of regulation.

It was clear from this work that toxin A production began in early stationary phase and carried on and accumulated by the end of stationary phase. This onset has also

been seen by others both in the toxin transcripts and in the production of the toxins. Production of the toxins at this point in the growth phase has been reported by Haslam *et al.* (1986), Ketley *et al.* (1984), Osgood *et al.* (1993) and Dupuy & Sonenshein (1998). Transcription of the toxin genes at this point has been demonstrated by Hundsberger *et al.* (1997), Dupuy & Sonenshein (1998), Hammond *et al.* (1997), Moncrief *et al.* (1997), Mani & Dupuy (2001) and Mani *et al.* (2002). The last two papers have both shown the onset of the transcript for the *tcdD* gene (the alternative sigma factor) to mirror that of toxin transcripts.

Why strains differ in their levels and timing of toxin production is poorly understood but one paper by Spigaglia and Mastrantonio (2002) showed strains with variants of TcdC, the putative negative regulator of toxin production. The pattern of disease severity associated with these strains showed no correlation between the mutations in TcdC and the virulence of the strain. It is conceivable that changes in this protein would affect toxin production. For example, they found one nonsense mutation which reduced the TcdC protein from 232 to 61 amino acids. Lack of a functional protein may lead to abrogated repression of the toxin genes. This may be a partial explanation for the differences common between strains of *C. difficile*. PCR would show if the strains used in this work differed in *tcdC*. Levels of toxin production was not studied in these strains which may have given a idea of the effects of containing a mutation in this gene.

This work has clearly shown that production of toxin A in the three strains differed in the time it began and the levels of toxin A achieved. However, the pattern of growth phase dependence of toxin production was the same in all three strains with it

beginning in early stationary phase and reaching the highest levels of toxin when the strains were heading towards decline.

This work also demonstrated the usefulness and relative accuracy of using OD_{600} as a tool to predict the growth phase of the culture. After the initial delay of ca. 4h behind the viable and total counts the OD_{600} values followed the same pattern and timings as the viable and total counts curves. This delay was likely due to the differences generated when using different scales (linear for OD and log for cell counts) and the lack of sensitivity until a density of ca. 10^6 cells/ml is reached. OD_{600} values were also useful in predicting the onset of decline and sporulation as the values decreased as the viable count decreased and the spore count increased. As most of the subsequent work in this thesis would require accurate and immediate predictions of late log and early stationary phase boundaries then the use of OD_{600} was more than adequate to fulfil this role. The ease of using OD_{600} over viable and total counts in both time (allows immediate count estimates) and resources allowed many more time points to be sampled in the subsequent experiments.

CHAPTER 4 Minimum Inhibitory Concentrations

AIMS

1. To obtain current information on the sensitivity (as MICs) of a representative number of strains to a variety of precipitating and treatment antibiotics during an 18-month epidemiological study.
2. To look for any differences in resistance profiles between S-types.
3. To use repeat samples to relate antibiotic susceptibility within isolates from individual patients over time and to look for evidence of infections with more than one strain of *C. difficile*.
4. To confirm the presence of the *ermB* gene in strains demonstrating high-level resistance to macrolide, lincosamide, streptogramin B (MLS) antibiotics i.e. clindamycin in this study.

The epidemiological study funded by the Chief Scientist Office, Scottish Executive, was already underway at the start of this work. Over the course of the study over 1000 faecal samples (from 390 patients) were collected from two geriatric wards at the Royal Victoria Hospital in Edinburgh. One hundred of these patients tested positive for *C. difficile* by culture and up to six isolates from each positive faecal sample were collected. More than one faecal sample from many patients was taken during the 18 months as they were readmitted to the hospital, had a recurrence/reinfection or simply carried *C. difficile* for a long period of time. Normally one isolate from each faecal sample was typed by detecting its S-layer proteins. A database was set-up to include the patients' details including their age, sex, antibiotic regimes and underlying illness along with the information collected

about the strains. Isolates were chosen at random to carry out MICs and the results related to strain and patient data present in the database. Randomly-chosen isolates from 90 patients were tested.

RESULTS

4.1 Minimum Inhibitory Concentrations

4.1.1 MIC set-up and strain data

The agar dilution protocol from the NCCLS (1997) was followed to establish the MICs of the strains to six different antibiotics. The antibiotics selected for this study were not meant to be extensive, but representative: the two agents used for treatment of *C. difficile* associated disease, vancomycin and metronidazole, and four of the agents with known association with CDD, amoxycillin, clindamycin, ceftriaxone and cefoxitin; the latter is also used in the CCEY selective medium at 8μg/ml. Cefotaxime would have been a wiser choice of antibiotic instead of cefoxitin as it is commonly implicated in CDD but this antibiotic was included as it is one of the agents used in the selective medium. The addition of cefotaxime would have yielded more clinically useful results due to its propensity to cause disease and its widespread use. The non-treatment agents other than cefoxitin were chosen because they are common precipitating agents of CDD – they have poor in-vitro activity against *C. difficile*. The concentrations used in the study were vancomycin (8-0.125μg/ml), metronidazole (8-0.125μg/ml), amoxycillin (64-1μg/ml), clindamycin (128-2μg/ml), cefoxitin (256-8μg/ml) and ceftriaxone (256-8μg/ml). These ranges were chosen using results and guidance from the following papers; Barbut *et al.*

(1999), Bianchini (1999), Ednie *et al.* (1997), Freeman & Wilcox (2001), Goldstein *et al.* (1999); Hoellman *et al.* (1998), Jang *et al.* (1997), Jamal *et al.* (2002), Jang *et al.* (1997), Johnson *et al.* (1999), Johnson *et al.* (2000), Marchese *et al.* (2000), Nord (1996), Poliane *et al.* (2000), Sanchez *et al.* (1999), Spangler *et al.* (1994), Wilcox *et al.* (2000).

4.1.2 MICs to 186 strains

In total 186 representative isolates were investigated. Table 4.1 shows the ranges of MICs among the isolates for the six antibiotics used, together with MIC_{50} and MIC_{90} data, and where known the break points for the antibiotics. The two antibiotics used for treatment (vancomycin and metronidazole) both showed a narrow range between 0.5 and 4μg/ml. Cefoxitin, the antibiotic used in the selective medium (at 8μg/ml), showed a range of MICs from 64-256μg/ml. The other three precipitating antibiotics all showed a wider range of MICs.

Table 4.1 Range of MIC values from 186 isolates.

ANTIBIOTIC	MIC RANGE in μg/ml	MIC_{50}	MIC_{90}	BREAKPOINT
Vancomycin	0.5-4	1	2	8
Metronidazole	0.5-4	1	2	8
Amoxycillin	≤1-16	4	4	?
Clindamycin	≤2->128	8	16	8
Cefoxitin	64-256	256	256	64
Ceftriaxone	16-256	64	64	64

The MIC_{50} and the MIC_{90} for the six antibiotics used were either the same or two-fold different. This highlights the closeness in sensitivity of the majority of isolates. MIC_{50} and the MIC_{90} for vancomycin and metronidazole were low (2μg/ml) and only five strains (2.7%) had a MIC of 4μg/ml to vancomycin and two (1.1%) had a MIC of 4μg/ml to metronidazole. None of the isolates tested were resistant to the two treatment agents for CDD. Both the MIC_{50} and the MIC_{90} values for amoxycillin were 4μg/ml. This shows that even though the range of MICs to this antibiotic was relatively broad (≤1-16μg/ml) the majority of the isolates had very similar sensitivity. Clindamycin produced a large range of sensitivities within the tested isolates (≤2 - >128μg/ml). For this antibiotic MIC_{50} and the MIC_{90} values were 8μg/ml and 16μg/ml respectively. The NCCLS breakpoint for clindamycin resistance is ≥8μg/ml thus 66.7% (n=124) of isolates were resistant to clindamycin, 24.7% (n=46) had intermediate resistance (MIC= 4μg/ml) and the rest were sensitive. Twelve *C. difficile* isolates with MICs to clindamycin of ≥128μg/ml from six patients were found. The MIC_{50} and MIC_{90} of cefoxitin were the same at 256μg/ml. The NCCLS (1997) guidelines state that MICs of 64μg/ml or higher are resistant to cefoxitin therefore none of the 186 isolates tested was sensitive. According to the NCCLS guidelines MICs of ≥64μg/ml are resistant to ceftriaxone. Isolates had MIC_{50} and the MIC_{90} values of 64μg/ml to ceftriaxone. Thirty-three strains (17.7%) had intermediate resistance to ceftriaxone at 32μg/ml (NCCLS). Only two strains (1.1%) were sensitive with MICs of 16μg/ml.

4.2 S-layer types and sensitivities

Of the 186 strains included in the collection for MIC determinations 145 had been phenotyped by analysis of their S-layer proteins on SDS-PAGE and included in the database. To complete the set it was decided to S-type the remaining 41 strains. Two strains could not be recovered so 39 strains were successfully typed resulting in 184 of the 186 strains (98.9%) being S-typed. Figures 4.1-3 show the S-layer proteins from the 39 strains. Table 4.2 shows these results along with the MIC_{90} of the different S-types. Most strains (76.6%; n=141) belonged to the common S-type 5236, with most of the others being S-type 5242 (13.6%; n=25). Of the remainder 2.7% (n=5) were S-type 5140, 2.2% (n=4) S-type 5438, 1.6% (n=3) S-type 5046, with single isolates of S-types 5739 and 5043. Four strains collected were non-typeable: they did not show the typical two major S-layer bands on SDS-PAGE.

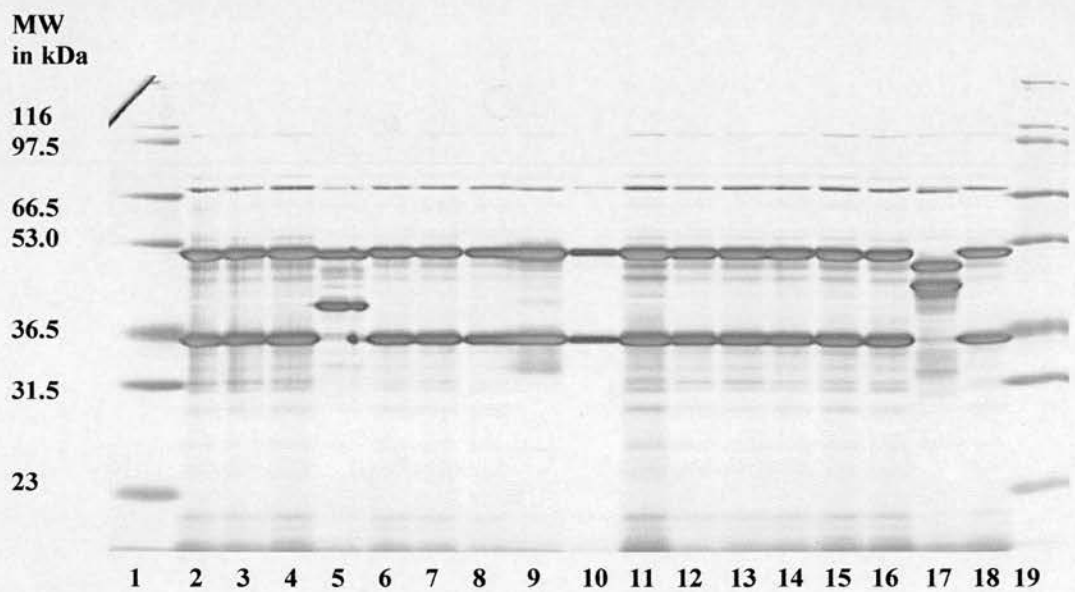

Figure 4.1 ***C. difficile*** **S-layer types**

Lanes 1 and 19 contain the Mark 12 MW marker; lanes 2-4, 6-16 and 18 contain strains with the S-type 5236. The strain in lane 5 has an S-type of 5242 and lane 17 of 5046.

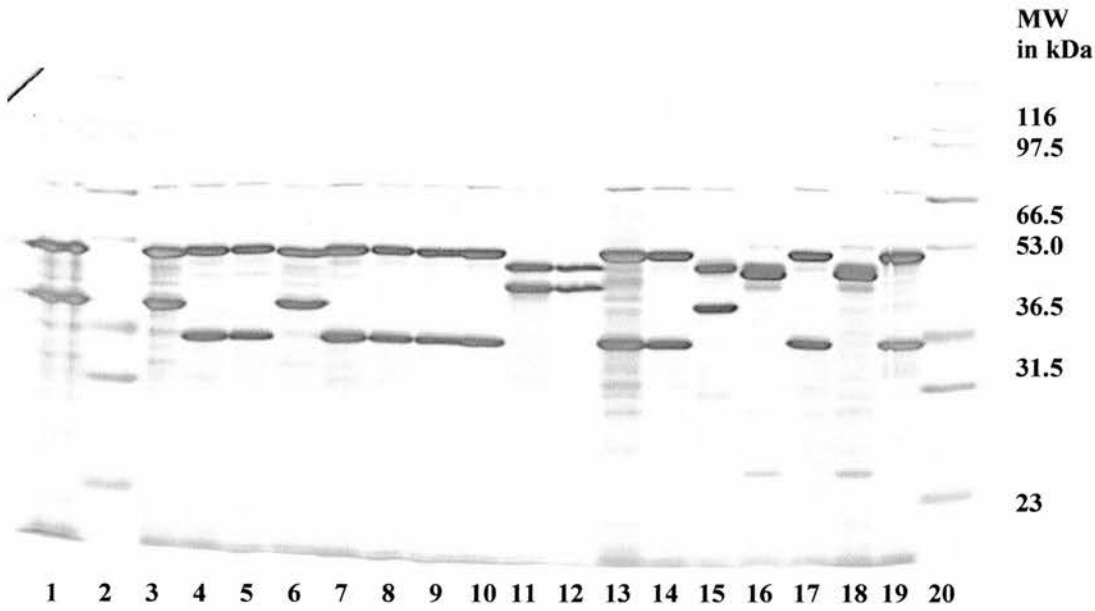

Figure 4.2 ***C. difficile*** **S-layer types**

Lanes 2 and 20 contain the MW marker. Lanes 4-5, 7-10, 13-14, 17 and 19 all contain the S-type 5236. Lanes 1, 3 and 6 contain strains with S-type 5242. Strains of S-type 5046 can be found in lanes 11 and 12. Lane 15 contains S-type 5043 and 2 untypeable strains are in lanes 16 and 18.

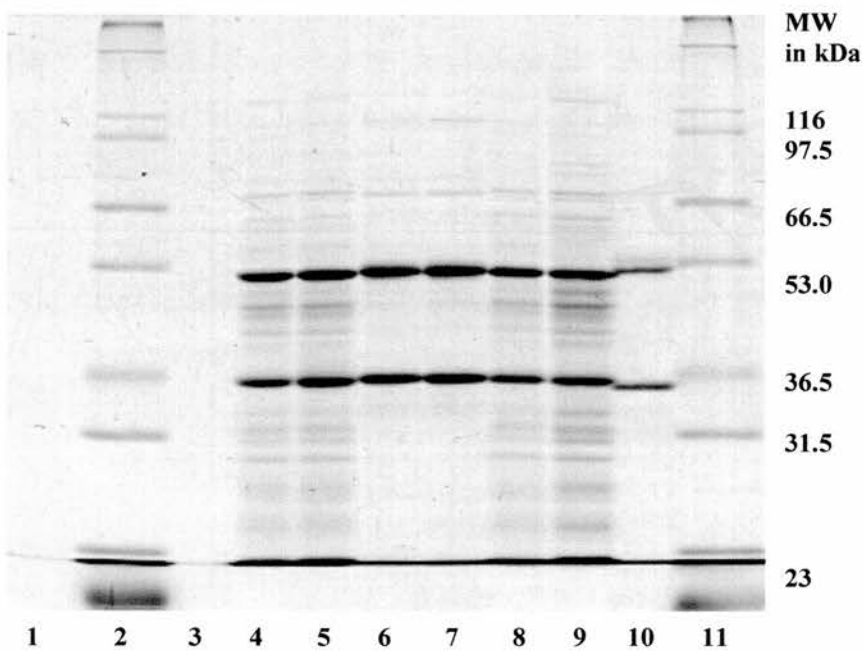

Figure 4.3 ***C. difficile*** **S-layer types**

Lanes 2 and 11 contain the MW marker. Lanes 4-9 contain strains of S-type 5236. Lane 10 contains the type strain 11223 which has a slightly different S-type of 5335.

Table 4.2 Variation in MICs among different S-types

S-type	% of pop (n)	MIC range in μg/ml (MIC_{90})					
		Van	Met	Amox	Clin	Cefo	Ceft
All	100 (184)	0.5-4 (2)	0.25-4 (2)	≤1-16 (4)	≤2->128 (16)	64-256 (256)	16-256 (64)
5236	76.6 (141)	1-4 (2)	0.5-2 (2)	≤1-16 (4)	≤2->128 (16)	64-256 (256)	16-64 (64)
5242	13.6 (25)	1-4 (4)	0.5-4 (2)	≤1-8 (8)	≤2-16 (8)	64-256 (128)	32-64 (64)
5140	2.7 (5)	1-2 (2)	0.25-1 (1)	1-4 (4)	4-16 (16)	64-128 (128)	32-64 (64)
5438	2.2 (4)	1-2 (2)	0.5-1 (1)	2-4 (4)	4-16 (16)	64-256 (256)	32-64 (64)
5046	1.6 (3)	2	1	2	>128	64	16
5043	0.5 (1)	2	2	4	≤2	64	32
5739	0.5 (1)	1	1	2	8	128	32
0*	2.2 (4)	2	1	2-4 (4)	≤2-8 (8)	64	32

* = Non-typeable

There was a degree of variation in sensitivity to antibiotics depending to which S-type the isolate belonged. This is summarised in Table 4.2. The common S-type 5236 had a large range of MICs and there were no differences in the overall pattern between this and the total population. However the less common S-types did show some variations, in particular with respect to clindamycin sensitivity. Three of the non-typeable strains were extremely sensitive to clindamycin with MICs of ≤2μg/ml and had lower than average MICs to cefoxitin and ceftriaxone at 64μg/ml and 32μg/ml respectively. Three strains of S-type 5046 were found during this work and they were all highly resistant to clindamycin with MICs of >128μg/ml.

4.3 Repeat samples and MICs over time

Forty patients were sampled more than once, with some up to 10 times. From those whose strains were S-typed 80% of patients (32/40) retained the same S-type throughout the study while 20% (8/40) definitely harboured different S-types over time, with one patient having three different types at different times. Isolates from 36 patients exhibited changing patterns of sensitivity to one or more of the six antibiotics. While some of these changes related to change of S-type, others did not. Typical changes in isolates that were all of the same S-type were no greater than 2-4-fold different and were therefore of little interest. However some major changes occurred but only in sensitivity to clindamycin. One noteworthy example of this was an isolate with a MIC to clindamycin of 8μg/ml. Two subsequent samples taken from the same patient 13 and 15 days later each produced a highly clindamycin-resistant strain with a MIC of >128μg/ml. The isolates from these samples were all S-type 5236. Another example of changing clindamycin sensitivity was in a patient who also harboured isolates of S-type 5236. The first sample produced an isolate with a MIC of >128μg/ml. A month later another sample contained a strain with a MIC of 16μg/ml followed three days later by one with a MIC to clindamycin of 8μg/ml. Neither of these patients was on clindamycin or any other macrolide. No significant changes in MIC of the patients' isolates were found to the other five antibiotics.

No clear patterns emerged from the data to suggest any link between prescribed antibiotics and specific sensitivities. For example patients on amoxycillin showed no propensity to produce isolates more resistant to that agent.

Overall 97% of the total isolates produced toxin (by the Techlab™ A+B kit). However there was no correlation between toxin production and antibiotic susceptibility or indeed S-type.

4.4 Clindamycin resistance and *ermB*

Twelve isolates were found to have a high level of resistance to clindamycin during this study. Strains with this high level resistance carry the Macrolide, Lincosamide, Streptogramin B (MLS) resistance determinant which contains the *ermB* gene (Farrow *et al.*, 2000). This encodes an RNA methyltransferase which alters the antibiotic target site by modifying the 23S rRNA molecule (Farrow *et al.*, 2000). The 12 isolates were tested for this gene as was the type strain 11223 and the sequenced strain 630 which also have high level resistance to this antibiotic.

PCR for clindamycin resistant isolates

The primers were taken from the paper by Farrow *et al.* (2001) with a product size of 493 base pairs. Table 4.3 shows the isolates tested and their product sizes in base pairs. The sequenced strain 630 was used as the positive control as the *ermB* gene from this strain was used as a template for the primers (Farrow *et al.*, 2001; Accession number - AF109075). The reference strain 11223 is clindamycin resistant and so was expected to have the *ermB* gene. Strain 338a is a representative of the "endemic" UK isolate (ca. 80% of isolates collected; McCoubrey, 2002) and has no resistance to clindamycin and was used as a negative control. As is clear from the subject numbers associated with these isolates, some of them were isolated from the same patient. Patient 81 produced four stool samples over eight months and all samples contained clindamycin-resistant isolates of S-type 5236. An interesting thing to note is that the first isolate from this patient was found in a stool sample collected

in October 1999 and it was toxin positive. The three subsequent samples, two collected in February 2000 and the final in May 2000, produced non-toxigenic isolates. The size of the product resulting from the *ermB* PCR is the same at 493bp for all four isolates. The two different product sizes for the *ermB* gene have been reported before in Farrow *et al.* (2001) so this was not unexpected. Clindamycin resistance was found in isolates of S-types 5236 and 5046. Strain 11223 is one of the positive controls for *ermB* and has S layer proteins of sizes ca. 53 and 35kDa. The gel picture resulting from the PCR for *ermB* is shown in Figure 4.4.

Table 4.3 Clindamycin resistance and *ermB*

Strain	Product size in base pairs	S-type	Subject number	Toxin producer?
630	493	5236	NA	Yes
11223	493	5335	NA	Yes
338a	-ve	5236	93	Yes
1124a	320	5046	688	No
1082a	493	5236	654	Yes
1076a	493	5236	654	Yes
1041a	493	5236	585	Yes
748a	320	5236	402	No
668a	320	5236	361	Yes
628b	320	5046	337	No
625b	320	5046	325	No
363b	493	5236	81	No
269b	493	5236	81	No
261a	493	5236	81	No
223a	493	5236	81	Yes

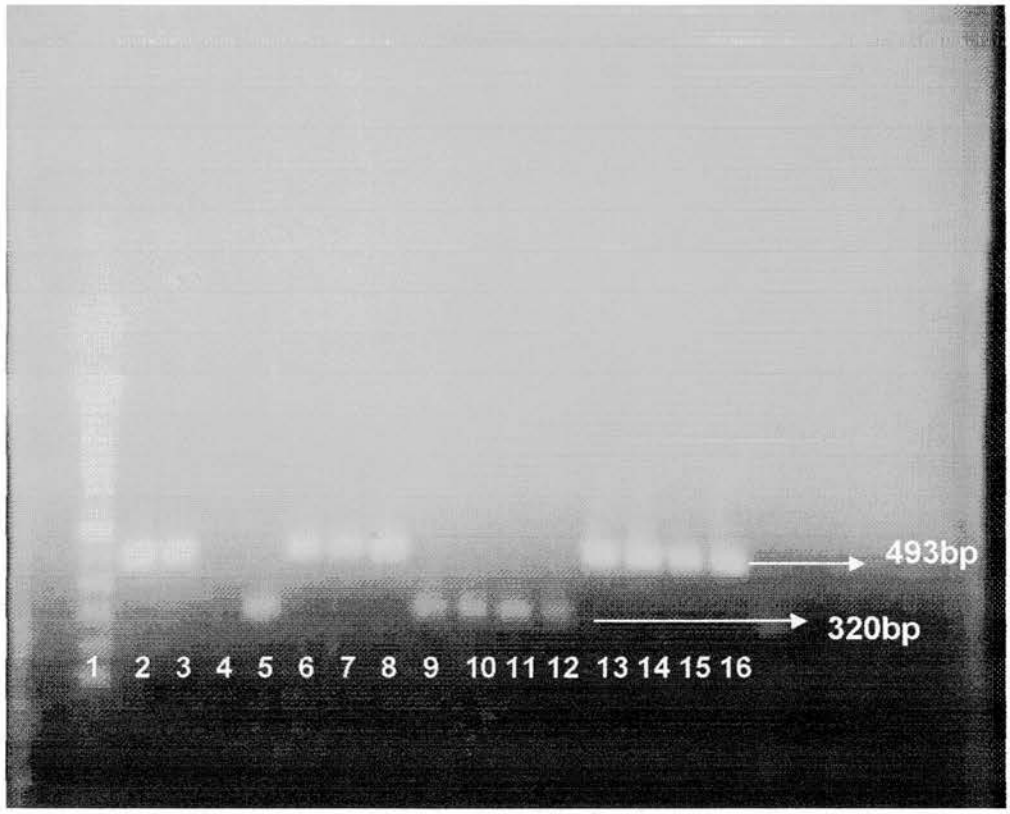

Figure 4.4 PCR gel picture for *ermB* gene.

This gel picture shows the PCR products from the 12 clindamycin-resistant isolates. Lane 1 contains the 100bp MW marker. Lanes 2 and 3 contain the positive controls 630 and 11223 and lane 4 the negative control from the endemic strain 338a. Lanes 5-16 contain the following isolates; 5) 1124a, 6) 1082a, 7) 1076a, 8) 1041a, 9) 748a, 10) 668a, 11) 628b, 12) 625b, 13) 363b, 14) 269b, 15) 261a, 16) 223a. Two product sizes are clearly seen, 320bp and 493bp.

4.5 Discussion

This 18-month study has investigated the susceptibility of *C. difficile* isolates to a range of antibiotics associated with development of CDAD, together with the two antibiotics used in therapy of the disease. Overall there was good correlation of sensitivities with those found in other studies (Ackermann *et al.*, 2003a and b; Alonso *et al.*, 2001; Barbut *et al.*, 1999; Brazier *et al.*, 2001; Ednie *et al.*, 1997; Freeman & Wilcox, 2001; Goldstein *et al.*, 1999; Hoellman *et al.*, 1998; Jamal *et al.*,

2002; Jang *et al.*, 1997; Johnson *et al.*, 1999; Johnson *et al.*, 2000; Marchese *et al.*, 2000; Marks & Kather, 2003; Nord, 1996; Noren *et al.*, 2002; Pelaez *et al.*, 2002; Poliane *et al.*, 2000; Sanchez *et al.*, 1999; Spangler *et al.*, 1994; Wilcox *et al.*, 2000). There was no evidence of any resistance occurring to vancomycin or metronidazole, the treatment agents. However, such strains, especially human isolates, are still extremely rare (Brazier *et al.*, 2001). Five strains (2.7%) had a slightly reduced susceptibility to vancomycin of 4 μg/ml. This low level of reduced susceptibility has also been reported by others (Pelaez *et al.*, 2002), also in small numbers. There was general resistance to the cephalosporin and cephamycin antibiotics, but not to the other beta-lactam amoxycillin. Resistance to clindamycin was common despite its infrequent use. In summary this shows that increased colonisation with *C. difficile* and subsequent disease may well be due to acquisition of resistant strains of the bacterium, but, as has been shown frequently in the past, other mechanisms must be operating as demonstrated by the apparent sensitivity to amoxycillin. The formation of spores by *C. difficile* allow the bacterium to survive in the presence of antibiotics until the levels fall and the spores can germinate. Amoxycillin is a widely used antibiotic both in the hospital environment and in the community and is one of the most common precipitating antibiotics (Freeman and Wilcox, 1999).

Isolates showed a wide range of sensitivities to clindamycin with MICs varying from ≤2μg/ml to >128μg/ml. *C. difficile* appears to be inherently resistant to the cephalosporin and cephamycin antibiotics as the majority of isolates had MICs to these agents of ≥32μg/ml (NCCLS, 1997). Strains resistant to clindamycin have been widely reported and some have been involved in epidemics (Johnson *et al.*, 1999).

Recently Fawley *et al.* (2003) have suggested that clindamycin resistance be used to further separate the endemic PCR ribotype 001 strains. Clindamycin usage has decreased dramatically due to its involvement in the precipitation of *C. difficile* diarrhoea. There is now little selective pressure for clindamycin resistance. The usual mechanisms by which clindamycin resistance is conferred also mediates resistance to other macrolide, lincosamide and streptogramin B antibiotics: this is known as the MLS resistance determinant (Mullany *et al.*, 1996; Farrow *et al.*, 2001). The MLS determinant is the major mechanism of multiple resistance among Gram-positive anaerobes (Noren *et al.*, 2002). The gene responsible (*ermB* in *C. difficile* 630) encodes a 23S ribosomal RNA methylase that modifies the target site for the antibiotic. The gene is 99% homologous to the *ermB* gene from *Clostridium perfringens* but unlike this gene it is not located on a plasmid (pIP402) but on a mobilisable non-conjugative transposon Tn*5398* (Farrow *et al.*, 2001). All the clindamycin-resistant strains tested were confirmed to contain this *ermB* gene though the product sizes differed. As is seen in Table 4.3 some strains produced a gene product of 320bp and others the expected product of 493bp. In the paper by Farrow *et al.* (2001), the source of the primers, two product sizes were also seen and this was due to the lack of a leader sequence in some strains. This is a further difference between the strains suggesting that the transposons may be from a different source. Sequencing the amplified products of *ermB* would confirm whether the difference in size was in fact due to the lack of a leader sequence as described in Farrow *et al.* (2001).

C. difficile possesses an outer cell coat termed the S-layer consisting of two polypeptides that form a regular crystalline array over the surface of the cell (Kawata *et al.*, 1984). The most common S-type in this study was 5236. The number corresponds to the molecular masses in kilodaltons of the two major polypeptides found on the cell surface. Out of all strains tested so far the molecular mass of the larger of the two proteins varies from 45-64kDa with the smaller ranging from 25-40kDa (Poxton *et al.*, 1999). S-layer typing is a quick and easy method of phenotyping and appears to correspond well with other typing techniques including ribotyping and serotyping (McCoubrey and Poxton, 2001). Toxigenic S-type 5236 is the same as PCR ribotype 001 (McCoubrey, 2002) which is the most common PCR ribotype (55%) in the UK (Stubbs *et al.*, 1999). The S-layer is a putative virulence factor that appears to have a role in adhesion of the bacterium to the host mucosal surface. Calabi *et al.* (2002) demonstrated this adhesion to gastrointestinal tissues mediated mainly by the high molecular weight S-layer protein. It may also have a role in immune evasion or impermeability to certain compounds-including antibiotics. Three of the four non-typeable strains appeared to be more sensitive to clindamycin, cefoxitin and ceftriaxone. They appear to have only one band but this may be two bands of the same or similar size. Though no firm conclusions can be made especially when this pattern was rare, it may be speculated that as they appear to lack a typical S-layer pattern on the gels they are more sensitive to some antibiotics (their membrane may be more permeable to antibiotics?). However, overall there were no obvious correlations between S-type and resistance to antibiotics.

Multiple isolates were obtained from 40 patients, and for some patients as many as 10 were available. These isolates permitted assessment of sensitivity patterns over time and within and between S-types. In the majority of cases isolates did not change either in S-type or in sensitivity pattern. The isolates from some patients did change in antibiotic sensitivity and in the S-type suggesting that there had been re-infection with a different strain, or possibly emergence of a minor strain from an initially mixed infection. In patients whose isolate did not change in S-type, resistance to clindamycin was the only significant difference observed. Resistance to clindamycin typically resides on a transposon, Tn*5398* (Mullany *et al.*, 1996; Farrow *et al.*, 2001) which could transfer between strains. It is feasible that the strain acquired this resistance determinant, or that the patient was reinfected with a clindamycin-resistant strain of the same, predominant S-type. In the patient whose strain appeared to lose clindamycin resistance it is possible that the resistance determinant was lost. More likely is the explanation that the patient had picked up another 5236 S-type that lacked the clindamycin-resistance determinant. In the patients who produced same-type isolates with changing resistance it would be interesting to use another typing method (sero- or ribotyping) to try and identify sub-types which may explain the sensitivity changes. There was no direct evidence that resistance to clindamycin was selected in strains despite the use of macrolides in many patients during the study. The presence of wide-spread use of clindamycin may be required for this type of selection but as clindamycin is so associated with the precipitation of CDD this situation is now extremely rare.

CHAPTER 5 The effect of sub-inhibitory concentrations of antibiotics

AIMS

1. To investigate the effects of sub-inhibitory concentrations of antibiotics on the growth of *C. difficile*.
2. To investigate toxin production in *C. difficile* in the presence/absence of sub-MIC antibiotics.
3. To compare effects between different antibiotics and different strains.

The effects of sub-inhibitory concentrations of antibiotics on the growth and toxin production of *C. difficile* have been studied little over the years. Early studies by Onderdonk *et al.* (1979) and Honda *et al.* (1983) discovered a possible role for certain antibiotics in the potentiation of toxin production. Onderdonk *et al.* (1979) found vancomycin and penicillin to affect cytotoxin production and Honda *et al.* (1983) found clindamycin and cephaloridine to affect cytotoxin and enterotoxin production. This work was undertaken to attempt to clarify the relationship of antibiotics and the growth and toxin production of *C. difficile*.

RESULTS

Three strains of *C. difficile* were used in this study: NCTC 11223, strain 630 (recently sequenced) and strain 338a a local endemic strain collected during a recent epidemiology study (McCoubrey, 2002). Strain 338a is of S-type 5236 (PCR ribotype 001) and was present in 78% of cases of *C. difficile* diarrhoea in a geriatric unit in the Royal Victoria Hospital, Edinburgh (McCoubrey, 2003). The antibiotics

chosen for the study were vancomycin (Sigma V2002) and metronidazole (Sigma M1547), two agents used for treatment of *C. difficile* associated disease, and four of the agents associated with precipitating *C. difficile* disease: amoxycillin (Sigma A8523); clindamycin (Sigma C5269); cefoxitin (Sigma C4786) and ceftriaxone (Sigma C5793). These are the same antibiotics used previously in the MIC chapter. The minimum inhibitory concentrations (MICs) of the six antibiotics for these strains were determined by broth macrodilution (NCCLS, 1997) and are shown in Table 1. The concentration of antibiotics used in this study corresponded to 1/2, 1/4 and 1/8 of the MIC except in the case of clindamycin with strain 11223. This strain was highly resistant and 512μg/ml, the highest concentration achievable in the study, allowed growth of this strain.

5.1 Growth and toxin production - controls

In preliminary experiments and in Chapter 3, OD_{600} values reflected viable counts and were therefore used to assess bacterial growth. Figure 5.1 shows the growth curves and toxin levels for the untreated controls of each strain. Values represent means (with standard errors) of six replicates grown on six different occasions and as can be seen growth varied little between strains. Each strain was clearly in log phase by 8h and stationary phase began by ca. 24h. Decline was then apparent from 32h with the OD stabilising by ca. 56h. However, toxin production differed between strains, both in how much and when it was produced relative to growth. Strain 11223 produced less toxin A than strains 338a and 630. Toxin A production by strain 11223 rarely exceeded the measurable levels of the assay (OD_{600} 3.0), whereas higher values (>3.0) were obtained with strains 338a and 630. A notable difference was the point in the growth phase at which each strain produced toxin. Strain 338a

produced toxin during stationary phase (by 24h), which preceded toxin production by both 11223 and 630 in late stationary phase and early decline. This further correlated with the times required to produce comparable levels of toxin i.e. 24, 32 and 48h for 338a, 630 and 11223, respectively. Levels of toxin A in strains 338a and 630 generally reached maximum readable levels by 48h.

Table 5.1 MICs of the three strains.

	Minimum Inhibitory Concentration (mg/L)					
Strain	**Van**	**Met**	**Amox**	**Clind**	**Cefo**	**Ceft**
11223	2	1	8	>512	256	64
338a	1	1	4	4	256	64
630	2	1	4	256	256	128

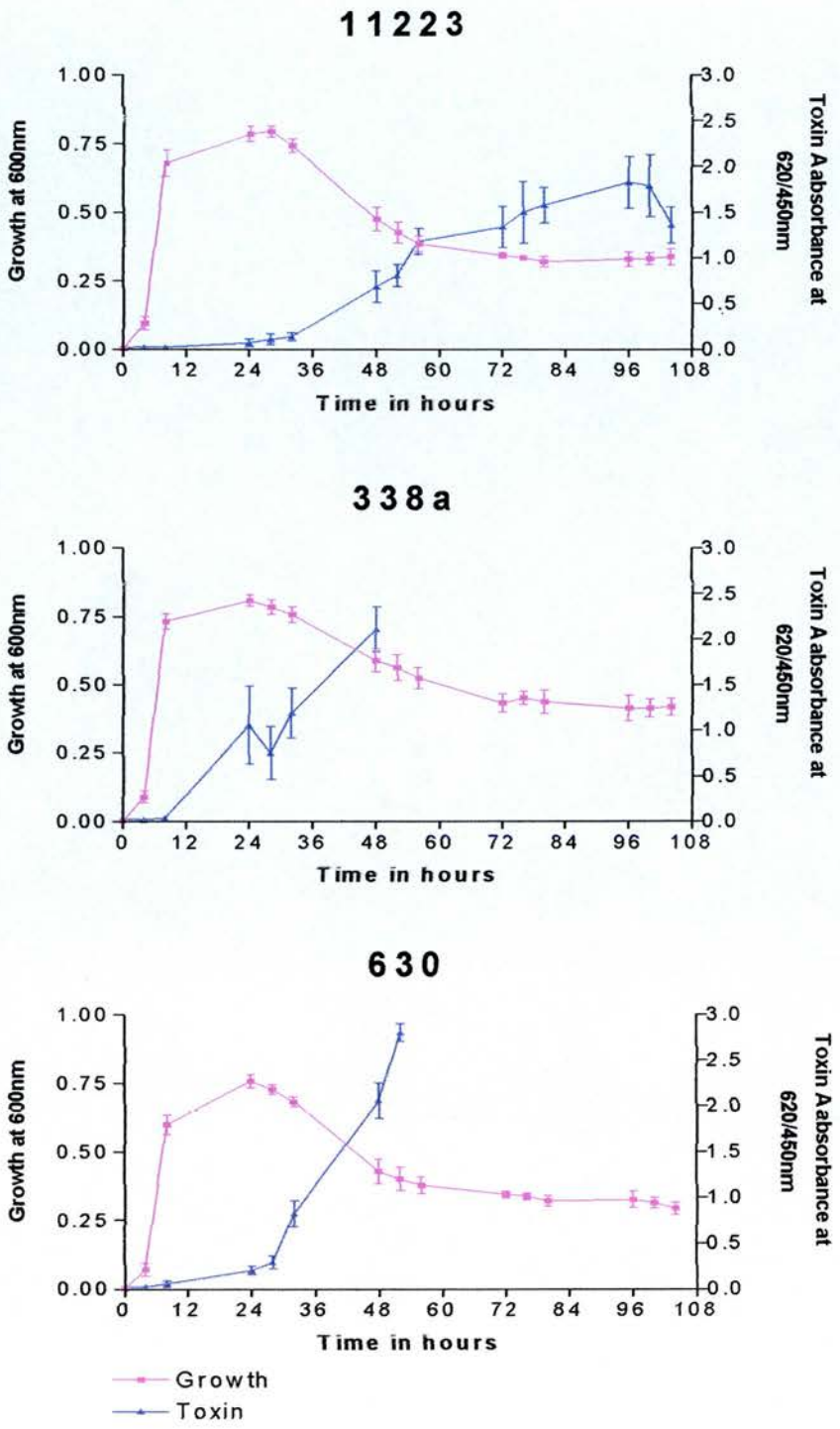

Figure 5.1 Control growth for the 3 strains.

These graphs show the controls used in the sub-MIC work. They represent the strains grown without antibiotics six times with standard error.

5.2 Effects of sub-MIC antibiotics on growth

The effects of sub-MIC levels of antibiotics on bacterial growth were very similar between antibiotics and strains. Sub-inhibitory concentrations of antibiotics tended to delay the growth of the bacteria – increasing the lag period, especially at the highest concentration (1/2 the MIC) of antibiotic. All three strains with every concentration of antibiotic showed a lag in growth compared to the control with one exception. This exception was strain 11223 with clindamycin. This highly resistant strain showed no growth lag whatsoever even at the highest concentration of antibiotic. With strain 11223 it was impossible to get the true 1/2 MIC as it grew at the highest achievable experimental concentration of 512mg/L. For the other combinations the growth lags were often a few hours though some were up to 24 hours.

5.3 Effects of sub-MIC antibiotics on toxin production

Figure 5.2 shows the three strains grown in vancomycin. As shown in Table 5.2 vancomycin only produced two examples of bringing forward onset of toxin production. The first example was at 1/8 MIC with strain 11223. There was a lag in growth of ca. 4h but toxin still appeared before the antibiotic-free control. In this case the levels of toxin produced were greater than the control as well as being produced quicker. The other example was strain 630 grown in 1/2 MIC. This showed a significant growth lag of ca. 24h but the toxin was produced very quickly after growth was first measurable.

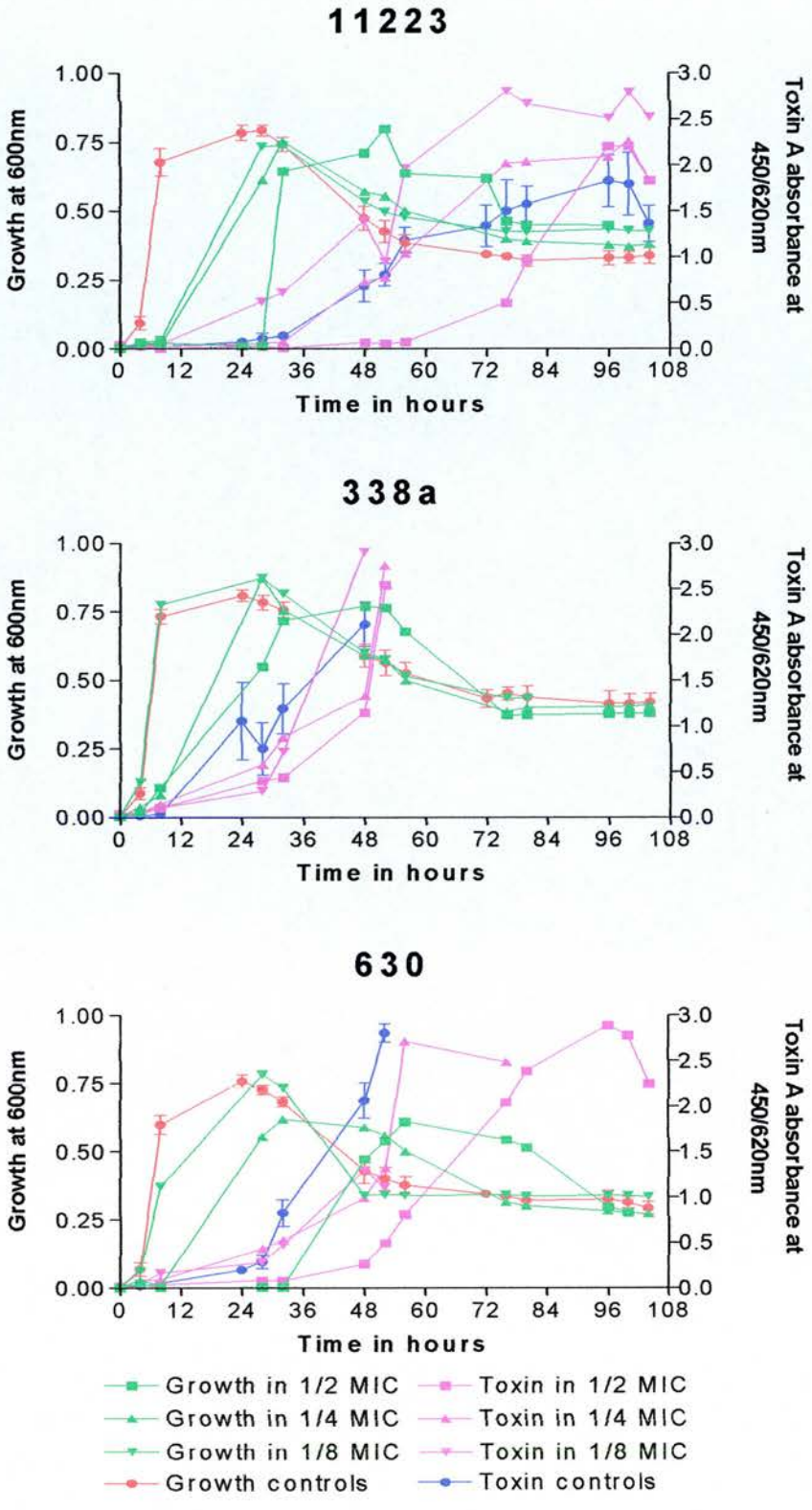

Figure 5.2 Strains with sub-MIC levels of vancomycin.

These graphs show the strains grown with sub-MIC concentrations of vancomycin.

Figure 5.3 shows the strains grown in the presence of metronidazole. In strain 11223 there was a growth lag with all three sub-MIC concentrations of metronidazole but in all cases the toxin was produced before the control (comparable levels in the control is 24h away). Strain 338a showed a similar pattern. When grown with 1/4 and 1/8 MIC of metronidazole toxin A was again produced before the control even with a lag in growth. In the graph this appeared as though toxin was being produced as soon as measurable (ca. 10^6 cells/ml) growth was seen. The concentration of 1/2 MIC in this strain produced a long lag of ca. 24h though soon after growth was seen toxin also appeared. Strain 630 shows the same profile as 338a. Concentrations of 1/4 and 1/8 produced a lag but the toxin still appeared before the control. Again there was a long lag with 1/2 MIC with toxin A appearing soon after growth was measurable.

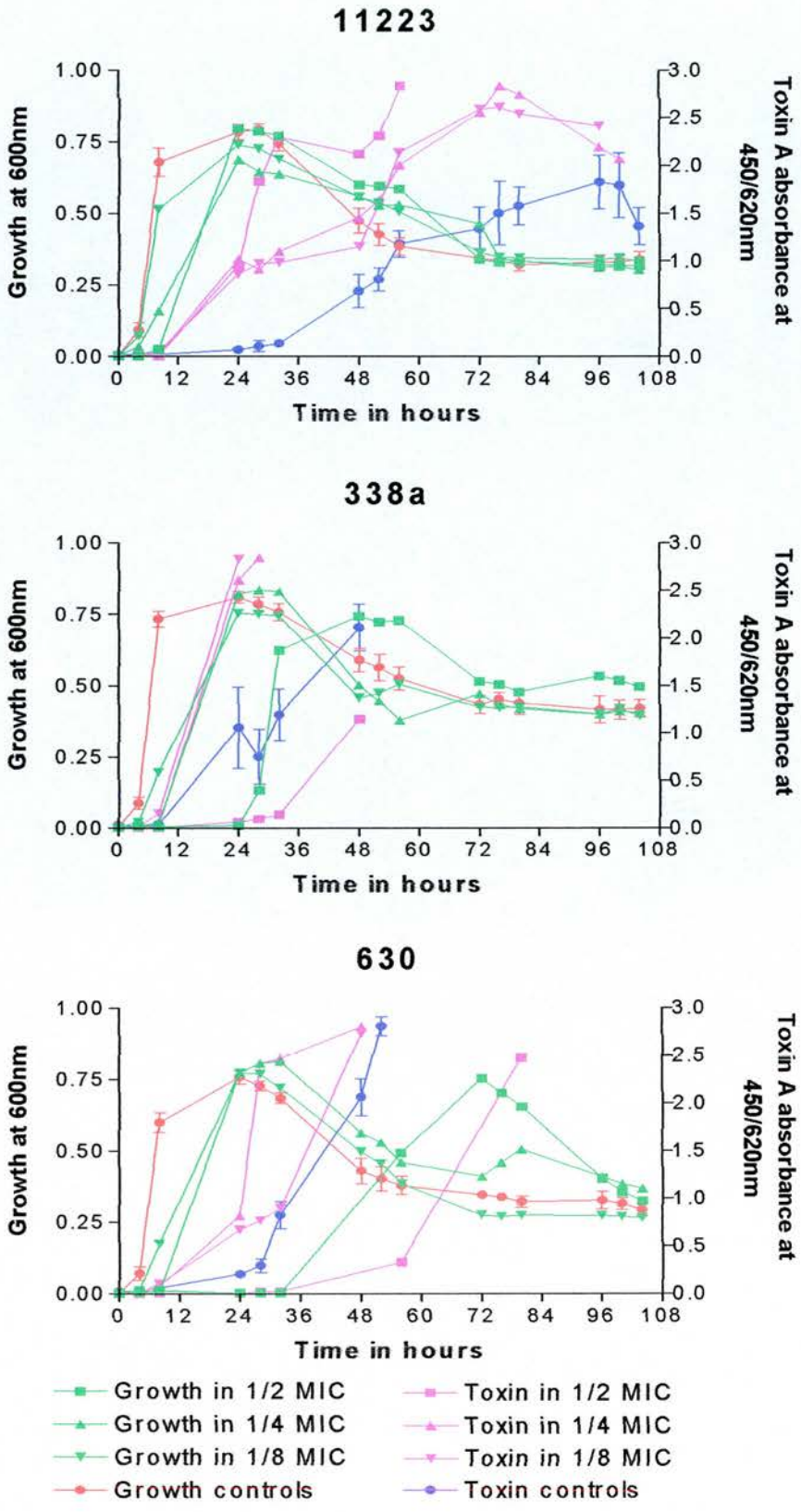

Figure 5.3 Strains with sub-MIC levels of metronidazole.

These graphs show the strains grown with metronidazole.

Figure 5.4 shows the results of the sub-MIC experiment with amoxycillin. All three concentrations of amoxycillin with strain 11223 produced a growth lag of ca. 4h and a shift forward in toxin A production. Toxin A appeared before the controls at every concentration but the most marked effect occurred at 1/8 MIC with it being produced almost as soon as growth was measurable. Toxin in the presence of 1/4 MIC appeared next followed by 1/2 MIC. Strains 338a and 630 show the same pattern when grown in amoxycillin with 1/2 MIC producing a significant lag of ca. 24h and the two other concentrations a short lag (ca. 4h). Toxin A appeared before the control in all three concentrations for both strains.

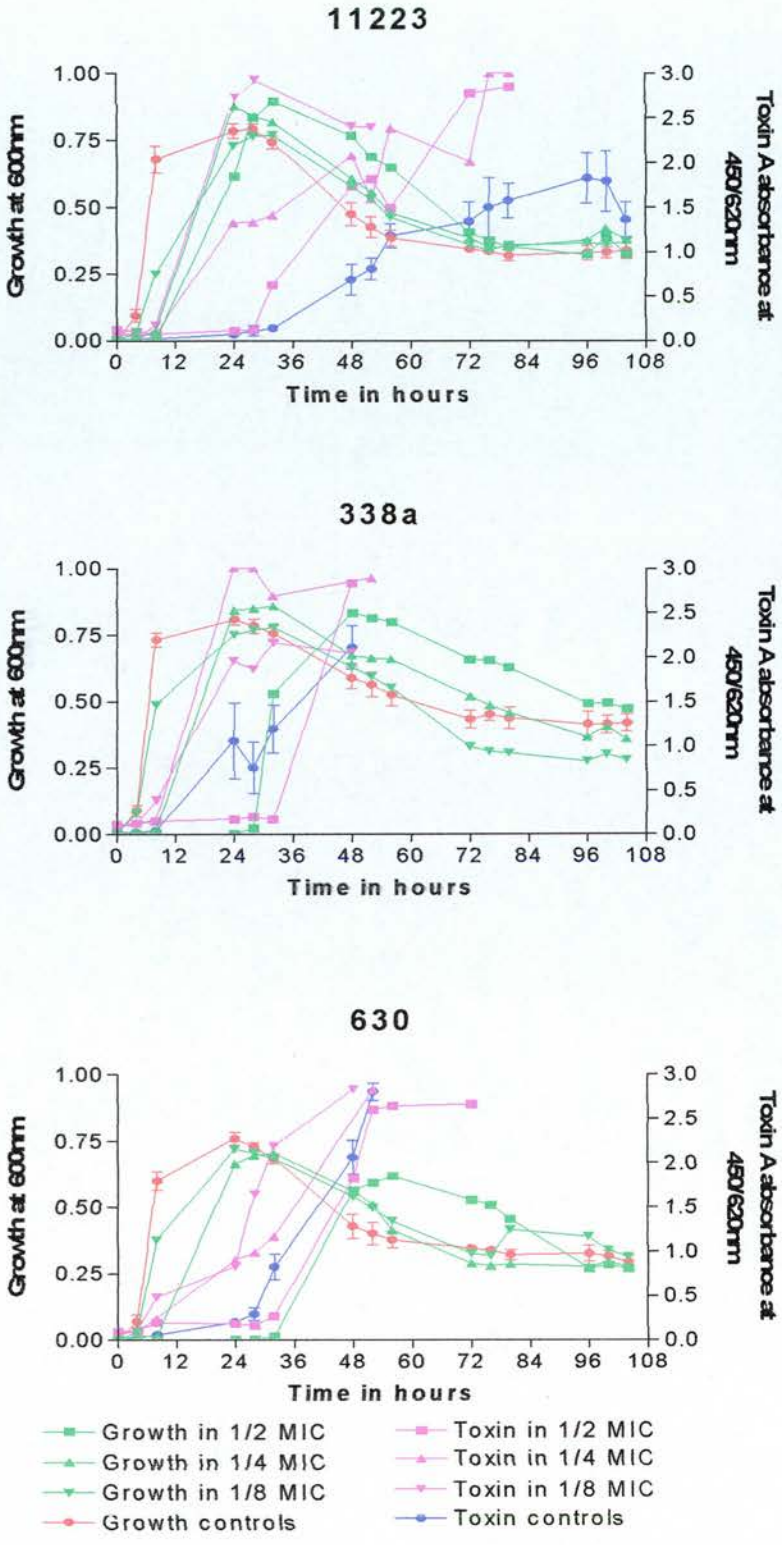

Figure 5.4 Strains with sub-MIC levels of amoxycillin.

The graphs represent the strains grown in sub-MIC concentrations of amoxycillin.

Figure 5.5 shows the results of the strains grown with clindamycin. It should be noted that strain 11223 is highly resistant to clindamycin (MIC of >512μg/ml) and for this strain only; the sub-MICs were not truly 1/2, 1/4 and 1/8, but fractions of 512μg/ml. When these sub-MIC levels of clindamycin were added, growth of *C. difficile* was not noticeably affected (Figure 5.5), but toxin production was affected. Compared to the antibiotic-free control the toxin was elaborated sooner and reached higher levels than in the absence of clindamycin. Thus this antibiotic potentiated toxin production by both acceleration and enhancement of production. The other two strains showed a significant lag of ca. 24h with 1/2 MIC and ca. 8h with 1/4 and 1/8 MIC. Strain 630 also contains the Macrolide, Lincosamide and Streptogramin B (MLS) resistance determinant and has a MIC of 512μg/ml though the sub-inhibitory concentrations had a more pronounced effect on this strain than on 11223. The effect of clindamycin on toxin production also differed between 338a and 630 compared with 11223. The situation was less clear in these two strains. Strain 338a in the presence of 1/2 MIC produced toxin very quickly after growth was detected. In 630 the toxin was produced later and there was no clear effect at this concentration. With 1/4 and 1/8 MIC in both strains toxin was produced earlier than expected and in the case of 630 before the controls even with a lag in growth.

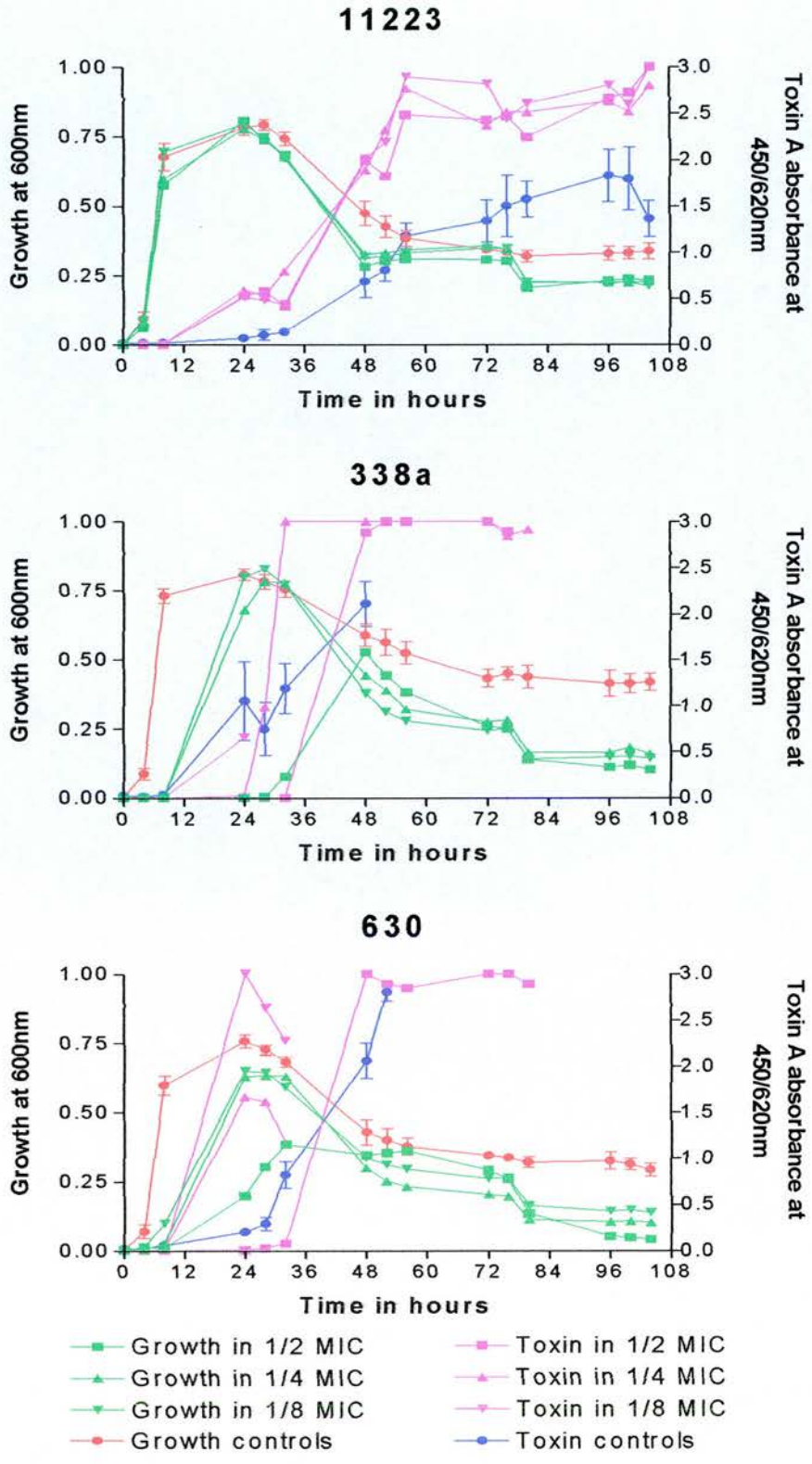

Figure 5.5 Strains with sub-MIC levels of clindamycin

The graphs show the three strains grown with clindamycin.

Figure 5.6 represents the strains grown in the presence of sub-inhibitory concentrations of cefoxitin. This 2nd generation cephalosporin (cephamycin) produced a lag in growth at every concentration with every strain. Each strain cultured with cefoxitin (at every concentration) showed an apparent inhibition in toxin production. In the case of 11223 the toxin levels did not reach the levels of the control and with strains 338a and 630 the levels remained within the measurable levels of the assay.

Figure 5.7 shows the strains grown with ceftriaxone. At 1/2 MIC with 11223 this antibiotic produced a growth lag of ca. 12h but no subsequent lag in toxin production. The other two concentrations had no effect on growth or toxin. Strain 338a at 1/2 MIC showed a growth lag of ca. 8h that produced a corresponding lag in toxin production. Ceftriaxone showed no obvious effect on toxin production in strain 338a. Strain 630 with 1/4 MIC of ceftriaxone produced a lag of ca. 8h but the toxin still appeared slightly before the controls.

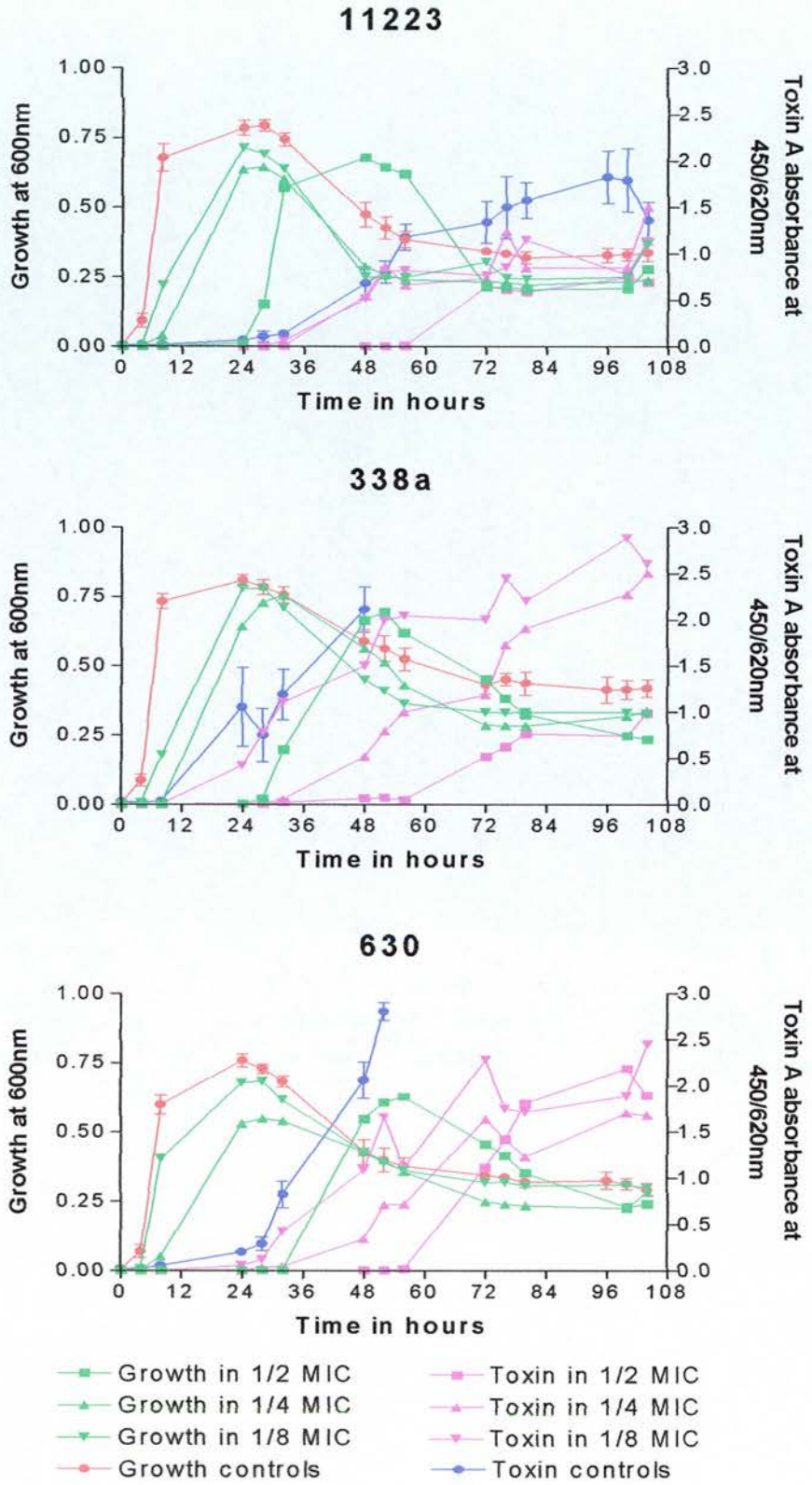

Figure 5.6 Strains with sub-MIC levels of cefoxitin.

These graphs represent the strains grown with cefoxitin.

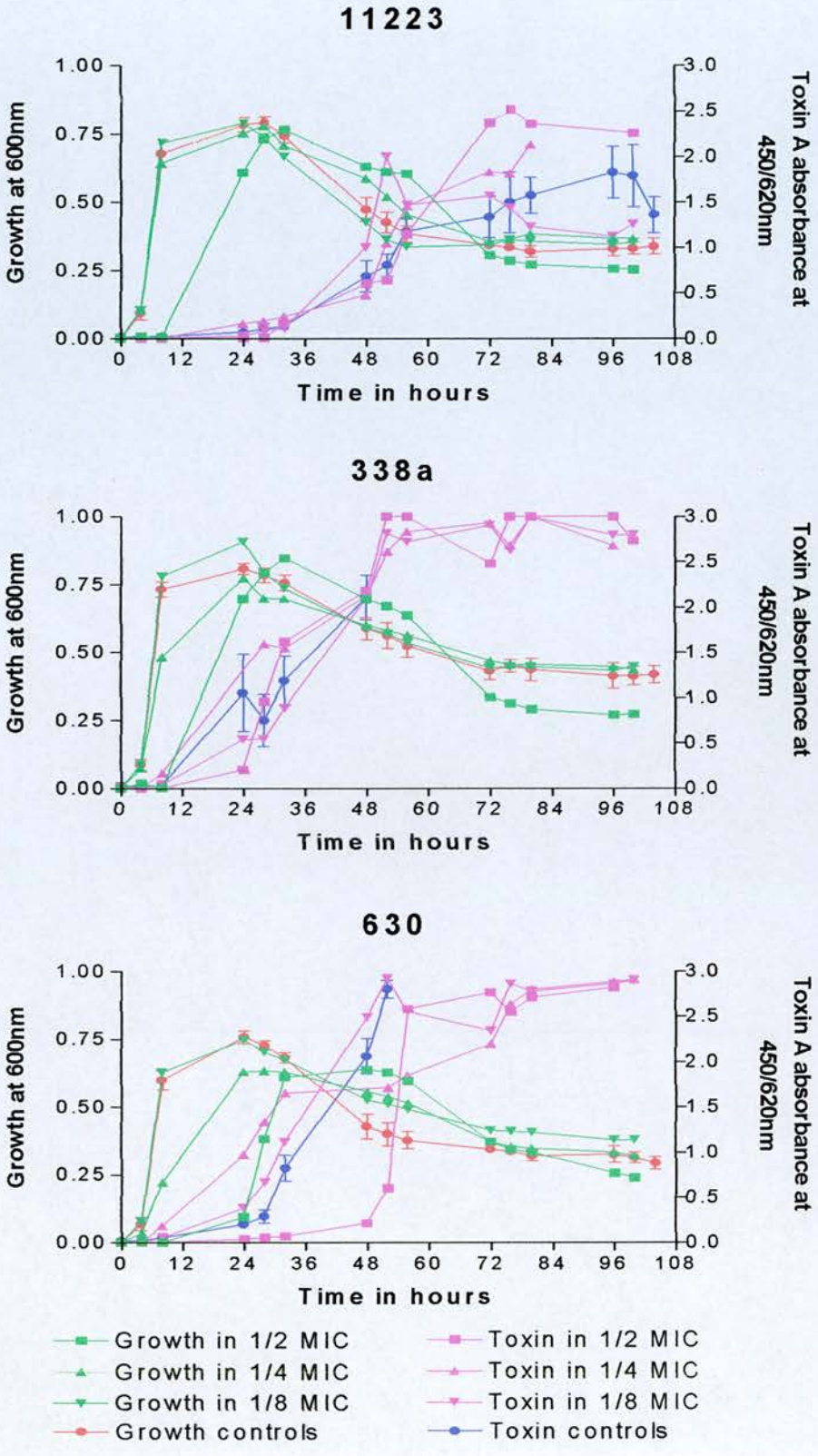

Figure 5.7 Strains with sub-MIC levels of ceftriaxone

The figure shows the strains grown in the presence of sub-MIC ceftriaxone.

The effects on toxin production for all strains and antibiotics are summarised in Table 5.2. For toxin production, three general consequences were evident: toxin level was increased; toxin was produced earlier; or toxin level was unaffected or reduced. The most common effect on toxin production was the shift forward of the onset to a point earlier in the growth phase. Even with a lag in growth some strains produced toxin earlier than the control with some antibiotics. As shown in the table there were four occasions where toxin A was produced sooner and reached greater levels than the control. This effect may in practice be more common but due to the high toxin levels in strains 338a and 630 exceeding the maximum of the assay these levels could not be measured. As is clear from the table some antibiotics were more likely to cause effects on toxin production than others.

Metronidazole, amoxycillin and clindamycin commonly caused these effects. Vancomycin, ceftriaxone and cefoxitin rarely affected toxin production.

Table 5.2 Summary of effects on toxin.

	Antibiotic and effect on toxin (T)					
Strain	**Van**	**Met**	**Amox**	**Clind**	**Cefo**	**Ceft**
11223						
1/2	-	E	E	+ E	-	E
1/4	-	E	E	+ E	-	-
1/8	+ E	E	E	+ E	-	-
338a						
1/2	-	-	E	E	-	-
1/4	-	E	E	E	-	-
1/8	-	E	E	E	-	-
630						
1/2	E	-	E	-	-	-
1/4	-	E	E	E	-	E
1/8	-	E	E	E	-	-

- = no effect or reduced, E = elaborated sooner, + = toxin level increased

1/2, 1/4 and 1/8 are the concentrations which correspond to 1/2, 1/4 and 1/8 of the MIC

5.4 Discussion

For *C. difficile* there is a clear relationship between disease and antibiotic usage such that antibiotics are most often a prerequisite for the disease. Broad-spectrum agents especially have been shown to predispose to *C. difficile* infection through depletion of the patient's normal protective bowel microbiota. The equilibrium of the gut, when disturbed, leaves the patient open to opportunistic infection possibly through the newfound availability of binding sites and nutrients. Thus suppression of colonisation resistance by antibiotics facilitates colonisation and promotes disease. Hence *C. difficile* is the commonest cause of nosocomial, antibiotic-associated diarrhoea.

It has been proposed that antibiotics may promote CDAD not solely by modulating commensal microorganisms, but also by physiological effects that affect pathogenicity (Lorian & Gemmell, 1994). Several early reports (Onderdonk *et al.*, 1979; Honda *et al.*, 1983) suggested that certain antibiotics potentiated production of toxins A and/or B, the main recognised virulence factors of *C. difficile*. Furthermore, antibiotics have been shown to affect the expression of virulence factors in other species including *Escherichia coli*, *Vibrio cholerae* and various staphylococci (Levner *et al.*, 1977; Yoh *et al.*, 1983 & Lorian *et al.*, 1971). Determining the effect of antibiotics on virulence factor expression in an organism for which antibiotics are important triggers of disease is therefore crucial. This work focused on the effect of sub-inhibitory concentrations of six antibiotics, including those that precipitate the disease and those used for treatment, on the production of toxin A by three strains of *C. difficile*.

This study has clearly shown that there is heterogeneity between strains in respect to growth, MICs and the toxin levels that are produced. A common effect on the bacteria in the presence of antibiotics was the slowing of growth in comparison to the controls. They took longer than the controls to reach stationary phase at all three sub-MIC concentrations or just at the higher concentrations of antibiotics. In addition to slower growth the bacteria sometimes failed to achieve the same OD that the controls had reached. This was seen in many cases with the strains cultured with cefoxitin. Even with sub-inhibitory concentrations it would still be expected that they have an affect on the bacterial systems, including growth. Strain 11223 is highly clindamycin resistant (MIC >512μg/ml) and the growth of this strain was not affected at all in the presence of this antibiotic. An explanation for this may be that it is so well adapted to this agent that it can function and grow as normal. This strain contains the macrolide, lincosamide and streptogramin B resistance determinant (MLS) that contains the *ermB* gene (encodes an RNA methyltransferase) which makes it resistant to these antibiotics (see Chapter 4.4). Strain 630 also carries the *ermB* gene (Farrow *et al.*, 2001) but it has a slightly lower MIC of 512μg/ml and its growth is affected by all three sub-MIC concentrations of clindamycin. The reasons for this are uncertain but heterogeneity between strains was common during this work.

As can be seen in table 5.2 and figures 5.2-5.7, the sub-MIC concentrations of antibiotics can cause quicker elaboration of toxin A compared to the antibiotic-free control. Antibiotics, even at sub-MIC concentrations, can be expected to cause stress on the bacteria. Bacteria under stress switch on a catalogue of genes and it is possible that the toxin promoters are affected by it. To support this, TcdD, the alternative

sigma factor of the toxin genes, shows similarity to UviA the UV-inducible regulator from *Clostridium botulinum* (Mani & Dupuy, 2001). Onderdonk *et al.* (1979) showed that the stress of increased temperature led to greater cytotoxin production. In the same paper they demonstrated an increase in toxin in the presence of sub-inhibitory concentrations of vancomycin and penicillin. Karlsson *et al.* (2003) also showed temperature as a controlling factor for toxin and TcdD expression. It has been shown by Hennequin *et al.* (2001) that *C. difficile* cultured in the presence of antibiotics produces greater levels of GroEL, a chaperone from the heat shock protein 60 (Hsp60) family. The examples all serve to suggest that the toxin promoters can respond to multiple environmental stresses. Inducing this stress response may enable *C. difficile* to survive the gut environment better after colonisation as GroEL functions as a 58kDa surface adhesin. This adhesin could help *C. difficile* to colonise the recently vacated binding sites left by the depletion of the normal gut flora.

Type strain 11223 produces lower levels of toxin than the sequenced strain 630 and the 'endemic' strain 338a. During the course of the experiments the 11223 samples almost never exceeded the limits of the ELISA plate reader (>3.0). The other two strains commonly reached levels greater than 3.0 after ca. 48 hours of growth. It was desirable to look at the trends of toxin production and this was achievable by comparing the OD values of the antibiotic-free control and the strain in the presence of antibiotics. Differences in toxin production are not well understood though a recent paper by Spigaglia and Mastrantonio (2002) showed strains with variants of TcdC, the putative negative regulator of toxin production. No correlation between

disease severity and variant TcdC strains was found, though it is possible that changes in this protein would affect toxin production. For example, they found one nonsense mutation which reduced the TcdC protein from 232 to 61 amino acids. Lack of a functional protein may lead to abrogated repression of the toxin genes. This may be an explanation for the differences common between strains of *C. difficile*. PCR would show if the strains used in this work differed in *tcdC*.

In addition to the disruption of the barrier flora in *C. difficile* disease, antibiotics also appear to increase the stress response in the bacteria. For example, upregulation of the adhesin GroEL may increase the virulence of the infecting *C. difficile* by aiding its utilisation of the new niche. The reason for producing toxins in the gut is unclear but as they are upregulated during glucose starvation their purpose may be to cause cell disruption for the acquisition of nutrients (Dupuy & Sonenshein, 1998).

CHAPTER 6 Sub-MIC effects on PaLoc and *groEL* mRNA

AIMS

1. To look for toxin transcripts in total RNA extracted using the Qiagen RNA extraction kit.
2. To attempt to detect toxin transcripts in conditions (sub-MIC antibiotics) which demonstrated an increase in toxin A in Chapter 5.
3. To investigate message levels of the alternative sigma factor (*tcdD*) and putative negative regulator (*tcdC*).
4. To investigate the theory that sub-MICs cause increased toxin production through a stress response by looking for transcript levels of the stress protein GroEL

After the interesting results garnered from Chapter 5 with the effect of sub-inhibitory antibiotics on toxin A production it was decided to attempt to analyse toxin transcripts under these conditions. Sub-inhibitory concentrations of clindamycin produced a remarkable effect on the growth and toxin production in strain 11223. The growth of this highly clindamycin-resistant strain (MIC >512μg/ml) was unaffected even at the highest concentration of antibiotic (512μg/ml) unlike the other strains used in the study (sequenced strain 630 and locally endemic strain 338a) which experienced a lag in growth. This was likely due to the fact that this strain was so highly resistant to this antibiotic. The effect on toxin production was also interesting in that it caused the elaboration of toxin A earlier than 11223 grown without antibiotics. There was also more toxin A produced in sub-inhibitory antibiotic conditions (measured by ELISA) than in antibiotic-free controls. It was

this clear-cut effect on toxin A that led to this strain being chosen for the RT-PCR study.

C. difficile produces toxins A and B upon entry to stationary phase and others have shown the toxin transcripts to be transcribed in the highest amounts in late log and early stationary phase.

Colleagues within the department had successfully used the Qiagen RNA extraction kit to look at mRNA transcripts in eukaryotic cells. It was considered easy to use and yielded good concentrations of RNA.

RESULTS

6.1 Toxin transcripts from strain 11223

The first step was to look for the toxin transcripts in control-growth *C. difficile* before moving on to the analysis of different conditions. This would clarify the best time points and procedures to use before moving on to investigate different environmental conditions.

Strain 11223 was grown to stationary phase (20h & 24h) in AIM under anaerobic conditions and two parts (2ml) of Qiagen Protect Bacteria Reagent was added to one part (1ml) *C. difficile* culture. The protect reagent stops the degradation of the RNA prior to extraction and is especially good for protecting mRNA species which have very short half lives. This also allows the mRNA profile at that time to be "frozen" and remain unaffected until the start of the extraction process. The resulting pellet can be stored for up to one month at -70°C without significant degradation. RNA was extracted from the two pellets (20h and 24h) using the Qiagen RNA extraction kit according to the manufacturer's instructions but with the addition of an on-

column DNase step. The Qiagen extraction column is designed to prevent DNA contamination of the extracted RNA but a colleague recommended the inclusion of a DNA digestion step as she regularly encountered problems with contamination. cDNA was synthesised using Qiagen Omniscipt Reverse Transcriptase (RT) with appropriate negative controls minus the RT enzyme. The cDNA produced was used in PCR reactions with three different primer sets. Primers for the two toxin genes (Braun *et al.*, 1996) and a control set specific to *C. difficile* 16S RNA were used. Figure 6.1 shows the gel resulting from this PCR and it is clear that DNA contamination is present in the negative control of the 24h sample (lane 8).

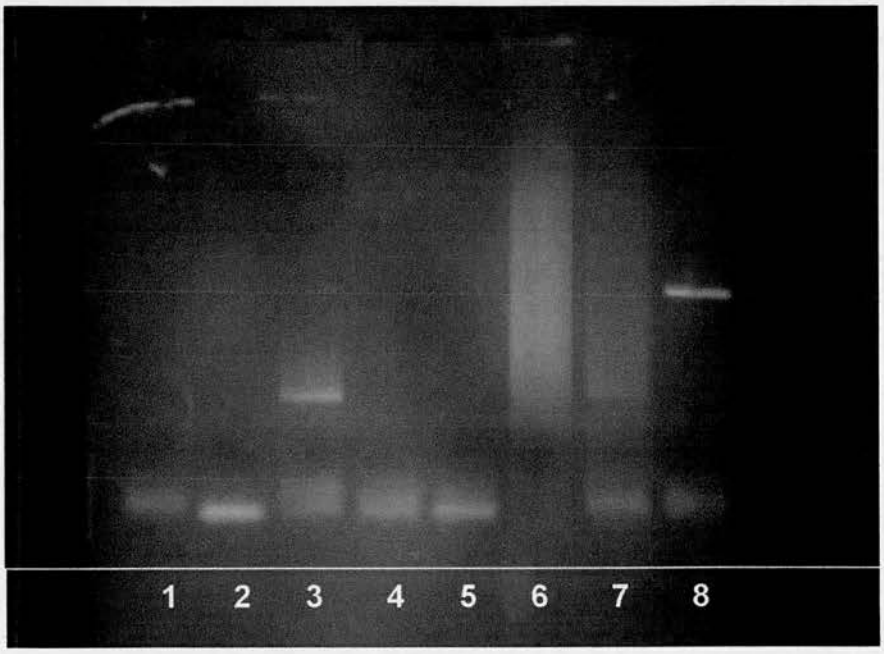

Figure 6.1 Preliminary RT-PCR

Lanes 1-4 contain samples taken at 20 hours and lanes 5-8 from 24 hours. Strain 11223 was used. Lanes 1 and 5 used toxin A primers; lanes 2 and 6 primers for toxin B; lanes 3 and 7 16S RNA primers (positive control) and lanes 4 and 8 negative controls using toxin A primers but no RT enzyme during the cDNA step.

The negative controls in lanes four and eight should be free of product as no RT enzyme was added, preventing any conversion of RNA into cDNA. If no RT enzyme is present then anything that reacts in the PCR has to have arisen from contaminating DNA. The contamination of the extracted RNA occurred even with the addition of the DNA removal step; a step which the Qiagen handbook says is optional. Only one out of the two samples contained DNA and it was thought that this may be an anomaly. Smears were also seen on this gel (24h sample) with the primers for toxin B and 16S. This may be due to RNA degradation within this sample and hereafter precautions and increased care was taken when handling the RNA and cDNA. Two further RT-PCRs were carried out using this RNA but the same results were found each time. Despite these flaws it was decided to go ahead and grow strain 11223 in the presence of clindamycin (an antibiotic which produced in this strain a marked effect on toxin A in Chapter 5). The RT-PCR experiment would be performed on these samples to analyse any difference in transcript levels (toxins A, B and 16S RNA) between controls and *C. difficile* 11223 grown with sub-MIC clindamycin.

6.2 Strain 11223, clindamycin and DNA

Strain 11223 with 1/2, 1/4 and 1/8 MICs of clindamycin was grown as in Chapter 5 to try to achieve continuity between experiments. Again as in Chapter 5 due to the highly resistant nature of this strain true MIC could not be achieved. A concentration of 512μg/ml was the highest concentration achievable in this study and strain 11223 grew at this concentration with ease. The samples were prepared as before with one part culture and two parts Protect reagent. Extra care was taken during the RNA extraction process to ensure that the protocol was religiously followed (application of reagents etc. directly to membrane, stringent RNase-free solutions and equipment).

This was to increase the likelihood of yielding good quality RNA for the subsequent steps of the experiment. RNA was extracted from the resulting pellets (with the on-column DNase step) and processed through the RT-PCR. The results were not good. The resulting gel showed all eight negative controls to contain very prominent bands where they should have been free of DNA. It was decided to run a PCR using the supposedly DNA-free RNA to look for contaminating DNA.

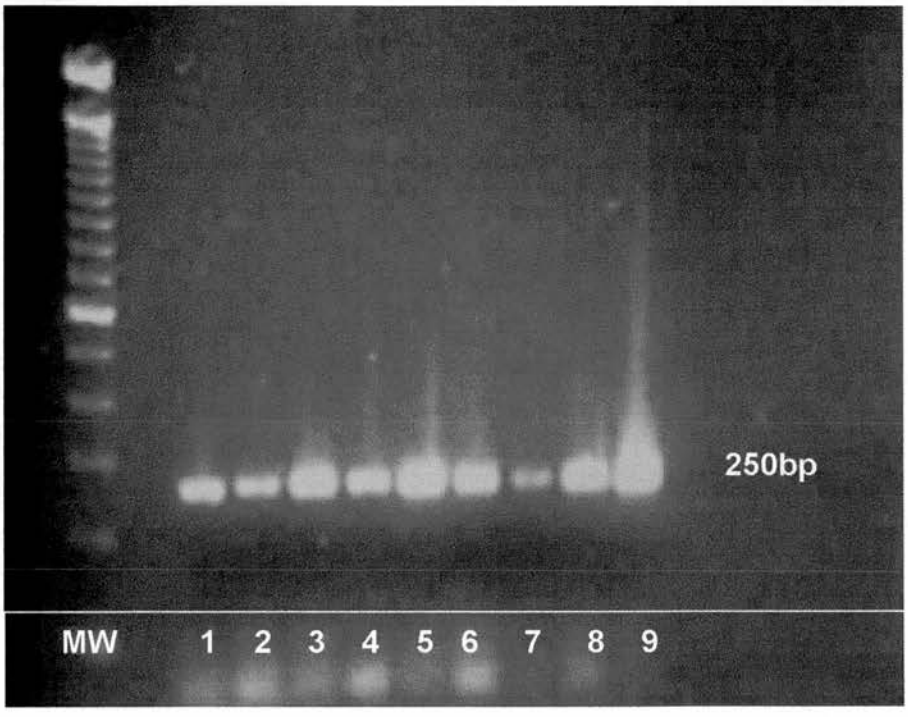

Figure 6.2 DNA contamination of RNA

This gel shows the extent to which the extracted RNA was contaminated with DNA. Lanes 1-8 contain RNA samples and lane 9 is a positive control. The primer set used was 16S RNA.

This gel clearly shows how much DNA was present in the RNA extracts. Many of the negative controls contain as strong a band as the positive control in lane 9. Clearly DNA was going to be a problem with this kit and procedure. At this point Qiagen were contacted to help in solving this problem. They provided an alternative

DNA removal step (see Materials and Methods for protocol) which involved digesting the DNA after RNA extraction and not during extraction on-column. The negative controls that contained DNA were treated using this protocol and 5µl used in a PCR reaction. This time no bands were seen in the negative controls but no bands were seen in the RT-PCR reactions either. No transcripts had been amplified though the DNA had been effectively removed. This protocol was used hereafter for every RNA extraction in place of the on-column DNA digestion.

This RNA was now free of DNA contamination but upon quantification it was revealed that very little RNA was present in the samples. The RNA was quantified using spectrometry (at 260nm) according to a Qiagen protocol (Qiagen bench guide). On average a yield of 50-150ng RNA was found in the extracted RNA samples. This is very low in comparison to *Bacillus subtilis*, which Qiagen states as producing a yield of 33µg on average. Despite this low RNA yield the extracts were tested using all three primer sets (toxin A, toxin B and 16S) and the resulting gel is seen in Figure 6.4. The only bands on this gel other than the positive controls appear in lanes 10-13 which contain the 16S RNA transcripts. No toxin transcripts were seen at all.

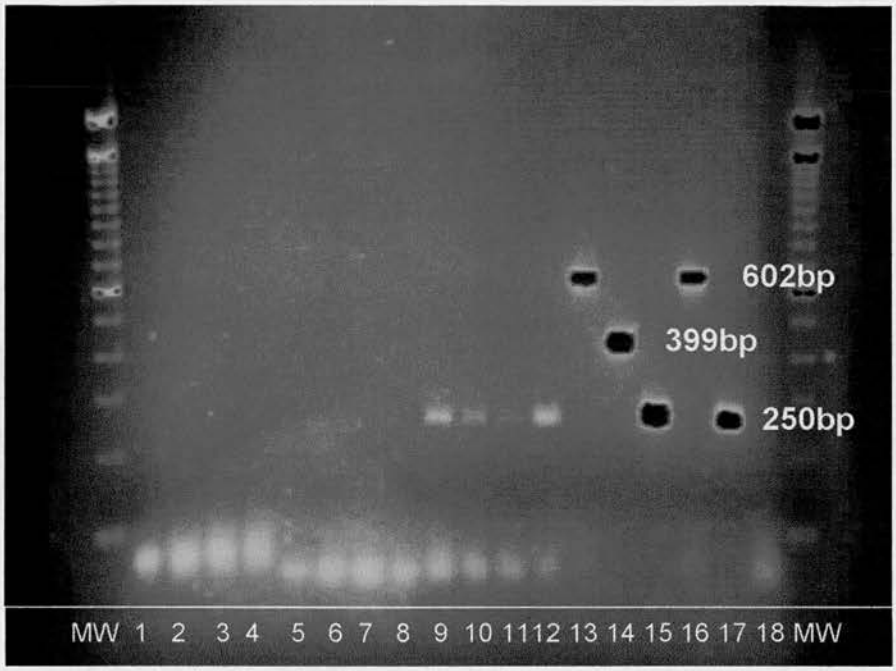

Figure 6.4 ***toxA*, *toxB* and *16S***

Lanes 1-4 contain samples from 1/2, 1/4, 1/8 MIC and control with the primers for *toxA*. Lanes 5-8 the same with *toxB* primers and lanes 9-12 16S RNA primers. The positive controls are found in lanes 13-17. *toxA* primers produce a product of 602bp and *toxB* and *16S* RNA products of 399bp and 250bp respectively.

6.3 Strain 11223, clindamycin and Sensiscript

Due to this low yield it was decided that a more sensitive reverse transcriptase would likely produce a better result. The Qiagen Sensiscript enzyme is suited for RT reactions using <50ng. A new experiment sampling strain 11223 in the presence of clindamycin was needed as the RNA from the previous experiments had all been used. At this point the lysozyme concentration was increased at the start of the protocol to ensure complete lysis of the cell and release of RNA (from 50mg/ml to 100mg/ml). As an extra precaution the RNA samples were passed through a Qiashredder to help remove debris and to break down DNA to help facilitate its

removal from the column. This was done as it was thought that the low RNA yield may have been partly due to poor release of RNA from the cells. This extracted RNA was DNase treated as before using the alternative protocol supplied by Qiagen. Upon quantification the RNA concentration was again low, between 50-150ng, as in the previous samples. The toxin primer sets were used here along with the 16S RNA primer set. Again no toxin transcripts were seen though the bands corresponding to the 16S RNA were clearer using Sensiscript than they were when Omniscript was used. Several more RT-PCR's were performed with a cDNA step extended to one hour to try to increase the likelihood of a positive result. Despite this lack of toxin transcripts it was decided to expand the targets to look for other gene transcripts that may be important in virulence.

6.4 *tcdC*, *tcdD* and *groEL*

New primer sets were ordered for the putative negative regulator of the PaLoc *tcdC*, and the alternative sigma factor *tcdD* (Mani & Dupuy, 2001). Primers for the stress protein gene *groEL* (Hennequin *et al.*, 2001) were also ordered. It was hoped that a difference in the *tcdD* transcripts alongside the toxin mRNA would be seen. Primers for *tcdC* were included, as higher levels of transcripts for this gene are usually present when the toxin mRNAs are low (Hundsberger *et al.*, 1997). As discussed in Chapter 5 the effect of antibiotics may be stress-related so transcripts for *groEL* was investigated to try to corroborate this theory. The same RNA that was used in section 6.3 was also used here in a PCR reaction with the three new primer sets and the control set of 16S. The gel in Figure 6.5 shows the results for this PCR.

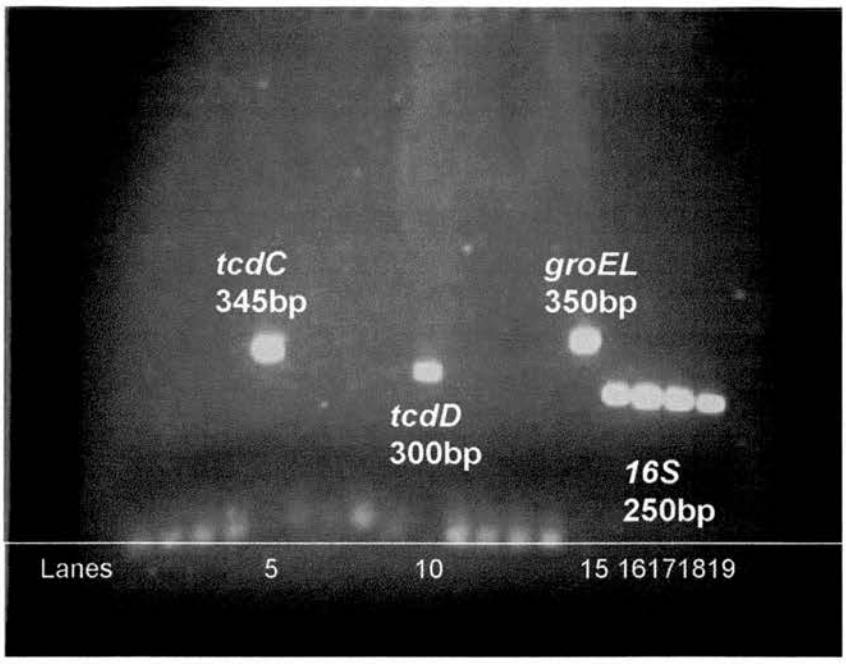

Figure 6.5 ***tcdC, tcdD, groEL* and *16S***

Lanes 5, 10 and 15 contain the positive controls for genes *tcdC*, *tcdD* and *groEL* respectively. No negative controls are shown on this gel as they were already shown to be clear of DNA on a separate gel. Lanes 1-4, 6-9,11-14 and 16-19 contain samples (1/2, 1/4, 1/8 and control) and the primer sets *tcdC*, *tcdD*, *groEL* and *16S* respectively. Again the negative controls had been certified DNA-free on a separate gel.

It is obvious from this gel that no transcripts of *tcdC*, *tcdD* or *groEL* genes were seen. The last four lanes of the gel contain cDNA resulting from an RT reaction with primers for 16S RNA. These are shown clearly as before when the Sensiscript enzyme was used for the first time (gel not shown) and this is likely due to the increased sensitivity of Sensiscript. Two more cDNA steps were performed but still no transcripts for genes other than 16S RNA were seen.

6.5 Discussion

It was hoped at the start of this work to see the toxin effect found in Chapter 5 mirrored in the toxin mRNA. That is, to see toxin transcripts in the antibiotic-containing experiments appear before mRNA in the controls. Despite the development involved in this work with the use of different primers, DNA digestion and the increase in sensitivity offered by Sensiscript, the only transcripts seen were those of 16S RNA. This was extremely disappointing as it would have been valuable to analyse the transcripts in different conditions even if a difference in these transcripts was not apparent. It can only be concluded that the levels of transcripts to toxins A and B, *tcdC*, *tcdD* and *groEL* are considerably lower than those of 16S RNA. Ribosomal mRNA is extremely prevelent in the bacterial cell and so the relative ease with which this is seen was unsurprising. The production of toxins by *C. difficile* is costly to cell in terms of energy output. The half-life of the toxin mRNA is perhaps quite short which would make the mRNA inherently unstable. The low concentration of RNA extracted using the Qiagen kit was also disappointing. In *Bacillus subtilis*, another Gram positive spore-former, vastly greater yields of RNA are extracted using the kit (55μg - Qiagen handbook) for the same number of bacterial cells (10^9 cells). A number of protocols were offered by Qiagen to test the efficacy of the kit and my handling of it. One of these was to use a sample with a known concentration of RNA and to process it through the extraction kit and DNA digestion to see if there was any loss in yield at the end. If the yield of RNA decreased after passage through the kit then it would indicate a loss of RNA at some point in the protocol. Unfortunately time was of the essence and this work, after months of development, was abandoned to allow time for other work.

It would have been interesting to look at mRNA transcripts using an alternative extraction, DNA digestion and RT-PCR method as RNA studies in *C. difficile* have been successfully done by others. The extraction steps in other protocols have the added benefit of being able to use larger volumes of culture – Qiagen could only accommodate a maximum of 10^9 cells in their mini-kit. This may allow the RT enzyme to pick up RNA from genes other than 16S RNA. A number of groups have successfully looked at mRNA transcripts from various genes in *C. difficile*. Many utilised the Trizol reagent from Gibco BRL, formerly Life Technologies, now incorporated into Invitrogen. This reagent contains phenol and guanidine hydrochloride for extraction of RNA. Song *et al.* (1999) and Dupuy and Sonenshein (1998) both used this reagent and they went on to successfully study *C. difficile* RNA. Calabi *et al.* (2001) used Tri reagent from Sigma which contains acid:phenol and the RNA is precipitated with ethanol. Another group that incorporated an ethanol precipitation in their methodology was Hennequin *et al* (2001). They pelleted the cells with Bentone Rheological additive: phenol SDS. The aqueous phase was recovered and extracted three times with phenol: chloroform and the precipitation with ethanol followed. In the paper by Hundsberger *et al.* (2001 it was demonstrated that *tcdC* transcripts are present during exponential phase and toxin transcripts appear in stationary phase. They showed that mRNA transcripts for the toxin genes and the accessory proteins *tcdD* and *tcdE* appeared in late log – early stationary phase. The times chosen for this work were 20 hours and 24 hours which represent early stationary phase in this strain and in these conditions. The toxin transcripts, although not visualised using RT-PCR, should have been present at this time. The transcripts for the stress protein GroEL should also have been present as they are

seen during the stress response and also during normal cell growth. We had hoped to see these transcripts increase in the experiment containing antibiotics compared to control conditions. Hennequin *et al.* (2001) managed to find mRNA transcripts of *groEL* so it should have been possible in this work.

One type of method that has successfully analysed mRNA transcripts is the use of RNA protection assays. This allows the digestion of unwanted RNA leaving only the mRNA desired for the particular study. If this method had been tried in this work it may have proved successful, as it would have increased the likelihood of the small numbers of mRNA transcripts being transformed into cDNA and subsequently amplified.

The conclusions from these experiments must be that there are significantly lower levels of transcripts of *toxA*, *toxB*, *tcdC*, *tcdD* and *groEL* than of 16S RNA. This undoubtedly led to the difficulty in analysing these messages and ultimately led to the unsuccessful outcome. In hindsight it would have been better to choose an established protocol for extraction and analysis of mRNA in *C. difficile*. The availability of the Qiagen kit and the presence of departmental expertise in its use led to this method being used. Perhaps if an established method had been used then this would have determined a different outcome.

CHAPTER 7 Proteomic study of total cell protein of *C. difficile*

AIMS

1. To establish a working protocol for the preparation of whole cell extracts of *C. difficile* 630 for two-dimensional (2D) gel electrophoresis.
2. To elucidate the pattern of proteins produced under standard laboratory growth conditions.
3. To compare protein profiles under control conditions and during exposure to sub-inhibitory concentrations of ceftriaxone.
4. To investigate protein spots on the 2D gels, including any that differ between conditions, and to attempt to identify them using matrix-assisted laser desorption/ionization –time of flight (MALDI-TOF) mass spectrometry and a *C. difficile* MASCOT database.

After the effects seen on toxin A in the presence of sub-inhibitory concentrations of antibiotics (Chapter 5) it was important to investigate the broader physiological effects antibiotics may have on *C. difficile*. Preliminary work had already shown sub-inhibitory concentrations of antibiotics to have no effect on the S-layer proteins of the bacterium, in a change in mass or in the amount produced. The secreted protein profile was studied using 1D-PAGE but it was difficult to analyse the complex pattern with one-dimensional separation. A proteomic study using 2D gel electrophoresis and MALDI-TOF was required to get an accurate and more complete picture of the total cell protein profile of *C. difficile*. Total cell protein extracts were used as they would give an overall picture of the profile of proteins from this

bacterium. It was expected that using total cell extracts would produce a complex picture of the proteins produced in this strain. In the studies by Karlsson *et al.* (1999) and Mukerhjee *et al.* (2002) they found ca. 500 proteins mostly with pIs between 4 and 7 in their samples.

Sample preparation is arguably the most important step in 2D gel electrophoresis. If the sample preparation is complete and reproducible then the separation stages become routine. Many different methods have been employed for the extraction of proteins for 2D electrophoresis. The presence of spores in clostridia require a more vigorous method of cell disruption to ensure complete lysis and recovery of proteins. Tip sonication was successfully used in the study by Sinchaikul *et al.* 2002 to study cold shock in *Bacillus stearothermophilus* and by Schaffer *et al.* (2002) to study solventogenesis in *Clostridium acetobutylicum*. The initial method used in this work did not include a sonication step but had previously been successful with mycobacteria so it was a useful starting point with *C. difficile*.

RESULTS

7.1 Preliminary set 1

C. difficile was cultured in AIM in volumes of 5ml, 10ml and 20ml and grown for 18 hours to early stationary phase (OD_{600} – 0.8). The pellets were washed three times in ice-cold PBS to remove the medium contaminants. The cultures and pellets were kept on ice to reduce the risk of protein degradation. The pellets resulting from 5 and 10ml were resuspended in 200µl of lysis buffer (see Appendix 1) and an additional 200µl of lysis buffer was added to the 20ml pellet to ensure complete lysis. Although this method has been used successfully for mycobacteria after 30 minutes of

vortexing there was a great amount of insoluble material still visible in all three samples. Further disruption (10 minutes) in a sonic bath was added into the protocol to aid the lysis of the cells. After this step there was still a great amount of insoluble material present and the samples were centrifuged and the supernatant (100µl; 5, 10 & 20ml pellets) processed through the Amersham Clean-up kit (Amersham Biosciences 80-6484-51). The precipitated proteins recovered from the Clean-up kit were resuspended directly into rehydration buffer (see Appendix 1) and 125µl of each used to rehydrate 7cm pH 3-10 linear IPG strips (Amersham Biosciences 17-6001-11). The strips were focused to 40000 volt hours. These strips were placed on a small 2D gel (250 × 110 × 0.5, Amersham Biosciences 80-1261-01) and the proteins separated according to their MW using the Amersham Biosciences MultiPhor II system. Figure 7.1 shows the resulting gel stained with colloidal coomassie blue.

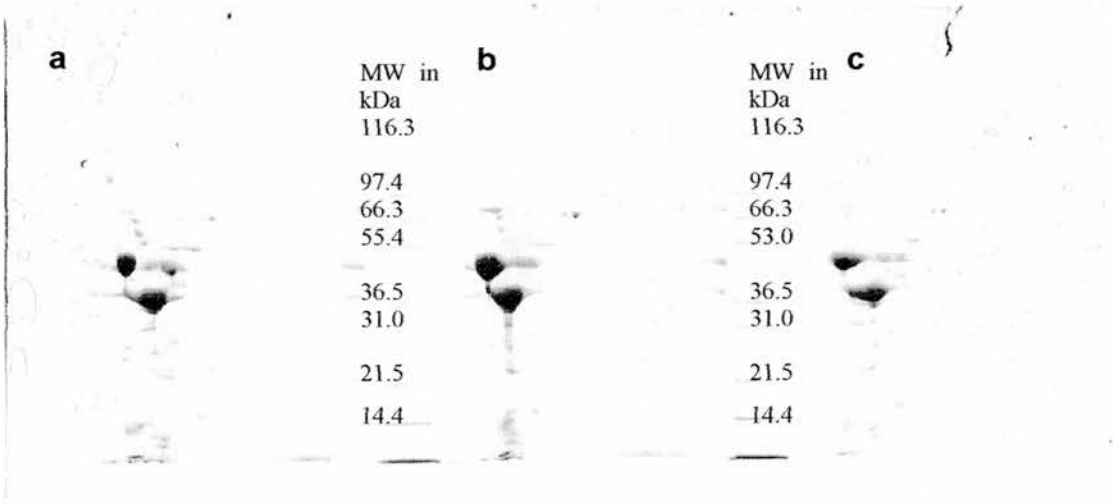

Figure 7.1 Preliminary sample preparation

This figure shows the three protein samples resulting from 5ml (a), 10ml (b) & 20ml (c) pellets, focused on 7cm 3-10 linear IPG strips and run on a small 2D gel. The gels were stained with Coomassie blue and the MW marker used was Mark 12 (Invitrogen LC5677).

These preparatory experiments were necessary to establish the proteomic pattern of *C. difficile* and determine the volumes of *C. difficile* culture required. As is clear from the figure, the pattern and quantity of proteins on the gels were visually similar between all three samples. It appeared that the sample preparation protocol was saturated by the size of pellets used since so much insoluble material remained after the lysis and sonication steps. From this it was clear that less material would be required in future experiments.

All three gels contained two large protein spots at ca. 46 and 36kDa which are likely to correspond to the S-layer proteins. The S-layer proteins are extremely abundant on the cell surface and are also the two major bands on 1D gels. On 1D gels using guanadine hydrochloride-extracted proteins the upper S-layer protein appears as ca. 52kDa. Others have shown using urea extracts the higher MW S-layer in this strain to be of ca. 46kDa. The extraction process appears to have some effect on the size or migration of this protein. Due to the saturation found by using large volumes of culture it is possible that incomplete solubilisation of total cell protein had occurred on this occasion. The next preliminary experiment was set-up using smaller pellets to try to alleviate this problem.

7.2 Preliminary set 2

For these experiments *C. difficile* was again grown in AIM for 18 hours but in smaller volumes of 1ml, 2ml and 3ml. The pellets from these were used in the sample preparation with one difference from the first set. After witnessing the amount of insoluble material in the previous preparations more vigorous cell disruption was employed (three rounds of tip sonication of 10s @ 50% output). After sonication there was little or no insoluble material left in the sample. These samples

after passage through the Clean-up kit were focused on 13cm non-linear pH 3-10 strips. This strip contains a pH range of 3-10 but it focuses on the range between 4 and 7 which covers the majority of isoelectric points of proteins. The IPG strips were again focused to 40000 volt hours on a small 2D gel. Figure 7.2a– c show these gels stained with colloidal coomassie blue.

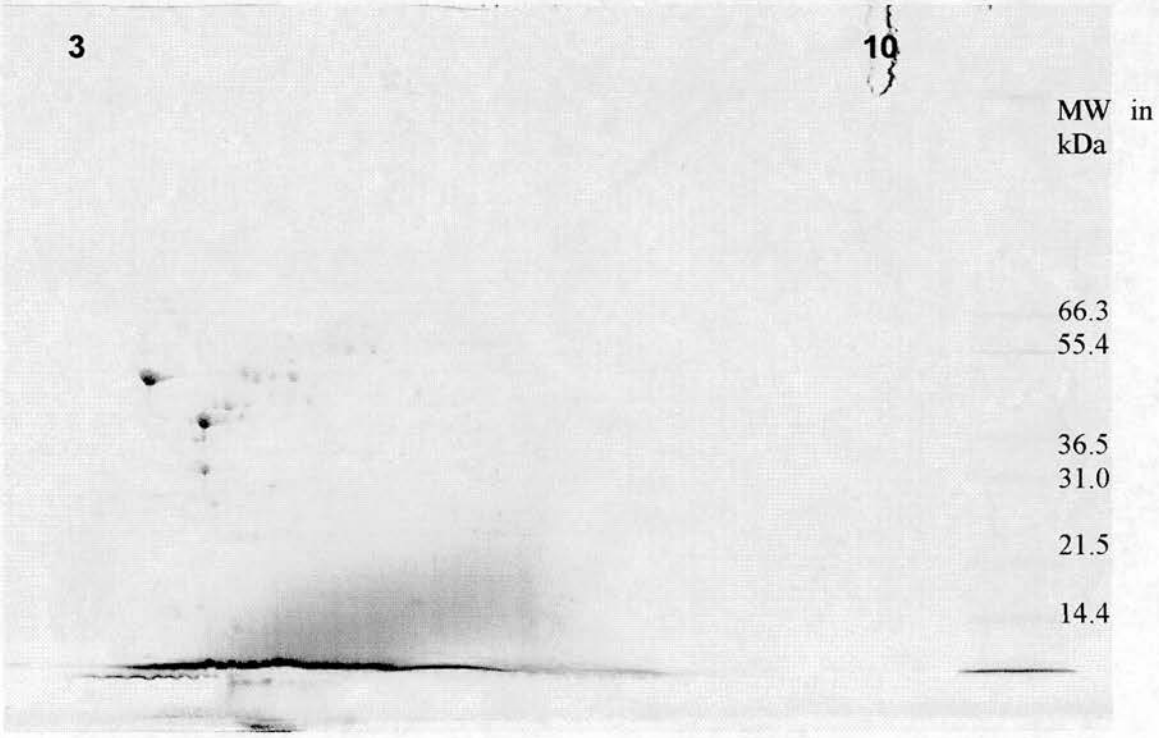

Figure 7.2a ***C. difficile*** **protein extractions from 1ml of culture**

The proteins extracted from 1ml of culture were focused on a 13cm IPG strip and separated on a small gel.

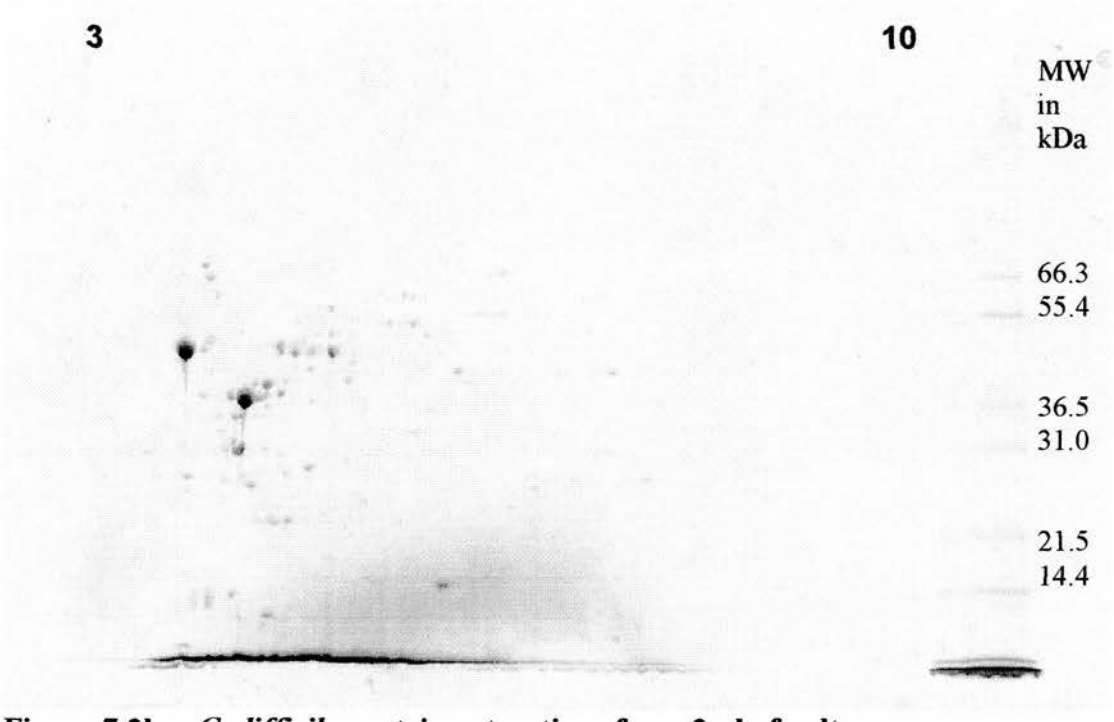

Figure 7.2b ***C. difficile*** **protein extractions from 2ml of culture**

The proteins extracted from 2ml of culture were focused on a 13cm IPG strip and separated on a small gel.

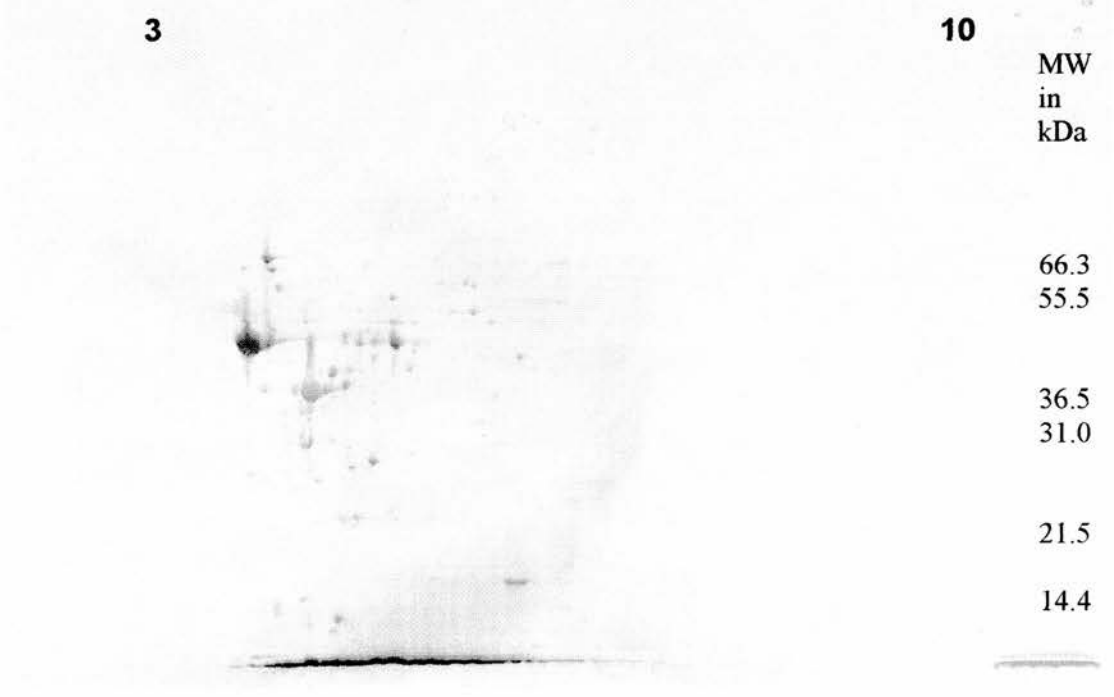

Figure 7.2c ***C. difficile*** **protein extractions from 3ml of culture**

The proteins extracted from 3ml of culture were focused on a 13cm IPG strip and separated on a small gel.

These gels again show the two large spots, which are the putative S-layer proteins, but as there was less protein in these samples these proteins no longer masked the lower abundance spots around them. Figure 7.2a using the pellet from 1ml culture clearly shows the majority of proteins and is ideal for identifying spots around the putative S-layer proteins. Figures 7.2b and c, the gels from the 2ml and 3ml pellets respectively, show a few more of the low abundance proteins but the larger protein loading has begun to mask the proteins around the putative S-layer spots. This set of gels clearly showed that the sample preparation had been successful as the same pattern of spots was seen each time from different experiments. One way of ensuring that the two large spots would not mask the lower abundance ones of similar size/p*I* was to use a longer IPG strip and a bigger gel to ensure maximum separation. The experimental set-up was now ready to begin a comparison of environmental conditions using the optimum conditions listed below.

- Protein extraction from 1ml of culture.
- Vigorous disruption of cells using tip sonication.
- 18cm IPG strips for maximum separation of proteins in the 1st dimension.
- Large 12-14% gels for maximum 2nd dimension migration of proteins.

7.3 Effect of sub-inhibitory concentrations of ceftriaxone on *C. difficile* 630

7.3.1 Growth set-up and preliminary gels

The combination of sub-inhibitory concentrations of ceftriaxone on strain 630 was chosen for proteome analysis. From the sub-MIC growth curves (Chapter 5), this antibiotic had profound effects on the growth and toxin A production of this strain.

For instance, a ceftriaxone concentration of 1/4 MIC produced a lag in growth and an increase in toxin A production. Typically, conditions which affect growth, in this case ceftriaxone, would be expected to result in stress on the bacteria which may in turn affect the protein profile.

The experimental proceedure for the growth of *C. difficile* in the presence of sub-inhibitory concentrations of ceftriaxone was as described in Chapter 5. In brief, bacteria were grown to an OD_{600} of 0.8 which represents the start of stationary phase in these conditions. The experiment was carried out in triplicate to try to achieve reproducibility and a valid protein profile under each condition. Although ceftriaxone at concentrations of 1/2, 1/4 and 1/8 MIC were sampled only the experiments at 1/2 MIC were used for the proteomic study as this produced the greatest effect on the growth of *C. difficile* in Chapter 5. To check the sample integrity and the profiles of the triplicate experiments two small 12.5% gels were run with each experimental condition focused on a 7cm, pH 3-10 non-linear strip (3 X controls and 3 X sub-MIC). These are seen in Figures 7.3a and b.

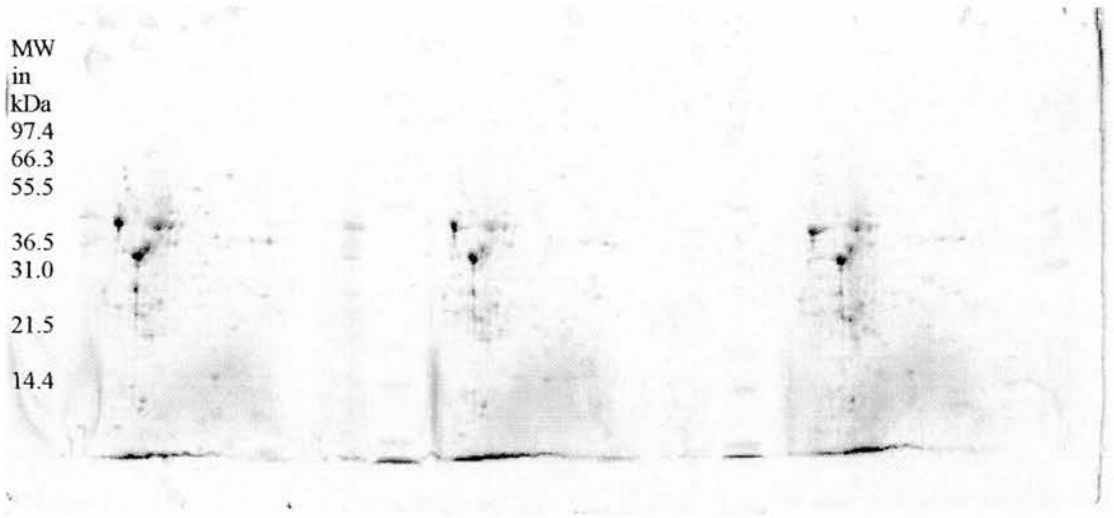

Figure 7.3a Controls from triplicate experiment

Three triplicate antibiotic-free controls from sub-MIC experiment separated on 7cm pH 3-10 strips.

Figure 7.3b Sub-MIC ceftriaxone in triplicate

This figure shows the sub-MIC pellets from three experiments.

Figure 7.3a shows the three triplicate control experiments. As is clear from the gel the pattern appears visually similar to samples from control-growth *C. difficile* 630 in previous experiments. Figure 7.3b shows the gels resulting from the growth in triplicate of *C. difficile* in the presence of 1/2 MIC ceftriaxone. The important thing

to note with these gels are that the proteins are not degraded or streaky and the three separate experiments show the same pattern – likely a valid proteome profile. At first analysis no visable differences between the two sets of conditions was seen.

The integrity of the samples had been established and the experiment proceeded with a comparison of control growth and growth in the presence of ceftriaxone.

7.3.2 Sub-MIC vs. controls

The next stage of the development was to separate these samples further on 18cm strips and larger 2D gels to get a more complete proteome picture for each experiment (control vs. sub-MIC ceftriaxone).

The same control and sub-ceftriaxone (1/2 MIC) samples used for gels 7.3a and b were examined in this experiment. To achieve better 1st dimensional separation of the proteins 18cm IPG strips were used, focusing until they reached 46000 volt hours. They were then separated on large 12-14% gels (245 × 180 × 0.5, Amersham Biosciences 17-1236-01). Figures 7.4a-c show the triplicate controls and as is clear from the gels they show a similar pattern and are also similar to the previous gels. Figure 7.5a-c show the samples from *C. difficile* grown with 1/2 the MIC. These gels are visually similar to each other, confirming the reproducibility of the experiment.

Figure 7.4a Control 1

This figure shows the pellet from 3ml of culture from experimental control 1.

Figure 7.4b Control 2

This figure shows the pellet from 3ml of culture from experimental control 2.

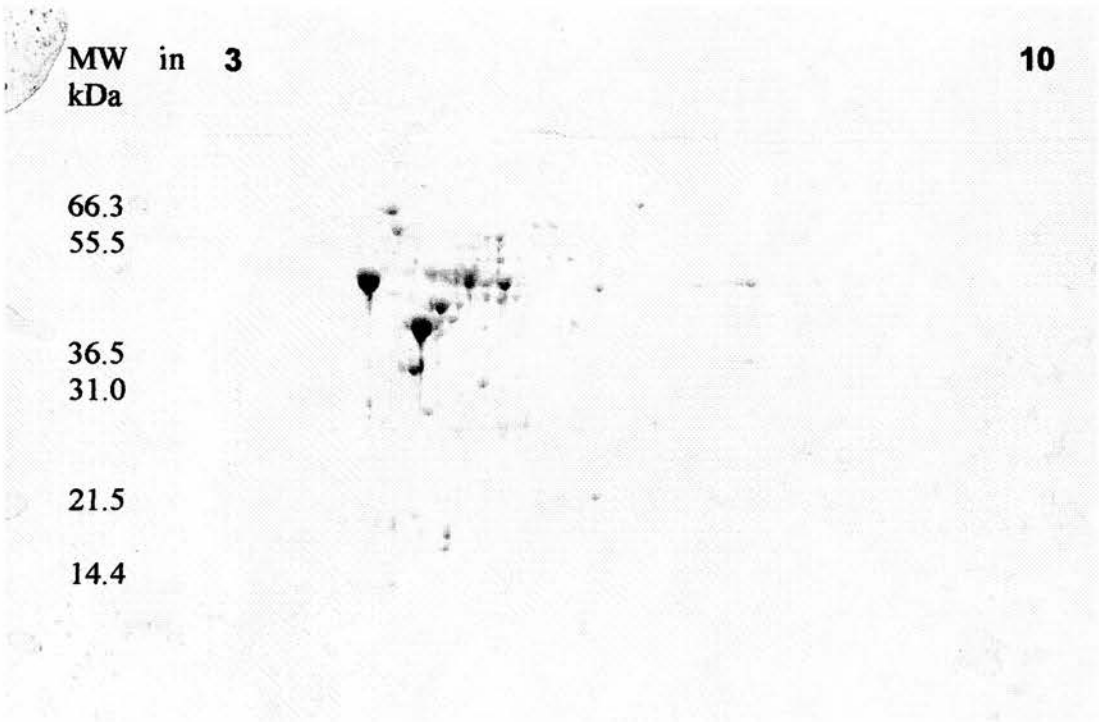

Figure 7.4c Control 3

This figure shows the pellet from 3ml of culture from experimental control 3.

Figure 7.5a Sub-ceftriaxone 1

This figure shows the pellet from 3ml of culture from sub-ceftriaxone experiment 1.

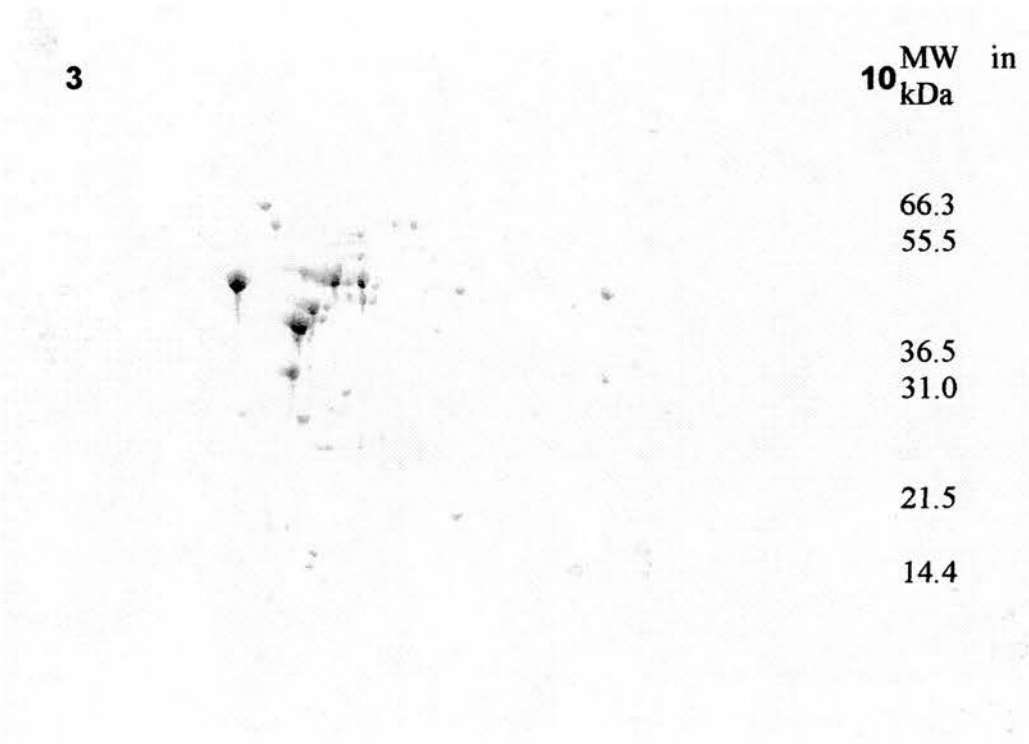

Figure 7.5b Sub-ceftriaxone 2

This figure shows the pellet from 3ml of culture from sub-ceftriaxone experiment 2.

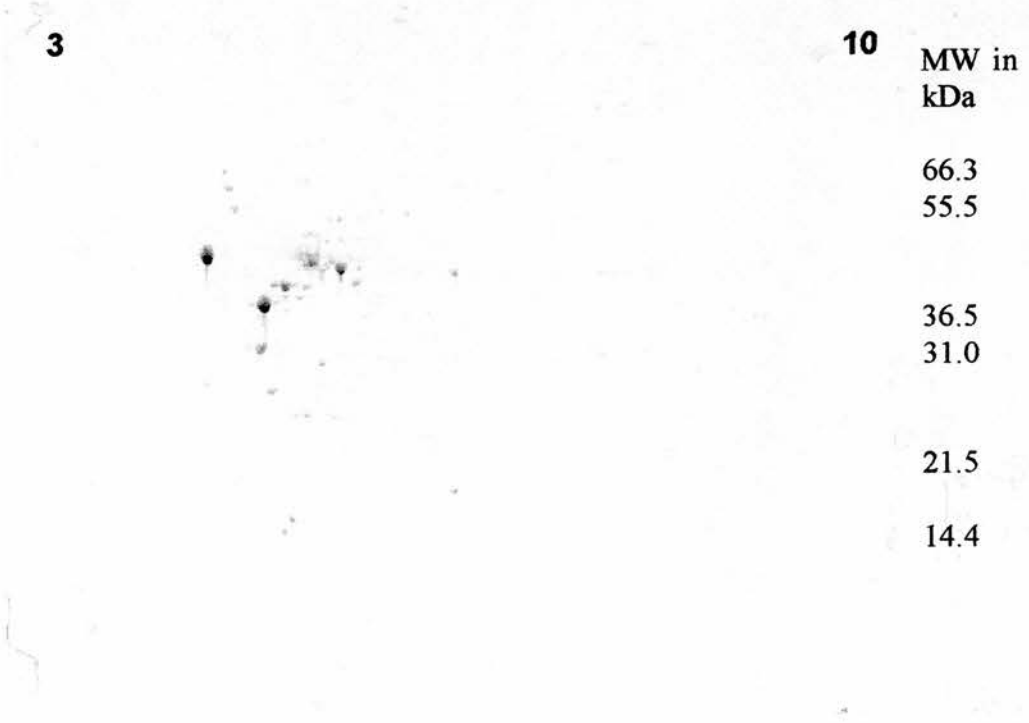

Figure 7.12 Sub-ceftriaxone 3

This figure shows the pellet from 3ml of culture from sub-ceftriaxone experiment 3.

Visual analysis of the two condition sets showed no obvious difference in protein profile between them. What was apparent from these gels was that the majority of proteins focused in a narrow pH range – between 4 and 7 – a situation mirrored in the study by Karlsson *et al.* (1999).

7.4 Trypsin digests and MALDI-TOF

Twenty representative protein spots from all six gels were selected for identification of peptides using matrix-assisted laser desorption/ionization –time of flight (MALDI-TOF) analysis. For control samples protein spots were numbered 1-20 (see Figure 7.6) and equivalent protein spots from the sub-ceftriaxone gels were designated 21-40 and all 40 spots digested with trypsin (see Materials and Methods). The predicted peptide masses for each spot were entered into the Swiss prot database as an initial search for a protein match.

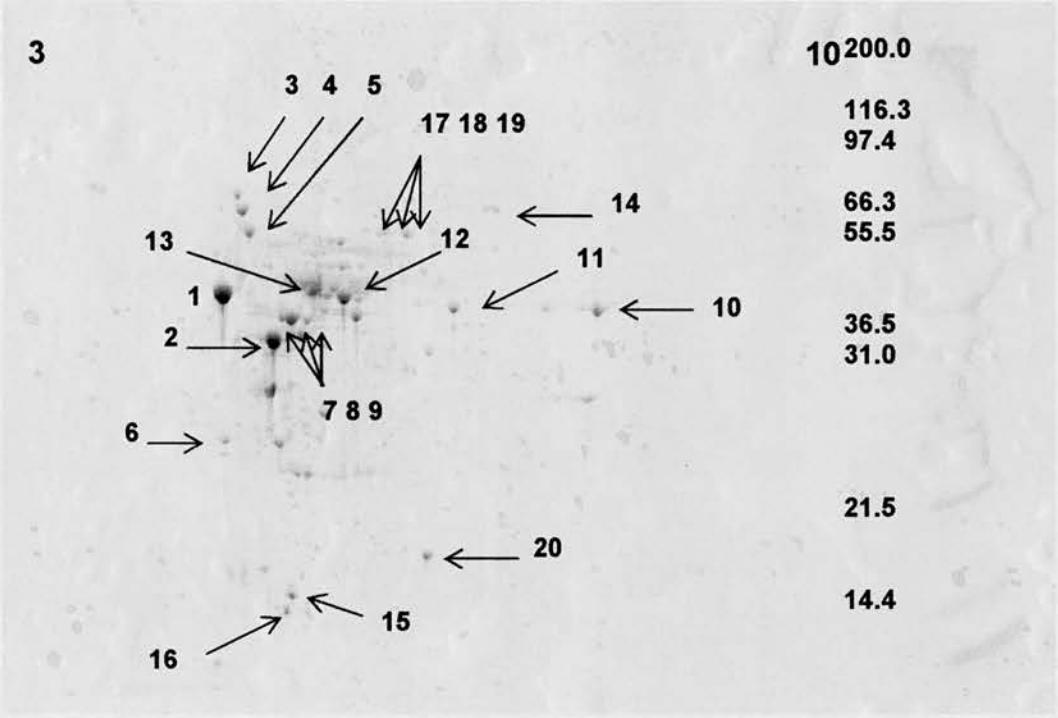

Figure 7.6 Proteins spots used in MALDI-TOF analysis.

The spots shown demonstrate the proteins excised from the gels and used in the proteomic analysis. Spots 1-20 were picked from the three control gels and 20 corresponding spots (21-40) from the sub-ceftriaxone gels.

C. difficile MASCOT database

To aid valid identification of these proteins it was crucial to gain access to a homologous database for *C. difficile*. The genome for strain 630 was kindly donated to Moredun Research Institute by the Wellcome Trust Sanger centre. The *C. difficile* MASCOT database was produced at Moredun from the genome of strain 630. Mascot is a powerful search engine which uses mass spectrometry data to identify proteins from primary sequence databases. Mass spectrometry data (peptide mass) from trypsin-digested protein spots was entered into the relevant protein database; heterologous (in this work NCBInr, SwissProt) or homologous (*C. difficile*) and this data used to identify the proteins. The experimental mass values are compared with

calculated peptide mass, obtained by applying cleavage rules (trypsin cut sites) to the entries in the database. NCBInr is a comprehensive, non-identical protein database. The entries have been compiled from many sources including GenBank and SWISS-PROT. SwissProt is a high quality, curated protein database which contains a minimal level of redundancy. Detailed information on the application of MALDI-TOF and MASCOT databases can be found at http://www.matrixscience.com.

The results of these searches are given in MOWSE scores. To get a MOWSE score an algorithm is applied which estimates the probability that the experimental mass data matches the theoretical (Pappin et al., 1993). Each calculated value which falls within a given mass tolerance of an experimental value counts as a match. Probability Based Mowse scoring incorporates the probability of such matches occurring and allows the user a degree of certainty in the match (http://www.matrixscience.com). High-scoring matches are more likely to be true matches. Matches using mass values (either peptide masses or MS/MS fragment ion masses) are always handled on a probabilistic basis. The total score is the absolute probability that the observed match is a random event. For a further explanation of MOWSE and probability scores see http://www.matrixscience.com.

Table 7.1 shows the outcome of these searches.

Table 7.1 Homologous and heterologous identifications of protein spots.

Spot Number	ID	Mowse score Cdiff/NCBInr/ Sprot	No. peptides matched	Predicted MW (kDa)	Experimental MW (kDa)	Predicted pI	Experimental pI
1	S-layer protein	112/104	28	75.5	46	4.78	4.6
21	S-layer protein	167/167	22	76	46	4.76	4.6
2	S-layer protein	99/153	23	38.3	36	4.97	4.9
22	S-layer protein	135/192	20	38.3	36	4.97	4.9
3	S-layer precursor protein	89/89	17	76	70	4.76	4.7
23	S-layer precursor protein	168/168	26	76	70	4.76	4.7
4	DNAk homologue	104	18	66.5	65	4.76	4.75
24	DNAk homologue	162	22	66.5	65	4.76	4.75
5	GroEL	158/158/139	23/23/16	57.7	57	4.74	4.8
25	GroEL	96/108/146	15/16/14	57.7	57	4.74	4.8
7	Electron transport flav homologue	62	13	37.3	38	5.03	4.95
8	Electron transport flav homologue	78	10	37.3	38	5.03	5
28	Electron transport flav homologue	55	10	37.3	38	5.03	5
9	Electron transport flav homologue	56	10	36.2	38	5.11	5.1
29	Electron transport flav homologue	104	14	36.2	38	5.11	5.1

Spot Number	ID	Mowse score Cdiff/NCBInr/ Sprot	No. peptides matched	Predicted MW (kDa)	Experimental MW (kDa)	Predicted pI	Experimental pI
10	Acyl-CoA dehyd.	73	12	41.5	40	5.89	6.5
30	Acyl-CoA dehyd.	90	12	41.5	40	5.89	6.5
11	Acyl-CoA dehyd.	114	16	41.4	42	5.71	5.8
31	Acyl-CoA dehyd.	131	16	41.4	42	5.71	5.8
12	Acyl-CoA dehyd.	84	12	43.3	42	5.56	5.4
32	Acyl-CoA dehyd.	123	18	43.3	42	5.56	5.4
13	Hypothetical protein y	155	21	45	44	5.12	5.1
33	Hypothetical protein y	144	20	45	44	5.12	5.1
14	S-layer homologue	106	18	66.4	66	6.2	6.1
34	S-layer homologue	53	14	66.4	66	6.2	6.1
16	10kDa chaperonin (GroES)	68/67	5/4	10.2	12	5.09	5.0
36	10kDa chaperonin (GroES)	89/76/95	7/6/6	10.2	12	5.09	5.0
17	NADH oxidase	95	14	59	58	5.49	5.5
37	NADH oxidase	128	18	59	58	5.49	5.5
18	NADH oxidase	125	16	59	58	5.49	5.55
38	NADH oxidase	144	18	59	58	5.49	5.55
19	NADH oxidase	55	9	59	58	5.49	5.6
39	NADH oxidase	136	4	59	58	5.49	5.6

As is clear from the table the majority of protein spots were identified to a degree of certainty (significant MOWSE scores) by the databases. The *C. difficile* MASCOT database produced the majority of hits which was unsurprising as it was generated using the sequence of strain 630. Few spots generated no significant hits. Pairs 6 and 26, 15 and 35 and 20 and 40 were unidentified by all three databases as was spot 27 (whose corresponding spot on the control gel was identified as a protein from the electron transport system). Many spots were shown to match to the same protein. Spots 1, 21, 2, 22, 3, 23, 14 and 34 were all shown to be S-layer proteins or precursors. Spots 7, 8 and 9 and 28 and 29 were matched with proteins from the electron transport system. The group of spots 17, 18 and 19, and 37, 38 and 39 scored hits for the protein NADH hydrogenase. Other proteins identified included a Dnak homologue, GroEL and GroES. The S-layer proteins were identified as *N*-acetylmuramoyl-L-alanine amidase (from *Bacillus subtilis*) in the *C. difficile* MASCOT database. Calabi *et al.* (2001) have previously shown the S-layer proteins to have weak homology to these proteins so the matched peptides from this amidase were used in a BLAST peptide search. Mukerhjee *et al.* (2002) have also shown *slpA* to have homology to this amidase. Short peptide fragments (shown below) that matched the *N*-acetylmuramoyl-L-alanine amidase were used in the BLAST search to try to get an identification.

DLKDYVDDLKTYNNTYSNVVTVAGEDRIETAIELSSKYYNSDDKNAITDK

and

DAAAEKLYNLVNTQLDKLGDGDYVDFSVDYNLENKIITNQADAEAIVTKLNSLNEKT

LIDIATKDTFGMVSK

These fragments and similar fragments for all proteins identified by the *C. difficile* database as being *N*-acetylmuramoyl-L-alanine amidase (Spots 1, 21, 2, 22, 3, 23, 14 & 34) were all shown to match *C. difficile* S-layer proteins in the NCBI database. Interestingly the matched peptides from the upper S-layer protein (spot 1, 21) match the latter half of the *N*-acetylmuramoyl-L-alanine amidase protein and the lower MW S-layer protein (spot 2, 22) matches the first half of the protein. This is shown below using spots 1 and 2 as examples.

Spot 1 (46kDa S-layer protein)

Match to: **3252562** Score: **112** Expect: **3e-08**
***N*-acetylmuramoyl-L-ala 2.66e-23 CWLB_BACSU**

Nominal mass (M_r): **76087**; Calculated pI value: **4.76**
NCBI BLAST search of 3252562 against nr
Unformatted sequence string for pasting into other applications

Fixed modifications: Carbamidomethyl (C)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Number of mass values searched: **60**
Number of mass values matched: **23**
Sequence Coverage: **28%**

Matched peptides shown in **Bold Red**

```
   1 MNKKNIAIAM SGLTVLASAA PVFAATTGTQ GYTVVKNDWK KAVKQLQDGL
  51 KDNSIGKITV SFNDGVVGEV APKSANKKAD RDAAAEKLYN LVNTQLDKLG
 101 DGDYVDFSVD YNLENKIITN QADAEAIVTK LNSLNEKTLI DIATKDTFGM
 151 VSKTQDSEGK NVAATKALKV KDVATFGLKS GGSEDTGYVV EMKAGAVEDK
 201 YGKVGDSTAG IAINLPSTGL EYAGKGTTID FNKTLKVDVT GGSTPSAVAV
 251 SGFVTKDDTD LAKSGTINVR VINAKEESID IDASSYTSAE NLAKRYVFDP
 301 DEISEAYKAI VALQNDGIES NLVQLVNGKY QVIFYPEGKR LETKSANDTI
 351 ASQDTPAKVV IKANKLKDLK DYVDDLKTYN NTYSNVVTVA GEDRIETAIE
 401 LSSKYYNSDD KNAITDKAVN DIVLVGSTSI VDGLVASPLA SEKTAPLLLT
 451 SKDKLDSSVK SEIKRVMNLK SDTGINTSKK VYLAGGVNSI SKDVENELKN
 501 MGLKVTRLSG EDRYETSLAI ADEIGLDNDK AFVVGGTGLA DAMSIAPVAS
 551 QLKDGDATPI VVVDGKAKEI SDDAKSFLGT SDVDIIGGKN SVSKEIEESI
 601 DSATGKTPDR ISGDDRQATN AEVLKEDDYF TDGEVVNYFV AKDGSTKEDQ
 651 LVDALAAAPI AGKFKESPAP IILATDTLSS DQNVAVSKAV PKDGGTNLVQ
 701 VGKGIASSVI NKMKDLLDM
```

Spot 2 (36kDa S-layer protein)

Match to: **3252562** Score: **99** Expect: **5.8e-07**
***N*-acetylmuramoyl-L-ala 2.66e-23 CWLB_BACSU**

Nominal mass (M_r): **76087**; Calculated pI value: **4.76**
NCBI BLAST search of 3252562 against nr
Unformatted sequence string for pasting into other applications

Fixed modifications: Carbamidomethyl (C)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Number of mass values searched: **60**
Number of mass values matched: **23**
Sequence Coverage: **29%**

Matched peptides shown in **Bold Red**

```
  1 MNKKNIAIAM SGLTVLASAA PVFAATTGTQ GYTVVKNDWK KAVKQLQDGL
 51 KDNSIGKITV SFNDGVVGEV APKSANKKAD RDAAAEKLYN LVNTQLDKLG
101 DGDYVDFSVD YNLENKIITN QADAEAIVTK LNSLNEKTLI DIATKDTFGM
151 VSKTQDSEGK NVAATKALKV KDVATFGLKS GGSEDTGYVV EMKAGAVEDK
201 YGKVGDSTAG IAINLPSTGL EYAGKGTTID FNKTLKVDVT GGSTPSAVAV
251 SGFVTKDDTD LAKSGTINVR VINAKEESID IDASSYTSAE NLAKRYVFDP
301 DEISEAYKAI VALQNDGIES NLVQLVNGKY QVIFYPEGKR LETKSANDTI
351 ASQDTPAKVV IKANKLKDLK DYVDDLKTYN NTYSNVVTVA GEDRIETAIE
401 LSSKYYNSDD KNAITDKAVN DIVLVGSTSI VDGLVASPLA SEKTAPLLLT
451 SKDKLDSSVK SEIKRVMNLK SDTGINTSKK VYLAGGVNSI SKDVENELKN
501 MGLKVTRLSG EDRYETSLAI ADEIGLDNDK AFVVGGTGLA DAMSIAPVAS
551 QLKDGDATPI VVVDGKAKEI SDDAKSFLGT SDVDIIGGKN SVSKEIEESI
601 DSATGKTPDR ISGDDRQATN AEVLKEDDYF TDGEVVNYFV AKDGSTKEDQ
651 LVDALAAAPI AGRFKESPAP IILATDTLSS DQNVAVSKAV PKDGGTNLVQ
701 VGKGIASSVI NKMKDLLDM
```

This is likely due to the fact that the S-layer proteins are the product of one gene (*slpA*) which is then processed to become two proteins.

7.5 Discussion

In Chapter five of this thesis sub-inhibitory concentrations of ceftriaxone were shown to affect the growth and toxin A production of *C. difficile* strain 630. A concentration of 1/2 MIC produced a lag in growth and also caused toxin A to be elaborated before toxin A in the control experiment. Due to this effect on growth and toxin production

in this strain these conditions were chosen to analyse 2D protein profiles. The use of 2D analysis coupled with identification of proteins using MALDI-TOF mass spectrometry would allow an insight into the broader physiological effects of antibiotics at sub-inhibitory levels.

The proteomic approach with *C. difficile* was based on evidence of reproducible sample preparation protocols in mycobacteria and various Gram positive bacteria. The addition of the sonication step was to aid the lysis of the cells. Although no obvious difference was apparent between the control conditions and cells grown with sub-MIC ceftriaxone this work demonstrated an easy, reproducible sample preparation and identification of a number of proteins for future experiments. It was interesting to note that 2D gels run by Karlsson *et al.* (1999) and Mukherjee *et al.* (2002) demonstrated a similar pattern of proteins to those found in this work. In strain 630 they also showed the putative S-layer proteins to have similar MW and pI as found in this work. They also demonstrated the S-layer proteins to be the most abundant spots present on the gels. Further investigation by this group showed that the N-terminal regions of these proteins matched the S-layer ORF.

In the study by Karlsson *et al.* (1999) they used proteomics to analyse the suppression of toxin production by amino acids. They also used stationary phase cells and they prepared the protein extracts using pellets from 10ml of culture. Approximately 500 proteins mostly with pIs ranging between 4 and 7 were identified in both defined medium and in Peptone Yeast Extract medium. During biotin limitation when the toxin levels were upregulated (discussed in section 1.8) they

found several 22kDa proteins with pIs ranging from 5 to 5.5 also upregulated. Several proteins (60-100kDa, pIs 5-7) were downregulated in the presence of excess amino acids or glucose in PY and defined medium.

In the follow-up study by Mukherjee *et al.* (2002) they analysed the proteins released during high toxin production. As in the previous study they discovered that limiting nutrient levels caused in increase in toxin production. In strain VPI 10463 they found that a toxin output of 50% was present with minimal lysis or spore formation proving that toxins are not released by this manner as had been previously proposed (Kamiya *et al.*, 1992). During this period of increased toxin production they discovered a 47kDa protein to be released with similar kinetics. It was also down-regulated with the addition of glucose to the medium as are the toxins. It matched an ORF in the *C. difficile* database and its C-terminus showed weak similarity to the outer-membrane efflux proteins of Gram –ve bacteria. A 40kDa protein which showed homology to XkdK encoded by the prophage PBSX in *B. subtilis* was located on a segment of the *C. difficile* chromosome that contained several ORFs homologous to those for PBSX. XkdK is apparently exported through a holin-like protein. *C. difficile* possesses a protein with holin-like properties in the form of TcdE, found in the Pathogenicity Locus and upregulated along with the toxins and TcdD (see Chapter 1.6.2). The PBSX prophage is induced by the SOS response though neither the toxins or the homologue showed altered levels after the addition of mitomycin C.

As in this work the most abundant proteins seen in the 2D gels by Mukherjee *et al.* (2002) were the putative S-layer proteins. The putative S-layers from VPI 10463 (50

and 36kDa) showed no homology to any in the strain 630 database though the 36kDa protein showed partial homology to the S-layer protein SplA. The gene segment containing *slpA* comprises *secA* and several additional genes similar to *slpA*. SlpA and *slpA*-like ORFs contained parts with significant homology to *N*-acetylmuramoyl-L-alanine amidase (CwlB/LytC) and amidase enhancer protein (LytB) from *B. subtilis*. They all contained a typical Sec-dependent signal peptide and the predicted cleavage site in the 630 SlpA was identical to that found in the extra-cellular form. Other serogroups were tested for the *slpA* gene and all strains apart from A, A5 and S4 produced one major product of 2900bp though the product of serogroup H was slightly larger. Upon digestion of the PCR fragments with *Sau*IIIA and *Rsa*I each serogroup yielded a different pattern demonstrating the heterogeneity of the S-layer genes.

During this work the higher S-layer protein migrated to ca. 46kDa on the gel. On 1D gels with guanidine hydrochloride-extracted surface proteins the weight appears as ca. 52kDa. The reasons for this are unclear but it is likely that gel conditions or different methods of S-layer extraction play a part. Urea, high salt, low-pH and EDTA extractions all show the higher MW S-layer protein of strain 630 to be of ca. 46kDa (Calabi *et al.*, 2001). It is unclear why the guanidine hydrochloride-extraction causes this difference in mobility of the higher MW S-layer protein and not the lower MW protein.

The spots chosen for MALDI analysis included the S-layer proteins but also protein spots chosen at random. Spots of the same size (but different pIs) were chosen to

investigate the presence of post-translational modifications. Other spots were chosen because they were abundant and represented a selection of different MWs and pIs.. The S-layer proteins were all identified by the NCBInr database as *C. difficile* S-layer proteins or through a match with the S-layer precursor protein. A 72kDa spot which generated a hit as an S-layer protein was seen on the gels and identified by the databases. Cerquetti *et al.* in 1992 investigated the 36kDa S-layer protein of strain C253 and found the native form of this protein (found using gel filtration) to have a MW of 72kDa which was unaffected by EDTA or SDS in the column. They hypothesised that it was a homodimeric form of the 36kDa protein. The other possibility for an identification of this protein in this work is as the S-layer precursor protein which was previously reported by Takeoka *et al.* (1991). For some reason the *C. difficile* database identified the S-layer proteins as *N*-acetylmuramoyl-L-alanine amidase from *B. subtilis*. Calabi *et al.* (2001) and Mukerhjee *et al.* (2002) have already noted this homology to the *B. subtilis* protein. Further annotation to the *C. difficile* database may result in the database recongising these proteins as S-layers ahead of *N*-acetylmuramoyl-L-alanine.

Of the spots that generated significant hits with the databases there was some evidence of the presence of post-translational modification in some of them. Spots 7, 8 and 9 on the control gels and 28 and 29 on the sub-MIC gels were all identified as proteins from the electron transport system (spot 27 was not a significant hit). These proteins all appeared at ca. 38kDa with pIs of ca. 4.95, 5 and 5.1. Spots 7 and 27 were very difficult to pick from the gels due to their proximity to other proteins including the lower S-layer protein. This may be why spot 27 was not identified – it

may not have been picked 'cleanly'. A group of spots with a greater difference in their pIs was spots 10, 11 and 12 and 30, 31 and 32 (at 6.5, 5.8 and 5.4 respectively). These spots all generated hits for the protein acyl-CoA-dehydrogenase. Spots 17, 18 and 19 and 37, 38 and 39 were all matched to an NADH oxidase by the *C. difficile* database – another possibiltiy for post-translational modification of proteins.

GroEL and GroES from the heat shock protein 60 (Hsp60) family of molecular chaperones were identified during this work. GroEL was identified as *C. difficile* GroEL by all three databases and GroES by all three for spot 56 and by the *C. difficile* and Swiss prot databases for spot 16. Proteins from this family of chaperones are often upregulated by stress (which possibly includes sub-MIC antibiotics) though no difference was seen during this work.

Spot 4 and 24 both scored significant hits for Dnak, a protein from the Hsp70 family of molecular chaperones.

Spots 13 and 33 scored well for an *E. coli* hypothetical protein y. This protein may no longer be hypothetical in *E. coli* as *C. difficile* appears to possess a homologue. This increases the possibility of it being a translated protein in *E. coli*.

Few pairs of spots generated no significant hits with the databases. Spot 27 has already been discussed and as its corresponding spot on the control gel was matched then it is likely that it was contaminated with other proteins or perhaps there was too little of the protein for the MALDI analysis. Spots 6 and 26, 15 and 35 and 20 and 40

remain unidentified. Further annotations and development of the *C. difficile* MASCOT database should enable further identifications in the future.

The outcome of this work has been to validate the procedures for proteomic analysis in *C. difficile*. There is now a clear framework present for analysing the protein profile in this organism and future work should include further comparison of conditions including different antibiotics and strains. It would be valuable to analyse different membrane fractions and secreted proteins as this would allow lower abundance proteins to be visualised and not masked by the high abundance proteins found in total cell extracts. The proteins identified in this work have been involved in many important cellular functions and so it is unsurprising that they appear to have been unaffected by the presence of ceftriaxone. Including different methods of sample preparation (membrane extracts, cytosolic protein fractions) may yield lower abundance proteins which are affected by sub-inhibitory concentrations of antibiotics.

CONCLUSIONS

C. difficile is a major problem in the developed world and is the cause of significant numbers of cases of diarrhoea and colitis among predominantly elderly patients. Antibiotics are the main risk factor for *C. difficile* disease in that they are most often a prerequisite for acquisition of the disease. Broad-spectrum antibiotics especially affect the normal gut flora leading to abrogation of colonisation resistance. The disappearance of the protective flora allows *C. difficile* access to this niche where they colonise and produce two high molecular weight exotoxins, A and B. The toxins glycosylate proteins of the Rho family and disrupt the actin cytoskeleton leading to cell rounding and cell death. This effect on the gut mucosa leads to diarrhoea and in some cases the more severe manifestation of pseudomembranous colitis. Only *C. difficile* strains which possess the toxins (one or both) cause disease. In the United Kingdom one strain (PCR ribotype 001, S-type 5236) is responsible for the majority of nosocomial cases of CDD and colonisation with *C. difficile* (55% - Stubbs *et al.*, 1999 (PCR ribotype 001); 73% - McCoubrey *et al.*, 2003 (S-type 5236).

In this study patient data and stool samples had been collected from two geriatric wards in the Royal Victoria Hospital in Edinburgh as part of a Scottish Office epidemiology study. Isolates from positive stool samples were typed according to the molecular weights of the two S-layer proteins. One isolate from each stool sample was tested for their antibiotic susceptibilities to six antibiotics and this data analysed in context to patient and strain data already available. It was clear that no resistance was found in any strain to the two treatment antibiotics vancomycin and metronidazole. This was unsurprising as reports of resistance to these agents is rare

or unsubstantiated. As expected, all strains were resistant to cefoxitin, one of the agents used in the *C. difficile* selective medium. The majority of isolates were resistant or had intermediate resistance to ceftriaxone, a third generation cephalosporin common in precipitating CDD. The range of MICs to amoxicillin and clindamycin was great but MIC_{50} and MIC_{90} values were similar (8 & 16μg/ml for amoxicillin and 16 & 16μg/ml for clindamycin). Twelve isolates from six patients contained the *ermB* gene which encodes high-level clindamycin resistance (>128μg/ml). This has been proposed as a way to further divide the PCR ribotype 001 group of isolates (Fawley *et al.*, 2003) and these isolates have been responsible for outbreaks of CDD (Johnson *et al.*, 1999; Noren *et al.*, 2002).

S-layer typing is a quick and easy method of phenotyping and appears to correspond well with other typing techniques including ribotyping and serotyping (McCoubrey and Poxton, 2001). In this study 76.6% of the isolates were of the type 5236. Toxigenic S-type 5236 correlates well to PCR ribotype 001 (McCoubrey, 2002) which is also the most common PCR ribotype (55%) in the UK as a whole (Stubbs *et al.*, 1999). The S-layer is a putative virulence factor that appears to have a role in adhesion of the bacterium to the host mucosal surface. Calabi *et al.* (2002) demonstrated this adhesion to gastrointestinal tissues mediated mainly by the high molecular weight S-layer protein. S-type 5236 had a large range of MICs to the six antibiotics and there were no differences in the overall pattern between this strain and that of the total population. In the less abundant S-types the sensitivity patterns to the antibiotics were not significantly different from one another or the whole population suggesting that the S-layer proteins do not affect the sensitivity of the strains to antibiotics. In the untypeable isolates (n=4) three of them were extremely sensitive to

clindamycin (MIC≥2μg/ml) but as the number was so small no conclusions can be drawn from this.

During this study many patients were sampled more than once due to long stays in hospital, readmission or the presence of diarrhoea. This allowed a comparison of the isolate profiles over a period of time. Isolates from 36 patients exhibited changing patterns of sensitivity to one or more of the six antibiotics. While some of these changes related to change of S-type, others did not. Typical changes in isolates that were all of the same S-type were no greater than 2-4-fold different and were therefore of little interest. Two patients however, produced isolates with differing clindamycin resistance at different times. The isolates were all of S-type 5236 and the changes would have probably occurred with the loss or reinfection with a clindamycin resistant clone rather than the loss or acquisition of the *ermB* resistance determinant.

Among the data collected during the epidemiological study was the antibiotics prescribed to the patients during their stay and any prescribed in the community. No links between antibiotics prescribed and susceptibility patterns were found.

At the beginning of this study it was necessary to understand the relationship of growth (measured using viable, total and spore counts), OD_{600}, and the production of toxin. Detailed growth curves on strains NCTC 11223, the sequenced strain 630 and an endemic isolate 338a showed toxin A to be produced upon entry to stationary phase in agreement with other studies. OD_{600} was found to be a good predictor of growth phase with discrepancy only at the very beginning of log phase before the culture turbidity reached 0.6. Until this point the OD_{600} lags behind the viable and

total counts by ca. 2 h. As the entry to stationary phase was to be the most important stage for this work, and the OD_{600} was sufficient for this prediction then it allowed this measurement to be used for subsequent experiments. Toxin A production varied greatly between the strains. Endemic strain 338a produced toxin A first, followed by strain 630 and then strain 11223. The levels of strain 11223 were also lower than those of 630 and 338a in that they rarely exceeded the tolerance of the ELISA assay. This work has clearly shown that production of toxin A in the three strains differed in the time it began and the levels of toxin A achieved. However, the pattern of growth phase dependence of toxin production was the same in all three strains with it beginning in early stationary phase and reaching the highest levels of toxin when the strains were heading towards decline.

Three strains (NCTC 11223, strain 630 and endemic isolate 338a) were cultured in sub-lethal concentrations of the six antibiotics (1/2, 1/4 and 1/8 of the MIC) over 104 hours and growth and toxin A measured three times a day. The effects varied between strain and antibiotic. The most common effect on the growth of the strains was to increase the initial lag period by ca. 4h compared to the antibiotic-free controls though the clindamycin resistant strain NCTC 11223, (MIC $\geq$512μg/ml) showed no lag whatsoever in comparison to the controls when grown in this antibiotic. An explanation for this may be that it is so well adapted to this agent that it can function and grow as normal. This strain contains the macrolide, lincosamide and streptogramin B resistance determinant (MLS) that contains the *ermB* gene (encodes an RNA methyltransferase) which makes it resistant to these antibiotics. Strain 630 also carries the *ermB* gene (Farrow *et al.*, 2001) but it has a slightly lower

MIC of 512μg/ml and its growth was affected by all three sub-MIC concentrations of clindamycin. The reasons for this are uncertain but heterogeneity between strains was common during this work. The most common effect on toxin A production was in the onset of toxin elaboration. Normally toxin began to appear in low levels in early stationary phase before accumulating to high levels by the start of decline. In the presence of sub-MIC antibiotics this onset appeared before that of the antibiotic-free controls. This effect was seen with metronidazole, amoxycillin and clindamycin, rarely with vancomycin and never with cefoxitin. Results suggest a very complex relationship between the effects of growth and toxin production, which is strain dependent. This study has clearly shown that there is heterogeneity between strains in respect to growth, MICs and the toxin levels that are produced. This effect on growth an toxin A production may be a result of stress. Bacteria under stress switch on a catalogue of genes and it is possible that the toxin promoters are affected by it. To support this, TcdD, the alternative sigma factor of the toxin genes, shows similarity to UviA the UV-inducible regulator from *Clostridium botulinum* (Mani & Dupuy, 2001). Onderdonk *et al.* (1979) showed that the stress of increased temperature led to greater cytotoxin production. In the same paper they demonstrated an increase in toxin in the presence of sub-inhibitory concentrations of vancomycin and penicillin. Karlsson *et al.* (2003) also showed temperature as a controlling factor for toxin and TcdD expression. It has been shown by Hennequin *et al.* (2001) that *C. difficile* cultured in the presence of antibiotics produces greater levels of GroEL, a chaperone from the heat shock protein 60 (Hsp60) family. The examples all serve to suggest that the toxin promoters can respond to multiple environmental stresses. Inducing this stress response may enable *C. difficile* to survive the gut environment

better after colonisation as GroEL functions as a 58kDa surface adhesin. This adhesin may help *C. difficile* to colonise the recently vacated binding sites left by the depletion of the normal gut flora.

mRNA transcripts of the two toxin genes, *tcdC*, *tcdD* and *groEL* were investigated in the presence and absence of sub-MIC clindamycin to see if the potentiation of toxin A production was mirrored in the toxin transcripts. During the sub-MIC study sub-inhibitory concentrations of clindamycin resulted in a shift forward and increase in toxin A production. RNA concentrations garnered from the Qiagen RNA extraction kit were disappointing and although message levels were enhanced by the use of a more sensitive reverse transcriptase, only mRNA from the positive control – 16S RNA – was seen during this work. Many problems were encountered and overcome with regards to DNA contamination of the RNA extracts. An additional DNase step was added into the protocol which effectively removed the contaminating DNA but resulted in a further dilution of already low levels of RNA. An alternative extraction method, using a protocol shown to be successful in *C. difficile*, would likely increase the possibility of success in this area.

The protein profile of strain 630 in the presence and absence of ceftriaxone was studied using proteomics. Strain 630 has recently been sequenced and was kindly donated by the Sanger Centre to Moredun Research Institute for the production of a MASCOT database. The combination of strain 630 and ceftriaxone in Chapter 5 produced a growth lag of ca. 4 h and a shift forward in toxin A production compared to the controls. 2D gels, MALDI-TOF analysis and a *C. difficile* MASCOT database

were utilised to identify proteins from total cell extracts of strain 630. No differences were found between the protein profiles with and without ceftriaxone but 40 spots were picked from the gels for further identification. The *C. difficile* S-layer proteins were identified along with GroEL and GroES, acetyl Co-A dehydrogenase, NADH oxidase and proteins from the electron transport system. This work has provided essential information on successful procedures for proteomic analysis in *C. difficile* and the MASCOT database will be invaluable for further studies.

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APPENDIX 1

Culture media and preparations

All culture media was autoclaved at 121°C for 15 minutes before the addition of blood or supplements unless otherwise stated.

Columbia blood agar with 5% horse blood (1L)

Columbia agar base (OXOID CM331, Basingstoke, Hampshire)	39g
Defibrinated horse blood	50ml

Anaerobic Investigation Medium (AIM) (1L)

Proteose peptone (OXOID L85, Basingstoke, Hampshire)	20g
Yeast extract (OXOID L21, Basingstoke, Hampshire)	5g
Trypticase (BBL 11921, MD, USA)	5g
NaCl	5g
Cysteine HCl (3.75% aqueous solution)	20ml
Na_2CO_3 (2% aqueous solution)	20ml
Haemin + menadione (1mg/l) See below for recipe	20ml
Distilled water	940ml

Adjust to pH 7.1 and make volume up to 1 litre with distilled water.

Haemin + Menadione
(Barnes & Impey, 1971)

Mix equal parts of haemin (500mg/L) and menadione (100mg/L) and store in the dark at 4°C. See below for recipes.

Haemin (500mg/L)

Dissolve 50mg haematin HCl (BDH) in 1 M NaOH solution in a bijou bottle overnight. Make up to 100ml with distilled water. Store at 4°C.

Menadione (100mg/L)

Dissolve 10mg menadione (Sigma) in 2ml ethanol in a bijou bottle in the dark overnight. Make up to 100ml with distilled water. Store in the dark at 4°C.

Enriched thioglycollate medium (1L)

Sigma thioglycollate medium (FTG) T9032 (St Louis, USA)	29.8g
Hemin stock solution (5mg/ml) (see below for recipe).	1ml
Vitamin K_1 working solution (1mg/ml) (see below for recipe).	1ml
$NaHCO_3$ stock solution (10mg/ml) to every 5ml of sterile medium.	0.25ml

Hemin stock solution (5mg/ml)

Dissolve 0.5g in 10ml of 1M/L NaOH

Bring volume to 100ml with distilled water and sterilise (121°C for 15 minutes).

Store at 4°C.

Add 1ml to 1 litre of medium.

Vitamin K_1 stock solution and working solution (10mg/ml and 1mg/ml respectively)

Add 0.2ml of vitamin K_1 to 20ml of 95% ethanol. Store at 4°C in a dark bottle.

Prepare the working solution (1mg/ml) by adding 1ml of the stock solution to 9ml of distilled water. Store at 4°C in a dark bottle for no longer than a month.

Add 1ml of the working solution to 1 litre of medium to achieve 1μg/ml.

Appendix 1

$NaHCO_3$ stock solution (10mg/ml)

Dissolve 2g of $NaHCO_3$ in 100ml distilled water.

Sterilise by filtration and store at 4°C.

Add 0.25ml to 5ml of sterile medium.

Brain heart infusion/ Proteose peptone yeast broth
(Brettle *et al.*, 1982)

Brain heart infusion broth (OXOID)	37g
Proteose peptone (OXOID)	10g
Distilled water	1000ml

Proteose peptone/ Yeast extract broth
(Deacon *et al.*, 1978- adapted by Poxton *et al.*, 1984)

Proteose peptone (OXOID)	20g
Yeast extract (OXOID)	10g
NaCl	5g
Cysteine HCL	750mg
Na_2CO_3	400mg
Haemin (250µl/L) & Menadione (100µg/L) solution	20ml

Nutrient broth (1L)

Nutrient broth (OXOID CM1, Basingstoke, Hampshire)	13g

Fastidious Anaerobe Agar with 5% blood (1L)

Anaerobe agar base (Bioconnections 143-2832)	46g
Defibrinated horse blood	50ml

0.85% saline (physiological) (100ml)

Sodium chloride	0.85g

Supplemented *Brucella* Blood Agar (1L)
(NCCLS, 1997)

Brucella Medium Base (OXOID CM169)	45g
Hemin stock solution (5mg/l)	1ml
Vitamin K_1 stock solution (1mg/l)	1ml

Sterilise at 121°C for 15 minutes.

Cool to 50°C and dispense 17ml into sterile glass universals and keep at 4°C until required (within 1 month).

Laked sheep blood

Freeze the defibrinated sheep blood below -20°C.

Thaw rapidly at 35°C in a waterbath. Store in the fridge for up to 1 month if not being used immediately.

Before addition to the medium, gently invert to ensure a uniform suspension.

Cooked meat broth (CMB)
(Collee & Marr, 1996)

Add 4ml of AIM to 3-4 particles of cooked meat (see below for preparation details).

Autoclave at 121°C for 15 minutes.

Cooked meat particles

Remove all fatty tissues from ca. 500g of fresh sheep or bull heart or beef. Mince and add 500ml of distilled water containing 1.5ml of 1M NaOH solution and simmer for 20 minutes. Drain off the liquid and dry the particles at 60°C. Store at -20°C.

Defined Medium (Karasawa *et al.*, 1995)

Once the components are dissolved they are sterilised through a 0.25μm pore filter (Whatman 6780-2502) and kept at 4°C.

Amino acids	**mg/L**
Arginine (IDH 194626)	100
Cysteine (IDH 101444)	500
Glycine (BDH 284586N)	100
Histidine (IDH 101954)	100
Isoleucine (IDH 194689)	100
Leucine (IDH 194694)	1000
Methionine (IDH 194707)	100
Proline (IDH 194728)	800
Threonine (IDH 194753)	100
Tryptophan (IDH 194758)	100
Valine (IDH 194769)	100
Vitamins	**μg/L**
Biotin	10
Pantothenate	1000
Pyridoxine	100

Appendix 1

Salts and glucose	**mg/L**
KH_2PO_4	300
Na_2HPO_4	1500
NaCl	900
$NaHCO_3$	5000
$(NH_4)_2SO_4$	40
$CaCl_2.2H_2O$	26
$MgCl_2.6H_2O$	20
$MnCl_2.4H_2O$	10
$FeSO_4.7H_2O$	4
$CoCl_2.6H_2O$	1

POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

BUFFERS (Laemmli, 1970)

Double strength separating gel buffer (0.75M TrisHCl, pH 8.8, 0.2% sodium lauryl (dodecyl) sulphate (SDS))

Tris (hydroxymethyl) methlyamine (BDH analar)	90.885g
Sodium lauryl sulphate (SDS) (BDH analar)	2g
Pyrogen-free water	1000ml

Dissolve the tris in 800ml of pyrogen-free water and adjust pH to 8.8 with 5M and 1M hydrochloric acid. Add the SDS and make up to 1000ml with pyrogen-free water.

Double strength stacking gel buffer (0.25M TrisHCl, pH 6.8, 0.2% SDS)

Tris (hydroxymethyl) methlyamine (BDH analar)	15.142g
Sodium lauryl sulphate (SDS) (BDH analar)	1g
Pyrogen-free water	500ml

Dissolve the tris in 400ml of pyrogen-free water and adjust pH to 6.8 with 5M and 1M hydrochloric acid. Add the SDS and make up to 500ml with pyrogen-free water.

SDS PAGE electrode buffer (0.025M Tris, 0.192M glycine, 0.1% SDS, pH 8.3)

Tris (hydroxymethyl) methlyamine (BDH analar)	6.057g
Glycine (BDH analar)	28.827g
Sodium lauryl sulphate (SDS) (BDH analar)	2g
Pyrogen-free water	2000ml

Dissolve the tris and the glycine in 1800ml of pyrogen-free water and check pH is 8.3. minor adjustments may be made with 1M NaOH. Add the SDS and make up to 2000ml with pyrogen-free water.

Sample buffer (Double strength) (0.125m Tris/HCl, pH 6.8, 4% SDS, 20% glycerol, 2% mercaptoethanol, 0.002% bromophenol blue)

Tris (hydroxymethyl) methlyamine (BDH)	1.514g
Sodium lauryl sulphate (SDS) (BDH)	4g
Glycerol (BDH analar) (≡ 20% v/v glycerol)	25.2g
2-mercaptoethanol (BDH)	2ml
Bromophenol blue (0.05% aq. sol.) (BDH)	4ml

Pyrogen-free water	100ml

Dissolve the Tris in 60ml pyrogen-free water and adjust to pH 6.8 with 1M HCl. Add the SDS. Add the bromophenol blue to the glycerol in a flask and transfer the Tris-HCl/SDS solution to it. Add the 2-mercaptoethanol and make up to 100ml. Store aliquots in the dark at -20°C.

SILVER STAIN FOR PROTEIN REAGENTS

Prefix A (50% methanol, 10% acetic acid

Methanol (BDH GPR)	1000ml
Glacial acetic acid (BDH)	200ml
Distilled water	800ml

Prefix B (5% methanol, 7% acetic acid)

Methanol (BDH GPR)	100ml
Glacial acetic acid (BDH)	140ml
Distilled water	1760ml

10% glutaraldehyde

Glutaraldehyde (50% sol. BDH)	200ml
Distilled water	800ml

Dithiothreitol (5mg/L)

Ditiothreitol	1-2mg
Distilled water	100-200ml

Use immediately.

Ammoniacal silver nitrate

Ammonia solution (BDH, SG 0.88)	1.4ml
Sodium hydroxide solution (BDH, 0.36%)	21ml

Silver nitrate solution (BDH)	4ml

Developer (0.005% citric acid in 0.019% formaldehyde solution)

Citric acid (BDH)	10mg
Formaldehyde solution (BDH, 38-40% sol.)	100µl
Distilled water	200ml

COOMASSIE STAINING FOR PAGE GELS
(Hancock and Poxton, 1988)

All the stains were made up in 2L volumes.

Coomassie 1

Coomassie brilliant blue R-250	1g
Propan-2-ol (GPR)	500ml
Acetic acid (Analar)	200ml
Distilled water	1300ml

Coomassie 2

Coomassie brilliant blue R-250	100mg
Propan-2-ol (GPR)	200ml
Acetic acid (Analar)	200ml
Distilled water	1600ml

Coomassie 3

Coomassie brilliant blue R-250	48mg
Acetic acid (Analar)	200ml
Distilled water	1800ml

Coomassie 4

Methanol (GPR)	800ml

Acetic acid (Analar)	200ml
Distilled water	1000ml

Coomassie 5

Acetic acid (Analar)	200ml
Distilled water	1800ml

TAE buffer (500ml at 10X)

This buffer is made up as a 10X solution and diluted when required.

Tris(hydroxymethyl)methylamine (BDH 103156X)	24.22g
Ethylenediamine-tetraacetic acid (EDTA) (Sigma E-6635)	3.72g

Add 500ml of distilled water and adjust pH to 8.0 using 30% acetic acid.

REAGENTS FOR PROTEIN ASSAY USING FOLIN AND CIOCALTEUS REAGENT (Lowry *et al.*, 1951)

Sodium carbonate (12.5% w/v)

Anhydrous Na_2CO_3	62.5g
Distilled water	500ml

Copper sulphate (0.1% w/v)

$CuSO_4.5H_2O$	100mg
Distilled water	100ml

Folin and Ciocalteu phenol reagent (BDH 19058)

Bovine serum albumin (2g/L)

REAGENTS FOR 2D GEL ELECTROPHORESIS

Lysis buffer (8M urea, 4% CHAPS, 40mM Tris)

Urea (BDH 102904W)	19.2g
CHAPS (Sigma C9426)	1.6g
Tris base (Promega H5131)	0.194g
Protease Inhibitor Cocktail	4 tablets

Dissolve in deionised water (dH_2O) and make up to 40ml final volume with dH_2O.

Rehydration solution (8M urea, 2% CHAPS, Bromophenol blue)

Urea (BDH 102904W)	24g
CHAPS (Sigma C9426)	1g
Bromophenol Blue	trace

Dissolve in dH_2O and make up to 50ml final volume with dH_2O. Aliquot into 1ml volumes and store at -20°C.

SDS Equilibration Buffer (50mM Tris-HCl, pH 8.8, 6M Urea, 2% SDS, Bromophenol blue)

Tris-HCl, pH8.8	50ml of 1.5M stock
Urea	360.35g
Glycerol	345ml
SDS	20g
Bromophenol Blue (0.002%)	2ml of 1% stock
Deionised water	up to 1000ml

Aliquot into 40ml volumes and store at -20°C.

Strain	Subject no.	S-type	Toxin A+B	van	mz	amox	clind	cefo	ceft
1205b		5236	pos						
1191a	785	5236	pos	1	1	2	8	128	64
1174a	785	5236	pos	1	1	2	4	128	64
1162a	549	5236	pos	1	1	2	4	128	64
1159a	560	5236	pos	1	1	2	4	128	64
1103a	560	5242	pos	1	0.5	2	4	128	64
1085a	560	5236	pos	1	0.5	2	4	128	64
1011a	560	5242	pos	1	1	2	4	128	64
989b	560	5438	pos	1	1	2	16	256	64
959a	560	5236	pos	1	1	2	4	128	64
845b	560	5236	pos	1	1	4	8	256	64
1154a	657	5236	pos	1	0.5	2	4	128	64
1130a	657	5236	pos	2	2	2	16	256	64
1149	704	5236	pos	1	0.5	2	4	128	64
1127a	685	5140	pos	1	1	4	4	128	64
1124a	688	5046	neg	2	1	2	>128	64	16
1107a	699	5242	pos	4	2	4	8	128	64
1082a	654	5236	pos	2	0.5	4	>128	128	64
1076a	654	5236	pos	2	1	4	>128	256	64
1060	654	5236	pos	2	2	4	8	128	64
1072	665	5242	pos	4	2	8	8	128	64
1056a	516	5236	pos	1	1	1	4	128	64
1043a	557	5236	pos	1	1	1	8	128	64
1041a	585	5236	pos	2	1	8	>128	128	64

Appendix 2 – Patient and strain data (symptom and toxin status, S-type, MIC).

Strain	Subject no.	S-type	Toxin A+B	van	mz	amox	clind	cefo	ceft
1030a	604	5242	pos	4	2	8	4	128	64
961a	604	5242	pos	1	0.5	2	8	64	32
1020a	627	5236	pos	1	1	4	4	256	64
990a	627	5236	pos	1	1	2	4	128	64
996a	596	5242	pos	1	1	1	8	64	32
992d	469	5438	pos	1	0.5	2	16	64	32
988a	610	5242	pos	1	4	8	4	128	64
982	597	5140	pos	2	0.5	2	16	64	32
969b	597	5140	pos	1	1	2	8	64	32
967a	597	5140	pos	1	0.5	2	8	64	32
981b	553	5236	pos	1	0.5	2	8	256	64
965a	553	5236	pos	1	0.5	2	4	128	64
973a	591	5236	pos	2	2	<1	16	128	64
972a	609	5242	pos	1	1	1	8	64	32
964a	609	5242	pos	1	1	2	8	64	32
960a	567	5242	pos	1	1	4	8	128	64
945a	567	5236	pos	1	1	2	8	128	64
926	567	5236	pos	2	1	2	8	256	64
958a	603	5242	pos	4	2	4	8	128	64
936	577	5140	pos	2	0.25	1	4	64	32
935	515	5236	pos	1	1	2	16	256	64
934a	562	5236	pos	2	0.5	2	4	64	32
918b	579	5242	pos	1	4	4	4	128	64
896b	537	5236	pos	2	1	4	16	256	64

Appendix 2 – Patient and strain data (symptom and toxin status, S-type, MIC).

Strain	Subject no.	S-type	Toxin A+B	van	mz	amox	clind	cefo	ceft
859b	472	5236	pos	1	1	4	16	256	64
836a	474	5236	pos	2	2	4	16	256	64
816b	468	5236	neg	1	1	4	16	64	64
804c	378	5438	pos	2	1	4	4	64	32
803a	458	5438	pos	2	1	4	4	128	32
797b	467	5236	pos	1	1	2	4	256	64
793b	368	5236	pos	1	1	4	8	128	64
788b	368	5236	pos	2	2	<1	16	128	64
751a	368	5236	pos	2	2	<1	16	128	64
791a	361	5236	pos	1	2	4	16	256	64
754a	361	5236	pos	1	1	2	16	256	64
681a	361	5236	pos	2	2	4	4	256	64
668a	361	5236	pos	2	1	2	>128	64	16
790c	354	5236	pos	1	2	4	16	256	64
785a	354	5236	pos	2	2	4	8	256	64
780b	398	5236	pos	2	2	4	16	256	64
779b	427	5236	pos	2	1	4	16	256	64
778b	449	5242	pos	1	0.5	<1	8	64	32
767	346	5236	pos	1	1	2	8	256	64
758	426	5236	pos	1	1	4	8	128	32
753a	347	5236	pos	2	2	4	16	256	64
750a	409	5236	pos	2	2	4	16	256	64
749a	411	5236	pos	2	2	4	16	256	64
712a	411	5236	pos	2	2	4	16	256	64
748a	402	5236	neg	2	1	2	>128	64	32

Appendix 2 – Patient and strain data (symptom and toxin status, S-type, MIC).

Strain	Subject no.	S-type	Toxin A+B	van	mz	amox	clind	cefo	ceft
711b	402	5236	pos	2	1	4	8	128	32
708b	366	5236	pos	2	2	4	16	256	64
702a	366	5236	pos	2	2	4	16	256	64
679a	366	5242	pos	2	2	4	16	256	64
705a	367	5242	pos	1	1	1	4	64	32
697b	367	5236	pos	2	1	2	8	64	32
669a	367	5236	pos	2	1	4	16	64	32
563a	367	5242	pos	1	1	1	8	64	64
659b	367	5242	pos	2	1	2	8	64	32
532a	449	5242	pos	1	0.5	<1	4	64	64
703	345	5236	pos	2	2	4	16	256	64
666a	206	5236	pos	2	2	4	16	256	64
665a	251	5236	pos	2	2	4	16	256	64
633b	251	5236	pos	2	2	4	16	256	64
622	251	5236	pos	2	2	4	8	256	64
605a	251	5236	pos	2	2	4	16	256	64
566c	251	5236	pos	1	1	2	8	256	64
561a	251	5236	pos	1	2	4	16	256	64
554c	251	5236	pos	1	1	2	16	256	64
661a	316	5236	pos	2	2	4	16	256	64
638b	316	5236	pos	2	2	4	16	256	64
643a	303	5236	pos	2	2	4	16	256	64
628b	337	5046	neg	2	1	2	>128	64	32
625b	325	5046	neg	2	1	2	>128	64	32
623a	170	5236	pos	2	2	4	4	256	64

Appendix 2 – Patient and strain data (symptom and toxin status, S-type, MIC).

Strain	Subject no.	S-type	Toxin A+B	van	mz	amox	clind	cefo	ceft
580f	170	5236	pos	1	2	4	4	256	64
564a	170	5236	pos	1	1	2	16	256	64
518a	170	5236	pos	1	2	2	8	256	64
414a	170	5236	pos	2	2	4	8	256	64
621	18	5236	pos	2	2	4	16	256	64
565a	18	5236	pos	2	2	4	8	256	64
247a	18	5236	pos	2	2	4	4	256	64
222a	18	5236	neg/pos*	1	2	4	4	256	64
602a	76	5236	pos	2	2	4	8	256	64
495a	76	5236	pos	1	1	2	8	256	64
485	76	5236	pos	1	1	4	8	256	64
439a	76	5236	pos	1	2	4	8	256	64
401b	76	5236	neg	1	2	4	8	256	64
386a	76	5236	pos	1	2	4	8	256	64
362a	76	5236	pos	2	2	4	8	256	64
348a	76	5236	pos	2	2	4	8	256	64
253a	76	5236	pos	1	2	4	4	256	64
224a	76	5236	pos	1	1	4	4	256	64
589a	93	5236	pos	1	1	2	8	256	64
545a	93	5236	pos	2	1	4	16	256	64
530a	93	5236	pos	1	2	2	16	256	64
501a	93	5236	pos	2	2	4	16	256	64
487	93	5236	pos	1	1	2	16	256	64
381a	93	5236	pos	2	2	4	8	256	64
360a	93	5236	pos	2	2	4	8	256	64

Appendix 2 – Patient and strain data (symptom and toxin status, S-type, MIC).

Strain	Subject no.	S-type	Toxin A+B	van	mz	amox	clind	cefo	ceft
344b	93	5236	pos	1	2	4	2	256	64
338a	93	5236	pos	1	1	2	2	256	64
588a	254	5236	pos	1	1	2	8	256	64
568a	233	5236	pos	1	1	4	16	256	64
551a	233	5236	pos	2	1	4	16	256	64
520a	233	5236	pos	2	2	4	16	256	64
541a	240	5739	pos	1	1	2	8	128	32
528a	250	5236	pos	2	1	2	4	64	32
524a	151	5236	pos	1	1	4	8	256	64
359b	151	5043	pos	2	2	4	<2	64	32
511a	201	5242	pos	1	1	2	8	128	64
473a	201	5242	pos	1	2	4	<2	128	64
484	205	5236	pos	1	0.5	2	4	256	64
483a	182	5236	pos	4	2	4	16	256	64
480a	144	5236	pos	2	2	4	4	256	64
461a	144	5236	pos	1	2	4	8	256	64
419a	144	5236	pos	2	2	4	8	256	64
366a	144	5236	pos	2	2	4	16	256	64
467a	103	5236	pos	1	1	8	<2	64	64
394b	103	5236	pos	1	1	16	<2	64	64
316a	103	5236	pos	1	1	4	2	128	64
447a	163	5236	pos	1	1	4	<2	256	64
445c	84	0	pos	2	1	4	8	64	32
391a	84	5236	pos	2	2	4	8	256	64
364a	84	5236	pos	2	1	4	8	256	64

Appendix 2 – Patient and strain data (symptom and toxin status, S-type, MIC).

Strain	Subject no.	S-type	Toxin A+B	van	mz	amox	clind	cefo	ceft
336b	84	5236	pos	1	2	4	4	256	64
322b	84	5236	pos	1	2	2	4	256	64
444a	119	5236	pos	1	2	2	8	256	64
440a	49	5236	pos	2	2	4	8	256	64
433a	49	5236	pos	2	2	4	8	256	64
277b	49	0	pos	2	1	2	<2	64	32
244a	49	0	pos	2	1	4	<2	64	32
218a	49	0	pos	2	1	2	<2	64	32
431b	137	5236	pos	2	2	4	8	256	64
332b	137	5046	pos	1	2	2	8	256	64
428	147	5236	pos	1	2	4	8	256	64
385	147	5236	pos	1	1	4	8	256	64
399a	172	5236	pos	1	1	16	2	128	64
392a	82	5236	pos	1	2	4	8	256	64
371a	82	5236	pos	2	2	4	16	256	64
375a	143	5236	pos	2	2	4	8	256	64
370a	68	5236	pos	2	2	4	8	256	64
369b		5236	pos	2	2	4	16	256	64
363b	81	5236	neg	2	2	8	128	64	64
269b	81	5236	neg	1	0.5	4	>128	64	64
261a	81	5236	neg	1	1	8	128	64	32
223a	81	5236	pos	1	1	4	>128	64	64
357a	138	5236	pos	1	2	4	4	256	64
346a	61	5236	pos	1	2	2	4	256	64
257a	61	5236	pos	2	1	2	4	256	64

Appendix 2 – Patient and strain data (symptom and toxin status, S-type, MIC).

Strain	sub. no.	S-type	sample date	process date	van	mz	amox	clind	cefo	ceft	antibiotics at sample time
1191a	785	5236	13/11/00	14/11/00	1	1	2	8	128	64	co-am
1174a	785	5236	07/11/00	08/11/00	1	1	2	4	128	64	0
1159a	560	5236	26/10/00	27/10/00	1	1	2	4	128	64	co-am, tri
1103a	560	5242	02/10/00	04/10/00	1	0.5	2	4	128	64	co-am
1085a	560	5236	18/09/00	22/09/00	1	0.5	2	4	128	64	co-am
1011a	560	5242	12/08/00	21/08/00	1	1	2	4	128	64	co-am
989b	560	5438	05/08/00	08/08/00	1	1	2	16	256	64	co-am
959a	560	5236	24/07/00	26/07/00	1	1	2	4	128	64	co-am
845b	560	5236			1	1	4	8	256	64	?
1154a	657	5236	27/10/00	27/10/00	1	0.5	2	4	128	64	0
1130a	657	5236	13/10/00	13/10/00	2	2	2	16	256	64	0
1082a	654	5236	19/09/00	22/09/00	2	0.5	4	>128	128	64	oral, cephs
1076a	654	5236	17/09/00		2	1	4	>128	256	64	oral, cephs
1060	654	5236	04/09/00	05/09/00	2	2	4	8	128	64	oral
961a	604	5242	25/07/00	26/07/00	1	0.5	2	8	64	32	0
1030a	604	5242	23/08/00	24/08/00	4	2	8	4	128	64	0
1020a	627	5236	21/08/00		1	1	4	4	256	64	co-am
990a	627	5236	07/08/00	08/08/00	1	1	2	4	128	64	co-am
982	597	5140	31/07/00	02/08/00	2	0.5	2	16	64	32	0
969b	597	5140	27/07/00	28/07/00	1	1	2	8	64	32	0
967a	597	5140	23/07/00	26/07/00	1	0.5	2	8	64	32	0
981b	553	5236	31/07/00	02/08/00	1	0.5	2	8	256	64	co-am
965a	553	5236	21/07/00	26/07/00	1	0.5	2	4	128	64	co-am
972a	609	5242	27/07/00	28/07/00	1	1	1	8	64	32	0
964a	609	5242	23/07/00	26/07/00	1	1	2	8	64	32	0
960a	567	5242	25/07/00	26/07/00	1	1	4	8	128	64	mac
945a	567	5236	12/07/00	12/07/00	1	1	2	8	128	64	mac
926	567	5236	28/06/00	04/07/00	2	1	2	8	256	64	mac

Appendix 3 – Patient repeat samples and antibiotic regime at sample date.

Strain	sub. no.	S-type	sample date	process date	van	mz	amox	clind	cefo	ceft	antibiotics at sample time
793b	368	5236	13/03/00	15/03/00	1	1	4	8	128	64	quins
788b	368	5236	03/03/00	10/03/00	2	2	<1	16	128	64	quins
751a	368	5236	22/02/00	24/02/00	2	2	<1	16	128	64	quins
791a	361	5236	04/03/00	10/03/00	1	2	4	16	256	64	
754a	361	5236	24/02/00	25/02/00	1	1	2	16	256	64	oral, amox, co-am
681a	361	5236	27/02/00	28/02/00	2	2	4	4	256	64	oral, amox, co-am
668a	361	5236	23/01/00	26/01/00	2	1	2	>128	64	16	amox
785a	354	5236	06/03/00	10/03/00	2	2	4	8	256	64	co-am, paren, oral, macro
790c	354	5236	04/03/00	10/03/00	1	2	4	16	256	64	co-am, paren, oral, macro
778b	449	5242	05/03/00	10/03/00	1	0.5	<1	8	64	32	0
532a	449	5242	11/11/99	15/11/99	1	0.5	<1	4	64	64	0
749a	411	5236	22/02/00	24/02/00	2	2	4	16	256	64	quins, co-am
712a	411	5236	09/02/00	11/02/00	2	2	4	16	256	64	quins
748a	402	5236	21/02/00	22/02/00	2	1	2	>128	64	32	amox,mac,pens,paren,oral
711b	402	5236	07/02/00	11/02/00	2	1	4	8	128	32	0
708b	366	5236	07/02/00	11/02/00	2	2	4	16	256	64	mac, co-am, oral
702a	366	5236	07/02/00	07/02/00	2	2	4	16	256	64	mac, co-am, oral
679a	366	5242	28/01/00	28/01/00	2	2	4	16	256	64	mac
705a	367	5242	07/02/00	07/02/00	1	1	1	4	64	32	0
697b	367	5236	04/02/00	07/02/00	2	1	2	8	64	32	0
669a	367	5236	24/01/00	28/01/00	2	1	4	16	64	32	0
659b	367	5242	18/01/00	19/01/00	2	1	2	8	64	32	0
563a	367	5242			1	1	1	8	64	64	0
665a	251	5236	22/01/00	26/01/00	2	2	4	16	256	64	co-am,mac,oral,ceft,paren,cephs
633b	251	5236	10/01/00	13/01/00	2	2	4	16	256	64	co-am,mac,oral,ceft,paren,cephs
622	251	5236	06/01/00	07/01/00	2	2	4	8	256	64	co-am,mac,oral,ceft,paren,cephs
605a	251	5236	09/12/99	10/12/99	2	2	4	16	256	64	co-am, mac, oral
566c	251	5236	22/11/99	24/11/99	1	1	2	8	256	64	co-am, mac
561a	251	5236			1	2	4	16	256	64	co-am, mac
554c	251	5236	18/11/99	19/11/99	1	1	2	16	256	64	co-am, mac

Appendix 3 – Patient repeat samples and antibiotic regime at sample date.

Strain	sub. no.	S-type	sample date	process date	van	mz	amox	clind	cefo	ceft	antibiotics at sample time
661a	316	5236	19/01/00	21/01/00	2	2	4	16	256	64	mac, cephs
638b	316	5236	11/01/00	14/01/00	2	2	4	16	256	64	mac
623a	170	5236	07/01/00	10/01/00	2	2	4	4	256	64	oral, mac
580f	170	5236	29/11/99	30/11/99	1	2	4	4	256	64	oral, mac
564a	170	5236			1	1	2	16	256	64	oral + - mac
518a	170	5236	01/11/99	03/11/99	1	2	2	8	256	64	oral
414a	170	5236	21/09/99	22/09/99	2	2	4	8	256	64	oral
621	18	5236	06/01/00	07/01/00	2	2	4	16	256	64	quins
565a	18	5236	24/11/99	24/11/99	2	2	4	8	256	64	quins
247a	18	5236	28/07/99	28/07/99	2	2	4	4	256	64	quins
222a	18	5236	20/07/99	20/07/99	1	2	4	4	256	64	quins
602a	76	5236	06/12/99	07/12/99	2	2	4	8	256	64	co-am,pens,quins,oral,amox,pens
495a	76	5236	18/10/99	20/10/99	1	1	2	8	256	64	co-am, pens, quins, oral, amox
485	76	5236	14/10/99	15/10/99	1	1	4	8	256	64	co-am, pens, quins, oral, amox
439a	76	5236	28/09/99	29/09/99	1	2	4	8	256	64	co-am, pens, quins, oral
401b	76	5236	15/09/99	19/09/99	1	2	4	8	256	64	co-am, pens, quins, oral
386a	76	5236	09/09/99	10/09/99	1	2	4	8	256	64	co-am, pens, quins
362a	76	5236	30/08/99	01/09/99	2	2	4	8	256	64	co-am, pens, quins
348a	76	5236	25/08/99	25/08/99	2	2	4	8	256	64	co-am, pens, quins
253a	76	5236	30/07/99	30/07/99	1	2	4	4	256	64	co-am, pens
224a	76	5236	21/07/99	21/07/99	1	1	4	4	256	64	co-am, pens
589a	93	5236	30/11/99	01/12/99	1	1	2	8	256	64	quins, co-am, amox, oral
545a	93	5236	12/11/99	17/11/99	2	1	4	16	256	64	quins, co-am, amox, oral
530a	93	5236	11/11/99	12/11/99	1	2	2	16	256	64	quins, co-am, amox, oral
501a	93	5236	19/10/99	20/10/99	2	2	4	16	256	64	quins, co-am, amox, oral
487	93	5236	14/10/99	15/10/99	1	1	2	16	256	64	quins, co-am, amox, oral
381a	93	5236	08/09/99	09/09/99	2	2	4	8	256	64	quins, co-am, amox, oral
360a	93	5236	31/08/99	31/08/99	2	2	4	8	256	64	quins, co-am, amox, oral
344b	93	5236	23/08/99	25/08/99	1	2	4	2	256	64	quins, co-am, amox
338a	93	5236	23/08/99	23/08/99	1	1	2	2	256	64	quins, co-am, amox

Appendix 3 – Patient repeat samples and antibiotic regime at sample date.

Strain	sub. no.	S-type	sample date	process date	van	mz	amox	clind	cefo	ceft	antibiotics at sample time
568a	233	5236	22/11/99	24/11/99	1	1	4	16	256	64	oral, co-am, mac, quins, paren
551a	233	5236	16/11/99	17/11/99	2	1	4	16	256	64	oral, co-am, mac, quins, paren
520a	233	5236	02/11/99	03/11/99	2	2	4	16	256	64	oral, co-am, mac, quins, paren
524a	151	5236	05/11/99	09/11/99	1	1	4	8	256	64	co-am
359b	151	5043	28/08/99	31/08/99	2	2	4	<2	64	32	co-am
511a	201	5242	25/10/99	26/10/99	1	1	2	8	128	64	co-am
473a	201	5242	09/10/99	13/10/99	1	2	4	<2	128	64	co-am
480a	144	5236	14/10/99	14/10/99	2	2	4	4	256	64	tri, amox, macro, oral
461a	144	5236	04/10/99	07/10/99	1	2	4	8	256	64	tri, amox, macro, oral
419a	144	5236	21/09/99	22/09/99	2	2	4	8	256	64	tri, amox, macro, oral
366a	144	5236	31/08/99	01/09/99	2	2	4	16	256	64	tri, amox, macro, oral
467a	103	5236	12/10/99	13/10/99	1	1	8	<2	64	64	co-am
394b	103	5236	13/09/99	15/09/99	1	1	16	<2	64	64	co-am
316a	103	5236	16/08/99	17/08/99	1	1	4	2	128	64	0
445c	84	0	01/10/99	04/10/99	2	1	4	8	64	32	am,mac,oral,quin,ceft,ceph,co-am
391a	84	5236	11/09/99	13/09/99	2	2	4	8	256	64	am,mac,oral,quin,ceft,ceph,co-am
364a	84	5236	30/08/99	01/09/99	2	1	4	8	256	64	amox,mac,oral,quins,ceft,cephs
336b	84	5236	19/08/99	23/08/99	1	2	4	4	256	64	amox,mac,oral,quins
322b	84	5236	17/08/99	17/08/99	1	2	2	4	256	64	amox,mac,oral,quins
440a	49	5236	29/09/99	29/09/99	2	2	4	8	256	64	oral
433a	49	5236	25/09/99	29/09/99	2	2	4	8	256	64	oral
277b	49	0	04/08/99	04/08/99	2	1	2	<2	64	32	0
244a	49	0	26/07/99	28/07/99	2	1	4	<2	64	32	0
218a	49	0	20/07/99	20/07/99	2	1	2	<2	64	32	0
431b	137	5236	28/09/99	29/09/99	2	2	4	8	256	64	co-am
332b	137	5046	20/08/99	23/08/99	1	2	2	8	256	64	0
428	147	5236	29/09/99	29/09/99	1	2	4	8	256	64	quins, oral, ceft
385	147	5236	09/09/99	10/09/99	1	1	4	8	256	64	
392a	82	5236	13/09/99	13/09/99	1	2	4	8	256	64	mac,co-am,quins,ceft,paren,cephs
371a	82	5236	05/09/99	06/09/99	2	2	4	16	256	64	mac,co-am,quins,ceft,paren,cephs

Appendix 3 – Patient repeat samples and antibiotic regime at sample date.

Strain	sub. no.	S-type	sample date	process date	van	mz	amox	clind	cefo	ceft	antibiotics at sample time
363b	81	5236	30/08/99	01/09/99	2	2	8	128	64	64	mac
269b	81	5236	02/08/99	03/08/99	1	0.5	4	>128	64	64	mac
261a	81	5236	30/07/99	02/08/99	1	1	8	128	64	32	mac
223a	81	5236	21/07/99	21/07/99	1	1	4	>128	64	64	mac
346a	61	5236	24/08/99	25/08/99	1	2	2	4	256	64	0
257a	61	5236	31/07/99	02/08/99	2	1	2	4	256	64	0
289b	59	5242	08/08/99	09/08/99	1	1	2	8	64	32	co-am
239a	59	5242	27/07/99	28/07/99	1	0.5	<1	<2	64	32	co-am
205a	59	5242	14/07/99	15/07/99	1	1	<1	4	64	32	co-am
281b	101	5236	04/08/99	04/08/99	2	2	4	4	256	64	oral
246a	101		26/07/99	28/07/99	1	1	2	<2	256	64	oral
271a	79	5236	03/08/99	03/08/99	2	2	4	4	256	64	co-am, quins
265a	79	5236	30/07/99	02/08/99	1	2	4	4	256	64	co-am, quins
210a	52	5236	14/07/99	15/07/99	2	1	2	<2	256	64	0
203a	52	5236	14/07/99	15/07/99	1	2	2	4	256	64	0

Appendix 3 – Patient repeat samples and antibiotic regime at sample date.

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Changes in sensitivity patterns to selected antibiotics in *Clostridium difficile* in geriatric in-patients over an 18-month period

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Clostridium difficile-associated disease continues to be a major problem in hospitals and long-term care facilities throughout the developed world. Administration of certain antibiotics such as amoxycillin, oral cephalosporins and clindamycin is associated with the greatest risk of developing *C. difficile* disease. The two antibiotics used for treatment of *C. difficile* disease are vancomycin and metronidazole, to which there is currently very little resistance. Randomly selected isolates (186) from 90 patients being investigated during an 18-month epidemiological study into the disease were tested for their susceptibility to vancomycin, metronidazole, amoxycillin, clindamycin, cefoxitin and ceftriaxone by the NCCLS agar dilution method. There was a narrow range of MIC for the two treatment agents (vancomycin and metronidazole), from 0·5 to 4 μg ml^{-1}, with no evidence of resistance. All strains were resistant to cefoxitin (MIC 64–256 μg ml^{-1}), the antibiotic used in most selective media. All strains were of similar sensitivity to amoxycillin (MIC_{90} = 4 μg ml^{-1}). Most strains were resistant to ceftriaxone (MIC ⩾ 64 μg ml^{-1}) or of intermediate resistance (MIC ⩾ 32 μg ml^{-1}), with only two sensitive strains (MIC 16 μg ml^{-1}). Clindamycin resistance was common, with 67 % of strains resistant (MIC ⩾ 8 μg ml^{-1}), 25 % with intermediate resistance (MIC ⩾ 4 μg ml^{-1}) and only 8 % sensitive (MIC ⩽ 2 μg ml^{-1}). Twelve isolates from six different patients had very high resistance to clindamycin (MIC ⩾ 128 μg ml^{-1}). Multiple isolates from the same patient, taken at different times, showed changes in susceptibility patterns over time. The only major change in susceptibility over the time-period was in clindamycin resistance; some strains appeared to become more resistant while others became less resistant. No differences were seen in the MIC_{50} and MIC_{90} of the different S-types of *C. difficile* identified, although some S-types were present in very small numbers. There was no correlation between the antibiotics prescribed and susceptibility.

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INTRODUCTION

Clostridium difficile is an important cause of nosocomial, antibiotic-associated diarrhoea (AAD) and pseudomembranous colitis. Its clinical manifestations range from asymptomatic carriage to severe diarrhoea and pseudomembranous colitis with toxic megacolon. Although it was first described in 1935, as a commensal in the gut flora of infants, it was implicated in antibiotic-associated colitis in the 1970s (Tedesco *et al.*, 1974; Bartlett *et al.*, 1978). *C. difficile* is prevalent in hospitals and long-term care facilities and increases costs to health services for the care of infected patients as well as in isolation and infection-control procedures (Spencer, 1998).

Disease is generally thought to occur after depletion of the patient's normal protective bowel microbiota following use of broad-spectrum antibiotics (Borriello & Barclay, 1986; Larson & Welch, 1993). This state leaves the patient vulnerable to overgrowth by *C. difficile* that is already in the patient in small numbers (endogenous) or, more commonly, from another patient or the environment (exogenous). The third-generation cephalosporins, clindamycin and amoxycillin are associated with the greatest risk for developing AAD because of their widespread use in the hospital as well as the community, but almost all antibiotics can cause the disease (Mylonakis *et al.*, 2001).

The antibiotics used to treat *C. difficile* diarrhoea are vancomycin and metronidazole, with metronidazole being the drug of choice because it has fewer side-effects, is cheaper and is not associated with selection of vancomycin-resistant enterococci (Wilcox & Dave, 2001). Few reports have appeared of decreased susceptibility to these therapeutic

agents (Barbut *et al.*, 1999; Johnson *et al.*, 2000; Brazier *et al.*, 2001; Peláez *et al.*, 2002). The majority of strains with reported decreased susceptibility to metronidazole have been non-toxigenic and are therefore considered clinically insignificant (Barbut *et al.*, 1999; Johnson *et al.*, 2000; Brazier *et al.*, 2001).

The aims of this study were to obtain current information on the sensitivity (as MICs) of a sample of *C. difficile* isolates to a variety of precipitating and treatment antibiotics during the 18-month period of a major epidemiological study. It also investigated the patterns of susceptibility in relationship to phenotype (S-type) and antibiotics prescribed. This work was not meant to be a comprehensive study of susceptibility. When more than one isolate was taken from a patient, they were used to determine changes in sensitivity patterns over time. Another paper (to be published elsewhere) will contain the demographical information together with computer modelling.

METHODS

***C. difficile* isolates and characterization of S-types.** Isolates were obtained through an 18-month epidemiological study (July 1999–December 2000) during which over 1000 faecal samples were taken from 390 patients, of whom 100 were culture-positive. The study was unable to determine whether *C. difficile* was acquired from the hospital or the community environment, as it was difficult to guarantee that a stool sample was taken immediately on admission to the ward. Stool samples were taken at least once during a patient's stay and weekly if possible. Patient data and stool samples were collected by a research nurse in wards 5 and 6 (care-for-the-elderly wards) of the Royal Victoria Hospital in Edinburgh. Full demographical details including patient age, antibiotic usage and health are to be published elsewhere. However, briefly, 1003 specimens were taken from 390 patients: mean age 82·5, SD 7·3, 65 % female, with 44 % being transferred from another hospital ward.

The stool samples were processed on cefoxitin/cycloserine/egg-yolk (CCEY) selective agar (Brazier, 1993). The isolates were identified by characteristic colony morphology, smell and appearance on a Gram film. From over 500 isolates (with up to six from a sample), a subset of 186 from 90 patients was randomly selected for study of antibiotic-sensitivity patterns. These included multiple isolates from 38 patients. Subcultures were stored in cooked meat+anaerobic investigation medium (AIM; Brown *et al.*, 1996) for maintenance.

One isolate from each sample was S-typed using guanidine hydrochloride to extract their S-layer proteins followed by analysis on SDS-PAGE (McCoubrey & Poxton, 2001). Where multiple isolates were available, differences were observed in the S-types present. Isolates were also tested for toxin production using Techlab Tox A+B ELISA kits. All the data collected were stored in a Microsoft Access database. Only one isolate from each sample was used for MIC determinations.

Antibiotics and MIC determinations. Details of all antibiotics used in the treatment of these patients were available in the database. The antibiotics selected for this study (all from Sigma) were not meant to be extensive, but representative: the two agents used for treatment of *C. difficile*-associated disease, vancomycin (concentrations used 8–0·125 μg ml^{-1}) and metronidazole (8–0·125 μg ml^{-1}), and four of the agents with known association with *C. difficile* disease, amoxycillin (64–1 μg ml^{-1}), clindamycin (128–2 μg ml^{-1}), ceftriaxone (256–8 μg ml^{-1}) and cefoxitin (256–8 μg ml^{-1}); the latter is also used in the CCEY selective medium, at 8 μg ml^{-1}. The non-treatment agents were chosen because they are the most common precipitating agents of *C. difficile* diarrhoea –they have poor *in vitro* activity against *C. difficile*.

MICs were determined using the agar dilution protocol in the NCCLS guidelines (NCCLS, 1997). The isolates were subcultured from spores in cooked-meat broth into pre-reduced (80 % N_2, 10 % H_2, 10 % CO_2 at 37 °C) thioglycollate medium (Sigma) enriched with 5 μg haemin, 1 μg vitamin K_1 and 1 mg $NaHCO_3$ ml^{-1} and incubated overnight anaerobically at 37 °C. This yielded approximately 1×10^8 bacteria ml^{-1}. Purity of the cultures was checked by Gram stain and was checked retrospectively by anaerobic and aerobic incubation for 48 h on Columbia blood agar (Oxoid; supplemented with 5 % horse blood). Aliquots (1–2 μl) of the cultures were spotted onto Brucella agar (Oxoid) supplemented with 5 % defibrinated sheep blood, 5 μg haemin ml^{-1} and 1 μg vitamin K_1 ml^{-1} using a multi-point inoculator.

MIC_{50} and MIC_{90} were calculated by pasting all the MICs for each strain into an Excel spreadsheet and sorting into ascending order. The MIC_{50} was taken as the MIC that was the median value. Of 186 isolates, this was cell 93. Similarly, the MIC_{90} was the value found in row 168 (90 % of 186) and represented the concentration of antibiotic that would inhibit 90 % of the isolates tested.

RESULTS

MICs

In total, 186 representative isolates were investigated. Table 1 shows the ranges of MICs among the isolates for the six antibiotics used, together with MIC_{50} and MIC_{90} values and, where known, the break-points for the antibiotics. The two antibiotics used for treatment (vancomycin and metronidazole) both showed a narrow range, between 0·5 and 4 μg ml^{-1}. Cefoxitin, the antibiotic used in the selective medium (at 8 μg ml^{-1}), showed a range of MICs from 64 to 256 μg ml^{-1}. The other three precipitating antibiotics all showed a wider range of MICs.

The MIC_{50} and MIC_{90} for the six antibiotics used were either the same or twofold different. This highlights the closeness in sensitivity of the majority of isolates. The MIC_{50} and MIC_{90} for vancomycin and metronidazole were low (2 μg ml^{-1}), and only five strains (2·7 %) had an MIC of 4 μg ml^{-1} to vancomycin and two (1·1 %) had an MIC of 4 μg ml^{-1} to metronidazole. None of the isolates tested was resistant to the two treatment agents for *C. difficile* diarrhoea. Both the MIC_{50} and MIC_{90} values for amoxycillin were 4 μg ml^{-1}. This shows that, even though the range of MICs to this antibiotic was relatively broad ($\leqslant$ 1–16 μg ml^{-1}), the majority of the isolates had very similar sensitivity. Clindamycin produced a range of sensitivities within the tested isolates ($\leqslant$ 2 to $>$ 128 μg ml^{-1}). For this antibiotic, the MIC_{50} and MIC_{90} values were respectively 8 and 16 μg ml^{-1}. The NCCLS break-point for clindamycin resistance is $\geqslant$ 8 μg ml^{-1}; therefore, 66·7 % ($n = 124$) of the isolates were resistant to clindamycin, 24·7 % ($n = 46$) had intermediate resistance (MIC 4 μg ml^{-1}) and the rest were sensitive. Twelve *C. difficile* isolates with MICs to clindamycin of $\geqslant$ 128 μg ml^{-1} from six patients were found. The MIC_{50} and MIC_{90} of cefoxitin were the same, at 256 μg ml^{-1}. The NCCLS guidelines state that MICs of $\geqslant$ 64 μg ml^{-1} indicate

Table 1. Range of MIC values from 186 isolates

Antibiotic	MIC range ($\mu g\ ml^{-1}$)	MIC_{50}	MIC_{90}	Break-point
Vancomycin	0·5–4	1	2	8
Metronidazole	0·5–4	1	2	8
Amoxycillin	⩽ 1–16	4	4	?
Clindamycin	⩽ 2–> 128	8	16	8
Cefoxitin	64–256	256	256	64
Ceftriaxone	16–256	64	64	64

resistance to cefoxitin (NCCLS, 1997); therefore, none of the 186 isolates tested was sensitive. According to the NCCLS guidelines, MICs of ⩾ 64 $\mu g\ ml^{-1}$ indicate resistance to ceftriaxone. Isolates had MIC_{50} and MIC_{90} values of 64 $\mu g\ ml^{-1}$ to ceftriaxone. Thirty-three strains (17·7 %) had intermediate resistance to ceftriaxone, with MICs of 32 $\mu g\ ml^{-1}$ (NCCLS, 1997). Only two strains (1·1 %) were sensitive, with MICs of 16 $\mu g\ ml^{-1}$.

Relationship of S-layer types to MICs

Of the 186 strains included in the collection for MIC determinations, 145 were phenotyped by analysis of their S-layer proteins on SDS-PAGE. Most strains (76·5 %; $n = 111$) belonged to the common S-type 5236, with most of the others being S-type 5242 (14·5 %; $n = 21$). Of the remainder, 3·4 % ($n = 5$) were S-type 5140 and 2·8 % ($n = 4$) were S-type 5438, with single isolates of S-types 5941 and 5144. Two strains collected were non-typable: they did not show the typical two major S-layer bands on SDS-PAGE.

There was a degree of variation in sensitivity to antibiotics depending on the S-type of the isolate (Table 2). The common S-type 5236 had a large range of MICs, and there was no difference in overall pattern between this and the total population. However, the less common S-types did show some variations, in particular in respect to clindamycin sensitivity. Both of the non-typable strains were extremely sensitive to clindamycin, with MICs of ⩽ 2 $\mu g\ ml^{-1}$, and had below-average MICs to cefoxitin and ceftriaxone, respectively 64 and 32 $\mu g\ ml^{-1}$.

Repeat samples and changes in antibiotic-sensitivity patterns over time

Thirty-eight patients were sampled more than once, with some being sampled up to 10 times. Of those S-typed, at least 50 % (19/38) retained the same S-type throughout the study, while 13 % (5/38) definitely harboured different S-types over time, with one patient having three different types at different times. Isolates from 19 patients exhibited changing patterns of sensitivity to one or more of the six antibiotics. While some of these changes related to changes of S-type, others did not. Typical changes in isolates that were all of the same S-type were no more than two- to fourfold differences in MIC. However, some major changes occurred in sensitivity to clindamycin. One noteworthy example of this was an isolate with an MIC to clindamycin of 8 $\mu g\ ml^{-1}$. Two subsequent samples taken from the same patient 13 and 15 days later each produced a highly clindamycin-resistant strain, with an MIC of > 128 $\mu g\ ml^{-1}$. The isolates from these samples were all S-type 5236. Another example of changing clindamycin sensitivity was in a patient who also harboured isolates of S-type 5236. The first sample produced an isolate with an MIC of > 128 $\mu g\ ml^{-1}$. A month later, another sample contained a

Table 2. Variation in MICs among different S-types

Abbreviations: Van, vancomycin; Met, metronidazole; Amox, amoxycillin; Clin, clindamycin; Cefo, cefoxitin; Ceft, ceftriaxone. NT, Not typable.

S-type	Isolates [% (n)]	MIC [range (MIC_{90})] ($\mu g\ ml^{-1}$)					
		Van	Met	Amox	Clin	Cefo	Ceft
All	100 (145)	0·5–4 (2)	0·25–4 (2)	⩽ 1–16 (4)	⩽ 2–> 128 (16)	64–256 (256)	16–256 (64)
5236	76·5 (111)	1–4 (2)	0·5–2 (2)	⩽ 1–16 (4)	⩽ 2–> 128 (16)	64–256 (256)	16–64 (64)
5242	14·5 (21)	1–4 (2)	0·5–4 (2)	⩽ 1–8 (8)	⩽2–16 (8)	64–128 (128)	32–64 (64)
5140	3·4 (5)	1–2 (2)	0·25–1 (1)	1–4 (4)	4–16 (16)	64–128 (128)	32–64 (64)
5438	2·8 (4)	1–2 (2)	0·5–1 (1)	2–4 (4)	4–16 (16)	64–256 (256)	32–64 (64)
5144	0·7 (1)	2	1	2	> 128	64	16
5941	0·7 (1)	1	1	2	8	128	32
NT	1·4 (2)	2	1	2–4 (4)	⩽ 2	64	32

strain with an MIC of 16 μg ml^{-1} followed, 3 days later, by one with an MIC to clindamycin of 8 μg ml^{-1}. Neither of these patients was on clindamycin or any other macrolide. No significant changes in the MIC of the patients' isolates were found to the other five antibiotics.

No clear patterns emerged from the data to suggest any link between prescribed antibiotics and specific sensitivities. For example, patients on amoxycillin showed no propensity to produce isolates more resistant to that agent.

Overall, 97 % of the total isolates produced toxin (by the Techlab A+B kit). However, there was no correlation between toxin production and antibiotic susceptibility or, indeed, S-type.

DISCUSSION

This 18-month study has investigated the susceptibility of *C. difficile* isolates to a range of antibiotics associated with development of *C. difficile*-associated diarrhoea, together with the two antibiotics used in therapy of the disease. There was no evidence of any resistance to vancomycin or metronidazole, the treatment agents. However, such strains, especially human isolates, are still extremely rare (Brazier *et al.*, 2001). Five strains (2·7 %) had slightly reduced susceptibility to vancomycin, with MICs of 4 μg ml^{-1}. This low level of reduced susceptibility has also been reported by others (Peláez *et al.*, 2002), also in small numbers. There was general resistance to the cephalosporin and cephamycin antibiotics, but not to the other beta-lactam amoxycillin. Resistance to clindamycin was common, despite its infrequent use. In summary, this shows that increased colonization with *C. difficile* and subsequent disease may well be due to acquisition of resistant strains of the bacterium, but, as has been shown frequently in the past, other mechanisms must also be operating, as demonstrated by the apparent sensitivity to amoxycillin. Amoxycillin is used widely, both in the hospital environment and in the community, and is one of the most common precipitating antibiotics (Freeman & Wilcox, 1999).

Isolates showed a wide range of sensitivities to clindamycin, with MICs varying from $\leqslant 2$ to > 128 μg ml^{-1}. Strains resistant to clindamycin have been widely reported, and some have been involved in epidemics (Johnson *et al.*, 1999). Clindamycin usage has decreased dramatically because of its involvement in the precipitation of *C. difficile* diarrhoea. There is now little selective pressure for clindamycin resistance. The usual mechanisms by which clindamycin resistance is conferred also mediate resistance to other macrolide, lincosamide and streptogramin B antibiotics: this is known as the MLS resistance determinant (Mullany *et al.*, 1996; Farrow *et al.*, 2001). The MLS determinant is the major mechanism of multiple resistance among Gram-positive anaerobes (Noren *et al.*, 2002). The gene responsible (*ermB* in *C. difficile* 630) encodes a 23S rRNA methylase that modifies the target site for the antibiotic. The sequence is 99 % similar to that of the *ermB* gene from *Clostridium perfringens* but, unlike this gene, it is not located on a plasmid (pIP402) but on a mobilizable, non-conjugative transposon, Tn*5398* (Farrow *et al.*, 2001). Whether the *C. difficile* isolates tested in this study have this gene has not yet been confirmed through PCR, though no other mechanism for such high-level resistance has been described in this species. *C. difficile* appears to be inherently resistant to the cephalosporin and cephamycin antibiotics, as the majority of isolates had MICs to these agents of $\geqslant 32$ μg ml^{-1} (NCCLS, 1997).

C. difficile possesses an outer cell coat, termed the S-layer, consisting of two polypeptides that form a regular crystalline array over the surface of the cell (Kawata *et al.*, 1984). The most common S-type in this study was 5236 (the number corresponds to the molecular masses in kDa of the two major polypeptides found on the cell surface). Of all strains tested so far, the molecular mass of the larger of the two proteins varies from 45 to 64 kDa, with the smaller ranging from 25 to 40 kDa (Poxton *et al.*, 1999). S-layer typing is a quick and easy method of phenotyping and appears to correspond well with other typing techniques, including ribotyping and serotyping (McCoubrey & Poxton, 2001). Toxigenic S-type 5236 is the same as ribotype 001 (McCoubrey, 2002), which is the most common ribotype in the UK (Stubbs *et al.*, 1999). The S-layer is a putative virulence factor that may have a role in adhesion of the bacterium to the host mucosal surface. It may also have a role in immune evasion or impermeability to certain compounds, including antibiotics. The two non-typable strains appeared to be more sensitive to clindamycin, cefoxitin and ceftriaxone. Though no firm conclusions can be made, especially when this pattern was rare, it may be speculated that, as they appear to lack a typical S-layer, they are more sensitive to some antibiotics. However, overall, there were no obvious correlations between S-type and resistance to antibiotics.

Multiple isolates were obtained from 37 patients and, for some patients, as many as 10 were available. These isolates permitted assessment of sensitivity patterns over time and within and between S-types. In the majority of cases, isolates did not change either in S-type or in sensitivity pattern. The isolates from some patients did change in antibiotic sensitivity and/or in S-type, suggesting that there had been reinfection with a different strain or, possibly, the emergence of a minor strain from an initially mixed infection. In patients whose isolate did not change in S-type, resistance to clindamycin was the only significant difference observed. Resistance to clindamycin typically resides on a transposon, Tn*5398* (Mullany *et al.*, 1996; Farrow *et al.*, 2001), which could transfer between strains. It is feasible that the strain acquired this resistance determinant or that the patient was reinfected with a clindamycin-resistant strain of the same, predominant S-type. In the patient whose strain appeared to lose clindamycin resistance, it is possible that the resistance determinant was lost. More likely is the explanation that the patient had picked up another S-type 5236 strain that lacked the clindamycin-resistance determinant. In the patients who produced same-type isolates with changing resistance, it would be interesting to use another typing method (sero- or

ribotyping) in order to try to identify subtypes, which may explain the sensitivity changes. There was no direct evidence that resistance to clindamycin was selected in strains, despite the use of macrolides in many patients during the study.

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Effects of sub-MIC concentrations of antibiotics on growth of and toxin production by *Clostridium difficile*

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Effects on growth and toxin A production of sub-MIC concentrations of six different antibiotics were investigated in three strains of *Clostridium difficile*: reference strain NCTC 11223, a fully sequenced strain (630) and a locally endemic isolate (strain 338a). The antibiotics chosen for investigation were the agents used to treat *C. difficile*-associated disease (CDAD), i.e. vancomycin and metronidazole, and four antibiotics that are commonly involved in precipitating CDAD (amoxycillin, clindamycin, cefoxitin and ceftriaxone). Strains were cultured in sublethal concentrations of antibiotics (1/2, 1/4 and 1/8 MIC) over 104 h and growth and toxin A production were measured three times a day. Effects varied between strain and antibiotic. The most common effect on growth of the strains was to increase the initial lag period by approximately 4 h, compared with antibiotic-free controls; however, strain NCTC 11223, which has high-level clindamycin resistance ($\geq$ 512 μg ml^{-1}), showed no lag whatsoever in comparison with the controls when grown in this antibiotic. The most common effect on production of toxin A was in the onset of toxin elaboration. Normally, toxins began to appear at low levels in the early stationary phase, before accumulating to high levels by the start of decline. In the presence of sub-MIC antibiotics, this onset appeared before that of the antibiotic-free controls. This effect was seen with metronidazole, amoxycillin and clindamycin, rarely with vancomycin and never with cefoxitin. These results suggest a very complex, strain-dependent relationship between the effects of growth and toxin production.

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INTRODUCTION

Clostridium difficile is the most common cause of antibiotic-associated diarrhoea and is the aetiological agent of pseudomembranous colitis. This Gram-positive, obligately anaerobic spore-former causes a wide spectrum of disease, ranging from mild, self-limiting diarrhoea to serious diarrhoea and, in some cases, complications such as pseudomembrane formation, toxic megacolon and peritonitis. The main bacterial factors that are recognized in *C. difficile*-associated disease (CDAD) are two high-molecular-mass toxins, A and B. Once released onto the gut mucosa, they act in concert to produce the characteristic pathology and symptoms. Antibiotics have a prominent role in *C. difficile* disease: it is hypothesized that depletion of bowel microbiota by antibiotics leads to the elimination of any resistance to colonization by *C. difficile* (Farrell & LaMont, 2000). Broad-spectrum agents, such as clindamycin, amoxycillin and third-generation cephalosporins, are associated with the greatest risk of developing *C. difficile* diarrhoea, although almost all antibiotics have been implicated at one time or another (Mylonakis *et al.*, 2001).

Abbreviation: CDAD, *Clostridium difficile*-associated disease.

Toxin production is affected by environmental factors that include temperature, glucose concentration, biotin limitation and amino acid concentration (Onderdonk *et al.*, 1979; Honda *et al.*, 1983; Barc *et al.*, 1992; Yamakawa *et al.*, 1996; Dupuy & Sonenshein, 1998; Ikeda *et al.*, 1998; Karlsson *et al.*, 1999). Studies have shown that subinhibitory levels of antibiotics have an effect on the production of toxin: Onderdonk *et al.* (1979) found that subinhibitory concentrations of vancomycin and penicillin increased toxin production by *C. difficile* and Honda *et al.* (1983) found that clindamycin and cephaloridine increased the production of toxin A. Other antibiotics may also affect this process. The aim of this study was to investigate and clarify the effect of antibiotics on growth and production of toxin A by *C. difficile*. Six different antibiotics – including agents that either precipitate CDAD or are used for its treatment – and three different strains of *C. difficile* were investigated.

METHODS

Strains of *C. difficile*. Three strains of *C. difficile* were used in this study: NCTC 11223, strain 630 (fully sequenced) and strain 338a (a local endemic strain that was collected during a recent epidemiology study).

Strain 338a is of S-type 5236 (ribotype 1) and was present in 78 % of cases of *C. difficile* diarrhoea in a geriatric unit in the Royal Victoria Hospital, Edinburgh (McCoubrey *et al.*, 2003). Strains were grown from spores stored in cooked meat broth [anaerobic investigation medium (AIM) with cooked meat particles (Brown *et al.*, 1996)]. A loopful (approx. 30 µl) was added to 3 ml pre-reduced AIM and incubated anaerobically overnight (80 % H_2, 10 % N_2, 10 % CO_2 at 37°C) to yield approximately 10^8 cells ml^{-1}. Appropriate purity checks were carried out on starter cultures before use.

Antibiotics. Antibiotics chosen for the study were vancomycin (V2002; Sigma) and metronidazole (M1547; Sigma), two agents that are used for the treatment of CDAD, and four agents that are associated with precipitation of the disease: amoxycillin (A8523; Sigma); clindamycin (C5269; Sigma); cefoxitin (C4786; Sigma) and ceftriaxone (C5793; Sigma). Second-generation cephalosporins (including cefoxitin) are less commonly associated with *C. difficile* disease, although they still possess good anti-anaerobe activity. Cefoxitin, present in the selective medium at 8 mg l^{-1}, was chosen for comparison with the third-generation cephalosporin ceftriaxone. MICs of these strains to the six antibiotics were determined by broth macrodilution (National Committee for Clinical Laboratory Standards, 1997) and are shown in Table 1. Concentrations of antibiotics used in this study corresponded to 1/2, 1/4 and 1/8 of the MIC, except in the case of clindamycin with strain NCTC 11223. This strain was highly resistant and 512 µg ml^{-1}, the highest concentration achievable in the study, allowed growth of this strain. Antibiotics were prepared in sterile distilled water as 100× solutions with reference to the highest concentration required. Doubling dilutions were made in sterile distilled water and 1 vol. antibiotic was added to 100 vols broth.

Growth curves and toxin production. Preliminary growth curves were determined to relate optical density (OD_{600}) to viable counts and toxin A production (by semi-quantitative analysis –see below) over a period of 104 h. To ensure that three sub-MIC concentrations of antibiotics were used for each strain, four additional concentrations of antibiotic were used, two above and two below the predicted MIC, to allow for any minor differences in MIC. Where necessary, the extra determinations were discarded retrospectively. An antibiotic-free control was used for each strain for comparison. An inoculum of 10^6 cells ml^{-1} was used and growth (OD_{600}) was measured three times a day at 0, 4 and 8 h for 5 days. A 1 ml sample was removed for OD measurement. After measuring the OD, the sample was transferred to a 3 ml Eppendorf tube, centrifuged for 2 min at 13 000 ***g*** and the supernate was transferred to a fresh Eppendorf tube and stored at −20°C for toxin analysis.

Toxin A ELISA. Toxin A levels were assayed by ELISA with a ToxA kit (Techlab) according to the manufacturer's instructions. Prior to assay, 100 µl of each sample was diluted in 100 µl of the buffer provided; 100 µl of this dilution was used in the assay. Plates were read at a wavelength of 450 nm, with automatic subtraction of the 620 nm value. The maximum OD value of the assay was 3·0, up to which OD was linear with respect to control toxin concentration. No further dilutions of supernates were made. Results (OD values) were plotted against time to evaluate when toxin was being elaborated and to show differences between antibiotic-free and -containing cultures.

Table 1. MICs of the six antibiotics

Abbreviations: Van, vancomycin; Met, metronidazole; Amox, amoxycillin; Clind, clindamycin; Cefo, cefoxitin; Ceft, ceftriaxone.

Strain	MIC (mg l^{-1})					
	Van	Met	Amox	Clind	Cefo	Ceft
NCTC 11223	2	1	8	> 512	256	64
338a	1	1	4	4	256	64
630	2	1	4	512	256	128

RESULTS AND DISCUSSION

Growth curves and toxin production

In preliminary experiments, both viable counts and OD_{600} values were measured and these confirmed that over the time-period of the experiment, the values mirrored one another. Subsequently, bacterial growth was assessed by OD_{600} only. Fig. 1 shows growth curves and toxin levels for untreated controls of each strain. Values represent means (with standard errors) of six replicates grown on six different occasions; there was little variation in growth between strains. Each strain was clearly in the exponential phase by 8 h and in the stationary phase by 24 h. Decline was then

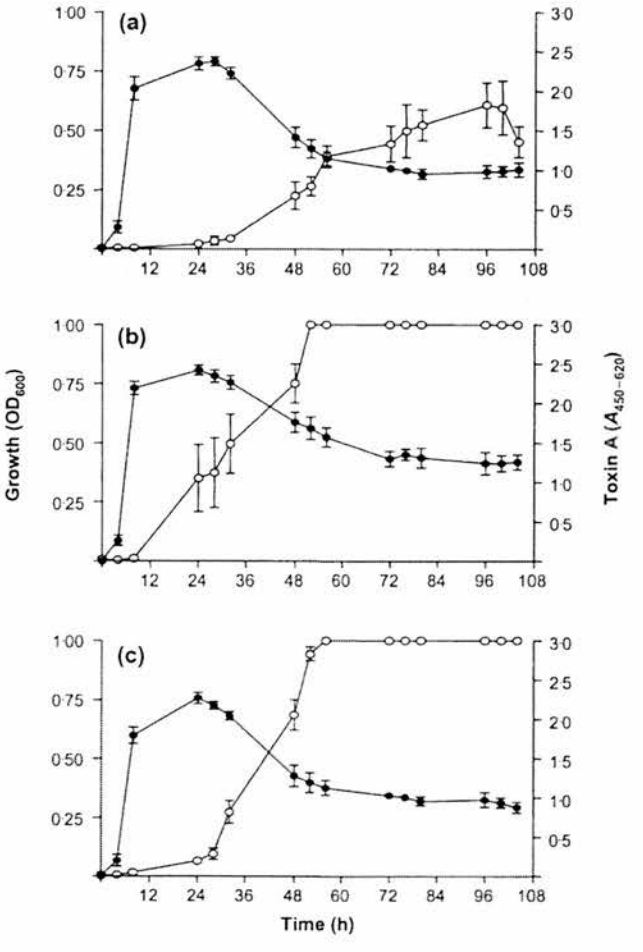

Fig. 1. Mean growth and toxin production of the strains over 104 hours: (a) strain NCTC 11223; (b) strain 338a; (c) strain 630. ●, Growth; ○, toxin A production. Mean and SE were calculated from six different experiments. A reading of 3·0 is the maximum value for the toxin assay.

apparent from 32 h, with the OD stabilizing by approximately 56 h. However, toxin production differed between strains, in both amount and timing of production relative to growth. Strain NCTC 11223 produced less toxin A than strains 338a and 630; toxin A production by strain NCTC 11223 rarely exceeded the measurable level of the assay (OD_{450}, 3·0), whereas higher values (>3·0) were obtained with strains 338a and 630. A notable difference was the point in the growth phase at which each strain produced toxin: strain 338a produced toxin during the stationary phase (by 24 h), which preceded toxin production by both strains NCTC 11223 and 630 (in late stationary phase). This further correlated with the time required to produce comparable levels of toxin A, i.e. 24, 32 and 48 h for strains 338a, 630 and NCTC 11223, respectively. Levels of toxin A in strains 338a and 630 generally reached maximum readable levels by approximately 52 h.

Effects of antibiotics on growth and toxin A production

The effects of sub-MIC levels of antibiotics on bacterial growth and toxin production varied with both antibiotic and strain. Examples of the effects of antibiotics on the kinetics of growth and toxin production are shown in Fig. 2(a–d). These were selected to show the range of variation in phenotype following sub-MIC antibiotic treatment. Effects on toxin production for all strains and antibiotics are summarized in Table 2. Subinhibitory concentrations of antibiotics tended to delay the growth of the bacteria by increasing the lag period, especially at the highest concentration of antibiotic (1/2 MIC) (strain NCTC 11223 in the presence of clindamycin was an exception to this and is described later). For toxin production, three general consequences were evident: toxin level was increased (+), toxin was produced earlier (E) or toxin level was unaffected or reduced (−).

It should be noted that strain NCTC 11223 is highly resistant to clindamycin (MIC >512 μg ml^{-1}) and for this strain only, the sub-MICs are not truly 1/2, 1/4 and 1/8, but are fractions of 512 μg ml^{-1}. When these sub-MIC levels of clindamycin were added, growth of *C. difficile* was not affected (Fig. 2a), but toxin production was affected noticeably. Compared with the antibiotic-free control, toxin was elaborated sooner and reached higher levels than in the absence of clindamycin. Thus, this antibiotic potentiated toxin production by both acceleration and enhancement of production.

Strain 630 grown with amoxycillin is shown in Fig. 2(b). Sub-MICs resulted in an increased lag period and for 1/2 MIC,

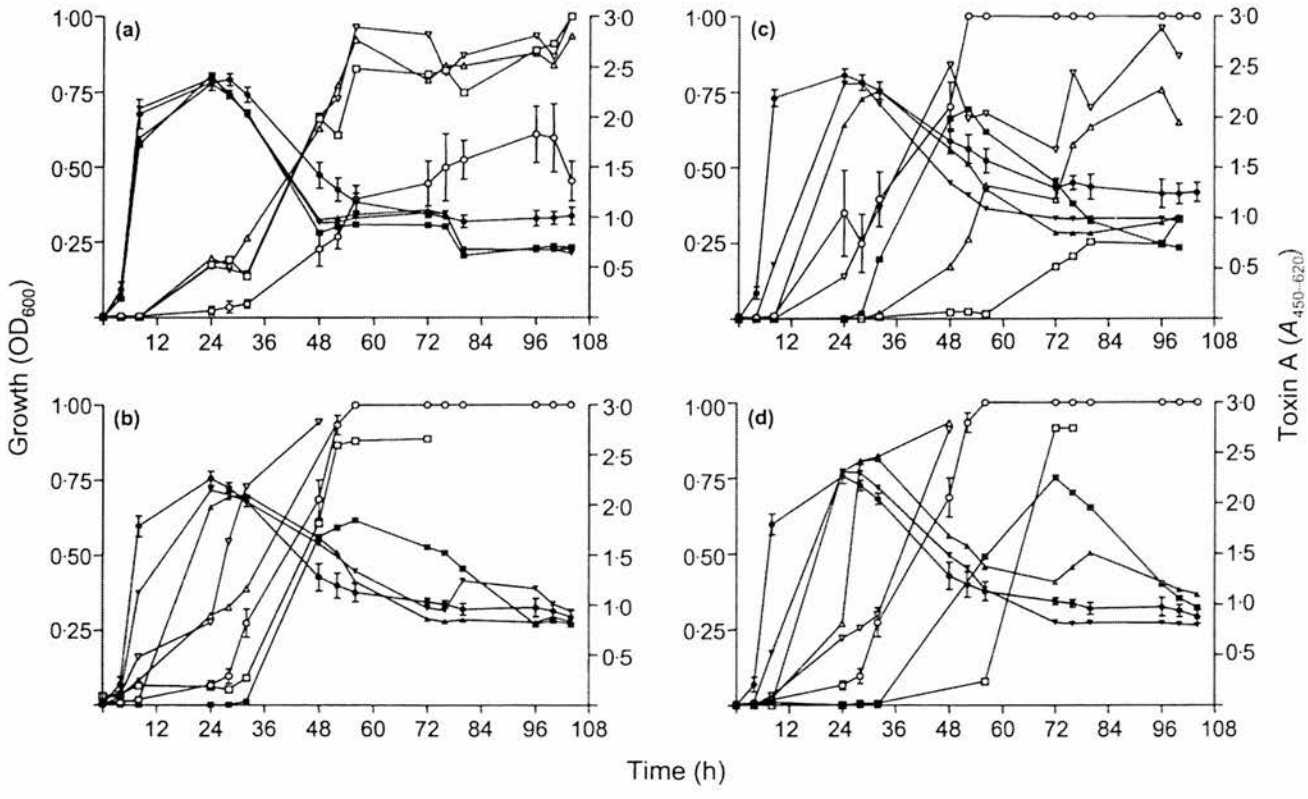

Fig. 2. Growth and toxin production by: (a) strain NCTC 11223 with clindamycin; (b) strain 630 with amoxycillin; (c) strain 338a with cefoxitin; and (d) strain 630 with metronidazole. Controls from Fig. 1 are also shown on these graphs. Closed symbols, growth; open symbols, toxin A. Circles, controls; squares, 1/2 MIC; triangles, 1/4 MIC; inverted triangles, 1/8 MIC.

Table 2. Summary of effects on toxin production

1/2, 1/4 and 1/8 are the concentrations that correspond to 1/2, 1/4 and 1/8 of the MIC. Abbreviations: Van, vancomycin; Met, metronidazole; Amox, amoxycillin; Clind, clindamycin; Cefo, cefoxitin; Ceft, ceftriaxone; –, no effect; E, elaborated sooner; +, toxin level increased.

Strain	Effect on toxin production					
	Van	Met	Amox	Clind	Cefo	Ceft
NCTC 11223:						
1/2	–	E	–	+, E	–	E
1/4	–	E	E	+, E	–	–
1/8	+, E	E	E	+, E	–	–
338a:						
1/2	–		E	E	–	E
1/4	–	E	E	E	–	E
1/8	–	E	–	E	–	–
630:						
1/2	E		E	–	–	–
1/4	–	E	E	E	–	E
1/8	–	E	E	E	–	–

toxin was produced as soon as growth was measurable, rather than later in the growth cycle. When compared to the control, toxin produced by this strain with 1/2 MIC was elaborated at the same time as by the strain without amoxycillin. This effect was less dramatic with the other two concentrations of amoxycillin. Even with a lag in growth, toxin was produced before the control for 1/4 and 1/8 MICs of amoxycillin.

Fig. 2(c) shows the results for strain 338a cultured with cefoxitin; antibiotic appears to inhibit toxin production. The growth rate in 1/2 and 1/4 MICs was lower than that of the control. At 1/2 MIC, there was a lag of approximately 24 h compared with the control, with toxin levels reaching maximum after 48–72 h.

A final example of the effect of sub-MIC amounts of antibiotic is shown in Fig. 2(d), which depicts strain 630 with metronidazole. This was similar to those for strain 338a with amoxycillin (results not shown), where toxin appeared to be elaborated before the control, even though growth was delayed.

For *C. difficile*, there is a clear relationship between disease and antibiotic usage, such that antibiotics are often a prerequisite for the disease. Broad-spectrum agents have been shown to particularly predispose to *C. difficile* infection, through depletion of the patient's normal protective bowel microbiota. The equilibrium of the gut, when disturbed, leaves the patient open to opportunistic infection, possibly via the newfound availability of binding sites and nutrients. Thus, suppression of colonization resistance by antibiotics facilitates colonization and promotes disease. Hence, *C. difficile* is the most common cause of nosocomial antibiotic-associated diarrhoea.

It has been proposed that antibiotics may promote CDAD not solely by modulating commensal micro-organisms, but also by physiological effects that influence pathogenicity (Lorian & Gemmell, 1994). Several early reports (Onderdonk *et al.*, 1979; Honda *et al.*, 1983) suggested that certain antibiotics potentiated the production of toxins A and/or B, the main recognized virulence factors of *C. difficile*. Furthermore, antibiotics have been shown to affect the expression of virulence factors in other species, including *Escherichia coli*, *Vibrio cholerae* and various staphylococci (Levner *et al.*, 1977; Lorian, 1971; Yoh *et al.*, 1983). Determining the effect of antibiotics on virulence factor expression in an organism for which antibiotics are important triggers of disease is therefore crucial. Our work has focused on the effect of subinhibitory concentrations of six antibiotics, including those that precipitate disease and those used for treatment, on the growth and production of toxin A by three strains of *C. difficile*.

This study has shown clearly that there is heterogeneity between strains with respect to growth, MICs and toxin levels that are produced. A common effect on the bacterial strains in the presence of antibiotics was the slowing of growth in comparison with the controls; they took longer than the controls to reach stationary phase, either at all three sub-MIC concentrations, or just at the higher concentrations, of antibiotics. In addition to slower growth, the bacteria sometimes failed to achieve the OD that the controls reached. This was seen in many cases with strains cultured with cefoxitin. Even with subinhibitory concentrations, it would still be expected that antibiotics would have an effect on bacterial systems, including growth. Strain NCTC 11223 is highly clindamycin-resistant and the growth of this strain was not at all affected in the presence of this antibiotic. An explanation for this may be that this strain is so well-adapted to this agent that it can function and grow as normal. This strain contains the macrolide, lincosamide and streptogramin B resistance determinant (MLS) that contains the *ermB* gene (encoding an RNA methyltransferase), which makes it resistant to these antibiotics (L. J. Drummond, unpublished data). Strain 630 also carries the *ermB* gene (Farrow *et al.*, 2001), but it has a slightly lower MIC of 512 μg ml^{-1} and its growth is affected by all three sub-MIC concentrations of clindamycin. The reasons for this are uncertain, but heterogeneity between strains was common during this work.

As can be seen in Table 2 and Fig. 2(a, b and d), sub-MIC concentrations of antibiotics can cause quicker elaboration of toxin A when compared with the antibiotic-free control. Antibiotics, even at sub-MIC concentrations, can be expected to cause stress to the bacteria. Bacteria under stress 'switch on' a catalogue of genes, by which the toxin promoters may be affected. To support this, TcdD – the putative positive regulator of toxin genes – shows similarity to UviA, the UV-inducible regulator from *Clostridium botulinum* (Mani & Dupuy, 2001). Onderdonk *et al.* (1979) showed that the stress of increased temperature led to greater cytotoxin production. In the same paper, the authors demonstrated an increase in toxin in the presence of

subinhibitory concentrations of vancomycin and penicillin. Karlsson *et al.* (2003) also showed temperature to be a controlling factor for toxin and TcdD expression. It has been shown by Hennequin *et al.* (2001) that *C. difficile* cultured in the presence of antibiotics produces greater levels of GroEL, a chaperone from the heat-shock protein 60 (Hsp60) family. These examples all serve to suggest that toxin promoters can respond to multiple environmental stresses. Inducing this stress response may enable *C. difficile* to survive the gut environment, as GroEL functions as a 58 kDa surface adhesin that may help *C. difficile* to colonize recently vacated binding sites left by the depletion of normal gut flora.

Reference strain NCTC 11223 produces lower levels of toxin than the fully sequenced strain (630) and the 'endemic' strain (338a). During the course of the experiments, samples of strain NCTC 11223 rarely exceeded the limits of the ELISA plate reader (>3), whereas the other two strains commonly reached levels >3 after approximately 48 h growth. It was desirable to look at the trends of toxin production; this was achievable by comparing the OD values of the antibiotic-free control and the strain in the presence of antibiotics. Differences in toxin production are not well understood, although Spigaglia & Manstrantonio (2002) demonstrated strains with variants of TcdC, the putative negative regulator of toxin production. No correlation between disease severity and variant TcdC strains was found, although it is possible that changes in this protein would affect toxin production. For example, they found one allele with a nonsense mutation that reduced the TcdC protein from 232 to 61 aa. Lack of functional protein may lead to abrogated repression of the toxin genes. This may be an explanation for the differences that are common between strains of *C. difficile*. PCR-based analysis would show whether the strains used differed in their *tcdC* alleles.

In addition to disruption of the barrier flora in *C. difficile* disease, antibiotics also appear to increase the stress response in the bacteria. For example, upregulation of the adhesin GroEL may increase the virulence of the infecting *C. difficile* by aiding its utilization of the new niche. The reason for producing toxins in the gut is unclear but, as they are upregulated during glucose starvation, their purpose may be to cause cell disruption for the acquisition of nutrients (Dupuy & Sonenshein, 1998).

The results presented here show that there is no consistent relationship between antibiotics and growth or toxin production by *C. difficile*. Antibiotics that are considered to be important for precipitation of the disease appear to have different effects on different strains; this is also true for antibiotics used for treatment, so the relationship appears to be much more complicated than thought previously. The effects of subinhibitory levels of precipitating agents on colonic flora may well allow the overgrowth of *C. difficile* and may also have a significant effect on the rate and/or level of toxins produced once colonization occurs. The impact of this in the patient is unclear, but production of higher levels of toxin earlier in the disease could prove to be detrimental. Perhaps the most important aspect of this study is the clear demonstration of variation in the response of strains to the same antibiotic. This may have important implications for the virulence potential of different strains.

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