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The Effect of the Proton Pump Inhibitor Pantoprazole on the Biology of *Campylobacter jejuni*

by

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Abstract

Campylobacter is a major cause of acute bacterial gastroenteritis worldwide, with the highest number of infections being attributed to *Campylobacter jejuni*. *C. jejuni* is a Gram negative, spiral, motile bacterium that belongs to the campylobacterales order and is related to both *Helicobacter* spp. and *Wolinella* sp.. It has long been established that proton pump inhibitors (PPIs) and other benzimidazole derivatives display anti-*Helicobacter* activity *in vitro*. PPIs have in the past been shown to affect *Helicobacter pylori* growth, survival, motility, morphology, adhesion/invasion potential and susceptibility to conventional antibiotics.

PPIs are highly effective drugs that are well tolerated, safe for prolonged daily use and are therefore in high demand. Both the PPIs omeprazole and lansoprazole featured in the top ten drugs prescribed in England in 2014. In 2014 *Campylobacter* was also the most commonly diagnosed gastrointestinal infection in Scotland, in England and Wales and also in Europe. It has previously been generally accepted that patients who are being treated with PPIs are more susceptible to enteric infections such as *Campylobacter* than people not taking PPIs. The effect of PPI exposure on *H. pylori* has been investigated rigorously in the past. A single previous study has hinted that PPIs may also be capable of affecting the related organism *C. jejuni*, but investigations have been extremely limited in comparison to those investigating the effect of PPIs on *H. pylori*. This study has investigated the *in vitro* effects of direct contact with PPIs on the biology of *C. jejuni*.

Exposure to the PPI pantoprazole was found to affect *C. jejuni* growth/survival, motility, morphology, biofilm formation, invasion potential and susceptibility to some conventional antibiotics. Microarray studies showed that the *cmeA* and *Cj0561c* genes were significantly up-regulated in response to pantoprazole exposure and a CmeABC deficient mutant was found to be significantly more susceptible to killing by pantoprazole than was the parent strain. Proteomic analysis indicated that the oxidative stress response of *C. jejuni* was induced following exposure to sub-lethal concentrations of pantoprazole. *C. jejuni* gene expression was assessed using qRT-PCR and the genes encoding for thiol peroxidase and GroEL co-chaperonin (both involved in the *C. jejuni* oxidative stress response) were found to be around four times higher in response to exposure to sub-lethal concentrations of pantoprazole. Experiments using the oxidative stress inhibitors thiourea (a hydroxyl radical quencher) and bipyridyl (a ferrous iron chelator) showed that killing by pantoprazole was not mediated by hydroxyl radical production.

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Author's Declaration

I, Kareen Macleod, confirm that the work presented in this thesis is my own. Information, which has been derived from other sources, has been indicated as such throughout. I, unless otherwise stated, performed all experimental work at the University of Glasgow.

List of Abbreviations

A	adenine
A. baumannii	*Acinetobacter baumannii*
ADP	adenosine diphosphate
ATP	adenosine triphosphate
BNF	British National Formulary
B. subtilis	*Bacillus subtilis*
C	cytosine
Caco-2	colon cancer derived epithelial cells
cAMP	cyclic adenosine monophosphate
CapA	*Campylobacter* adhesion protein A – an autotransporter lipoprotein
C. coli	*Campylobacter coli*
C. difficile	*Clostridium difficile*
cDNA	complementary DNA
CDT	cytolethal distending toxin
CFU	colony forming units
C. hyointestinalis	*Campylobacter hyointestinalis*
C. jejuni	*Campylobacter jejuni*
C. lari	*Campylobacter lari*
Cl^-	chloride ion
Cme	*Campylobacter* multidrug efflux
CmeABC	three subunit multidrug efflux pump of *C. jejuni*
CmeA	subunit A of the Cme pump – a periplasmic membrane fusion protein
CmeB	subunit B of the Cme pump – an inner membrane efflux transporter
CmeC	subunit C of the Cme pump – an outer membrane channel forming protein
CmeR	repressor of the Cme pump
CO_2	carbon dioxide
CT	cycle threshold
C. upsaliensis	*Campylobacter upsaliensis*
°C	degrees Celsius
DMEM	Dulbecco's modified eagle media with GlutaMAX™
DMSO	Dimethyl sulfoxide
DNA	deoxyribonucleic acid
dsDNA	double stranded deoxyribonucleic acid
ECL	entero-chromaffine-like
E. cloacae	*Enterobacter cloacae*
E. coli	*Escherichia coli*
E. histolytica	*Entamoeba histolytica*
FCS	foetal calf serum
g	gram
× g	relative centrifugal force
G	guanine
GBS	Guillain-Barré syndrome
G. duodenalis	*Giardia duodenalis*
GI	gastrointestinal
G. intestinalis	*Giardia intestinalis*
G. lamblia	*Giardia lamblia*
GORD	gastro-oesophageal reflux disease
H_2	hydrogen

H^+	hydrogen ion or proton
HCl	hydrochloric acid
HCO_3^-	bicarbonate ion
H^+/K^+-ATPase	proton pump enzyme that utilises ATP to move protons in exchange for potassium
HLA	human leukocyte antigen
H_2O	water
H_2O_2	hydrogen peroxide
H. pylori	*Helicobacter pylori*
IBS	irritable bowel syndrome
K^+	potassium ion
K. aerogenes	*Klebsiella aerogenes*
kan^R	kanamycin resistance cassette
KatA	catalase enzyme of *C. jejuni*
kb	kilobase
KCl	potassium chloride
KdpA	transmembrane subunit of the KDP ATPase
KdpB	catalytic subunit of the KDP ATPase
KdpC	inner membrane subunit of the KDP ATPase
KdpD	inner membrane bound protein that controls expression of the KDP ATPase
KdpE	soluble cytoplasmic protein that controls expression of the KDP ATPase
K. pneumoniae	*Klebsiella pneumoniae*
l	litre
LB	lysogeny broth
L. donovani	*Leishmania donovani*
log	logarithmic
LOS	lipo-oligosaccharide
LSHTM	London School of Hygiene and Tropical Medicine
MALDI	matrix-assisted laser desorption/ionisation
MBC	minimum bactericidal concentration
MEM	minimal essential media
MFS	Miller Fisher syndrome
mg	milligram
MHA	Mueller-Hinton agar
MHA + B	Mueller-Hinton agar supplemented with 7% horse blood
MHA + B + kan	Mueller-Hinton agar supplemented with 7% horse blood and kanamycin at 50 µg/ml
MHB	Mueller-Hinton broth
MIC	minimum inhibitory concentration
µg	microgram
µl	microlitre
ml	millilitre
mM	milimolar
M. morganii	*Morganella morganii*
MS	mass spectrometry
N_2	nitrogen
Na^+	sodium ion
NaCl	sodium chloride
O_2	oxygen
O_2^-	superoxide
OD	optical density

OD_{595}	optical density at 595 nanometres
OD_{600}	optical density at 600 nanometres
$OH^{\bullet}$	hydroxyl radical
OH^{-}	hydroxyl ion
PABA	4-aminobenzoic acid
P. aeruginosa	*Pseudomonas aeruginosa*
PBS	phosphate buffered saline
PCR	polymerase chain reaction
P_i	phosphate produced when ATP breaks down to ADP
P. mirabilis	*Proteus mirabilis*
pmol	picomole
PPI	proton pump inhibitor
ppm	parts per million
pTet	a *Campylobacter* plasmid which encodes for the *tetO* gene and confers tetracycline resistance
P. vulgaris	*Proteus vulgaris*
rRNA	ribosomal ribonucleic acid
RNA	ribonucleic acid
RND	resistance-nodulation-cell division
ROS	reactive oxygen species
rpm	revolutions per minute
S. aureus	*Staphylococcus aureus*
S. boydii	*Shigella boydii*
SD	standard deviation
SDS	sodium doecyl sulfate
S. enterica	*Salmonella enterica*
S. marcescens	*Serratia marcescens*
S. mutans	*Streptococcus mutans*
SOD	superoxide dismutase enzyme
SodB	superoxide dismutase enzyme of *C. jejuni*
S. pyogenes	*Streptococcus pyogenes*
SSA	semi-solid agar
STA	soft top agar
S. Typhimurium	*Salmonella enterica*, subsp *enterica*, serovar Typhimurium
T	thymine
TIF	Tagged Image File
TSB	tryptic soy broth
TOF	time of flight
T. spiralis	*Trichinella spiralis*
T. vaginalis	*Trichomonas vaginalis*
UK	United Kingdom
V	volts
vhrs	volt hours
VAIN	variable atmosphere incubator
VBNC	viable but non culturable
V. cholerae	*Vibrio cholerae*
V. parahaemolyticus	*Vibrio parahaemolyticus*
W	watts
2D	two dimensional
3D	three dimensional

Chapter 1

General Introduction

1 INTRODUCTION

1.1 History of *Campylobacter* Discovery

The first reported discovery of a pathogenic bacterium, which likely belonged in the genus currently known as *Campylobacter*, was in 1886. A German bacteriologist named Theodor Escherich observed spiral shaped organisms in the colon and faeces of children that had died of enteric infection, which he was notably unable to culture (Kist, 1986). Because he was unable to isolate the bacteria, the significance of the organism in patients with enteric infections was overlooked for many years.

In 1913, John McFadyean and Stewart Stockman reported that they had observed peculiarly shaped organisms in the uterine mucus of sheep and cattle that had suffered abortions (Skirrow, 2006). They classified them as vibrios because of their shape, but they had more than likely actually isolated *Campylobacter fetus* (*C. fetus*). In 1919, Theobald Smith and Marian Taylor isolated spiral shaped bacteria, similar to those observed by McFadyean and Stockman, from aborted bovine foetuses and the species name *Vibrio fetus* was proposed to describe these organisms (Smith & Taylor, 1919). The species name *jejuni* was first introduced by Jones *et al* in 1931 when they isolated a bacterium they called *Vibrio jejuni* (originally found in the jejunum), from calves with dysentery (Jones *et al.*, 1931) and the species name *coli* was first introduced by Doyle in 1944 to describe organisms isolated from pigs.

These difficult to isolate and culture organisms were considered pathogens of mainly veterinary importance for many years. In 1946, AJ Levy reported that *Vibrio jejuni* was the likely causative agent in a milk-borne enteritis outbreak that had occurred in Illinois in 1938 (Levy, 1946). This signalled the initiation of interest in these organisms in relation to human disease. In the 1950s, Elizabeth King noted that organisms isolated from the blood cultures of patients with diarrhoeal disease could be separated into two groups: those that grew best at 37°C and those that were thermophilic and grew best at 42°C. She correctly hypothesised that the thermophilic organisms could be the cause of the diarrhoeal illness and that they might occur more commonly than their isolation from blood might suggest (King, 1957).

In 1963, Sebald and Veron reported notable differences in the growth conditions of the organisms previously classified in the *Vibrio* genus. Differences in the deoxyribonucleic acid (DNA) guanine and cytosine content were also observed and the new genus *Campylobacter* (from the Greek campylo; meaning curved) was proposed for a number of

the organisms (Sebald & Veron, 1963) including *Vibrio fetus*. However, the complex growth requirements of the fastidious *Campylobacter* organisms were not fully understood and isolation and growth of the bacteria remained a challenge.

In 1972, Dekeyser *et al* made their own growth media using thioglycolate-agar base with 15% defibrinated ovine blood and the antibiotics novobiocin, bacitracin and polymixin B. They combined centrifugation and filtration techniques and were the first to report successful isolation of "related vibrios" from the stools of patients with enteritis (Dekeyser *et al.*, 1972). In 1977, a less "burdensome to laboratory staff" method of isolating campylobacters was developed by Skirrow (Skirrow, 1977). A selective media that contained trimethoprim, vancomycin and polymixin B finally enabled widespread isolation of the organisms under laboratory conditions using "only a vacuum jar and an incubator set at about 43°C". The importance, in human gastrointestinal (GI) disease, of organisms within the *Campylobacter* genus soon became apparent. Advances in culturing techniques and rising awareness have meant that *Campylobacter* is now recognised as the leading cause of human bacterial gastroenteritis in the world.

1.2 Campylobacter Genus and Campylobacter jejuni

The genus *Campylobacter* belongs to the epsilon class of *Proteobacteria* in the order campylobacterales. The related genera *Helicobacter* and *Wolinella* are also included in the campylobacterales order. The *Campylobacter* genus currently consists of seventeen species and six subspecies, of which the most frequently reported in human disease are *Campylobacter jejuni* subspecies *jejuni* (*C. jejuni*) and *Campylobacter coli* (*C. coli*) (Cody *et al.*, 2013). Other species such as *Campylobacter lari* (*C. lari*) and *Campylobacter upsaliensis* (*C. upsaliensis*) have also been, although much less frequently, isolated from patients with diarrhoeal disease (Kaneko *et al.*, 1999, Couturier *et al.*, 2012) and species such as *C. fetus* and *Campylobacter hyointestinalis (C. hyointestinalis*) remain mainly of veterinary importance. *C. jejuni* causes around 90% of human infections, *C. coli* around 8% and other species account for only around 2% of all human *Campylobacter* infections (Cody *et al.*, 2013). *C. jejuni* is therefore the most significant species with regard to human illness and is therefore the species used for the majority of experimental work detailed within this thesis.

C. jejuni is a small (0.2-0.8 µm wide and 0.5-5.0 µm long) spiral-shaped, Gram negative bacterium. It has a single unsheathed flagellum at one or both ends of the cell and exhibits a characteristic rapid darting or spinning motility. It is microaerophilic and cannot

normally be grown in the laboratory under ambient gaseous atmospheric conditions; it grows best at around 5-10% oxygen (O_2).

C. jejuni has a comparatively small genome (around 1.6-1.8 megabases) which is rich in adenine and thymine (Fouts *et al.*, 2005). Having such a small genome may explain some of *C. jejuni's* phenotypic properties e.g. their inability to metabolise carbohydrates or to degrade complex substances and their need for complex growth media (Dasti *et al.*, 2010). *C. jejuni* has only a single superoxide dismutase (SOD) enzyme (SodB) and a single catalase enzyme (KatA) encoded for in its genome (Stead & Park, 2000). The aerobic Gram negative enteric pathogen *Escherichia coli* (*E. coli*), in contrast, has three SODs and two catalase enzymes (Hwang *et al.*, 2012). *C. jejuni* is therefore extremely sensitive to the action of free radicals and superoxide, more so than some other aerobic enteric pathogens that are better equipped to manage oxidative stress. Some strains of *C. jejuni* can grow in atmospheric oxygen if blood or pyruvate has been added to growth media, as these are able to scavenge oxygen. Charcoal can also be added to agar used for the isolation of *C. jejuni* as it prevents the accumulation of reactive oxygen species (ROS) (John *et al.*, 2011).

1.3 Campylobacteriosis

Human disease is often food or waterborne and occurs via the oral route, where ingested *Campylobacter* must survive the acid environment of the stomach and the activity of proteolytic enzymes in order to reach the intestines. The surface of the intestinal tract is covered in a thick layer of mucus and *Campylobacter* must colonise the mucus layer in order to establish themselves in the colon and distal ileum. Black *et al* demonstrated that an infectious dose of only 500-800 organisms was sufficient to cause human disease (Black *et al.*, 1988).

In Scotland in 2014 there were 6,636 cases of campylobacteriosis diagnosed (Health-Protection-Scotland., 2015), which corresponds to an incidence of 124.6 cases per 100,000 population (Browning *et al.*, 2015). In comparison, there were only 717 cases of salmonellosis reported in Scotland for the same time period (Health-Protection-Scotland, 2015), with an incidence of only 13.5 cases per 100,000 population (Browning *et al.*, 2015).

Campylobacter was also by far the most common cause of GI illness for the same time period in England and Wales, causing more diagnosed infections than for *Salmonella*, *Shigella*, *E. coli* O157, *Norovirus*, *Rotavirus*, *Giardia* and *Cryptosporidium* combined (see **Table 1**). *Campylobacter* is therefore the most common cause of acute bacterial gastroenteritis in the United Kingdom (UK) and is of great clinical importance.

Table 1. The most commonly diagnosed GI infections in England and Wales in 2013 and 2014.

Laboratory Reports for England and Wales		
GI Pathogen	**Cumulative Totals 2013**	**Cumulative Totals 2014**
Campylobacter	58,742	58,722
Salmonella	7,255	6,672
Norovirus	6,922	5,734
Rotavirus	14,943	4,315
Giardia	3,584	3,779
Cryptosporidium	3,481	3,587
Shigella sonnei	986	1,088
***E. coli* O157**	770	891

Laboratory diagnosed infections listed in descending order for 2014. Compiled using data from (Public-Health-England, 2015).

C. jejuni has been the most commonly reported bacterial GI pathogen of humans in the European Union since 2005. In 2014 the number of confirmed human campylobacteriosis cases reported was 236,851 with an incidence of 71 per 100,000 population, an increase of 9.6% compared with the rate in 2013 (EFSA, 2015). Salmonellosis was the second most commonly reported infection in the European Union with a total of 88,715 confirmed cases. In the United States, *Campylobacter* is the second most common cause of food-borne bacterial gastroenteritis (with *Salmonella* being the commonest) and in 2013 the incidence of campylobacteriosis was 13.73 cases per 100,000 population and for salmonellosis was 15.15 cases per 100,000 population (CDC, 2013). In 2013 *Campylobacter* resulted in fewer hospitalisations than *Salmonella* (1,028 versus 2,029) and resulted in fewer deaths than *Salmonella* (11 versus 30) in the United States.

1.3.1 Symptoms and Management

The incubation period can be long and quite variable (between 1 and 7 days) but is often between 1 and 3 days. Symptoms can range from mild watery diarrhoea to severe bloody diarrhoea with fever and leukocytes in the stools (Yabe *et al.*, 2010). Patients may also experience malaise, fatigue, abdominal cramps, headaches or dizziness. Vomiting is rare

but prolonged infections may result in weight-loss. *Campylobacter* causes an inflammatory type diarrhoea that can lead to GI tissue damage but infections are usually self-limiting. Symptoms often resolve after around 7 days and patients often do not require any treatment other than rehydration and replacement of electrolytes.

1.3.2 Antibiotic Treatment of *Campylobacter* Infection

Antibiotic treatment is not normally required, especially in the immunocompetent. Immunocompromised individuals, those with persistent disease, patients with severe bloody diarrhoea, paediatric cases and infections in the elderly may however benefit from antibiotic treatment in order to shorten the duration of symptoms. Serious and systemic infections such as meningitis, endocarditis and bacteraemia can occur and also require antibiotic treatment, but they are rare and occur mostly in immunocompromised hosts. As a result campylobacteriosis has a relatively low mortality rate (Yabe *et al.*, 2010). If required, campylobacteriosis can be treated with fluoroquinolones (e.g. ciprofloxacin) or macrolides (e.g. erythromycin). Tetracycline may be considered as an alternative, although resistance rates can be high (for further information on the antibiotic resistance mechanisms of *Campylobacter* see **Section 5.1.2**). In severe cases or in the case of systemic infection, intravenous treatment with an aminoglycoside (e.g. gentamicin) may be considered (Quinn *et al.*, 2007).

1.4 Complications Associated with *Campylobacter jejuni* Infection

Occasionally, in the weeks or months following *C. jejuni* infection, "complications" can occur in some patients. The severity, seriousness and duration of which, can be extremely variable. These post-infectious complications can be easily divided into two groups: intestinal and extra-intestinal sequelae.

1.4.1 Intestinal Sequelae

Irritable bowel syndrome (IBS) is increasingly being implicated as a post-infectious complication of *C. jejuni* infection (Smith & Bayles, 2007, Spiller, 2007, Zilbauer *et al.*, 2008). Patients with IBS suffer from abdominal pain, bloating and altered bowel habits. Although IBS is not life-threatening, it is life changing and can in some cases be life-long.

C. jejuni has also been linked to inflammatory bowel diseases like Crohn's disease (Weber *et al.*, 1992, Berberian *et al.*, 1994). Crohn's patients have inflamed lining of the digestive tract that can cause abdominal pain, diarrhoea, weight-loss and fatigue. Crohn's disease can again be a long-term condition, although patients may undergo periods of remission when they have mild or absent symptoms, followed by debilitating flare ups.

Campylobacteriosis is known to cause acute exacerbation of Crohn's disease symptoms and can lead to these flare ups. Although the intestinal sequelae linked with preceding *C. jejuni* infection are long-term complications, they are not life-threatening conditions. However, some of the extra-intestinal sequelae linked with preceding *C. jejuni* infection can be life-threatening.

1.4.2 Extra-intestinal Sequelae

Extra-intestinal complications that may result following *C. jejuni* infection are often, like campylobacteriosis, self-limiting. However, they can occasionally result in serious long-term deficits in patients or be life-threatening (Wassenaar & Blaser, 1999). Extra-intestinal complications that can occur following *C. jejuni* infection include Guillain-Barré syndrome (GBS), which is the most commonly reported, the related Miller Fisher syndrome (MFS) and reactive arthritis (Reuter *et al.*, 2010).

1.4.2.1 Guillain-Barré Syndrome

GBS, which can occur around 7-21 days following *C. jejuni* infection, is an acute neurological disease caused by the demyelination of peripheral nerves. This nerve demyelination leads to a rapidly progressing ascending weakness of the limbs, with feet and legs usually being the first to display acute flaccid paralysis. If the paralysis ascends and reaches the respiratory muscles then mechanical ventilation may be required. The worldwide annual incidence of GBS is 0.6-4 cases per 100,000 population (Nyati & Nyati, 2013) and preceding *C. jejuni* infection is associated with around 20-40% of GBS cases (Nyati & Nyati, 2013). GBS is normally self-limiting but can occasionally lead to a long-term neurological deficit in some patients (due to irreversible nerve damage) and can be life-threatening if respiratory muscles are affected.

GBS is the result of an auto-immune response which can develop when antibodies to the lipo-oligosaccharide (LOS) present on certain serotypes of *C. jejuni* attack gangliosides found on human nerve tissue. Sialylated LOS structures of *C. jejuni* in particular are close mimics of human peripheral nerve gangliosides (Louwen *et al.*, 2012) and the sialyltransferase of *C. jejuni*, cst-II is involved in the synthesis of the sialylated LOS structures that induce the production of the cross-reacting antibodies. A cst-II knockout mutant which lacks the sialyltransferase and cannot sialylate LOS has been shown to be unable to induce the production of anti-ganglioside antibodies (Heikema *et al.*, 2013).

1.4.2.2 Miller Fisher Syndrome

MFS is an uncommon variant of GBS, which can also be caused by molecular mimicry, by certain strains of *C. jejuni*. MFS is characterised by a descending paralysis, which is the opposite of that seen in GBS. MFS often begins with paralysis of the eye muscles (ophthalmoplegia), progressing to facial asymmetry, slurred speech, general weakness and loss of motor co-ordination (Lo, 2007). In some cases the paralysis can descend to the respiratory muscles, which occurs most often in children. MFS can then be life-threatening and mechanical ventilation may be required. The annual incidence of MFS is low, at around 0.09 per 100,000 population (Lo, 2007). The symptoms of MFS are again normally self-limiting, although plasmapheresis (whereby cross-reacting antibodies can be removed from the bloodstream) may shorten the duration of both GBS and MFS symptoms.

1.4.2.3 Reactive Arthritis

Reactive arthritis is a condition where painful joints can also be accompanied by conjunctivitis, urethritis, fatigue, fever, weight-loss and dermatology symptoms. The arthritis occurs in the absence of antinuclear antibody or rheumatoid factor, affects multiple joints (knees and ankles being the most commonly affected) and is notably non-symmetrical. Reactive arthritis usually develops within 4 weeks of initial *C. jejuni* infection and symptoms persist for around 3-12 months, before spontaneously resolving (Wu & Schwartz, 2008). Reactive arthritis is more likely to occur in adults than in children and is more common in males than in females (Mortensen *et al.*, 2009).

1.5 Epidemiology

C. jejuni is the most common species of *Campylobacter* found in poultry, *C. coli* is the predominant species found in pigs, *C. upsaliensis* is commonly found in domestic pets and *C. lari* is the predominant species found in wild birds (particularly seagulls) and is also found in shellfish and crustaceans (Fouts *et al.*, 2005). Transmission of *C. jejuni* to humans is often the result of contact with, or consumption of, contaminated foodstuffs such as raw or undercooked chicken or unpasteurised dairy products. *C. jejuni* can also be transmitted to humans via contaminated water. Pork, veal and ham are significant sources of *C. coli* and *C. upsaliensis* can cause infections in humans that have been in contact with domestic pets, such as puppies or kittens, with diarrhoea. Person-to-person spread of *Campylobacter* spp. is rare, even though large numbers of viable organisms are often shed in the faeces of infected patients and the infectious dose is low (Everest, 2002). A notable exception to this is *C. upsaliensis*, which is rarely found in foodstuffs, but is transmittable

to people by pets and can also be transmitted via person-to-person spread (Fouts *et al.*, 2005).

The self-limiting nature of campylobacteriosis contributes to the predicted true incidence of disease being much higher than reported (O'Brien *et al.*, 2010) as many of those suffering from food-borne campylobacteriosis do not seek medical assistance. Yet *Campylobacter* enteritis is one of the most common forms of acute bacterial enteritis in the developed world. It affects people of all ages, but is most common in children less than 5 years old and in 15-24 year old adults (Zilbauer *et al.*, 2008). In developing countries campylobacters are hyper-endemic in children under 2 years old (where they are also associated with significant mortality) and asymptomatic infections are common in adults (Nyati & Nyati, 2013). *Campylobacter* enteritis is rarely seen in adults from areas where campylobacters are hyper-endemic, as immunity is usually acquired early in life due to frequent exposure (Konkel *et al.*, 1996). The high rate of asymptomatic infections seen in developing countries raised the question of whether *Campylobacter* strains isolated in developing countries should be considered pathogenic (Black *et al.*, 1988). However, many cases of campylobacteriosis are linked to foreign travel and the consumption of contaminated food or water in the areas visited. As such *Campylobacter* is a significant cause of travellers' diarrhoea and strains from developing areas are indeed pathogenic.

1.6 Virulence Factors of *Campylobacter jejuni*

C. jejuni is a very successful human pathogen and yet lacks many of the well-known virulence factors found in other successful human pathogens (Elmi *et al.*, 2012). Known virulence factors of *C. jejuni* include the production of cytolethal distending toxin (CDT), the presence of a polysaccharide capsule, the ability to invade cells and motility due to functioning flagella (Hendrixson *et al.*, 2001, Karlyshev *et al.*, 2002, Guerry, 2007). These will be discussed in further detail in the sections below.

1.6.1 Cytolethal Distending Toxin

CDT is the only known, fully defined exotoxin produced by *C. jejuni* and it is an antigenic protein toxin (Parkhill *et al.*, 2000). CDT was first characterised in *E. coli* but is known to be produced by strains of *C. jejuni* as well as other enteric pathogens like *Salmonella enterica* and *Shigella* (Johnson & Lior, 1988, Dasti *et al.*, 2010). CDT was first identified in *C. jejuni* in 1987 and it is now known that *C. jejuni* makes more CDT than *C. coli* (Castillo *et al.*, 2011). CDT is made up of three subunits which are all membrane associated, CdtA, CdtB and CdtC. CdtB is known to be the active component of the toxin

and has some similarity to DNase I-like proteins. It is thought to act like a DNase by damaging DNA in the nucleus where it localises. The specific functions of CdtA and CdtC are less well defined but they may play a role in host cell binding and delivery of the catalytic subunit CdtB into host cells (Young *et al.*, 2007). CDT interrupts the cell cycle and induces cell death (Ismaeel *et al.*, 2005) because it causes elongation and swelling of cells. CDT added to various cell lines *in vitro* induces apoptosis of the cells (Young *et al.*, 2007). CDT also causes the production of interleukin 8 from intestinal epithelial cells, which recruits dendritic cells, neutrophils and macrophages to the site of *C. jejuni* infection and induces inflammation (Dasti *et al.*, 2010). Strains lacking CDT are however also capable of causing disease and there therefore exists a CDT-independent mechanism for eliciting interleukin 8 production and inflammatory diarrhoea (Elmi *et al.*, 2012).

1.6.2 Capsule

C. jejuni has a number of hyper-variable regions (sometimes referred to as phase-variable) within its small genome which gain their variability as a result of slipped-strand mispairing. Certain areas of the *Campylobacter* genome contain strands of single nucleotide repeats (or homopolymeric tracts). These are prone to slipped-strand mispairing, which alters the length of the homopolymeric tract and can then influence the expression of downstream genes. These homopolymeric tracts are often found in areas of the genome upstream of genes linked to flagella, capsule and LOS production and so the expression of flagella, capsule and LOS genes can be affected (Dasti *et al.*, 2010). This method of altering surface structures such as capsule contributes to antigenic variation in *Campylobacter*, which can be useful e.g. for evading host immune responses.

The capsule of *C. jejuni* is thought to be a virulence factor and protects *C. jejuni* from environmental stress. The capsule is made up of polysaccharides and it interacts with the extracellular environment of *C. jejuni*. *C. jejuni* without a capsule have been shown to be less invasive, less virulent in ferrets, less able to colonise chickens and more sensitive to complement-mediated killing than *C. jejuni* with a capsule (Corcionivoschi *et al.*, 2012). Capsular polysaccharide was suggested in one study to be important for *C. jejuni* survival of osmotic stress (Cameron *et al.*, 2012). The presence or absence of a capsule can also affect the autoagglutination (see **Section 1.6.3**) ability of different *Campylobacter* strains (Guerry, 2007).

1.6.3 Flagella

C. jejuni has a single polar flagella at one or both ends of the cell, which is unsheathed, glycosylated and is also immunogenic (Wassenaar & Blaser, 1999). The flagella of

C. jejuni are used to penetrate the mucous layer of the gut and are also required for the adhesion to and invasion of epithelial cells (Guerry, 2007). Flagella are also used by *C. jejuni* to secrete non-flagellar proteins, which may play a role in invasion and autoagglutination (Almofti *et al.*, 2011). The flagella themselves are also important for autoagglutination and biofilm formation, which are both important for the survival of *C. jejuni* in the environment (Kalmokoff *et al.*, 2006). Flagella play an important role in the pathogenicity of *C. jejuni* and are essential for colonisation in animal models and for the colonisation of humans (Tsutsui *et al.*, 2000, Friis *et al.*, 2005, Mills *et al.*, 2012).

1.6.3.1 Role of Flagella in Invasion and Adhesion

It has long been acknowledged that entry into host cells provides a means for pathogenic bacteria to evade the host's immune system and gain access to a niche where the pathogen does not have to compete with other resident bacterial flora. The human GI tract is lined with a continuously secreted layer of mucus which acts as a physical barrier to infection and contains a mixture of glycoproteins that are responsible for its viscosity (Wisessombat *et al.*, 2010). Colonic biopsies from patients with stool cultures positive for *C. jejuni* and suffering from colitis were shown to contain bacteria associated with the mucous layer as well as within the intestinal epithelial cells, suggesting that adherence and invasion of host epithelial cells is a hallmark of *Campylobacter* infection (van Spreeuwel *et al.*, 1985).

The *in vivo* findings of van Spreeuwel *et al* were later followed by the discovery that various *C. jejuni* isolates adhered to and invaded a variety of epithelial cell lines *in vitro* (Fauchere *et al.*, 1986, Konkel & Joens, 1989, Everest, 2002). *Campylobacter* adhesion protein A (CapA) is an autotransporter lipoprotein of *C. jejuni* and insertional mutagenesis of *capA* has been shown to significantly reduce the adhesion to and invasion of Caco-2 cells and an inability to colonise or persist in chickens (Ashgar *et al.*, 2007).

1.6.3.2 Role of Flagella in Biofilm Formation

Quorum sensing is important in autoagglutination but autoagglutination is mediated by the glycans which are found on the flagella of *C. jejuni*. Biofilm formation requires flagella expression (Guerry, 2007). Autoagglutination is often one of the first steps leading to microcolony formation and the start of biofilm formation. Autoagglutination is affected by three major surface carbohydrates, the LOS core, the capsule and flagella. The genes for these three carbohydrate structures are found in the hyper-variable regions of the *C. jejuni* genome (see **Section 1.6.2**) and this may explain why autoagglutination varies markedly between different strains. Strains which readily autoagglutinate will associate in higher numbers to eukaryotic cells (Guerry, 2007). Multiple non-motile mutant *Campylobacter*

strains have also been found to be deficient in both pellicle and aggregate type biofilm formation (Joshua *et al.*, 2006).

1.6.3.3 Role of Flagella in Translocation/Transcytosis

The ability of selected pathogens to migrate across an intact cell barrier by invading cells can be an important virulence factor, as it allows access to underlying tissues as well as possible dissemination throughout the host. *C. jejuni* can also translocate across the epithelial cell barrier via an alternative mechanism and can migrate from the intestinal mucosa to a variety of extra-intestinal sites, resulting in complications such as meningitis, endocarditis and bacteraemia. This mechanism of translocation involves the bacteria moving down in between the host cells rather than being internalised by them.

It has been reported that *C. jejuni* motility, as well as contributing to adherence and invasion ability also contributes to the translocation ability of different strains and that *de novo* protein synthesis is also required (Bras & Ketley, 1999). Strains of *C. jejuni* which have a mutation in *flaA*, have a truncated flagella and the motility of these organisms is severely affected (Everest, 2002). FlaA mutants of *C. jejuni* have been shown to be unable to cross epithelial cell monolayers and so FlaA and related motility must be required for translocation (Grant *et al.*, 1993). It has also been shown that strains of *C. jejuni* which express sialylated ganglioside-like LOS (see **Section 1.4.2.1**) translocate through epithelial cells with greater efficiency than strains lacking ganglioside-like LOS (Louwen *et al.*, 2012).

1.7 Susceptibility to *Campylobacter*

The human host has a number of defences against potential colonisation by enteric pathogens, these include: stomach acid released by parietal cells, resident bacterial flora, the action of gut peristalsis, presence of an intact epithelial barrier, local gut immunity and the secretion of mucus (Bavishi & DuPont, 2011). Patients who have disturbed their resident bacterial flora (e.g. by taking antibiotics) can be more susceptible to enteric infections like campylobacteriosis, as can people with diabetes, people that have been in contact with pets or farm animals, people that have recently travelled internationally and those that have consumed unpasteurised dairy products like milk (Tam *et al.*, 2009). Numerous strains of pathogenic bacteria secrete virulence factors which damage cells and disrupt the intact epithelial barrier of the gut, thereby increasing their survival *in vivo* (Elmi *et al.*, 2012).

1.7.1 Proton Pump Inhibitors as a Risk Factor for Enteric Infection

It is generally accepted that patients being treated with PPIs are more susceptible to enteric infections such as *Campylobacter* than patients not taking PPIs (Lodato *et al.*, 2010, Bavishi & DuPont, 2011). The stomach contents of patients taking PPIs will be less acidic than those not taking PPIs. This is thought to result in increased survival of ingested bacteria that might not otherwise have survived the acidity of the stomach. The infectious dose of enteric pathogens may therefore be less for people taking PPIs. The taking of proton pump inhibitors (PPIs) is known to result in hypochlorhydria, which allows increased bacterial translocation across the epithelial cell barrier (Bavishi & DuPont, 2011). It has been proposed that severe diarrhoea in cases of campylobacteriosis could be a result of higher numbers of bacteria being endocytosed and translocating in between intestinal epithelial cells (Louwen *et al.*, 2012). The use of PPIs has also been associated with increased susceptibility to colonisation by *Clostridium difficile* (*C. difficile*) (Strachan *et al.*, 2013). Singh *et al* commented on the possibility that PPI use as a risk factor for *C. difficile* infection might be due to PPIs affecting the ability of normal resident bacterial flora to form protective biofilm in the GI tract, hence making it easier for *C. difficile* to colonise (Singh *et al.*, 2012).

1.8 Stomach Acid

1.8.1 Acid Production

H^+/K^+-ATPases (or proton pumps) can be found at rest within tubovesicles inside the parietal cells of the stomach. When a parietal cell is stimulated, e.g. by histamine, gastrin or acetylcholine, the proton pumps migrate to the apical surface of the parietal cell and fuse with the plasma membrane. This causes the intracellular membrane structure known as the canaliculus to undergo massive expansion, forming long microvilli and vastly increasing the secretory surface area of the parietal cell (Sachs *et al.*, 1995).

Hydrogen ions (H^+ or protons) and hydroxyl ions (OH^-) are generated within parietal cells from the dissociation of water molecules (see **Figure 1**). Carbon dioxide (CO_2) diffuses into parietal cells from the bloodstream and reacts rapidly with these hydroxyl ions, via a carbonic anhydrase enzyme, to produce bicarbonate ions (HCO_3^-). These bicarbonate ions are removed from the parietal cell via an anion exchanger, in exchange for incoming chloride ions (Cl^-). Chloride ions move quickly through the parietal cell to the area near the apical surface called the canaliculus. The hydrogen ions left over from the water dissociation are pumped into the canaliculus, via the H^+/K^+-ATPase, as potassium ions

move inside the cell (Shin & Sachs, 2008). Hydrochloric acid (HCl) is formed within the many canaliculi of parietal cells and then released into the stomach (**Figure 1**).

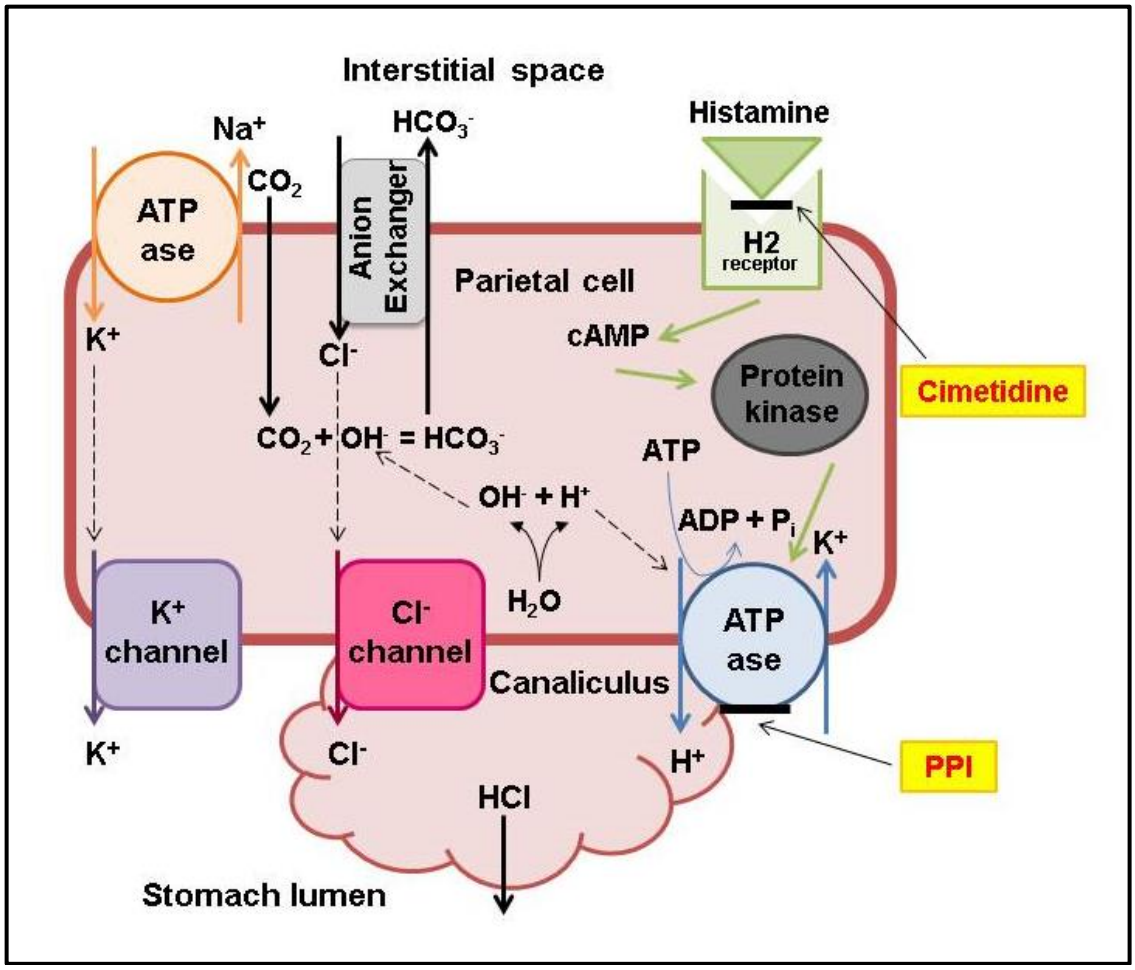


Figure 1. How hydrochloric acid is made and released from parietal cells. *Histamine release activates the H^+/K^+-ATPase via cyclic AMP (cAMP) to allow protons (H^+) into the canaliculus, where they react with chloride ions (Cl^-) to produce hydrochloric acid (HCl). HCl is then released from the canaliculus into the milieu of the stomach. Activation of the H^+/K^+-ATPase can be blocked by the H2-receptor antagonist cimetidine. Proton pump inhibitors (PPIs) can bind to and inhibit the activity of the H^+/K^+-ATPase, thereby also blocking the release of HCl, but at a later stage in the process.*

1.8.2 Excess Acid Production

The acidic environment of the stomach is effective in preventing bacterial infections via the oral route, as many pathogenic bacteria do not survive exposure to the low pH in the stomach. Stomach acid is also important for the digestion of foodstuffs, particularly protein and for the absorption of iron and calcium. Excess production of stomach acid can however lead to the development of gastro-oesophageal reflux disease (GORD) or peptic/duodenal ulcers. GORD occurs when the lower oesophageal sphincter muscle allows acid to leak out of the stomach and up into the oesophagus. Patients often experience difficulty or pain when swallowing and feel a burning pain in their chest after

eating. As recently as 50 years ago severe cases of excess acid production were sometimes life-threatening (Olbe *et al.*, 2003). Antacids could be given to temporarily neutralise stomach acid and offer symptom relief, but antacids were unable to block the continued production of acid. Treatment options were therefore limited and surgical removal of stomach nerves or partial stomach resection were common interventions (Olbe *et al.*, 2003).

Cimetidine was introduced in the 1970s and it was the first intervention capable of halting the production of stomach acid. Cimetidine is a H2-receptor antagonist which blocks the release of histamine. Histamine release is a trigger (by activating the H^+/K^+-ATPase) for stomach acid release from parietal cells and as such, cimetidine blocks stomach acid production via an indirect route (see **Figure 1**).

1.9 Development of Proton Pump Inhibitors

1.9.1 Benzimidazole Derivatives Inhibit Proton Pumps

Throughout the 1960s a pharmaceutical company, then known as Astra, was searching for prospective treatments for excess acid production and in the 1970s they discovered the first compounds that inhibited the proton pumps of parietal cells (Olbe *et al.*, 2003). The compounds they found were derivatives of benzimidazole, which is a bi-cyclic compound that results from the fusion of benzene and imidazole (**Figure 2**).

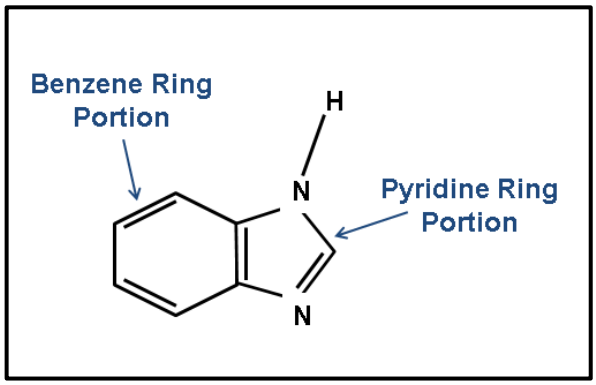


Figure 2. Core benzimidazole structure. *Benzene fuses with imidazole to form the core bi-cyclic structure found in all benzimidazole derivatives.*

In 1973, Astra discovered that a non-toxic benzimidazole called H124/26 was able to block the secretion of hydrochloric acid, by inhibiting the H^+/K^+-ATPase of parietal cells. Soon after, it was discovered that a sulphoxide metabolite of H124/26, called timoprazole (**Figure 3a**), was an even more potent inhibitor of the H^+/K^+-ATPase and of subsequent acid secretion.

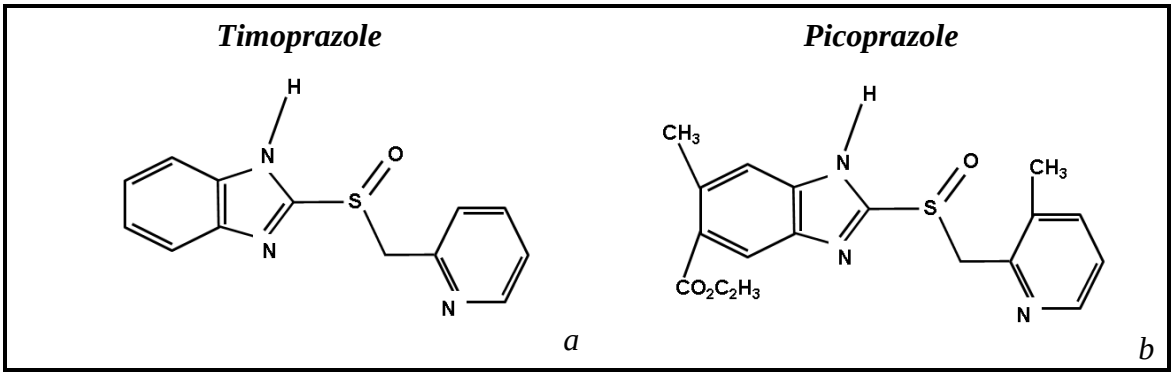


Figure 3. The chemical structures of timoprazole and picoprazole. *H124/26 was the first non-toxic benzimidazole found that inhibited proton pumps, but its sulphoxide metabolite timoprazole (a) was found to be a much more potent inhibitor of proton pumps. Timoprazole was however found to be toxic and picoprazole (b) was the chemically modified, non-toxic structure, which retained most inhibitory activity.*

However it was later found that timoprazole inhibited iodine uptake in the thyroid gland and caused thymus atrophy in animals and so it was unsuitable for clinical use. The core structure of timoprazole, including the benzimidazole portion was retained, with different substitutions and side chain configurations being added and the resulting structures tested for H^+/K^+-ATPase inhibitory activity. The most potent benzimidazole found, that did not inhibit iodine uptake, was called picoprazole (**Figure 3b**).

1.9.2 Importance of the H^+/K^+-ATPase

Around the same time it was becoming clear that the final step required for stomach acid production was the activation of the H^+/K^+-ATPase in parietal cells. This active transport mechanism is required to move protons into the canalicular region of the parietal cell, where they then combine with chloride ions to produce the hydrochloric acid that is subsequently released into the stomach (**Figure 1**). Without the H^+/K^+-ATPase activity, hydrochloric acid cannot be produced by parietal cells. The H^+/K^+-ATPase of parietal cells was therefore an excellent prospective target for new anti-secretory therapies.

There are also H^+/K^+-ATPases found in the kidneys, which the benzimidazole derivatives being developed by Astra could potentially also inhibit. However, it was known that the canaliculi of parietal cells was the only area of the body that reaches a pH of ≤ 1.0 and this property was exploited during the drug design stages (Sachs *et al.*, 1995). Different substituents were systematically added to the pyridine ring (see **Figure 2**) of the benzimidazoles in an attempt to increase the pKa of the drugs and maximise their ability, as weak bases, to accumulate in the acidic canalicular compartments of parietal cells. There, they would be in close proximity to the proton pump they were being specifically

designed to inhibit. Forming benzimidazole derivative structures with a low pKa would maximise their accumulation in parietal cells over the kidneys and reduce the likelihood of the wrong proton pumps being targeted. In 1979, a chemical molecule, which would later become known as omeprazole (**Figure 4a**) was discovered and the first human clinical trials testing it, began in 1982 (Olbe *et al.*, 2003).

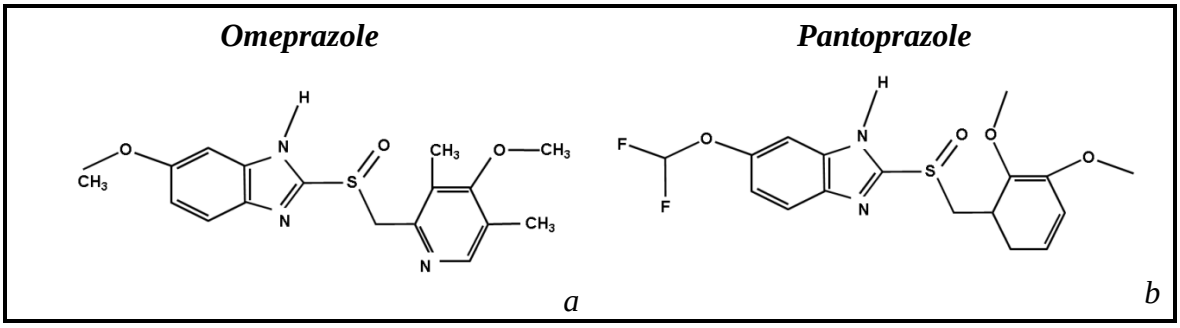


Figure 4. The chemical structures of omeprazole and pantoprazole. *Omeprazole (a) was the first commercially available PPI which has a core structure very similar to those of timoprazole and picoprazole. Pantoprazole (b) is also a benzimidazole derivative and is the PPI used in this study.*

1.9.3 Omeprazole Trials

Whilst the human trials were underway, animal studies were also being performed. In 1984, a long-term toxicology study using extremely high doses in rats resulted in endocrine tumour formation and all omeprazole human clinical trials were halted. On further investigation it was found that the tumours were developing in entero-chromaffine-like (ECL) cells which are specific to rats and there was no similar risk to humans (Olbe *et al.*, 2003). Human trials resumed and it was concluded that omeprazole was safe for human use and that omeprazole had a much longer duration of activity and was therefore superior to cimetidine. Because omeprazole was so efficacious, it was requested by some physicians for patients with severe disease on the grounds of "compassionate-use", even before it became commercially available (Klinkenberg-Knol *et al.*, 2000). Omeprazole was finally launched in Europe in 1988 and in the USA in 1990; over 15 years after Astra discovered the first benzimidazole derivative capable of inhibiting proton pumps.

1.9.4 Extended Proton Pump Inhibitor Family

1.9.4.1 Omeprazole

Although omeprazole was extremely potent and safe for use, it was known that making chemical substitutions to the core benzimidazole structure could produce other structures that were able to inhibit the proton pumps of parietal cells and that the properties of such structures might be subtly different from those of omeprazole. Omeprazole is almost

entirely eliminated through hepatic clearance (Ching *et al.*, 1991) and there was therefore potential for interactions with other drugs that were metabolised by the same liver enzymes. In 1987 the search for a PPI with increased bioavailability and therefore less liver clearance, was renewed by Astra. This heralded the creation of a family of chemically related benzimidazole derivatives known as PPIs with lansoprazole being launched in 1991, pantoprazole (see **Figure 4b**) in 1994, rabeprazole in 1999 and esomeprazole in 2000.

1.9.4.2 Lansoprazole

Lansoprazole in its inactive pro-drug form (see **Section 1.10**) was first known as AG-1749 and some of the most potent activated forms, capable of binding to and inhibiting proton pumps were known as AG-1789 and AG-2000 (Nagata *et al.*, 1995). Lansoprazole has a pKa around 4.0, which is similar to that of omeprazole and it therefore also preferentially accumulates in parietal cells rather than in the kidneys. It undergoes acid activation at a similar rate to omeprazole but has the added advantage of being available as an oro-dispersible tablet (Joint-Formulary-Committee., 2015). Lansoprazole is however not very water soluble (Nguyen *et al.*, 2005).

1.9.4.3 Pantoprazole

Pantoprazole has a similar potency to the first commercially available PPI omeprazole, but it interacts less with cytochrome P-450 (therefore has less potential for harmful interactions with other drugs metabolised by the same enzymes) than omeprazole and has a better pH dependent activation profile (Beil *et al.*, 1992). At a pH of 2.0 the half-life of pantoprazole is comparable with that of omeprazole at around 9 and 5 minutes respectively. The PPIs have been specifically designed to act on the H^+/K^+-ATPase of parietal cells where the pH is low and therefore both PPIs have short half-lives at this low pH, as they are quick to become acid activated when they preferentially accumulate in parietal cells. However the half-life of omeprazole at a pH of 5.0, where activation would be unwanted and potentially lead to targeting of the kidney cell H^+/K^+-ATPase rather than the parietal cell H^+/K^+-ATPase, is around 55 minutes. Pantoprazole was designed to be more stable at high pH and the half-life of pantoprazole at pH 5.0 was improved to > 90 minutes.

1.9.4.4 Rabeprazole

Rabeprazole was originally known as E-3810 and is the least stable PPI at neutral pH; it converts to its active form more quickly than the other PPIs at high pH (Besancon *et al.*, 1997). Rabeprazole also differs from the other members of the PPI family, in that it has a pKa of around five, whereas the others have a pKa around four (Horn, 2000). Rabeprazole

was however the first PPI to be recommended for "on demand" therapy regimes (Joint-Formulary-Committee., 2015).

1.9.4.5 Esomeprazole

Esomeprazole is in fact the S-isomer of the original PPI launched by Astra, omeprazole. Omeprazole is now known to be a racemic composition of its two optical isomers, S-omeprazole (later known as esomeprazole) and R-omeprazole. The S-isomer was found to inhibit gastric acid secretion to a greater degree than that of both the R-isomer and the original racemic mixture omeprazole (Andersson *et al.*, 2001). The S-isomer is also metabolised to a lesser degree and at a lower rate than the R-isomer and so has greater bioavailability and reaches higher peak plasma concentrations. The S-isomer is also better tolerated by patients with impaired liver-function than other PPIs. For these reasons, the omeprazole S-isomer alone was launched as esomeprazole by AstraZeneca, over a decade after the racemic mixture omeprazole was first launched.

1.10 Chemistry of Proton Pump Inhibitor Activity

PPIs are administered as tri-cyclic (see **Figure 4**) inactive pro-drugs (Andersson *et al.*, 2001) that are weak bases and have pKas (apart from rabeprazole) of around four (Besancon *et al.*, 1997). As such, they preferentially move inside the parietal cells of the stomach and accumulate in the canaliculus because the environment there is highly acidic as a result of the H^+/K^+-ATPase activity and the accumulation of protons. The first step required for the activation of all PPIs is protonation (acid activation) and this occurs quickly in the acidic environment of the canaliculus where protons are freely available. The free nitrogen of the pyridine ring (see **Figures 2**, **3** and **4**) becomes protonated (Shin *et al.*, 1993) and a sulfenic acid is formed. The sulfenic acid can undergo dehydration and the chemical structure rearranges quickly to form a tetra-cyclic sulfenamide (Olbe *et al.*, 2003).

Both the sulfenic acid form and the tetra-cyclic sulfenamide are active forms of PPIs and as both of these forms are cationic, they are therefore both also fairly membrane impermeable. Hence the sites of action available to activated PPIs are limited (Shin *et al.*, 2004). The activated forms of PPIs form strong disulphide bonds with thiol groups on exposed cysteine residues of the H^+/K^+-ATPase of parietal cells (Shin *et al.*, 1993). This binding renders the proton pump inactive and the production of hydrochloric acid is blocked because protons are unable to move to the canaliculus and react with chloride ions to make hydrochloric acid (Beil *et al.*, 1992).

The active PPI forms (the sulfenic acids and the tetra-cyclic sulfenamides) are highly reactive and are capable of self-reacting (Shin *et al.*, 2004). The stability of the activated forms is dependent on the pH and time. Hence, if for example the pH changes and an active form remains unbound to a H^+/K^+-ATPase then it can be broken down into inactive sulphides or multiple other products (Besancon *et al.*, 1997). Benzimidazole derivatives, like the pro-drug forms of PPIs, are usually colourless but many of the activated benzimidazole intermediates, like sulfenic acids and sulfenamides are yellow in colour (Nguyen *et al.*, 2005). The compounds that may be generated by the break down of unbound activated PPI forms are more than can be accurately quantitated or identified (Shin *et al.*, 2004).

The binding and subsequent inactivation of the H^+/K^+-ATPase by sulfenic acids or tetra-cyclic sulfenamides is irreversible and the production of hydrochloric acid only resumes when new proton pumps are synthesised by the parietal cells and these move to the plasma membrane (Ali *et al.*, 2009). The acid activated tetra-cyclic sulphenamide form is however known to be non-selective and is capable of binding to and inactivating the adenylate cyclase or Na^+/K^+-ATPase (see **Figure 1**) of parietal cells also (Beil *et al.*, 1992).

1.11 Proton Pump Inhibitor Uses and Dosages

1.11.1 Proton Pump Inhibitor Use

PPIs are frequently taken medications and are prescribed to treat common conditions such as GORD or peptic/duodenal ulcers. They are also often prescribed prophylactically to prevent the development of ulcers in patients being treated with non-steroidal anti-inflammatory drugs and in combination with antibiotics for the eradication of *Helicobacter pylori* (*H. pylori*). Diseases such as Zollinger-Ellisson syndrome and Barrett's oesophagus are much rarer conditions and these require a much higher daily dose of PPI. In Barrett's oesophagus the columnar epithelial cells in the lower oesophagus have become severely damaged, usually as a result of the long standing reflux of stomach acid and if left untreated, the cells can become cancerous. Patients with Zollinger-Ellison Syndrome have, sometimes multiple, gastrin-secreting tumours (or gastrinomas) in the duodenum or pancreas which leads to hyperstimulation of the parietal cell H^+/K^+-ATPase and sustained hydrochloric acid release.

1.11.2 The British National Formulary

The British National Formulary (BNF) is used by prescribers, pharmacists and healthcare professionals for guidance on uses and the recommended doses of medicines available by

prescription in the UK (Shen *et al.*, 2011). **Table 2** lists the daily dose of various PPIs recommended for the treatment of a variety of conditions according to the BNF. Typical daily dosages range from 20-80 mg per day, usually taken as a single oral dose, but in complicated cases, PPIs may require to be taken before every meal (Shen *et al.*, 2011). In a report by Klinkenberg-Knol *et al* one exceptional patient required a daily dose of 120 mg omeprazole to manage their symptoms (Klinkenberg-Knol *et al.*, 2000).

Table 2. Daily dosages of PPIs as recommended in the British National Formulary (Joint-Formulary-Committee., 2015).

PPI	Daily Dose (mg/ml) for Different Conditions		
	BNF Indication		
	Benign Ulcer	Severe Ulcer	Zollinger-Ellison Syndrome
Omeprazole	20	40	20-120*****
Lansoprazole	30	30	60-160******
Pantoprazole	40	80	80-160*****
Rabeprazole	20	20	60-120*******
Esomeprazole	20	40	80-160*****

PPIs are listed in the table in the order in which they were licenced for use in the UK and the severity of symptoms increases towards the right-hand side of the table. *******Doses of omeprazole, pantoprazole or esomeprazole over 80 mg per day to be divided into two doses.* ********Doses of lansoprazole over 120 mg per day to be divided into two doses.* *********Doses of rabeprazole over 100 mg per day to be divided into two doses.*

GORD and uncomplicated (or benign) ulcers are usually treated with the lowest recommended dose of PPIs. Complicated (or severe) ulcers include ulcers which are actively bleeding and in such cases the recommended dose of PPIs often increases. Zollinger-Ellison syndrome is the most severe condition that PPIs may be prescribed to treat and the recommended PPI dose in such cases is often over 100 mg per day (**Table 2**).

The maximum single dose that is recommended in the BNF for omeprazole, lansoprazole, rabeprazole or esomeprazole is 80, 120, 100 and 80 mg respectively. Pantoprazole is the PPI used for the *in vitro* testing in this study (due to its superior ability to dissolve in water) and the maximum single dose of pantoprazole that is recommended is 80 mg.

1.11.3 People Taking Proton Pump Inhibitors

PPIs are very effective drugs, with few serious side effects and are available in the UK both by prescription and over the counter. According to data published in April 2015 both

the PPIs omeprazole and lansoprazole featured in the top ten drugs prescribed in England in 2014 (Health and Social Care Information Centre). Omeprazole was the third most commonly prescribed drug with 28.8 million items being prescribed and lansoprazole the tenth most commonly prescribed with 21.6 million items being prescribed in England in 2014. It is clear that in the UK there are a great number of people taking PPIs and the numbers are increasing (see **Figure 5**). The numbers will likely be even higher than these data suggest, because data is unavailable for PPIs bought over the counter and taken without a prescription. It has been noted that PPIs are amongst the most widely prescribed family of drugs worldwide and that more and more people throughout the world are taking PPIs (Ali *et al.*, 2009, Bavishi & DuPont, 2011).

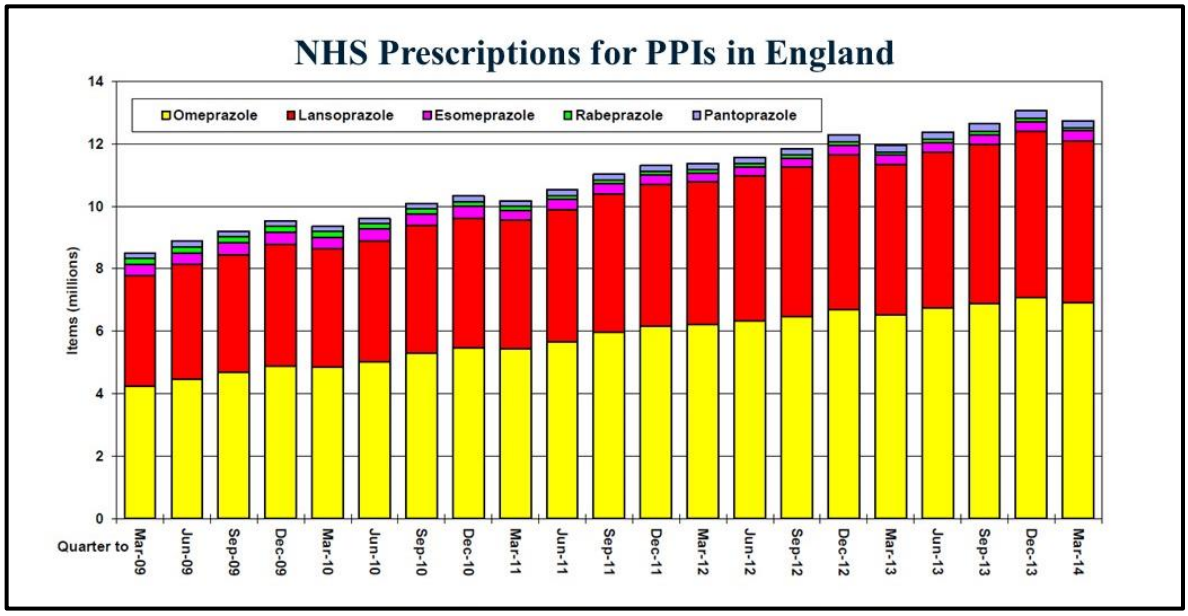


Figure 5. Quarterly data for PPI prescriptions in England. *Available from http://www.nhsbsa.nhs.uk/PrescriptionServices/Documents/PPDPrescribingAnalysisCharts/Gastro_National_June_2014.pdf.*

1.11.4 Proton Pump Inhibitors in the Gastrointestinal Tract

The PPI concentration which is clinically achievable in the mucous layer of the human gut is unknown (Megraud *et al.*, 1991). It has also been stated that the PPI concentration that can be found in the GI tract or stomach is unknown (Mirshahi *et al.*, 1998, Trautmann *et al.*, 1999). In what is believed to be one of the only reports of its kind Caselli *et al* used high liquid chromatography to measure the lansoprazole concentration in gastric juice. A very small group of patients were given 15, 30 or 60 mg lansoprazole at around 10pm and blood samples were taken 2 hours later. Peak plasma concentrations are reached around 2 hours post PPI dose and are dose dependent. Twelve hours post dose, 1 ml of gastric juice was collected from the patients and the concentrations of lansoprazole found

in the juices were in the range 0.3-0.5, 1.2-2.0 and 2.9-3.4 µg/ml respectively for the different dosing regimes. Following a 60 mg dose of lansoprazole, peak plasma concentration was reached 2 hours post dose and was found to be 1 µg/ml, but the concentration in gastric juice (12 hours post dose) was around three fold higher (2.9-3.4 µg/ml). The authors noted that rather than increasing linearly (as might be expected) the concentrations achieved in gastric juice appeared to increase "exponentially" with increasing PPI doses.

It is noteworthy to point out that the peak plasma concentration was reached around 2 hours post PPI dose, however, the lansoprazole concentration in gastric juice was measured many hours later, at 12 hours post dose, with no indication of why this time point was chosen or indeed what effect time-post-dose had on the concentrations detected in gastric juice. This study nevertheless provides evidence that the residual PPI concentration in the GI tract may be higher than peak plasma concentrations suggest and that PPIs remain in the GI tract at detectable levels for a prolonged period of time post dose.

It has been stated in another study that an acid activated form of rabeprazole can be detected in gastric juice (Ohara *et al.*, 2001) but the reference for this information is "unpublished data". It has also been estimated that the concentration of PPI found in the luminal surface of parietal cells may be as high as 1,000 times that of the concentration found in the blood (Shin & Sachs, 2008). The data on the PPI concentration that can be found in the GI tract or stomach is indeed scarce, perhaps at least in part due to the difficulties in acquiring samples of gastric juice from patients following PPI dosing regimes. Peak plasma concentrations are conversely easy to determine, as blood samples can be easily collected from patients. It is therefore difficult to ascertain the concentrations of PPI which are likely to be achieved in the human GI tract.

Table 3 lists the concentrations of PPI that might theoretically be achieved in the stomach following different PPI doses taken on a full or empty stomach. Results of calculations show that if all of the PPI remained in the stomach that the maximum achievable concentrations vary greatly depending on what dose is taken and whether on a full or empty stomach. The maximum single dose of pantoprazole recommended in the BNF is 80 mg and if taken on an empty stomach, the maximum achievable concentration might be around 1 mg/ml (or 1,000 µg/ml). Of course, the whole PPI dose is unlikely to remain free floating in the juices of the GI tract and an unknown proportion would instead be expected to accumulate in canaliculi and bind to ATPases. It is extremely difficult therefore to determine the concentrations of PPI that might be found in the GI tract, but they are likely

to be in the µg/ml range and (at least in the case of pantoprazole) unlikely to exceed 1 mg/ml (or 1,000 µg/ml). They are therefore likely to be present in higher concentrations than the concentrations of conventional antibiotics required to inhibit bacterial growth. The length of time that a concentration might be sustained for in the GI tract is also unknown. Worth additional consideration is that the volume of stomach contents is unlikely to remain steady over a period of 24 hours.

Table 3. Concentrations of PPI that might be achieved in the stomach following different dosages.

Daily Dose of PPI (mg)	BNF Indication	Concentration That Might Be Achieved (µg/ml)	
		Empty Stomach	Full Stomach
20	GORD	250	10
40	Benign Ulcer	500	30
80	Complicated Ulcer	1,000	50
120	Exceptional Cases	1,500	80

Calculations performed on the empty stomach containing 80 ml of residual fluid and a full stomach 1.5 l. The highest single dose recommended for pantoprazole is 80 mg.

1.12 The Anti-*Helicobacter* Activity of Proton Pump Inhibitors

A summary of studies relevant to the anti-*Helicobacter* properties of PPIs can be found in **Table 4** and selected details are discussed further in the sections below.

1.12.1 Early Studies

In 1964, Bishop *et al* were the first to report on the anti-bacterial activity of benzimidazole derivatives (Bishop *et al.*, 1964). In 1991, in the first report of its kind and only a few years after the global launch of omeprazole, Iwahi *et al* used both agar dilution and broth macrodilution to show that the PPIs omeprazole and lansoprazole inhibited the growth of *H. pylori* (Iwahi *et al.*, 1991). Exposure to lansoprazole in liquid media was shown to cause changes to *H. pylori* morphology and it was concluded that, at concentrations higher than those required to inhibit the growth of *H. pylori*, lansoprazole had a bactericidal effect. Iwahi *et al* also reported in this early study that the bactericidal activity of the PPIs appeared to have a "taxonomic boundary" as up to 100 µg/ml, lansoprazole was unable to inhibit the growth of a wide range of laboratory standard bacterial strains and also of 27 clinical isolates of *C. jejuni* (Iwahi *et al.*, 1991).

Table 4. Chronological list summarising the results of studies investigating the anti-Helicobacter properties of various PPIs.

Reference	PPIs Tested	Methods Employed	Organisms Used	Notable Results
Iwahi (Iwahi *et al.*, 1991)	Omeprazole Lansoprazole	Agar dilution Broth macrodilution	*H. pylori* *C. jejuni* *E. cloacae* *M. morganii* *Proteus* spp. *P. aeruginosa* *S. marcescens*	PPIs inhibited *H. pylori* growth Coccal forms and blebs seen in membranes of H. *pylori* No inhibition of growth of the other organisms tested up to 100 µg/ml
Megraud (Megraud *et al.*, 1991)	Omeprazole Lansoprazole	Agar dilution	*H. pylori* *C. jejuni* *E. coli*	PPIs inhibited *H. pylori* growth Coccal forms of *H. pylori* seen No effect on *C. jejuni* or *E. coli* growth
Suerbaum (Suerbaum *et al.*, 1991)	Omeprazole Pantoprazole	Agar dilution Broth macrodilution	*H. pylori*	PPIs inhibited *H. pylori* growth
Nagata (Nagata *et al.*, 1993)	Omeprazole Lansoprazole	Broth microdilution	*H. pylori* *Proteus* spp.	PPIs inhibited *H. pylori* growth No inhibition of *Proteus spp*. up to 1,000 µM (around 350 µg/ml)
Figura (Figura *et al.*, 1994)	Omeprazole	Agar dilution	*H. pylori*	PPI inhibited *H. pylori* growth
Hirai (Hirai *et al.*, 1995)	Omeprazole Lansoprazole Rabeprazole	Agar dilution	*H. pylori*	PPIs inhibited *H. pylori* growth
Nagata (Nagata *et al.*, 1995)	Omeprazole Lansoprazole	Broth macrodilution	*H. pylori*	PPIs inhibited *H. pylori* growth
Nakao (Nakao *et al.*, 1995)	Omeprazole Lansoprazole	Agar dilution	*H. pylori*	Motility of *H. pylori* affected above the MIC PPI exposure affected adherence to Hep-2 cells Morphology affected with blebs seen
Shibata (Shibata *et al.*, 1995)	Omeprazole Lansoprazole	Agar dilution	*H. pylori*	PPIs inhibited *H. pylori* growth
Sjostrom (Sjostrom *et al.*, 1996)	Omeprazole	Broth dilution	*Helicobacter* spp. *Campylobacter* spp. *B. subtilis* *E. coli* *P. vulgaris* *P. aeruginosa* *S. aureus*	PPI inhibited *H. pylori* growth No inhibition of growth of the other organisms tested up to 256 µg/ml

Reference	PPIs Tested	Methods Employed	Organisms Used	Notable Results
Bamba (Bamba *et al.*, 1997)	Omeprazole Lansoprazole	Broth microdilution	*H. pylori*	PPIs inhibited *H. pylori* growth PPIs additive to some conventional antibiotics
Midolo (Midolo *et al.*, 1997)	Omeprazole Lansoprazole	Agar dilution	*H. pylori*	PPIs additive to some conventional antibiotics
Mirshahi (Mirshahi *et al.*, 1998)	Omeprazole	Agar dilution Broth macrodilution	*H. pylori*	PPI inhibited *H. pylori* growth
Nakao and Malfertheiner (Nakao & Malfertheiner, 1998)	Omeprazole Lansoprazole Pantoprazole	Agar dilution Broth macrodilution	*H. pylori*	PPIs inhibited *H. pylori* growth Some PPIs were bactericidal Blebs seen on bacterial surface
Vogt (Vogt & Hahn, 1998)	Omeprazole Lansoprazole	Agar dilution Broth macrodilution	*H. pylori*	PPIs inhibited *H. pylori* growth
Woo (Woo *et al.*, 1998)	Omeprazole YJA20379*****	Agar dilution	*H. pylori*	PPIs inhibited *H. pylori* growth
Trautmann (Trautmann *et al.*, 1999)	Lansoprazole	Agar dilution Broth macrodilution	*H. pylori*	PPI inhibited *H. pylori* growth PPI additive to azithromycin killing
Tsutsui (Tsutsui *et al.*, 2000)	Omeprazole Lansoprazole Rabeprazole	Agar dilution	*H. pylori* *Campylobacter* spp. *P. mirabilis* *S. enterica* *V. cholerae* *V. parahaemolyticus*	PPIs inhibited *H. pylori* growth No inhibition of growth of the other organisms tested up to 256 µg/ml Inhibition of *H. pylori* and *Campylobacter* motility, but not of non-spiral organisms
Ohara (Ohara *et al.*, 2001)	Omeprazole Lansoprazole Rabeprazole	Agar dilution	*H. pylori*	Motility of *H. pylori* affected at sub-MIC levels
Tanaka (Tanaka *et al.*, 2002)	Omeprazole Lansoprazole Pantoprazole	Agar dilution	*H. pylori*	PPIs inhibited *H. pylori* growth PPIs additive or synergistic to conventional antibiotics
Spengler (Spengler *et al.*, 2004)	TF18******	Broth microdilution	*H. pylori*	PPI inhibited *H. pylori* growth Motility of *H. pylori* affected at sub-MIC levels

***** *YJA20379 = a newly synthesised PPI developed by Yung-Jin Pharmaceutical Company.*

****** *TF18 = a trifluoromethyl ketone derivative (1-(2-benzoxazolyl)-3,3,3-trifluoro-2-propanone).*

Also in 1991, agar dilution was used by Megraud *et al* to confirm that the PPIs omeprazole and lansoprazole inhibited the growth of *H. pylori*. Bactericidal activity at concentrations higher than those required to inhibit growth and changes to *H. pylori* morphology were also confirmed (Megraud *et al.*, 1991). Suerbaum *et al* also confirmed in 1991 that the PPI omeprazole inhibited the growth of *H. pylori* and reported that the, as yet not clinically available, PPI pantoprazole also inhibited the growth of *H. pylori* (Suerbaum *et al.*, 1991). They used a broth macrodilution method, with a short incubation period, to show that the acid activated forms of the PPIs were better able to inhibit the growth of *H. pylori* than the pro-drug forms were (Suerbaum *et al.*, 1991). The authors commented that inhibition of *H. pylori* growth, found when using agar dilution methods, was probably the result of PPI activation over the long incubation periods used (72-96 hours).

1.12.2 Later Work

In the years that followed, numerous studies confirmed that various PPIs were capable of inhibiting *H. pylori* growth (Nagata *et al.*, 1993, Figura *et al.*, 1994, Hirai *et al.*, 1995, Nagata *et al.*, 1995, Shibata *et al.*, 1995, Sjostrom *et al.*, 1996, Bamba *et al.*, 1997, Mirshahi *et al.*, 1998, Nakao & Malfertheiner, 1998, Vogt & Hahn, 1998, Woo *et al.*, 1998, Trautmann *et al.*, 1999, Tsutsui *et al.*, 2000, Tanaka *et al.*, 2002, Spengler *et al.*, 2004), inducing morphological changes (Nakao *et al.*, 1995, Nakao & Malfertheiner, 1998), were bactericidal (Nakao & Malfertheiner, 1998) and that growth inhibition of other bacterial genera was not apparent (Nagata *et al.*, 1993, Sjostrom *et al.*, 1996, Tsutsui *et al.*, 2000). In 1994, Figura *et al* suggested that inhibition of *H. pylori* growth by omeprazole might infer that PPIs were affecting bacterial ATPases (Figura *et al.*, 1994). They postulated that if this was indeed the case, then PPIs could theoretically affect anything which required energy production by the bacterium. Bacterial motility is an energy requiring process, important for the pathogenicity of *H. pylori*, and yet Figura *et al* reported that the motility of *H. pylori* was not adversely affected at sub-inhibitory levels of PPI.

1.12.3 Proton Pump Inhibitors Affect Bacterial Motility

In disagreement with Figura *et al*, Nakao reported only 1 year later that the motility of *H. pylori* was adversely affected by exposure to PPIs at concentrations that affected bacterial growth (Nakao *et al.*, 1995). They noted that only slight motility was observed following exposure to the MIC of lansoprazole for 5 hours (MIC having been determined using agar dilution over 4 days). A complete lack of motility was not observed until *H. pylori* had been exposed to four times the MIC for 4 hours. Much later, *in vitro*

methods were improved and an adverse effect on *H. pylori* motility, at concentrations lower than those required to inhibit bacterial growth, were described by multiple authors (Tsutsui *et al.*, 2000, Ohara *et al.*, 2001, Spengler *et al.*, 2004). Of particular relevance to this study is the report by Tsutsui *et al* which showed that various PPIs (up to a concentration of 256 µg/ml) adversely affected the motility of both *H. pylori* and *Campylobacter* spp., even though no inhibition of *Campylobacter* spp. growth was observed (Tsutsui *et al.*, 2000).

1.13 Problems with Previous Research

Many preceding studies make use of a single PPI, (Megraud *et al.*, 1991, Figura *et al.*, 1994, Spengler *et al.*, 2004) whereas others use more than one (Iwahi *et al.*, 1991, Suerbaum *et al.*, 1991, Hirai *et al.*, 1995, Tsutsui *et al.*, 2000). In studies where a single PPI has been used, the PPI in question is often different to those used in other similar studies (Megraud *et al.*, 1991, Figura *et al.*, 1994). PPIs are known to be not very soluble in water (Shin *et al.*, 2004, Nguyen *et al.*, 2005) and the PPIs used in different studies have often been dissolved and in some cases further diluted in different liquids (Iwahi *et al.*, 1991, Figura *et al.*, 1994). Broth dilution has been used by some authors (Suerbaum *et al.*, 1991, Spengler *et al.*, 2004) and agar dilution by others (Iwahi *et al.*, 1991, Megraud *et al.*, 1991, Figura *et al.*, 1994, Hirai *et al.*, 1995, Tsutsui *et al.*, 2000) even though the stability of PPIs in different agars is unknown (Trautmann *et al.*, 1999). Hence it is very difficult to compare the results of relevant studies directly with one another.

Suerbaum *et al* investigated the anti-bacterial properties of PPIs in pro-drug form, as well as in acid activated forms (Suerbaum *et al.*, 1991). Is it important to note that work carried out by Suerbaum *et al*, using acid activated PPIs, could only use very short incubation times (1 hour) and that acid activation of a PPI does not result in a single activated chemical structure, rather a number of different active forms can result (Iwahi *et al.*, 1991, Suerbaum *et al.*, 1991). Also pertinent is that these different activated forms will have varied anti-bacterial activities themselves, based on their own specific chemical structures (Tsutsui *et al.*, 2000).

1.14 Other Bacteria and Proton Pump Inhibitors

The majority of research into the anti-bacterial properties of PPIs has focused on *H. pylori*, but the activity of PPIs, and other benzimidazole derivatives, against other pathogenic and opportunistic bacteria have also been investigated. A summary of relevant studies can be found in **Table 5** and selected details are discussed further in the sections below.

Table 5. Chronological list summarising the results of studies investigating the properties of various PPIs against organisms other than Helicobacter and Campylobacter.

Reference	PPIs Tested	Methods Employed	Organisms Used	Notable Results
Bishop (Bishop *et al.*, 1964)	Various benzimidazoles	Broth macrodilution	*E. coli* *K. aerogenes* *S. aureus* *S. pyogenes*	Inhibition of growth was observed in some cases The Gram positive organisms were more sensitive to inhibitory activity than Gram negative Problems with benzimidazole solubility were common
Aeschlimann (Aeschlimann *et al.*, 1999)	Omeprazole Lansoprazole	Broth microdilution	*S. aureus*	PPIs able to lower MIC and MBC of conventional antibiotics (ciprofloxacin and norfloxacin) PPIs increase killing by levofloxacin
Nguyen (Nguyen *et al.*, 2005)	Omeprazole Lansoprazole	Glass slide biofilm	*S. mutans*	PPIs found to be bactericidal against *S. mutans* PPIs also able to inhibit biofilm formation
Vidaillac (Vidaillac *et al.*, 2007)	Omeprazole Omeprazole analogues	Agar dilution Broth dilution	*S. aureus*	PPI MICs were > 512 µg/ml PPIs can reduce MIC of norfloxacin PPIs supplement killing by norfloxacin
Sambanthamoorthy (Sambanthamoorthy *et al.*, 2011)	Omeprazole Lansoprazole ABC-1*****	Crystal violet staining	*K. pneumoniae* *P. aeruginosa* *S. boydii* *S. aureus* *V. cholerae*	ABC-1 did not to inhibit growth of the organisms tested ABC-1 did prevent biofilm formation ABC-1 was more potent at preventing biofilm formation than omeprazole Pre formed biofilm was not dispersed by ABC-1
Singh (Singh *et al.*, 2012)	Esomeprazole	Crystal violet staining	*P. aeruginosa* *S. aureus*	Exposure to PPI decreased ability to form biofilm PPI was able to supplement the killing by conventional antibiotics (meropenem and vancomycin)

***** *ABC-1 = antibiofilm compound 1, a novel low molecular weight benzimidazole similar in structure to omeprazole and lansoprazole.*

1.14.1 Proton Pump Inhibitors Enhance Conventional Antibiotic Activity

In 1999, Aeschlimann *et al* reported that the PPIs omeprazole and lansoprazole improved the *in vitro* activity of various fluoroquinolones against multiple strains of *Staphylococcus aureus* (*S. aureus*) (Aeschlimann *et al.*, 1999). Co-exposure to PPIs and the fluoroquinolones ciprofloxacin and norfloxacin lead to a reduction in the minimum inhibitory and minimum bactericidal concentrations (MBCs) of the fluoroquinolones and increased the killing of bacteria by the fluoroquinolone levofloxacin. Further work investigating the activities of PPIs, and other benzimidazole derivatives, on *S. aureus* confirmed the ability to reduce the minimum inhibitory concentration (MIC) of conventional antibiotics and proposed a role for PPIs as potential inhibitors of bacterial efflux pumps (Vidaillac *et al.*, 2007).

In a later study Singh *et al* showed that pre-exposure to PPI enhanced killing of *S. aureus* by vancomycin and of *Pseudomonas aeruginosa* (*P. aeruginosa*) by meropenem (Singh *et al.*, 2012). They also reported that the PPI esomeprazole inhibited biofilm formation in both *S. aureus* and *P. aeruginosa*.

1.14.2 Proton Pump Inhibitors Inhibit Biofilm Formation

Susceptibility to conventional antibiotics and bacterial ability to form biofilm are linked. It is well known that planktonic bacteria are (sometimes up to 1,000×) more susceptible to antibiotics than bacteria in biofilms are and most antibiotics are developed to target planktonic bacteria rather than those in biofilms (Sambanthamoorthy *et al.*, 2011). In 2005, Nguyen *et al* reported that the PPIs omeprazole and lansoprazole inhibited biofilm formation in *Streptococcus mutans* (*S. mutans*) (Nguyen *et al.*, 2005). Later work by Sambanthamoorthy *et al* distinguished between the ability of PPIs, and other benzimidazole derivatives, to prevent biofilm formation but not to disrupt pre-formed biofilm (Sambanthamoorthy *et al.*, 2011).

1.15 Anti-parasitic Activity of Proton Pump Inhibitors

Albendazole and mebendazole (**Figure 6**) are benzimidazole carbamate derivatives that are used to treat helminth infections. They both have the same 2-ringed benzimidazole core structure that can be seen in **Figure 2** and have adapted sidechain configurations that resemble the early structures in PPI development shown in **Figure 3** and those of modern PPIs shown in **Figure 4**.

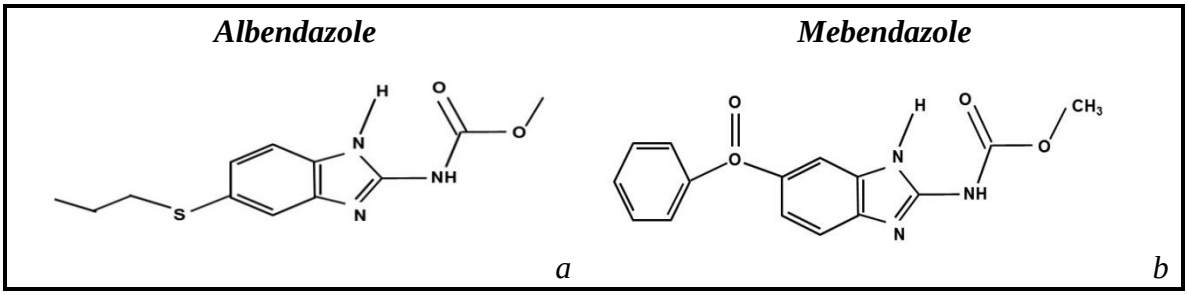


Figure 6. The chemical structures of albendazole and mebendazole. *Albendazole (a) and mebendazole (b) are benzimidazole carbamate derivatives which are used in the treatment of various helminth infections.*

In 1992, Cedillo-Rivera and Munoz reported that albendazole and mebendazole inhibited the growth of *Giardia lamblia* (*G. lamblia*) and, at even higher concentrations, the benzimidazoles were capable of killing the protozoan (Cedillo-Rivera & Munoz, 1992). They showed that the benzimidazoles were active at concentrations lower than that of the recommended treatment for *G. lamblia* infection (metronidazole) and suggested that tubulin was the target of the benzimidazoles. The observation was however overlooked for a number of years following the publication and the search for new anti-parasitic treatments was thought by many to be of little importance as they were rather "neglected" infections (Perez-Villanueva *et al.*, 2011). However, resistance to recommended treatments began to emerge and interest was renewed in identifying potential novel targets for the treatment of parasitic infections and in identifying potential novel treatments. The early 2000s saw a rush of articles being published, most of which focused on testing the anti-parasitic properties of newly synthesised or chemically modified benzimidazoles (a summary of relevant studies can be found in **Table 6**).

Navarrete-Vazquez *et al* extended the range of parasites used in their experiments to include the protozoan *Entamoeba histolytica* (*E. histolytica*) and the helminth *Trichinella spiralis* (*T. spiralis*). The major component of the cytoskeleton of *G. lamblia* and *T. spiralis* is tubulin but the major component of the cytoskeleton of *E. histolytica* is actin. Navarrete-Vazquez *et al* reported that albendazole was inactive against *E. histolytica*, but that some of the benzimidazole structures that they had created inhibited *E. histolytica* growth. They determined that albendazole inhibited the polymerization of tubulin but that other benzimidazole structures had anti-parasitic properties that were independent of tubulin polymerisation and that these required further study.

Table 6. Chronological list summarising the results of studies investigating the anti-parasitic properties of benzimidazole derivatives.

Reference	Benzimidazoles Tested	Methods Employed	Organisms Used	Notable Results
Cedillo-Rivera (Cedillo-Rivera & Munoz, 1992)	Albendazole Mebendazole	Broth macrodilution	*G. lamblia*	Inhibition of growth was observed as was killing Tubulin is likely target of benzimidazoles
Navarrete-Vazquez (Navarrete-Vazquez *et al*., 2001)	Albendazole Benzimidazole derivatives	Broth dilution	*G. lamblia* *E. histolytica* *T. spiralis*	Inhibition of *G. lamblia* and *T. spiralis* growth was observed as was killing Albendazole was inactive against *E. histolytica* Albendazole inhibited tubulin polymerisation but other benzimidazoles did not Binding to tubulin is not required for all anti-parasitic activity
Andrzejewska (Andrzejewska *et al.*, 2002)	Albendazole Benzimidazole derivatives	Broth dilution	*G. intestinalis* *E. histolytica* *T. vaginalis*	Inhibition of *G. intestinalis* and *T. vaginalis* growth was observed Albendazole was inactive against *E. histolytica* Benzimidazole derivatives inhibited growth of *E. histolytica* Benzimidazole carbamates bind to tubulin and inhibit polymerisation.
Cedillo-Rivera (Cedillo-Rivera *et al.*, 2002)	Albendazole	Broth macrodilution	*G. intestinalis* *E. histolytica* *T. vaginalis*	Inhibition of *G. intestinalis* and *T. vaginalis* growth was observed Albendazole was inactive against *E. histolytica* Possible that benzimidazole induce changes to the plasma membrane
Jiang (Jiang *et al.*, 2002)	Omeprazole	Broth dilution	*L. donovani*	Inhibition of growth was observed Due to inhibition of the K^+/H^+-ATPase on the surface membrane Interferes with pH homeostasis ability and disrupts proton motive force
Riel (Riel *et al.*, 2002)	Omeprazole Lansoprazole Pantoprazole Rabeprazole	Broth microdilution	*P. falciparum*	Lansoprazole and rabeprazole were best at inhibiting growth Omeprazole and quinine were found to be synergistic A V-type H^+-ATPase in the plasma membrane is unlikely to be the target
Valdez (Valdez *et al.*, 2002)	Albendazole Benzimidazole derivatives	Broth dilution	*G. lamblia* *E. histolytica* *T. spiralis*	Inhibition of *G. lamblia* and *T. spiralis* growth by albendazole was observed Albendazole was inactive against *E. histolytica* Benzimidazole derivatives inhibited growth of *E. histolytica* Not all benzimidazole derivatives inhibited tubulin polymerisation

Reference	Benzimidazoles Tested	Methods Employed	Organisms Used	Notable Results
Kazimierczuk (Kazimierczuk *et al.*, 2002)	Albendazole Benzimidazole derivatives	Broth microdilution	*G. duodenalis* *E. histolytica* *T. vaginalis*	Albendazole was inactive against *E. histolytica* and *T. vaginalis* (up to 200 µM) Benzimidazoles affect oxidative phosphorylation in mitochondria Issues with solubility and crystallisation were noted with some structures
Navarrete-Vazquez (Navarrete-Vazquez *et al.*, 2003)	Albendazole Mebendazole and their analogues	Broth macrodilution	*G. lamblia* *T. vaginalis* *T. spiralis* *C. elegans*	Inhibition of growth was observed Not all benzimidazole derivatives inhibited tubulin polymerisation Other structures have a different mechanism of action
Andrzejewska (Andrzejewska *et al.*, 2004)	Benzimidazole derivatives	Broth macrodilution	*G. intestinalis* *T. vaginalis*	Inhibition of growth was observed Benzimidazoles inhibit protein kinases (CK1, CK2, and others) They may interfere with a wide spectrum of cell regulatory mechanisms
Navarrete-Vazquez (Navarrete-Vazquez *et al.*, 2006)	Benzimidazole derivatives	Broth macrodilution Broth microdilution	*G. intestinalis* *T. vaginalis* *P. falciparum*	Inhibition of growth was observed
Valdez-Padilla (Valdez-Padilla *et al.*, 2009)	Benzimidazole derivatives	Broth dilution	*G. intestinalis* *T. vaginalis*	Inhibition of growth was observed
Hernandez-Luis (Hernandez-Luis *et al.*, 2010)	Benzimidazole derivatives	Broth macrodilution	*G. intestinalis* *E. histolytica* *T. vaginalis* *T. spiralis* *L. mexicana*	Inhibition of growth was observed Albendazole was inactive against *E. histolytica*
Perez-Villanueva (Perez-Villanueva *et al.*, 2011)	Omeprazole Lansoprazole Pantoprazole Rabeprazole	Unknown	*G. intestinalis* *E. histolytica* *T. vaginalis*	Inhibition of growth was observed Pantoprazole showed good activity against all three protozoa PPIs may make good candidates for drug repurposing Mechanism of anti-protozoal activity is yet to be described

The main component of the cytoskeleton of G. lamblia, G. intestinalis, G. duodenalis, T. spiralis and T. vaginalis is tubulin.
The main component of the cytoskeleton of E. histolytica is actin.

In 2002, Jiang *et al* were the first to test a clinically used formulation of a PPI for antileishmanial activity (Jiang *et al.*, 2002). They noted that at pH 7.2 there was no adverse effect on the protozoan, but at pH 5.5 omeprazole inhibited the growth of *Leishmania donovani* (*L. donovani*). They suggested that the prodrug form was therefore inactive and that the antileishmanial activity of omeprazole was due to one or more of the protonated active forms of PPI. They also postulated that omeprazole was inhibiting the P-type H^+/K^+-ATPase on the membrane surface of *L. donovani*. This enzyme is known to be important for pH homeostasis and maintenance of the proton motive force across the membrane of *L. donovani*.

It is known that acid activated forms of PPIs form strong disulphide bonds with thiol groups on exposed cysteine residues of the H^+/K^+-ATPase found in parietal cells and that acid activated tetra-cyclic sulphenamide forms can bind to and inactivate the adenylate cyclase or Na^+/K^+-ATPase of parietal cells (see **Figure 1** and **Section 1.10**). If acid activated forms of PPIs were present in the kidneys it is also known that binding to and inactivation of the kidney H^+/K^+-ATPases is also possible (see **Section 1.9.2**). It is therefore proven that PPIs can bind to and inhibit a variety of enzymes that they encounter and therefore plausible that PPIs could bind to bacterial or parasitic enzymes (particularly perhaps ATPases).

Riel *et al* also used clinically used formulations of PPIs to inhibit the growth of *Plasmodium falciparum* (*P. falciparum*) but they concluded that the V-type H^+-ATPase in the plasma membrane of *P. falciparum* was not the target (Riel *et al.*, 2002). In 2011 it was reported that after around 20 years of research, the mechanism of the tubulin polymerisation independent anti-protozoal action of benzimidazoles was "yet to be described" (Perez-Villanueva *et al.*, 2011). The properties of structures containing the core benzimidazole backbone are therefore hugely diverse, with some reportedly also having anti-viral, anti-fungal and anti-cancer activities (Andrzejewska *et al.*, 2002, Kazimierczuk *et al.*, 2002, Navarrete-Vazquez *et al.*, 2006).

1.16 Proton Pump Inhibitors and *Campylobacter*

Early studies reporting on the inhibitory effect of PPIs on *H. pylori* stated that PPIs had no similar inhibitory effect on *C. jejuni* (Iwahi *et al.*, 1991, Megraud *et al.*, 1991). Almost a decade later, the first report of PPIs affecting both *H. pylori* and *C. jejuni* motility was published (Tsutsui *et al.*, 2000). *C. jejuni* is very acid sensitive (Lodato *et al.*, 2010) and attempting to differentiate between the anti-bacterial properties of pro-drug versus acid

activated forms of PPI by artificially manipulating the pH might therefore be problematic in the case of *C. jejuni*. When Suerbaum *et al* attempted to do this with *H. pylori* they could only use short incubation times of 1 hour. The standard measures of antimicrobial activity (MIC and MBC) are however usually expressed using 24 hours exposure for rapidly growing organisms.

Considering that *C. jejuni* is one of the most important causes of bacterial gastroenteritis in the world (see **Section 1.3**); that there are increasing numbers of people taking PPIs worldwide (see **Section 1.11.3** and **Figure 5**); that there is a proposed link between taking PPIs and increased susceptibility to enteric infections (see **Section 1.7.1**); and that it has been suggested that taking PPIs allows increased bacterial translocation across the epithelial cell barrier (see **Section 1.6.3.3**), it seems prudent to use methods similar to those employed previously by others to thoroughly investigate the effect of exposure to PPIs on *C. jejuni*. If PPIs are truly able to affect *C. jejuni* motility as described by Tsutsui *et al* it therefore seems reasonable to suggest that the pathogenicity of the organism should also be adversely affected (see **Section 1.6.3**).

1.17 Summary and Aims

The exact method by which PPIs exert their anti-bacterial effect on *Helicobacter* and whether it is in fact a true bactericidal effect, are as yet unknown. PPIs are benzimidazole derivatives and such compounds have been shown by others to affect *H. pylori* morphology (Ikeda & Karlyshev, 2012), *H. pylori* motility (Ohara *et al.*, 2001), adherence of *H. pylori* to epithelial cells (Nakao *et al.*, 1995) and biofilm formation in organisms such as *S. mutans* (Nguyen *et al.*, 2005). PPIs have also been shown by others to supplement the killing of organisms such as *H. pylori* by conventional antibiotics (Bamba *et al.*, 1997). Previous work has hinted that PPIs may also be capable of affecting *C. jejuni* (Tsutsui *et al.*, 2000), but investigations have been extremely limited in comparison to those investigating the effect of PPIs on the related organism *H. pylori*. Indeed as *Campylobacter* and *Helicobacter* both belong in the campylobacterales order, adverse affects following exposure of *C. jejuni* to PPIs might in fact be expected.

The project aims were to determine whether direct exposure to PPIs affected *C. jejuni* in ways similar to those reported by others using other bacterial genera. Whether PPIs could affect *C. jejuni* growth/survival, motility, morphology, biofilm formation, adhesion and invasion of cultured epithelial cells and the effect of PPIs on the susceptibility to conventional antibiotics were investigated.

Microarrays, proteomics and metabolomics were used to investigate changes to *C. jejuni* gene expression, proteome and metabolome respectively, in response to PPI exposure. Selected *C. jejuni* genes were mutated to investigate the response to PPI exposure of deficient mutants in comparison to parent strains.

Chapter 2

In Vitro Effects of PPI Exposure on *Campylobacter jejuni*

2 INTRODUCTION

2.1.1 *Helicobacter* and Proton Pump Inhibitors

It has been widely reported that exposure to PPIs affects the growth and survival of *H. pylori in vitro*. The first study to report that exposure to PPIs inhibited the growth of *H. pylori* was published only a few years after the first PPI, omeprazole, was made commercially available (Iwahi *et al.*, 1991). The authors proposed that the anti-bacterial properties of PPIs were "selective" against *H. pylori* as 100 µg/ml had no inhibitory effect on *Bacteroides fragilis*, *Bifidobacterium bifidum*, *C. jejuni*, *Citrobacter freundii*, *Clostridium perfringens*, *Enterobacter cloacae* (*E. cloacae*), *Enterococcus faecalis*, *E. coli*, *Eubacterium alactolyticum*, *Eubacterium limosum*, *Fusobacterium mortiferum*, *Fusobacterium nucleatum*, *Klebsiella pneumoniae* (*K. pneumoniae*), *Lactobacillus acidophilus*, *Morganella morganii* (*M. morganii*), *Peptostreptococcus anaerobius*, *Proteus mirabilis* (*P. mirabilis*), *Proteus vulgaris* (*P. vulgaris*), *P. aeruginosa*, *Serratia marcescens* (*S. marcescens*), *S. aureus*, *Streptococcus pneumoniae* or *Streptococcus pyogenes* (*S. pyogenes*).

This early study reported that, as well as inhibiting the growth of *H. pylori*, PPI exposure caused membrane blebbing and changes to the morphology of bacterial cells, with bacilliform and coccal forms of *H. pylori* being observed (Iwahi *et al.*, 1991). These reported changes to *H. pylori* morphology were supported by later studies (Megraud *et al.*, 1991, Nakao *et al.*, 1995, Nakao & Malfertheiner, 1998). Exposure to PPIs was also reported in some studies to affect the motility of *H. pylori* (Nakao *et al.*, 1995, Tsutsui *et al.*, 2000, Ohara *et al.*, 2001, Spengler *et al.*, 2004) and its ability to adhere to cultured cells (Nakao *et al.*, 1995).

Over the years, a number of proposed targets were investigated to explain the anti-*Helicobacter* properties of PPIs. These included the potent urease enzyme of *H. pylori* (Nagata *et al.*, 1995, Logan, 1996), the organisms cytotoxin (Figura *et al.*, 1994) and various bacterial ATPase enzymes (Belli & Fryklund, 1995, Park *et al.*, 1996). However, the target/s responsible for the *in vitro* activity of PPIs against *H. pylori* remained elusive even after over a decade of research (Mills *et al.*, 2004), with urease, cytotoxin and specific ATPase enzymes all ruled out as targets. A more comprehensive review of relevant literature can be found in **Section 1.12** and in **Table 4**.

2.1.2 *Campylobacter* and Proton Pump Inhibitors

Iwahi *et al* were also the first to test the PPIs omeprazole and lansoprazole for inhibitory activity against *Campylobacter* sp. (Iwahi *et al.*, 1991). They tested 27 clinically isolated strains of *C. jejuni* and reported that no inhibition of growth was observed up to a concentration of 100 µg/ml of PPIs. This observation was supported by later studies reporting that the growth of *Campylobacter* spp. was not inhibited up to 256 µg/ml of omeprazole, lansoprazole or rabeprazole (Megraud *et al.*, 1991, Sjostrom *et al.*, 1996, Tsutsui *et al.*, 2000). However in one crucial study, where PPI exposure was found to affect the survival and motility of *H. pylori*, PPI exposure was reported to affect *Campylobacter* motility even though no effect on *Campylobacter* survival was found (Tsutsui *et al.*, 2000).

Motility is considered an important virulence factor (see **Section 1.6.3**) of *C. jejuni* (van Alphen *et al.*, 2012) and is required for host colonisation (Cullen *et al.*, 2013), for biofilm formation (Guerry, 2007) as well as for attachment to and invasion of epithelial cells (Mills *et al.*, 2012). In 1988, Black *et al* reported that after using a mixture of motile and non-motile *Campylobacter* strains in experimental human infections only motile strains were recovered from stools (Black *et al.*, 1988). Therefore, if exposure to PPIs can indeed adversely affect *C. jejuni* motility then adverse effects on the ability to form biofilm and to adhere to and invade epithelial cells could also result following PPI exposure. As discussed previously having an adverse effect on *C. jejuni* motility could have serious implications for the pathogenicity of the organism and its ability to cause disease.

2.1.3 Other Bacteria and Proton Pump Inhibitors

Whilst the bactericidal activity of PPIs seems to be mostly limited to *H. pylori* it seems likely that the bacterial target for PPIs would be an ATPase, or multiple ATPase enzymes, with the possibility of hampering any process which requires energy production (Figura *et al.*, 1994). It is therefore possible that energy requiring processes, in other bacteria, might also be affected by exposure to PPIs. A more comprehensive review of relevant literature can be found in **Section 1.14** and in **Table 5.** A number of studies concluded that PPIs, or structurally similar benzimidazoles, inhibit biofilm formation in various Gram positive and Gram negative bacteria (Nguyen *et al.*, 2011, Sambanthamoorthy *et al.*, 2011, Singh *et al.*, 2012). Co-exposure to PPIs and conventional antibiotics was also shown to increase bacterial killing and reduce MICs and MBCs (Aeschlimann *et al.*, 1999).

2.1.4 Chapter Aims

It is generally accepted that patients being treated with PPIs are more susceptible to all enteric infections (including campylobacteriosis and *C. difficile*) than patients who are not taking PPIs (Bavishi & DuPont, 2011). The numbers of people taking PPIs worldwide is also ever increasing. The link between PPI use and susceptibility to enteric infections has been investigated in countries including the Netherlands (Doorduyn *et al.*, 2010, Bavishi & DuPont, 2011, Bouwknegt *et al.*, 2014). The effect of PPI exposure on *H. pylori* has been investigated rigorously in the past, but the effect on *C. jejuni* remains unclear. With *C. jejuni* being the most common cause of acute bacterial gastroenteritis in the UK and in Europe it may prove useful to study the effects that direct contact with PPIs has on the pathogen.

Experiments presented in this chapter were performed to determine whether exposure to PPI had any effects on *Campylobacter*. Methods similar to those used in various *H. pylori* studies were employed to investigate if *in vitro* exposure to PPI inhibited the growth of, or indeed was bactericidal to *C. jejuni*. Any changes to *C. jejuni* motility, morphology, ability to form biofilm and ability to adhere to or invade epithelial cells were also investigated. Selected experiments were also performed using the Gram negative enteric pathogen *Salmonella enterica*, subsp *enterica*, serovar Typhimurium (*S.* Typhimurium) and a mouse commensal strain of *Lactobacillus* to investigate whether the *in vitro* effects of PPI exposure are likely "selective" and limited to the spiral Gram negative enteric pathogens.

This study has utilised the PPI pantoprazole, due to its superior ability to dissolve in water and give accurate concentrations, compared to other PPIs like omeprazole or lansoprazole (personal observations). Pantoprazole is also generally prescribed at a higher dose than some of the other PPIs (see **Table 2**) and the resulting concentration that might be physically achievable in the GI tract is therefore likely to be higher for pantoprazole than for other PPIs (see **Table 3**).

2.2 Materials and Methods

2.2.1 Bacterial Strains and Culture Conditions

The *C. jejuni*, *S.* Typhimurium and *Lactobacillus* strains used in this chapter of the study are listed in **Table 7**. All strains were stored at -80°C on Microbank™ beads (Prolab). To revive strains from frozen stocks, beads were thawed on ice and one bead removed and streaked onto a plate to obtain single colonies. After 48 hours, one or more colonies were re-streaked onto a fresh plate and this was termed passage number one. Strains were routinely passaged onto a fresh plate every 2-3 days, up to a maximum passage of ten. Strains from overnight growths were used in all individual experiments. Strains were routinely grown on either Mueller-Hinton agar (MHA; Oxoid) or MHA with 7% horse blood (MHA + B; see **Appendix 1**). *C. jejuni* incubations were carried out at 37°C, in a variable-atmosphere incubator (VAIN; Don Whitley Scientific) in an atmosphere of 5% H_2, 5% CO_2, 5% O_2 and 85% N_2. *S.* Typhimurium and *Lactobacillus sp.* incubations were carried out in a standard aerobic incubator at 37°C. The *Lactobacillus* strain was isolated from a 6-8 week old healthy C57BL/6 (Harlan Laboratories) control mouse and was identified using 16S ribosomal DNA typing.

Table 7. Bacterial strains used in this chapter.

Strain	Features	Origin/Reference
C. jejuni 11168-O	Minimally passaged strain, first *C. jejuni* to later have its genome sequenced	(Gaynor *et al.*, 2004)
C. jejuni 81-176	Human clinically isolated strain	(Korlath *et al.*, 1985)
C. jejuni 81116	Human clinically isolated strain	(Palmer *et al.*, 1983)
S. Typhimurium SL1344	Pathogenic laboratory strain	(Hoiseth & Stocker, 1981)
Lactobacillus sp.	Normal gut flora strain	Isolated from a healthy C57BL/6 mouse

2.2.2 Proton Pump Inhibitor

Unless otherwise stated the PPI pantoprazole sodium hydrate powder (Sigma) was dissolved in sterile water and sterilised using a 0.2 µm syringe filter (Sartorius). When required, PPI was further diluted in sterile water to achieve desired concentrations. Neat PPI solution and the most dilute concentration (in the case of serial dilutions) were routinely cultured aerobically and microaerophillicaly on MHA to check for sterility.

2.2.3 Growth on Solid Agar

C. jejuni colonies from an overnight plate growth were harvested into Mueller-Hinton broth (MHB; Oxoid) and around 1×10^8 CFU/ml added to 10 ml molten soft top agar (STA; see **Appendix 1**) and poured over the surface of an MHA plate. After cooling, prepared concentrations of pantoprazole (20-0 mg/ml or 20,000-0 µg/ml) were spotted onto the surface and plates incubated for 24 hours before being checked for inhibition of growth.

2.2.4 Pantoprazole Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

MIC and MBC experiments were performed using a broth microdilution method in sterile 96 well microtitre plates (Corning). *Campylobacter* or *Salmonella* colonies from an overnight plate growth were harvested into MHB or minimal essential media (MEM; Invitrogen); Dulbecco's modified eagle media with GlutaMAX™ (DMEM; Invitrogen) or tryptic soy broth (TSB; LabM) to around 5×10^5 CFU/ml. An equal volume of this bacterial suspension was added to the same volume of PPI (at concentrations ranging from 40-0 mg/ml or 40,000-0 µg/ml). The microtitre plate was covered with a sterile lid before being incubated for 4 or 24 hours. The microtitre plate was then placed in an automatic plate reader (BMG LabTech-FluoStar-Optima), shaken and the optical density at 600 nm (OD_{600}) measured. MIC was also assessed visually using a light box. 10 µl aliquots were taken from each well and spotted onto MHA + B plates and plates incubated for 24 hours before being examined for the growth of *Salmonella* or 48 hours before being examined for the growth of *Campylobacter*. PPI was replaced with an equal volume of sterile water for controls. Controls were also performed to ensure the sterility of water, MHB, MEM, DMEM, TSB and PPI. In a similar manner *Lactobacillus* colonies from an overnight MHA plate growth were harvested into MHB and pantoprazole MBC determined following exposure to pantoprazole for 4 or 24 hours by culturing 10 µl aliquots on MHA + B plates for 24 hours.

Following exposure to varying concentrations of PPI for 4 or 24 hours, aliquots were removed from the wells and inoculated into fresh broths containing no PPI. These were then incubated for a further 24 hours before aliquots were removed and plated onto MHA + B to determine if live bacteria could be revived in the absence of PPI following exposures to specific PPI concentrations.

2.2.5 Survival Studies

Following exposure to varying concentrations of PPI for 4 or 24 hours in a broth microdilution method similar to that used in **Section 2.2.4**, aliquots were serially diluted in phosphate buffered saline (PBS; Invitrogen) and remaining viable bacteria were enumerated by viable plate surface colony counting and calculating CFU/ml.

2.2.6 Motility Testing

Semi-solid agar (SSA, see **Appendix 1**) was prepared once weekly and plates refrigerated until required. *Campylobacter* or *Salmonella* colonies from an overnight plate growth were harvested into MHB to 1×10^7 CFU/ml. Aliquots of this suspension were added to an equal volume of PPI (at concentrations ranging from 5-0 mg/ml or 5,000-0 µg/ml). These were then incubated for either 4 or 24 hours before 1 µl aliquots were stabbed into the centre of SSA plates. Plates were then incubated for 24 (for *Salmonella*) or 48 hours (for *Campylobacter*) and the diameter of the zones of spread were measured in mm. Following each of the exposure times serial dilutions were made in PBS and spotted onto plates to monitor bacterial survival at the different concentrations of PPI tested. These plates were incubated for 24 (for *Salmonella*) or 48 hours (for *Campylobacter*) and surviving CFU/ml calculated.

2.2.7 Biofilm Formation

$25cm^2$ flasks (Corning) containing 10 ml MHB were primed overnight in the VAIN. They were then inoculated with a few colonies from an overnight plate growth of *Campylobacter* and incubated overnight. Cultures were pelleted (Sigma 4K15 centrifuge) at 4,500 × g for 10 minutes at 4^oC and resuspended to an OD_{600} of 0.6 using an Eppendorf Biophotometer. Equal volumes of this were aliquoted into 5 ml Eppendorf tubes (Starlab) and PPI, or water for no PPI controls, added to final concentrations of 500, 250, 125 and 0 µg/ml. The Eppendorf tubes were vortexed and incubated in the VAIN with loose lids for 2 hours. To remove the PPI, Eppendorf tubes were centrifuged as described above and bacterial pellets washed with 1 ml PBS, vortexed and re-centrifuged. The remaining bacteria were resuspended in MHB to an OD_{600} of 0.3. 200 µl of these suspensions was added to replicate wells of a 96 well microtitre plate before being covered with a lid and incubated in the VAIN to allow biofilm to form for 1, 2, or 3 days.

Following 1, 2 or 3 days incubation selected wells, covering a range of PPI concentrations, were observed using an inverted microscope (Zeiss Axiovert 25) at 400× magnification. 5 µl was taken from selected wells onto glass microscopy slides, stained using Live/Dead® BacLight™ (Invitrogen) and viewed using fluorescent microscopy (Axio Imager.A1) at

400× magnification. In both cases images were captured using Axiovision release 4.7.1 software (Carl Zeiss, Germany). Aliquots were removed, plated on MHA and incubated aerobically to check for contamination. The remaining media was removed from all wells and wells washed four times with PBS, plates inverted and dried in a 42°C incubator for 15 minutes. 1% crystal violet was added to wells and the plates placed on a rotary shaker (Stuart Scientific S03) at room temperature for 10 minutes at 180 revolutions per minute (rpm). Crystal violet was then removed and wells washed four times with PBS. Plates were again inverted and dried for 15 minutes before 70% ethanol was added to wells to elute the crystal violet stain and plates placed again on a rotary shaker for 10 minutes. OD_{600} was then measured using a BMG LabTech-FluoStar-Optima plate reader. If values were above the maximum detected by the plate reader, then 1:2 and 1:5 dilutions were made in water from appropriate wells, the values corrected according to the dilution factor and the average of both dilutions taken as the final result.

2.2.8 Adhesion and Invasion

In all of the references stated below, DMEM was supplemented with 10% foetal calf serum (FCS; Gibco). *C. jejuni* 81-176 was harvested from overnight plate growths into DMEM and the suspension diluted as required in fresh DMEM (see below). Pantoprazole for use in these experiments was dissolved and further diluted, when required, in PBS with PBS alone being used for no PPI controls.

Caco-2 cells were grown in DMEM. The cells were grown routinely in vented cap tissue culture flasks (Corning) at 37°C in a 5% CO_2 humidified atmosphere. For all assays, 12 well tissue culture plates (Corning) were seeded with approximately 5×10^5 cells per ml, and incubated until fully confluent (usually around 48 hours). Before infection with *C. jejuni* the monolayers were covered with 1 ml fresh DMEM. Approximately 1×10^7 CFU/ml of *C. jejuni* in DMEM was added to monolayers with pantoprazole or PBS alone being added to a final concentration of 1,000, 500, 250 or 0 µg/ml. The infected monolayers were incubated for 4 hours before 5 µl aliquots were stabbed into SSA and motility following exposures to varying PPI concentrations assessed as described in **Section 2.2.6**. Serial dilutions were also prepared from the wells to ensure PPI exposure concentrations tested did not significantly alter the CFU/ml surviving the PPI exposures.

For measurement of adhesion, the infected monolayers were incubated for 4 hours and then gently washed three times with PBS, before being lysed using 1% (v/v) Triton X-100 (Sigma) in water and ten fold serial dilutions made in PBS. Ten µl aliquots of each dilution were plated on MHA + B and agar plates incubated for 48 hours in the VAIN to

enumerate and calculate CFU/ml. For measurement of invasion, the infected monolayers were incubated for 4 hours as for the adhesion assay and then gently washed three times with PBS, before adding DMEM containing gentamicin (Sigma) 200 μg/ml for 2 hours to kill any extracellular bacteria. Following incubation with gentamicin the infected monolayers were gently washed, lysed and serial dilutions plated as described for the adhesion assay.

The total number of bacteria associated with the monolayers (adhered and internalised) was determined using the method described first above and internalised only bacteria determined by using gentamicin in the invasion assay. The difference between the total number of associated bacteria and the number of intracellular bacteria was calculated to obtain the number of adherent *C. jejuni*. One mg/ml (or 1,000 μg/ml) final concentration of pantoprazole, dissolved in PBS and added to wells containing fully confluent Caco-2 cells in DMEM, for 12 hours looked microscopically indistinguishable from Caco-2 cells not exposed to PPI. So the presence of PPI in the assay over 4 hours likely did not affect the cell morphology.

2.2.9 Electron Microscopy

Following exposure, for 24 hours, of *C. jejuni* to various concentrations of pantoprazole, 50 μl aliquots were removed and fixed for 1 hour at room temperature, in 2.5% glutaraldehyde in 0.1 M phosphate buffer. They were then rinsed three times, for 5 minutes each, with 0.1 M phosphate buffer. Specimens were then fixed for 1 hour in 1% osmium tetroxide. After three 10 minute washes with distilled water, specimens were dehydrated through an ascending series of acetone solutions (30, 50, 70, 90 and 100%) twice for 10 minutes each. Specimens were then dried in a critical point dryer (Polaron E3000) for 80 minutes and mounted on stubs using double-sided copper tape and silver paint. A Polaron SC515 SEM coating system was used to coat the specimens with gold–palladium (20 nm thickness) and they were viewed on a JEOL 6400 scanning electron microscope.

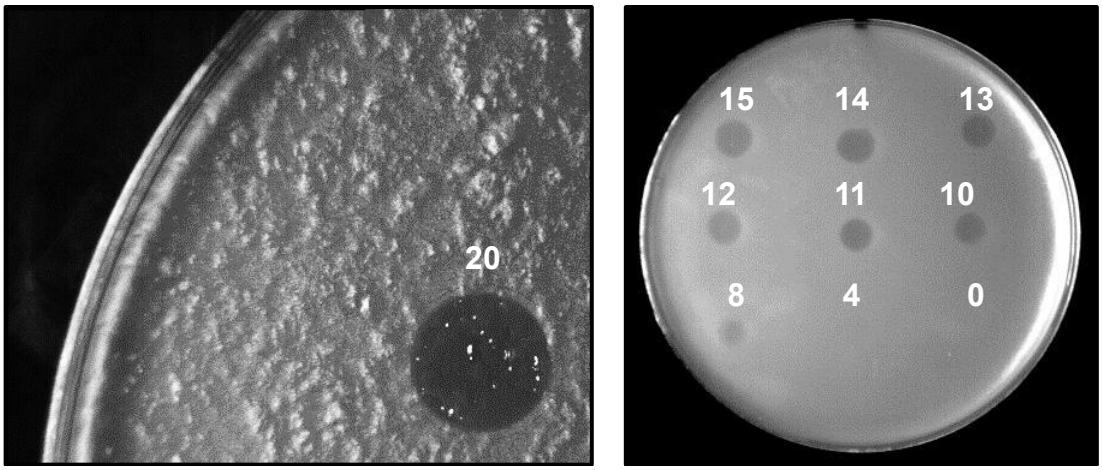
2.2.10 Replicates and Data Analysis

Each assay was conducted in triplicate and was independently repeated at least twice. Results are expressed as means +/- standard deviations (SD; error bars) of all replicate experiments. The unpaired Students t test was used to determine statistical significance. A P value of > 0.01 but < 0.05 was considered significant (*****) and a P value of < 0.01 highly significant (******).

2.3 Results

2.3.1 Growth on Solid Agar

Various prepared concentrations of pantoprazole were spotted onto plates inoculated with a lawn of *C. jejuni* to visually determine whether the PPI pantoprazole was able to inhibit the growth of *Campylobacter*. At a concentration of 20 mg/ml pantoprazole (or 20,000 µg/ml) a clear zone of inhibition was visible in the agar (**Figure 7a**) and zones of inhibition were observed down to 8 mg/ml (or 8,000 µg/ml) pantoprazole (**Figure 7b**), with the edges of the zones becoming progressively less clearly defined as the PPI concentration was lowered. At 4 mg/ml pantoprazole (or 4,000 µg/ml) no inhibition was observed and the growth of *C. jejuni* was undisturbed (**Figure 7b**) as it was for the no PPI control. The PPI appeared not to diffuse through the agar and inhibition was limited to the area of direct exposure.



a *b*

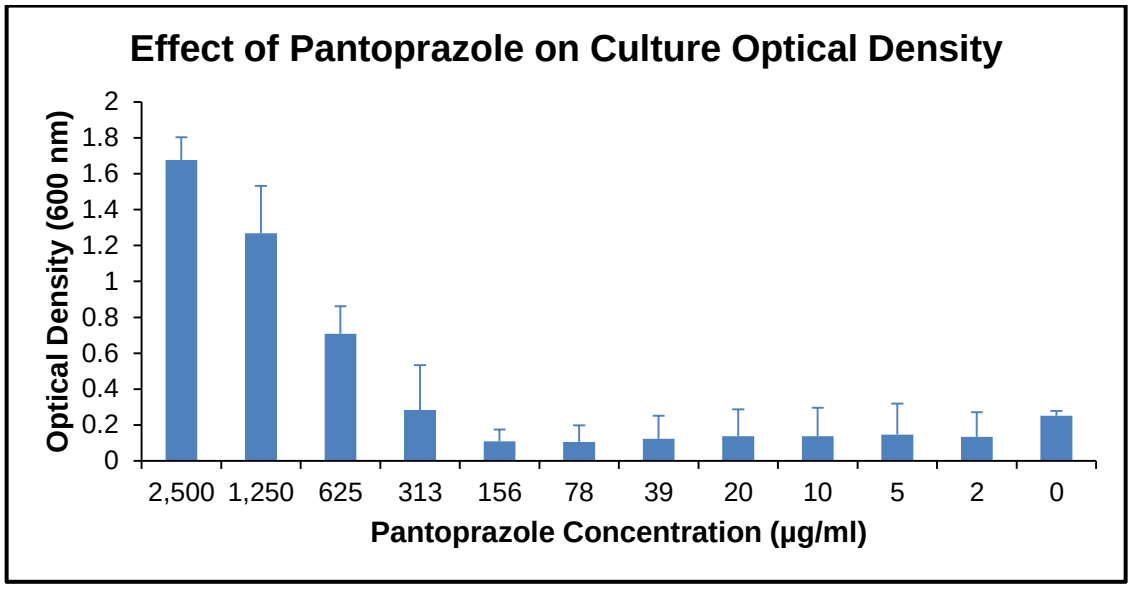
Figure 7. The PPI pantoprazole inhibits the growth of C. jejuni. *Pantoprazole solution, at the concentrations (mg/ml) indicated in white text above the relevant spots, was spotted onto a lawn of C. jejuni strain 81-176. Plates were then incubated for 24 hours before being inspected for zones of inhibition.*

Similar results were observed, with mg/ml concentrations of PPI inhibiting the growth of *C. jejuni* strains 11168-O and 81116 (data not shown). Direct inhibition of the growth of multiple strains of *C. jejuni*, observed using simple spot testing, prompted further investigation using standard methods for MIC and MBC determination.

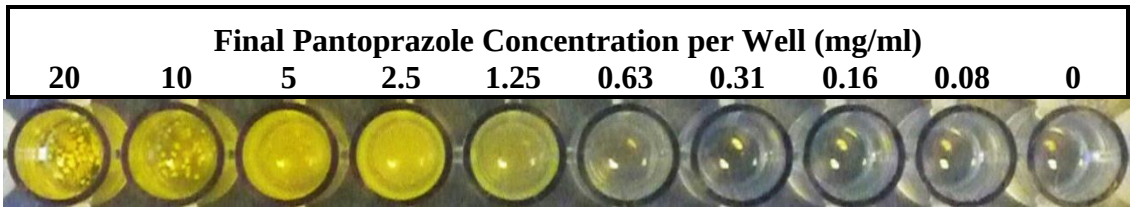
2.3.2 Pantoprazole Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

A standard MIC is determined by visually inspecting broth cultures, at various drug concentrations, for the lowest concentration at which growth inhibition can be observed,

indicated by the lack of turbidity caused by bacterial growth (van Alphen *et al.*, 2012). In some cases, a more objective result can be obtained by using an automatic plate reader to measure the optical density (OD) (Bamba *et al.*, 1997). Broth microdilution was used to try and determine the pantoprazole MIC for various strains of *C. jejuni*. The maximum achievable pantoprazole concentration in water was 40 mg/ml (personal observations) hence the maximum final PPI concentration in a standard broth MIC or MBC experiment, where equal volumes of bacterial suspension are mixed with an equal volume of the test agent, was 20 mg/ml. Results in **Figure 8** show that an MIC cannot be accurately determined for pantoprazole. Instead of turbidity increasing as the drug concentration decreases (as is normally the case in an MIC experiment) the opposite is true and the presence of the PPI itself causes an increase in turbidity.



a



b

Figure 8. Turbidity cannot be used to determine the MIC of pantoprazole for wild-type C. jejuni strains. *C. jejuni strain 11168-O was exposed to halving dilutions of PPI, in a broth microdilution experiment, for 24 hours, before OD_{600} was determined (a). Pantoprazole above 10 mg/ml comes out of solution after prolonged incubation and above 1.25 mg/ml a yellow colour develops, making it difficult to determine where lack of turbidity is due to lack of bacterial growth (b).*

It was found that, following 24 hours incubation at ≥ 1.25 mg/ml (or 1,250 µg/ml) pantoprazole, a yellow colour developed within the wells and at ≥ 10 mg/ml (or 10,000 µg/ml) problems with solubility developed (**Figure 8**). This made it difficult to determine a MIC by visual inspection or by reading the OD spectrophotometrically as cloudiness in the wells occurred at high PPI concentrations. Results show that ODs are much higher at high concentrations of PPI than even for the no PPI positive control.

In order to determine if the turbidity at high concentrations of PPI was due to solubility issues in MHB, the same test method was employed substituting MHB with MEM, DMEM or TSB. In all cases the results were similar to those achieved using MHB (data not shown) and an MIC could not be reliably determined either using an automatic plate reader or by visually inspecting wells. For this reason, MHB continued to be used for subsequent bacterial growth experiments, including MBC determinations.

The MBC is the concentration at which > 99.9% of a bacterial population are killed and this can be determined by exposing strains to varying concentrations of a drug and then plating aliquots onto agar which is free from the drug being tested (van Alphen *et al.*, 2012). MBC results obtained using broth microdilution for various strains of *C. jejuni* are shown in **Table 8**.

Table 8. The MBC of the PPI pantoprazole for different strains of C. jejuni falls within a similar range.

***C. jejuni* Strain**	**Pantoprazole MBC (mg/ml)**			
	4 Hour Exposure		**24 Hour Exposure**	
	Mean +/- SD	**Range**	**Mean +/- SD**	**Range**
11168-O	3.30 +/- 1.30	4.50-2.00	0.83 +/- 0.32	0.63-1.25
81-176	2.90 +/- 1.00	4.00-2.00	0.93 +/- 0.34	0.63-1.25
81116	3.30 +/- 1.30	4.50-2.00	0.99 +/- 0.42	0.63-1.25

Following exposure to PPI in MHB for 4 or 24 hours in an MIC experiment, 10 µl aliquots were removed from the wells and spotted onto MHA + B plates to determine the MBC.

These data support the observation in **Figure 7** that direct contact between *C. jejuni* and the PPI pantoprazole is deleterious to *Campylobacter* survival. The PPI MBC following 4 hours exposure was found to be 3.3 mg/ml (or 3,300 µg/ml) for two of the *C. jejuni* strains tested and 2.9 (or 2,900 µg/ml) for another (**Table 8**). The concentration required to kill lowers when the exposure time is extended, such that following 24 hours exposure, around 1 mg/ml (or 1,000 µg/ml), is bactericidal to *C. jejuni* strains (**Table 8**).

Following exposure to concentrations of PPI above 1.25 mg/ml (or 1,250 µg/ml) for 24 hours, no live *Campylobacter* could be revived when aliquots were removed and inoculated into fresh broths containing no PPI and incubated for another 24 hours in the absence of PPI.

Neither *S.* Typhimurium or *Lactobacillus* sp. demonstrated any susceptibility to PPI (**Table 9**) and the bacterial populations were able to survive exposures up to 20 mg/ml (or 20,000 µg/ml) pantoprazole, which is over 20× the MBC for *C. jejuni*. A similar number of colonies were isolated from 10 µl aliquots that had been exposed to 20 mg/ml (or 20,000 µg/ml) pantoprazole for 24 hours as were isolated from the no PPI control (**Figure 9**).

Table 9. The PPI pantoprazole does not kill S. Typhimurium or Lactobacillus.

Strain	**Pantoprazole MBC (mg/ml)**	
	4 Hour Exposure	**24 Hour Exposure**
***S.* Typhimurium SL1344**	> 20	> 20
***Lactobacillus* sp.**	> 20	> 20

Following exposure to PPI in MHB for 4 or 24 hours in an MIC experiment, aliquots were removed from the wells and spotted onto MHA + B plates to determine the MBC.

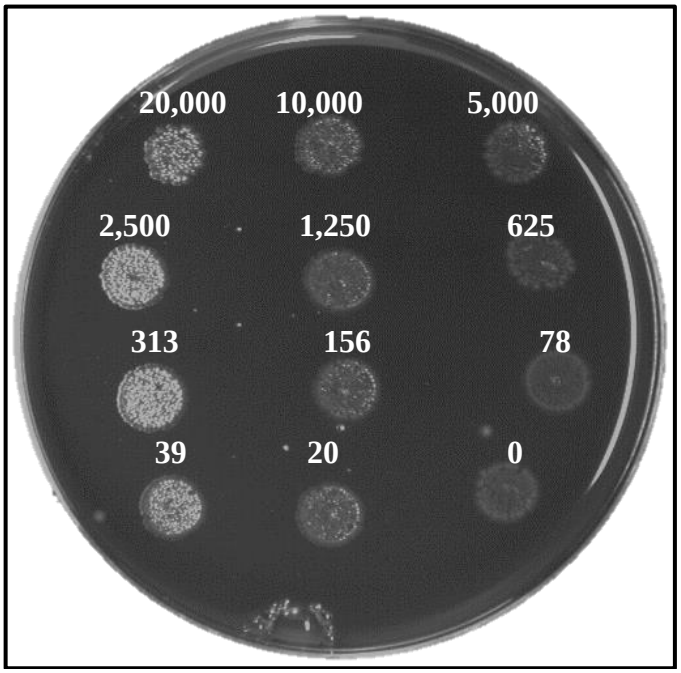
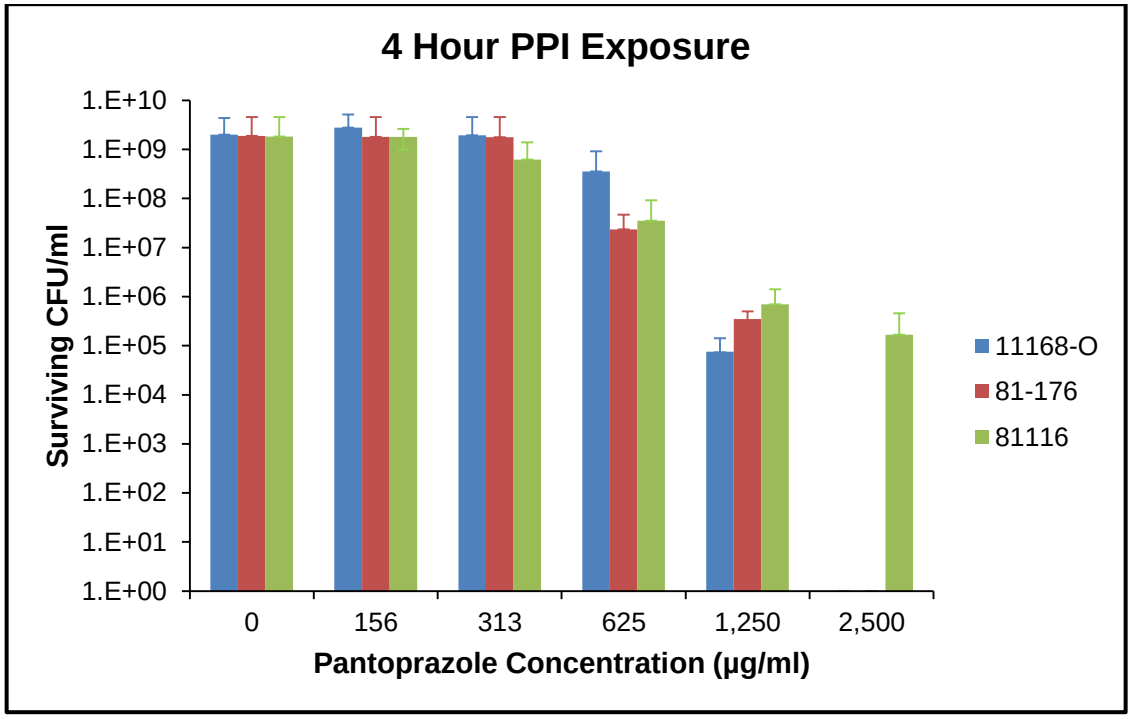


Figure 9. Lactobacillus displays no susceptibility to pantoprazole up to a concentration of 20 mg/ml (or 20,000 µg/ml). *Lactobacillus sp. in MHB were exposed to varying concentrations of pantoprazole (as indicated in µg/ml in the white text above individual spots) for 24 hours before 10 µl aliquots were removed and plated onto MHA + B. Plates were incubated aerobically for 24 hours before being examined for the presence of Lactobacillus and photographed using a Gel Doc system.*

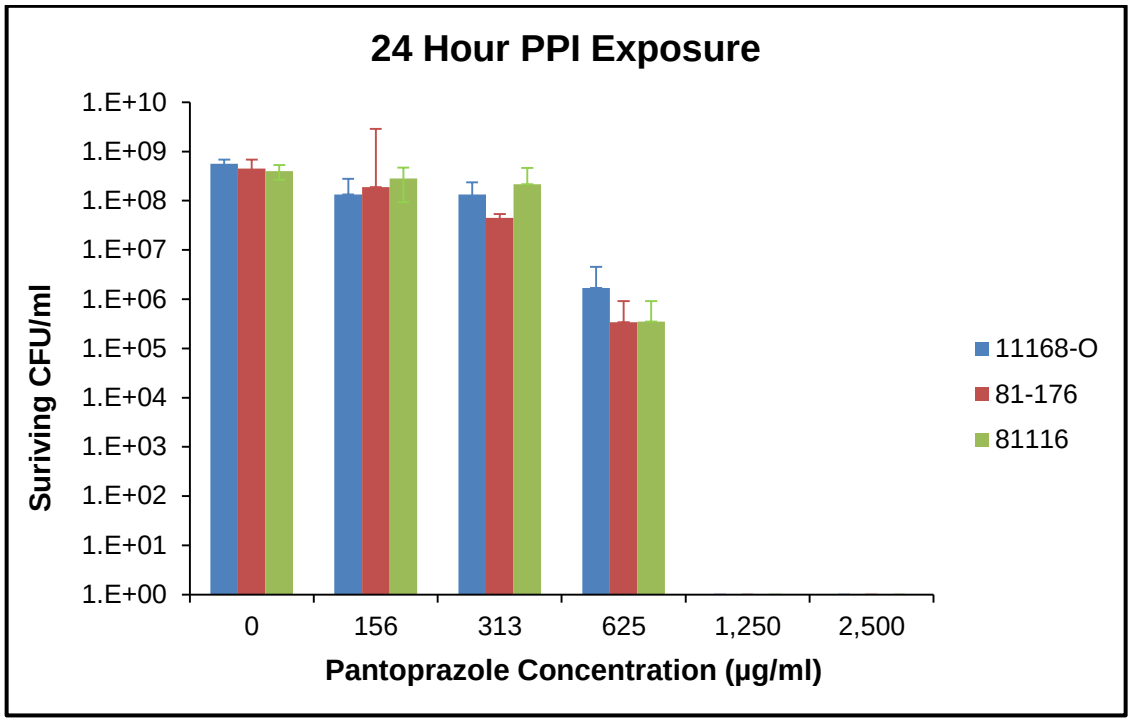
2.3.3 Survival Studies

To investigate if exposure to PPI at concentrations lower than the MBC had an effect on *C. jejuni* survival, samples were diluted in PBS and remaining viable bacteria were enumerated, following exposure to various concentrations of PPI for 4 or 24 hours. Results in **Figure 10a** show that following 4 hours PPI exposure there was more variability in the susceptibility of *C. jejuni* strains, but following 24 hours exposure, multiple strains of *C. jejuni* show very similar susceptibility patterns (**Figure 10b**). At the higher concentrations of pantoprazole tested, there are fewer *C. jejuni* surviving than at lower concentrations (**Figure 10a** and **b**). In **Figure 10b** where strains had been exposed to the PPI for 24 hours there was no bacterial survival above 1.25 mg/ml (or 1,250 µg/ml) for any of the three *C. jejuni* strains tested and this was to be expected as this exceeds the MBC.

Having determined that *C. jejuni* was killed by exposure to mg/ml concentrations of PPI, experiments were performed using *S.* Typhimurium to investigate whether another, non-spiral, Gram negative enteric pathogen might be similarly affected. Results in **Figure 11** show that, even following 24 hours exposure to concentrations of PPI four times higher than those required to kill *C. jejuni*, no effect on *S.* Typhimurium survival was evident. This is in support of the MBC data in **Section 2.3.2**, where the MBC of pantoprazole was > 20 mg/ml (or 20,000 µg/ml) pantoprazole for *S.* Typhimurium.



a



b

Figure 10. Exposure to the PPI pantoprazole affects C. jejuni strain survival in a dose dependent manner. *Aliquots were removed, serially diluted and surviving bacteria were calculated following exposure in MHB to varying concentrations of PPI for 4 hours (a) or 24 hours (b).*

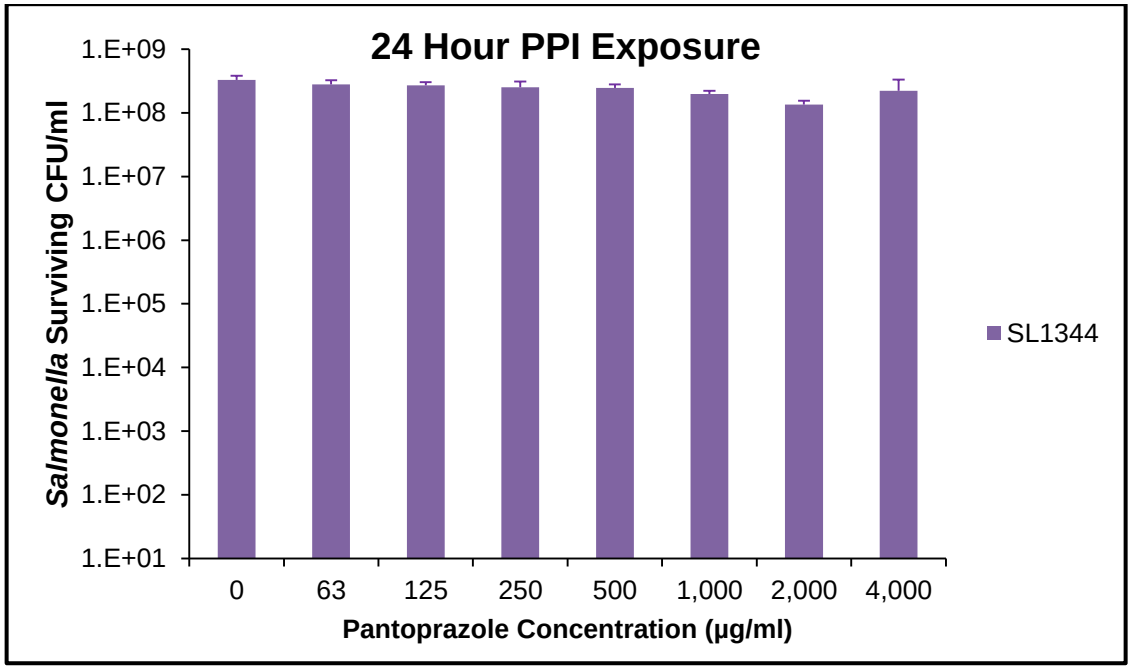
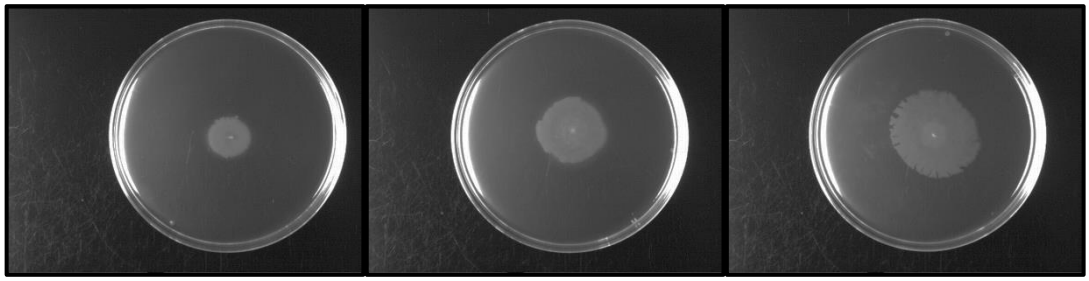


Figure 11. Exposure to the PPI pantoprazole does not affect S. Typhimurium survival. *Aliquots were removed, serially diluted and surviving bacteria were calculated following exposure to varying concentrations of PPI for 24 hours.*

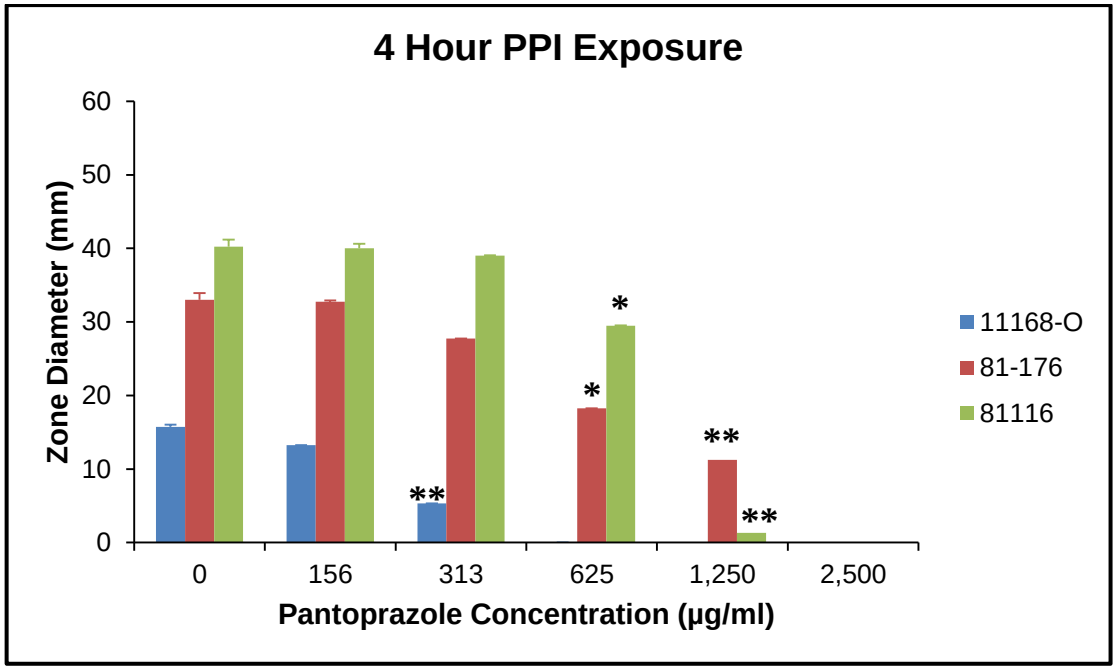
2.3.4 Motility Testing

Experiments were performed using soft agar motility to determine whether exposure to sub lethal levels of PPI could interfere with the motility of *C. jejuni.* Results in **Figure 12** confirm that exposure to PPI does inhibit the motility of *C. jejuni* strains as motility decreases as the PPI concentration increases.

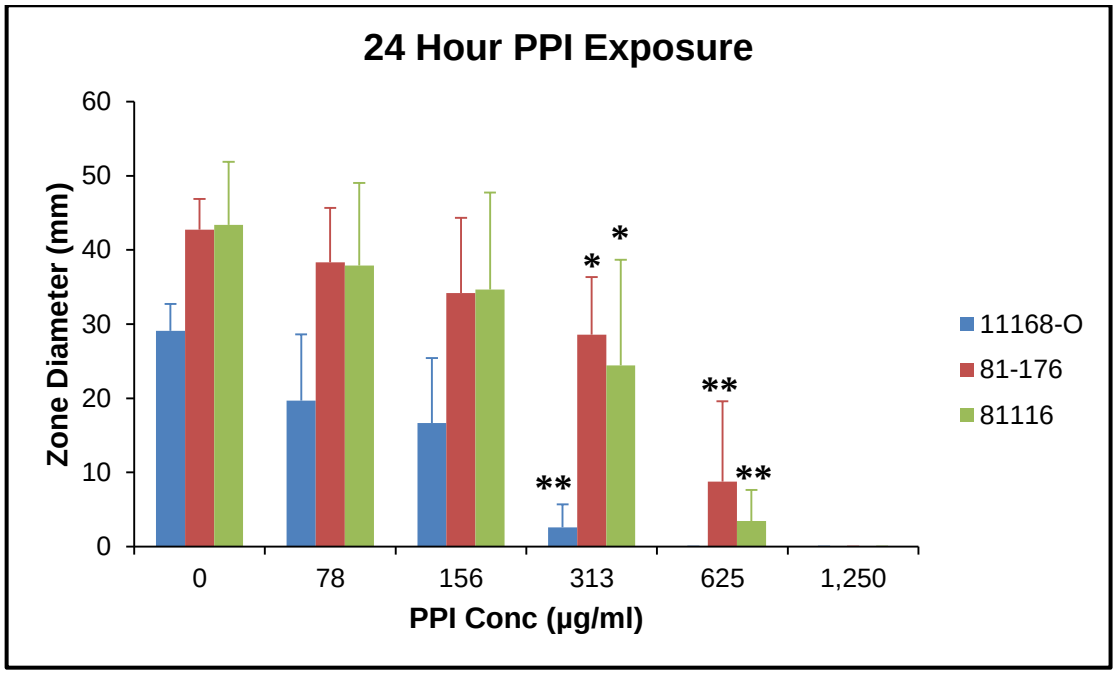
At the same time as the testing was performed to assess the motility of *C. jejuni* strains, serial dilutions were made to determine the numbers of bacteria surviving the exposures to the various PPI concentrations. In **Figure 10a**, the log CFU/ml counts for strain 11168-O (shown in **blue**) remains quite steady from the concentration 0 to 313 µg/ml. Yet when the motility of these surviving bacteria was measured (**Figure 12**) a highly significant difference in the motility of 11168-O exposed to 313 µg/ml was seen compared to the no PPI exposed control (P =.0.0046). Following exposure of 11168-O to 625, 313 and 156 µg/ml pantoprazole for 4 hours, the average zone diameter was found to be 0, 5.3 and 13.3 mm respectively whilst the remaining viable CFU/ml were 1.5×10^7, 3.0×10^7 and 3.5×10^7 respectively.



a *b* *c*



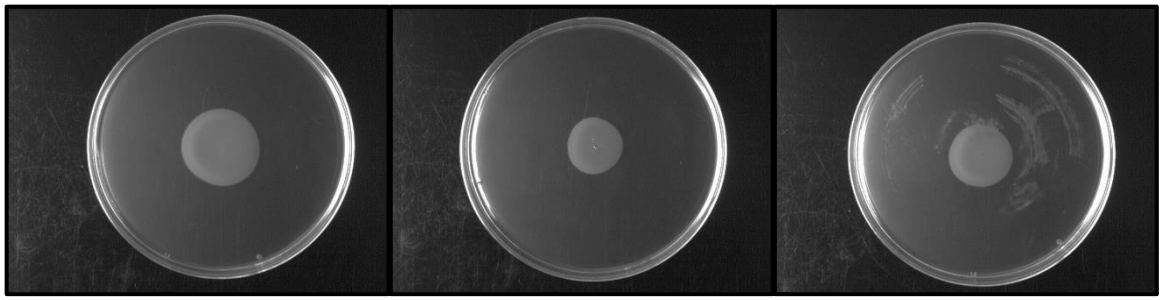
d



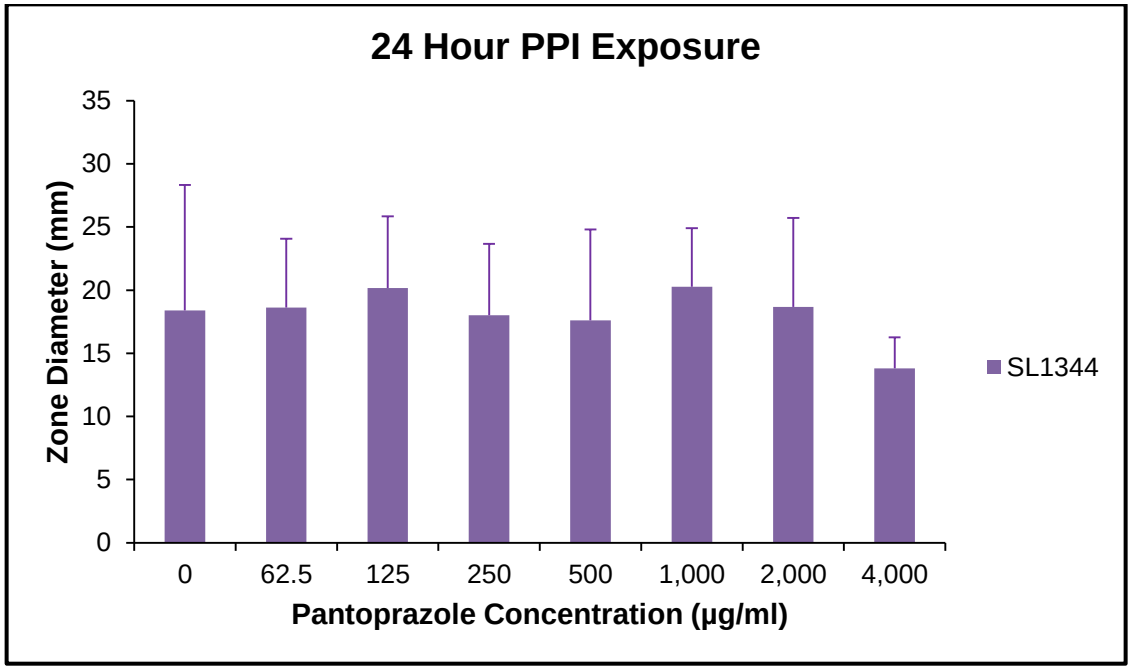
e

Figure 12. Exposure to the PPI pantoprazole adversely affects C. jejuni motility. *C. jejuni strain 11168-O was exposed to PPI at 1,000 (a), 500 (b) and 0 µg/ml (c) for 4 hours before aliquots were stabbed into 0.4% SSA. Three strains of C. jejuni were exposed to various concentrations of the PPI pantoprazole for 4 (d) or 24 hours (e) before having 1 µl aliquots stabbed into SSA. Plates were then incubated for 48 hours before the diameters of the zones were measured in mm and mean +/- SD plotted. Levels of significance, as indicated by* ***** *(P value > 0.01 but < 0.05) or* ****** *(P value < 0.01) relate to the individual test conditions compared to the no PPI control for the same strain.*

Similar experiments performed using *Salmonella* in contrast, found that *Salmonella* motility was not affected by exposure to PPIs (**Figure 13**).



a *b* *c*



d

Figure 13. Exposure to the PPI pantoprazole does not affect the motility of S. Typhimurium. *S. Typhimurium strain SL1344 was exposed to PPI at 1,000 (a), 500 (b) and 0 µg/ml (c) for 4 hours before aliquots were stabbed into SSA. SL1344 was exposed to various concentrations of the PPI pantoprazole for 24 hours (d) before having 1 µl aliquots stabbed into SSA. Plates were then incubated for 24 hours before the diameter of the zones was measured in mm and mean +/- SD plotted.*

2.3.5 Biofilm Formation

Crystal violet assays were used to assess the ability of *C. jejuni* (pre-exposed to PPI for 2 hours) to then form biofilms. Microscopy was also used to visualise the biofilm, with and without staining. Results in **Figure 14** show that in the absence of PPI, *C. jejuni* produced more biofilm at 48 hours than at 24 hours and still more at 72 hours (the **blue**, **red** and **green** bars on the far right of the graph). At 24 hours there was no significant difference in the ability to form biofilm at any of the concentrations of PPI tested (**blue** bars), but the biofilm formed in only 24 hours was quite small, even for the no PPI control.

At 48 hours however (**red** bars), compared to the no PPI control, pre-exposure to 500, 250 and 125 µg/ml PPI for 2 hours significantly reduced ability to form biofilm (P = 0.005, P = 0.003 and P = 0.011 respectively). At 72 hours (**green** bars), pre-exposure to 500 µg/ml PPI for 2 hours was the only concentration to significantly decrease biofilm (P = 0.021) Similar results were seen for *C. jejuni* 11168-O and 81-176, with pre-exposure to some PPI concentrations affecting ability to form biofilm (data not shown).

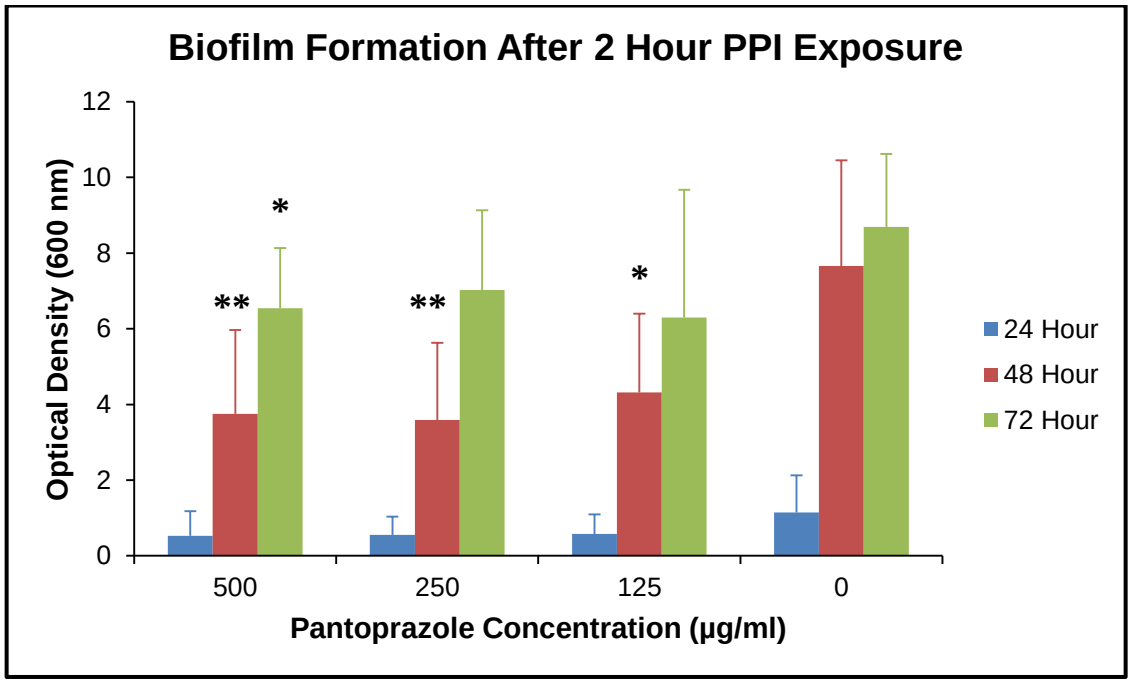


Figure 14. Effect of pre-exposure to the PPI pantoprazole on the ability of C. jejuni to form biofilm. *C. jejuni strain 81116 was exposed to varying concentrations of PPI in MHB for 2 hours before PPI was removed by pelleting and washing bacteria. Samples were then resuspended in fresh broth and corrected to the same* OD_{600} *before being allowed to form biofilm in wells of 96 well microtitre plates for 24, 48 or 72 hours. Levels of significance, as indicated by* ***** *(P value > 0.01 but < 0.05) or* ****** *(P value < 0.01) relate to the individual test conditions compared to the no PPI control for the same incubation time.*

Results in **Figure 14** indicate that biofilm formation at 48 hours was most affected by pre-exposure to PPI and **Figure 15** shows that these results were confirmed using microscopy. The no PPI control shows a dense structured biofilm using fluorescent microscopy (**Figure 15f**) and multiple patches of heavily clumped bacteria using inverted light microscopy (**Figure 15e**). *C. jejuni* pre-exposed to 250 µg/ml displays disruption to the biofilm with fewer patches of heavily clumped bacteria and large spaces between clumps (**Figure 15c** and **d**). *C. jejuni* pre-exposed to 500 µg/ml shows no apparent biofilm structure with individual bacterial cells rather than clumps or structured biofilm (**Figure 15a** and **b**).

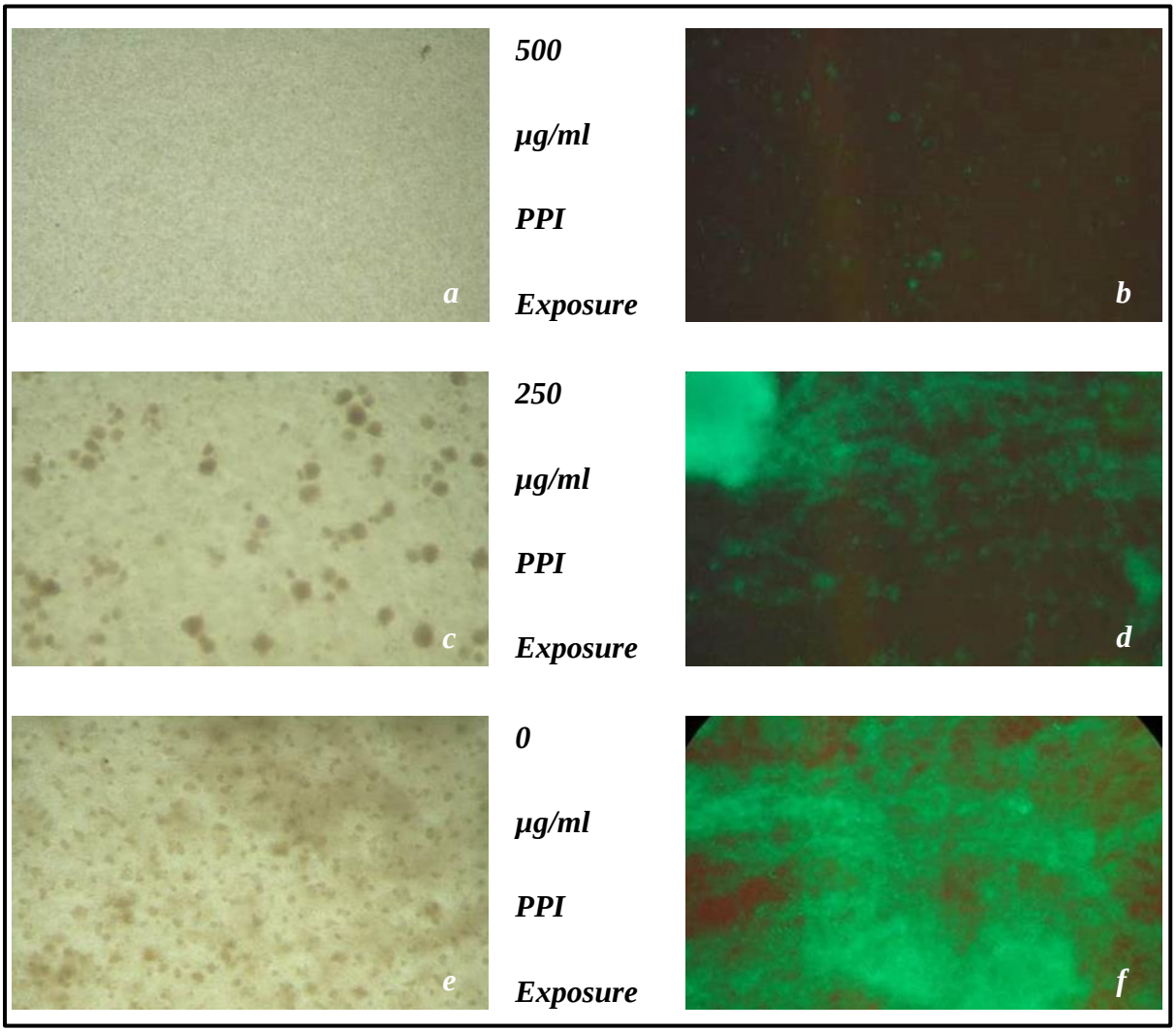


Figure 15. Pre exposure to the PPI pantoprazole for 2 hours affects C. jejuni ability to form biofilm at 48 hours. *C. jejuni 81-176 was exposed to 500 (a and b), 250 (c and d) and 0 µg/ml (e and f) pantoprazole in MHB for 2 hours before being allowed to form biofilm in wells of 96 well microtitre plates for 48 hours. Biofilms were viewed using a normal inverted light microscope at x400 magnification (a, c and e) and stained with LIVE/DEAD® BacLight™ stain then viewed using a fluorescent microscope (b, d and f) at x400 magnification.*

2.3.6 Adhesion and Invasion

Gentamicin protection assays were used to determine if exposure to PPI affected ability of *C. jejuni* to adhere to and/or invade Caco-2 cells. Caco-2 cells originated from a human colonic adenocarcinoma (Louwen *et al.*, 2012) and form polarised monolayers (Friis *et al.*, 2005) which can be used as models for the absorptive epithelial cells of the gut (MacCallum *et al.*, 2005). Results in **Figure 16** show that the decrease in adherent *C. jejuni* on exposure to pantoprazole does not reach statistical significance. The reduction in invasion of Caco-2 cells was highly significant at all concentrations of PPI tested (**Figure 16**). The P values for 250, 500 and 1,000 µg/ml pantoprazole exposed versus the no PPI exposed invasion control were 0.00009, 0.00008 and 0.00006 respectively.

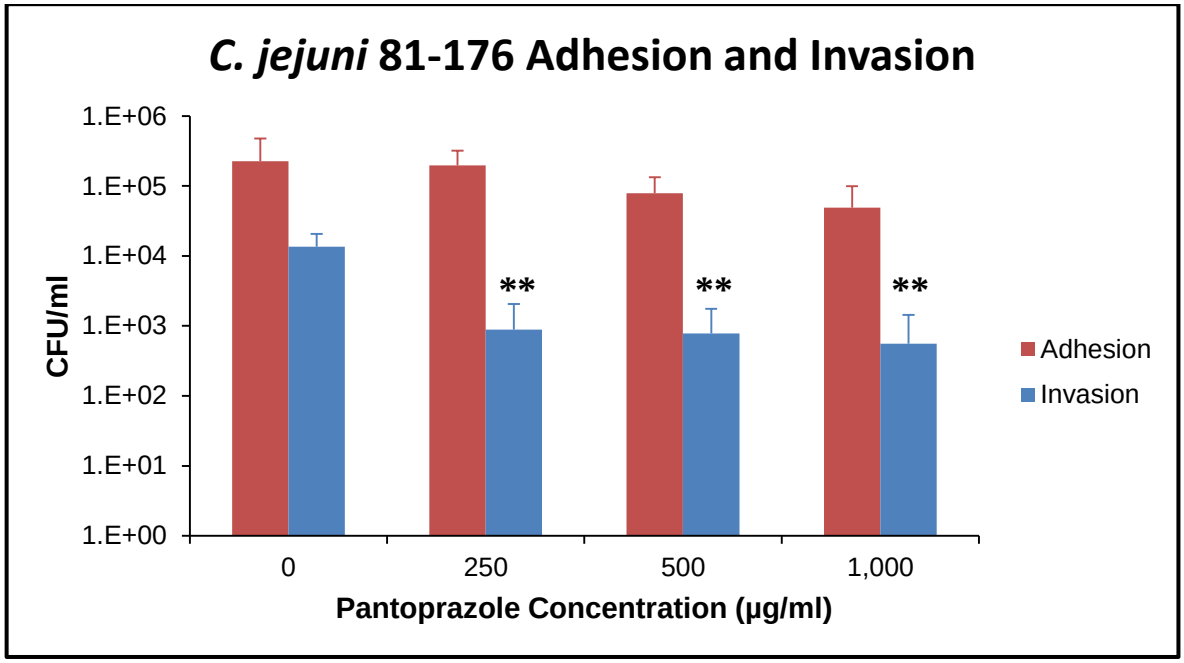


Figure 16. Exposure to the PPI pantoprazole significantly decreases invasion of Caco-2 cells by C. jejuni. *Fully confluent monolayers of Caco-2 cells were infected with C. jejuni 81-176 at varying concentrations of PPI for 4 hours before adherent and invaded organisms were quantified. Levels of significance, as indicated by* ** *(P value < 0.01) relate to the individual test conditions compared to the relevant no PPI control.*

2.3.7 Electron Microscopy

Results in **Figure 17a** show that prolonged exposure to concentrations of pantoprazole well above the MBC causes *C. jejuni* to change into atypical coccal forms which have lost their flagella. At around two times the MBC (**Figure 17b**) we see the population is a mixture of cells in typical spiral morphology, some with intact long smooth flagella and we also see atypical coccal forms which have shortened or absent flagella. In some cells we see evidence of membrane blebbing (indicated by a white arrow in **Figure 17b**). When the PPI concentration that *C. jejuni* has been exposed to, is lowered to below the MBC (**Figure 17c**), we find that most of the population is in typical spiral morphology with a few coccal forms also being present. *C. jejuni* cells which have not been exposed to PPI (**Figure 17d**) appear spiral, with intact flagella and smooth, bleb free membranes. Similar results were also obtained for strains 81-176 and 81116 (data not shown).

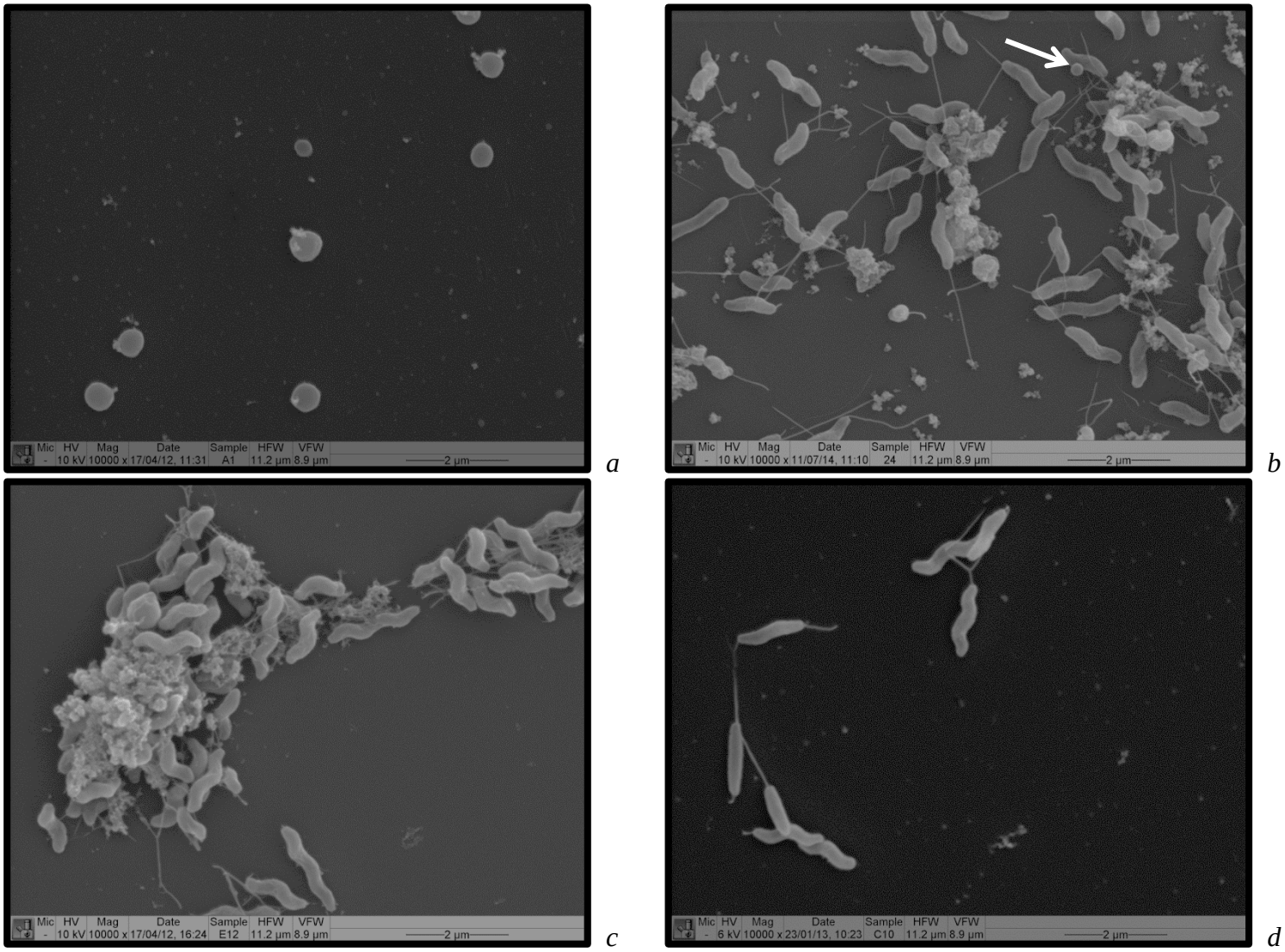


Figure 17. Prolonged exposure to high concentrations of PPI affects C. jejuni morphology. *C. jejuni 11168-O in MHB was exposed to 10,000 (a), 2,000 (b), 600 (c) and 0 µg/ml (d) pantoprazole for 24 hours. Morphology switches from atypical coccal forms following exposure to high concentrations of PPI to more typical spiral morphology at low PPI concentrations. A white arrow is used to highlight membrane blebbing.*

2.4 Discussion

2.4.1 Growth on Solid Agar

Spotting of pantoprazole onto solid agar clearly shows that direct application of PPI inhibits the growth of *C. jejuni* at mg/ml concentrations (**Figure 7**). It is however worth noting that the concentrations of conventional antibiotics generally required to inhibit bacteria are usually within the µg/ml range and so the concentrations of PPI shown here to inhibit growth are much higher than those required for conventional antibiotics. Other authors have concluded in the past that PPIs were not able to kill *Campylobacter* spp. (Iwahi *et al.*, 1991, Megraud *et al.*, 1991, Sjostrom *et al.*, 1996, Tsutsui *et al.*, 2000), but the maximum documented concentration previously tested was 0.256 mg/ml (or 256 µg/ml). Results obtained by direct spotting, suggest that the concentration required to inhibit *C. jejuni* growth is much higher than the concentration previously tested by others.

The molecular weight of pantoprazole is 383 and the molecular weight of the pantoprazole sodium hydrate used in this study is 405 and this is comparable with conventional antibiotics that commonly diffuse through agar. However results in **Figure 7** make it clear that the PPI does not diffuse through the agar and *C. jejuni* inhibition is limited to areas of direct contact.

It is thought that agar dilution methods may be less accurate than broth microdilution (Klancnik *et al.*, 2010) in determining inhibitory concentrations and that broth culture more closely resembles the *in vivo* environment (Trautmann *et al.*, 1999). The stability of PPIs in agar has also been questioned (Trautmann *et al.*, 1999). Agar dilution also requires far greater quantities of drug than broth microdilution and is therefore not often used when the cost of the drug is high, as is the case with pantoprazole sodium hydrate. For these reasons MIC and MBCs were performed using broth microdilution in this study.

2.4.2 Pantoprazole Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

Dimethyl sulfoxide (DMSO) has been used extensively by others in the past, as the solvent for PPIs, with some researchers further diluting in DMSO (Trautmann *et al.*, 1999) and others diluting in water (Midolo *et al.*, 1997). The use of DMSO is unlikely to closely mimic the *in vivo* environment as prescription drugs are likely to be taken with water. This study has utilised the PPI pantoprazole (due to its superior ability to dissolve in water and give accurate concentrations) but in other anti-bacterial studies it is one of the least commonly tested PPIs (see **Tables 4** and **5**).

PPIs such as pantoprazole are prodrugs that undergo acid activation to form sulfenic acids and subsequent dehydration to form cyclic sulfenamides (Shin *et al.*, 2004). The conversion occurs more quickly at acidic pH than at neutral pH, but can occur *in vitro* at neutral pH given time (Sachs *et al.*, 1995). The sulfenic acids and cyclic sulfenamides are highly reactive and change form quickly to generate more compounds than can be quantitated accurately (Shin *et al.*, 2004). Acid activated PPIs at high concentrations are not very soluble in water (Shin *et al.*, 2004). Acid activation of the prodrug probably occurs *in vitro* during long incubation periods (Suerbaum *et al.*, 1991).

It is known that a number of the intermediates that PPIs become converted to, upon protonation, are yellow in colour (Nguyen *et al.*, 2005). At high *in vitro* (and therefore in the absence of parietal cell H^+/K^+-ATPases to bind to) concentrations of PPI presumably more of the PPI is converted to yellow coloured intermediates and this caused the problems with determining MIC using visual inspection or automated OD detection as shown in **Figure 8**. The insolubility of pantoprazole and its acid activated intermediates at very high concentrations also contributed to the difficulty in determining the MIC. MIC has been determined by others by inoculating an actively growing culture onto agar plates containing a range of PPI concentrations (Suerbaum *et al.*, 1991, Shibata *et al.*, 1995), but for reasons discussed at the end of **Section 2.4.1** agar dilution was not used in this study and MBC was investigated instead.

Whether the anti-bacterial activity of PPIs was bacteriostatic (Megraud *et al.*, 1991), bactericidal (Midolo *et al.*, 1997) or both (Mirshahi *et al.*, 1998) for *H. pylori* has been heavily disputed. Results presented here demonstrate that an accurate MBC can be determined and pantoprazole was found to be bactericidal, at 24 hours, to multiple strains of *C. jejuni* at around 1 mg/ml (or 1,000 µg/ml) (**Table 8**). It is likely that the activity of PPIs is indeed truly bactericidal as attempts to revive any live *C. jejuni* from broths containing concentrations of PPI higher than the cidal level, repeatedly failed to produce any live bacteria. No killing, inhibition of *S.* Typhimurium growth or motility was observed using the same methods used for *C. jejuni* up to a maximum concentration of 20 mg/ml (or 20,000 µg/ml). A strain of *Lactobacillus* isolated from a young C57BL/6 healthy mouse also demonstrated no susceptibility to the bactericidal action of pantoprazole.

2.4.3 Motility Testing

When the motility of 11168-O exposed to 625 µg/ml pantoprazole is compared to the same strain exposed to 313 µg/ml pantoprazole, where surviving CFU/ml remains fairly steady,

we find that bacteria exposed to the lower PPI concentration for 4 hours are highly significantly more motile than those exposed to the higher PPI concentration (P = 0.005). The reduction in the zone diameters seen in **Figure 12** is therefore not merely a reflection of the number of live bacteria present in the 1 µl aliquot being stabbed into the semi-solid agar, but rather a display of reduced bacterial motility. We also see in **Figure 12** that strains 81-176 and 81116 are naturally more motile than strain 11168-O. Results indicate that the motility of *C. jejuni* strains is severely affected following exposure to pantoprazole for 4 or 24 hours. The effect is more pronounced the higher the PPI concentration present, with no growth at all being observed at concentrations above the MBC. For all three strains tested, as the PPI concentration decreases, motility increases. It is possible that the energy dependent motion of the flagellar motor is being targeted by the pantoprazole and that is why motility decreases as PPI concentration rises or exposure time increases. The minimum pantoprazole concentration which consistently significantly reduced bacterial motility in all three of the *C. jejuni* strains tested was 625 µg/ml following 4 hours pantoprazole exposure and 313 µg/ml following 24 hours pantoprazole exposure.

Tsutsui *et al* reported that the PPIs lansoprazole, omeprazole and rabeprazole adversely affected the motility of *Campylobacter* strains, at a concentration of 16 µg/ml (Tsutsui *et al.*, 2000). This study has demonstrated that the PPI pantoprazole is also able to adversely affect the motility of *Campylobacter* strains and that this effect becomes more pronounced with exposure to even higher concentrations of PPI than were tested by Tsutsui *et al*.

2.4.4 Biofilm Formation

The ability of short pre-exposures to PPI to affect biofilm formation were most apparent following 48 hours of biofilm formation (**Figure 15**). Exposure to concentrations of PPI as low as 125 µg/ml was shown to significantly reduce ability to form biofilm. *C. jejuni* forms biofilms on the surfaces of chicken meat and biofilm formation is known to be important for survival in the environment (Gundogdu *et al.*, 2011). Some bacteria exist in the colon in biofilms (Slonczewski *et al.*, 2009) where they are protected and better able to persist. Motility is known to be important for biofilm formation (Kalmokoff *et al.*, 2006) and defects in motility are known to cause defects in biofilm formation (Reuter *et al.*, 2010). Results in **Figure 12** show that the motility of *C. jejuni* was adversely affected by exposure to sub-lethal concentrations of PPI and it is therefore unsurprising that biofilm formation was also found to be impaired. Loss of biofilm formation ability may also suggest that pantoprazole exposure affects the quorum sensing ability of *C. jejuni*.

2.4.5 Adhesion and Invasion

Nakao *et al* reported in 1995 that exposure to even sub-MIC levels of lansoprazole for 1 hour resulted in a decreased ability of *H. pylori* to adhere to HEp-2 cells (Nakao *et al.*, 1995). Motility is required by *C. jejuni* for invasion of epithelial cells (Everest, 2002) and we have already demonstrated that PPI exposure affects the motility of *C. jejuni* (**Figure 12**). Results in **Figure 16** show that ability of *C. jejuni* to adhere to Caco-2 cells is not significantly affected by PPI exposure but that the ability to invade them was. The reduction in invasion of Caco-2 cells was highly significant at all concentrations of PPI tested, even as low as 250 µg/ml. The ability of *C. jejuni* to invade cells is considered a virulence factor (Zilbauer *et al.*, 2008), with some strains being notably more invasive than others and highly invasive strains are considered more pathogenic (Pesci *et al.*, 1994). It has also been suggested that severity of disease following infection with *C. jejuni* may be linked to differences in strain ability to invade epithelial cells (Friis *et al.*, 2005, Dasti *et al.*, 2010). It is clear that, even at sub lethal levels, the invasion potential of *C. jejuni*, and therefore also its pathogenicity or ability to cause invasive disease, is significantly diminished in the presence of PPIs.

2.4.6 Electron Microscopy

Results suggest that at concentrations of pantoprazole well above the MBC, cell lysis occurs as far fewer cells could be detected per field compared to the no PPI exposed control and those samples exposed to low concentrations of pantoprazole. Bacteriolysis can be indicated by a loss of turbidity and/or an increase in viscosity in broth cultures (Iwahi *et al.*, 1991) but due to issues with colour, insolubility and turbidity at concentrations of pantoprazole well above the MBC (**Figure 8**) this was unable to be confirmed. The only cells which can be seen at concentrations of pantoprazole around 10× the MBC are atypical coccal forms, which are thought to be degenerate forms of *Campylobacter* (Ikeda & Karlyshev, 2012).

In studies using *Helicobacter*, changes in morphology following PPI exposure (Megraud *et al.*, 1991, Nakao *et al.*, 1995) were noted as well as membrane blebbing (Iwahi *et al.*, 1991, Nakao & Malfertheiner, 1998). We have shown similar effects in the related Gram negative spiral pathogen *C. jejuni* (**Figure 17a** and **b**).

2.5 Summary and Conclusions

Direct spotting of a PPI solution, at a concentration of ≥ 8 mg/ml (or 8,000 µg/ml) onto the surface of agar plates inoculated with *C. jejuni* results in inhibition of growth. Broth microdilution shows the PPI pantoprazole is bactericidal at 24 hours to multiple strains of *C. jejuni* at a concentration of around 1 mg/ml (or 1,000 µg/ml). Inhibition of *C. jejuni* growth by pantoprazole occurs at concentrations lower than the bactericidal concentration and anti-*Campylobacter* activity is both concentration and time dependent. Motility testing indicates that the motility of *C. jejuni* strains is affected in a dose dependent manner following exposure to sub lethal concentrations of PPI. No inhibition of *S.* Typhimurium growth or motility was observed using the same methods. No growth inhibition or killing was observed for a strain of *Lactobacillus* isolated from a healthy mouse. Pre-exposure to sub lethal concentrations of PPI affects the ability of *C. jejuni* to produce biofilm. Gentamicin protection assays show that *C. jejuni* exposed to sub lethal concentrations of PPI were significantly less able to invade Caco-2 cells than the no PPI exposed control. EM analysis shows that exposure to lethal concentrations of PPI leads to a change from *C. jejuni* typical spiral morphology to atypical coccal forms, often lacking flagella.

C. jejuni virulence is dependent on a number of factors including the flagella, related motility and ability to adhere to and/or invade epithelial cells (Wassenaar & Blaser, 1999). We have shown that the PPI pantoprazole is bactericidal to multiple strains of *C. jejuni* and that motility of the organism is reduced, even at sub lethal levels of PPI. It is possible that the PPI affects the energy dependent mechanism of the flagellar motor of *C. jejuni*, thereby affecting *C. jejuni* motility. The reduction in motility seen was also found to affect the ability of *C. jejuni* to form biofilm and invade epithelial cells. Exposure to high concentrations of PPI has been shown to result in a loss of flagella and conversion to degenerate coccal forms of *C. jejuni*. We have postulated that the concentrations required to achieve these outcomes might be achievable in the human stomach, especially in those patients taking higher doses of PPIs for severe disease on an empty stomach (see **Table 3**). It is therefore important to further investigate the interactions between PPIs and *C. jejuni* as these data suggest the pathogenicity of the organism might be affected by exposure to PPIs. People taking PPIs are reportedly at higher risk of developing enteric infections like campylobacteriosis (Tam *et al.*, 2009). However, our results suggest that some people may be protected from developing campylobacteriosis due to the bactericidal action of PPIs on the organism or sub-inhibitory effects on motility and invasion. Crucially, this would depend on the concentrations and duration of exposure encountered by the organism.

Chapter 3

Investigation of the Possible Mechanisms of Pantoprazole Action on *Campylobacter jejuni*

3 INTRODUCTION

Proton pumps are ATPases, which break down ATP (in an energy dependent process) to regulate the movement of ions across the cell wall. Bacteria have a variety of proton pumps on their cell surface that act to control processes like turgor pressure, intracellular pH and the proton motive force. Disruption of the proton motive force affects the rotation of bacterial flagella and subsequently affects motility (Manson *et al.*, 1977). Exposure to pantoprazole has been shown in **Chapter 2** to affect *C. jejuni* motility.

PPIs are known to covalently bind to sulfhydryl groups on accessible cysteine residues found on the H^+/K^+-ATPase of parietal cells. The urease of *H. pylori* has four cysteine residues in its sequence. PPIs are known to inhibit the urease enzyme of *H. pylori* and it has been suggested that this may be the result of PPIs targeting these cysteines and thereby inhibiting the urease (Park *et al.*, 1996). However, it is now known that targeting of the urease by PPIs is not responsible for the growth inhibition and/or killing of *H. pylori* by PPIs as urease negative strains of *H. pylori* are also susceptible to the anti-bacterial activity of PPIs (Nagata *et al.*, 1995, Logan, 1996, Mirshahi *et al.*, 1998).

C. jejuni has a number of ATPases on its surface with available cysteines that may be potential targets for PPIs. It is of course possible that PPIs have multiple *C. jejuni* targets (as appears to be the case for *H. pylori*), which may or may not include bacterial ATPases. A variety of experiments were therefore performed to investigate intracellular changes to *C. jejuni* in response to pantoprazole exposure.

3.1.1 Use of Proteomic Analysis

Protein production is vital to all living organisms and the term proteomics is used to describe the large scale study of proteins. One of the most common methods of analysing all of an organisms proteins (the proteome) is by using two dimensional (2D) gel electrophoresis. Diverse proteins will have specific isoelectric points depending on their own specific mass and structure (i.e. the numbers of positively charged and negatively charged side chains present in the sequence) and at all pH values other than their own isoelectric point, proteins will be either positively or negatively charged (depending on their own individual properties). A protein sample can therefore be applied to a gel strip which has been impregnated with an immobilised pH gradient. Then an electric current can be applied and used to separate proteins along the length of the strip where individual proteins with the same isoelectric point will migrate to the point where they are neither

negatively nor positively charged. This is commonly referred to as the first dimension of the protein separation.

The proteins on the gel strip can then be treated with sodium dodecyl sulfate (SDS) which denatures the proteins and proteins with a large mass will unfold into long molecules and proteins with a small mass will unfold into shorter molecules. The number of SDS molecules that bind to a protein is proportional to the length (and therefore the original mass) of a protein. So a protein with a large mass, will denature to a long strand and bind many SDS molecules and a protein with a small mass, will denature into a short strand and bind fewer SDS molecules. However, because the number of SDS molecules that bind to a protein is dependent on the length of the protein, then all proteins (large and small) will essentially have an equal mass-to-charge ratio.

SDS molecules are negatively charged and a gel strip containing the pH gradient from a first dimension separation experiment can then be applied to an electrophoresis gel where the now negatively charged proteins can be separated according to relative size when a second electric current is applied. Large proteins will move through the gel more slowly than smaller proteins. This is commonly referred to as the second dimension of the protein separation and is useful because it is unlikely that different proteins with the same isoelectric point will also be of identical size. Therefore by separating proteins using 2D methods, better separation is achieved. 2D proteomics has previously been used to investigate changes in the *C. jejuni* proteome in response to bile exposure (Fox *et al.*, 2007) and to growth in a low oxygen environment (Liu *et al.*, 2012). Examination of the proteome of *C. jejuni* following exposure to pantoprazole may therefore prove useful in an effort to define the bactericidal mechanism of action.

3.1.2 Transcriptomics

The production of cellular proteins is determined by the preceding processes of transcription and translation. A DNA sequence may first be transcribed into ribonucleic acid (RNA), which is then translated into protein. Transcriptomics is therefore the study of the complete set of RNA transcripts produced by a particular genome. Microarrays are a commonly used method of studying the expression levels of a large number of genes simultaneously. Total transcribed RNA can be harvested from cells and converted to complementary DNA (cDNA) which is labelled with a fluorescent dye (e.g. cyanine III (Cy3) or cyanine IV (Cy5)). An array of unique short DNA sequences (probes) are arranged on a solid surface where the position of each probe (and the gene of which it is a part) is known. Hybridisation of samples to the probes allows the binding of

complementary sequences. If a particular gene was therefore being transcribed under test conditions, then the corresponding RNA would be harvested and converted to labelled cDNA, labelled cDNA would be bound by the corresponding probe and the confirmation of gene expression obtained by detecting fluorescence in the position of the specific probe. Microarrays have previously been used to investigate the differences in *C. jejuni* gene expression in response to varying growth conditions (Corcionivoschi *et al.*, 2009, John *et al.*, 2011, Mills *et al.*, 2012) and following exposure to the highly alkaline compound trisodium phosphate (Riedel *et al.*, 2012).

3.1.3 Chapter Aims

Exposure to pantoprazole has been shown in **Chapter 2** to have an effect on *C. jejuni* growth, motility, biofilm formation, invasion potential and morphology. Prolonged exposure to mg/ml quantities of pantoprazole has also been shown to be bactericidal. 2D gel proteomics experiments presented in this chapter were performed to determine whether exposure to pantoprazole caused significant changes to the *C. jejuni* proteome. The predicted roles of proteins which were identified as differentially present under pantoprazole exposed and control conditions were researched in anticipation that a protein or proteins that might be essential for bacterial survival might be identified that could account for the killing of *C. jejuni* by pantoprazole. The up-regulation of selected genes (as indicated from proteomic analysis) was confirmed using qRT-PCR. Microarray experiments were performed to identify any changes in gene expression following exposure to pantoprazole. Insertional mutation of a component gene for an ATPase that was thought to be a prospective target for PPIs was also performed to assess if there was any change in the susceptibility to pantoprazole for the mutant.

3.2 Materials and Methods

3.2.1 Bacterial Strains and Culture Conditions

The *C. jejuni* and *E. coli* strains used in this chapter of the study are listed in **Table 10**. All strains were stored at -80°C, revived, cultured and incubated as detailed in **Section 2.2.1**. *E. coli* used in cloning experiments was grown either in lysogeny broth (LB) broth with shaking at 200 rpm or on LB agar plates in an aerobic 37°C incubator. Strains from overnight growths were used in all individual experiments unless otherwise stated.

Table 10. Bacterial strains used in this chapter.

Strain	Features	Origin/Reference
C. jejuni 11168-H	Hypermotile derivative of strain 11168	(Karlyshev *et al.*, 2002)
C. jejuni 81-176	Human clinically isolated strain	(Korlath *et al.*, 1985)
C. jejuni 81116	Human clinically isolated strain	(Palmer *et al.*, 1983)
E. coli XL-2-Blue	Competent cells	Stratagene

3.2.2 Antibiotics and Pantoprazole

Pantoprazole sodium hydrate was prepared as described in **Section 2.2.2**. Ampicillin (Sigma) and kanamycin (Sigma) were dissolved in water, sterilised using a 0.2 µm syringe filter and added to agar plates at final concentrations of 100 and 50 µg/ml respectively, when required.

3.2.3 Proteomics 1

The details of many of the solutions used during proteomic analyses are provided in **Appendix 1**. A suspension of *C. jejuni* 81-176 in MHB was prepared from 48 hour plate cultures and corrected to an OD_{595} of 0.35. 9.5 ml of this was added to 0.5 ml pantoprazole to give a final pantoprazole concentration of 2 mg/ml (or 2,000 µg/ml). This was mixed well and placed in a sterile universal with the cap loosened before incubating for 2 or 4 hours. Samples were then centrifuged at 3,000 × g for 15 minutes, supernatant was removed, the pellet re-suspended in 5 ml PBS and centrifuged again at 3,000 × g for 15 minutes. The pellet was again re-suspended in 5 ml PBS and centrifuged at 3,000 × g for 15 minutes before all of the supernatant was removed and the inside of the tube dried completely. Samples were then stored overnight at -20°C.

Samples were defrosted and all cells were lysed and acetone precipitated (three washes) and made up to approximately 250 µl in lysis buffer. The amount of protein in each sample was then measured and adjusted so that they were approximately equal. Samples were then added to individual 2D gel strips and run overnight to accumulate a total of between 70 and 80,000 vhrs. Strips were then washed in DTT and IOA and 2D-PAGE gels run overnight at 1 W per strip. Gels were then fixed and stained using colloidal coomassie before being scanned and visually examined. Seventeen gel spots were selected for identification using mass spectrometry. Fourteen spots were thought to be present under PPI free control conditions but absent from the PPI exposed sample and three spots were thought to be present under PPI exposed conditions but absent from the PPI free control. Excised gel bands were placed in 1.5 ml Eppendorf tubes and washed with 100 mM ammonium bicarbonate for 30 minutes at room temperature on a rotary shaker (100 rpm). Ammonium bicarbonate was then removed and 50% acetonitrile/100 mM ammonium bicarbonate added. Eppendorfs were again placed on a rotary shaker and left at room temperature for 35 minutes. The wash solvent was removed from the tubes and 50 µl acetonitrile added for 10 minutes to shrink gel slices. Gel slices were dried for 30 minutes in a vacuum centrifuge. Gel slices were rehydrated with trypsin in 25 mM ammonium bicarbonate. The proteins were digested at 37°C for 24 hours. Liquid was then removed from the Eppendorfs and added to 96 well plates. 5% FA was added to the remaining gel slices and incubated at room temperature on a rotary shaker for 20 minutes. Two times volume of acetonitrile was then added and tubes incubated on a rotary shaker for an additional 20 minutes. All the liquid was collected and pooled in the original 96 well plate. The 96 well plate was dried in a vacuum centrifuge to concentrate the samples. Proteins were identified by MALDI-TOF-MS. Experiments were performed at each exposure time and pantoprazole concentration (0 and 2 mg/ml) only once.

3.2.4 Proteomics 2

3.2.4.1 Determining Pantoprazole Exposure Conditions

Experiments were first performed to determine the time and concentration of pantoprazole exposure to be used. Colonies were harvested into DMEM to an OD_{600} of 1.0 from overnight plate growths of *C. jejuni* strain 81-176. Bacterial suspensions were then split into two equal volumes in sterile bijous and sterile water or pantoprazole (to give final concentrations of 1, 1.5 or 2 mg/ml) added. Bijous, with the caps loosened, were then incubated and serial dilutions performed each hour for a total of 6 hours incubation and CFU/ml calculated. This experiment was carried out only once in triplicate.

3.2.4.2 Preparation of Protein Extracts

Proteomics experiments were carried out at a final pantoprazole concentration of 1 mg/ml (or 1000 µg/ml) for 2 hours. Overnight growths of *C. jejuni* strain 81-176 were harvested from MHA + B plates into DMEM to an OD_{600} of 1.2. This was then divided equally between two sterile vented cap 25 cm^2 tissue culture flasks (Corning) and pantoprazole or sterile water (for the no pantoprazole control) added. Flasks were mixed gently and incubated in the VAIN for 2 hours before the entire contents of the flasks were transferred into cooled sterile 15 ml falcon tubes (Corning). Aliquots were removed and serially diluted to calculate surviving CFU/ml. Falcon tubes were centrifuged at 3,000 × g for 15 minutes at 4°C, supernatant removed, and the pellets resuspended in ice cold fresh DMEM. Falcon tubes were again centrifuged at 3,000 × g for 15 minutes at 4°C, supernatant removed and the pellets resuspended in 5 ml ice cold fresh DMEM. Falcon tubes were centrifuged for a third time at 3,000 × g for 15 minutes at 4°C, supernatant removed and the inside of the tubes dried with a sterile swab.

Harvested pellets under both pantoprazole exposed and control conditions were stored at -80°C until all three replicates of the experiment had been performed. Proteomics 2 experiments were therefore performed in biological triplicate but only one technical replicate of 2D gels were performed. Samples were transported from the University of Glasgow to Moredun Research Institute on dry ice. All of the work described in the remaining **Proteomics 2** sections below was carried out by me at the Moredun Research Institute with the help of those named in the **Acknowledgements** section.

Protein pellets were thawed and resuspended in extraction buffer at room temperature for 30 minutes, vortexed and then transferred into fresh 1.5 ml Eppendorfs. Tubes were centrifuged at 1,500 × g for 10 minutes and supernatant removed into fresh Eppendorfs. The protein content of all six samples was measured at 480 nm on a Novaspec II Visible Spectrophotometer (GE Healthcare) using 2-D Quant Kit (GE Healthcare) according to the manufacturer's instructions and adjusted to 400 µg/ml. Proteins were precipitated using Precipitant and Co-precipitant, vortexed and centrifuged at 1,800 × g for 5 minutes. The supernatant was removed and protein pellets stored at -20°C.

3.2.4.3 Two Dimensional Polyacrylamide Gel Electrophoresis and Image Acquisition

Pellets were removed from the freezer and air dried at room temperature for 5 minutes and then resuspended in extraction buffer. Tubes were vortexed for 30 seconds each to fully dissolve the protein pellet and samples were then centrifuged at 1,800 × g for 5 minutes.

Samples were cleaned using 2-D Clean-Up Kit (GE Healthcare) according to the manufacturer's "Procedure A" guidelines. After step 11 of the protocol, samples were stored overnight at -20°C (as indicated in the instructions). 450 µl of rehydration solution (with IPG pH 4-7 buffer; GE Healthcare) was added to each protein sample in the last step of the protocol.

Each of the six samples was then applied evenly along the length of six individual Immobiline DryStrip pH 4-7 (GE Healthcare) strips in a DryStrip Reswelling Tray (GE Healthcare) and left overnight at room temperature. The following morning proteins were separated on the gel strips according to relative charge (first dimension of separation) at the same time, under identical test conditions. Isoelectric focusing was performed at 20°C for 7 hours at 500 volts (V), then 1000 V for 1 hour, a gradient from 1000-8000 V over a period of 3 hours and then a final 5 hours at 8000 V (Ettan IPGphor 3 Isoelectric Focusing Unit; GE Healthcare). Following isoelectric focusing the strips were placed in individual equilibration tubes and first equilibrated using equilibration buffer with DDT on a gentle rocker for 15 minutes. Buffer was removed and strips were additionally equilibrated using equilibration buffer with iodoacetamide on a gentle rocker for 15 minutes.

Strips were removed from equilibration tubes, rinsed with double-distilled water then mounted onto precast 12.5% polyacrylamide gels cross-linked with bisacrylamide (GE Healthcare) and the second dimension of separation (according to protein size) also carried out in the same gel tank (Ettan DALTsix Electrophoresis Unit), under identical test conditions for all six samples. The unit was first set at 15 W for 1 hour to allow the proteins to transfer off the strip and onto the gel and then the setting adjusted to 100 W and separation of proteins allowed to run overnight. Gels were then transferred into individual trays for fixing and staining. All six gels were fixed in the same manner, for equal lengths of time (two repeats of 1 hour) in 500 ml fresh fixative each time, then rinsed five times in double-distilled water for 15 minutes each time, stained using Colloidal Coomassie Blue working solution (see **Section 7.2**) overnight and then washed twice for 30 minutes each time in double-distilled water before being scanned using a high sensitivity scanner under the same brightness and contrast conditions. Tagged Image File (TIF) files were then uploaded into the ImageMaster 2D Platinum software programme (GE Healthcare) for analysis.

3.2.4.4 Identification of Proteins by Mass Spectrometry

For identification of proteins by MALDI-TOF-MS each gel spot indicated as differentially present under the PPI exposed and control conditions by ImageMaster 2D Platinum was

excised from the gel and subjected to standard in-gel destaining and trypsinolysis procedures (Shevchenko *et al.*, 1996). Tryptic peptides were applied to a steel MALDI target plate in a solution of 10 mg/ml α-cyano-4-hydroxycinnamic acid (CHCA) in 0.1% trifluoroacetic acid and 50% ACN. Mass spectra were obtained using an Ultraflex II TOF/TOF instrument (Bruker Daltonics) operated in the reflectron mode. The instrument was calibrated using known peptide standards (Bruker Daltonics PepMix 2). Each spectrum was produced by accumulating data from 10 × 100 consecutive laser shots. Peptides were identified by matching the measured monoisotopic masses to theoretical monoisotopic masses generated using the MASCOT search engine (peptide mass fingerprinting, PMF). Selected peptides from proteins that remained unidentified by PMF were fragmented in MS/MS mode. The search parameters were: maximum of one missed cleavage by trypsin, variable modification of oxidation of methionine, modification of cysteine by propionamidation and carbamidomethylation, peptide tolerance of ±50 ppm. Using these parameters and searching the NCBI database, Mascot scores greater than 87 were considered significant ($P < 0.05$).

3.2.5 Quantitative Real-time Polymerase Chain Reaction

To further assess if the operons for selected proteins were subject to induction by exposure to pantoprazole, *C. jejuni* was exposed to 1 mg/ml (or 1,000 µg/ml) pantoprazole, for 1 or 2 hours. The *rpoA* gene was used for normalization. The qRT-PCR experiments were repeated three times, using RNA samples prepared from three independent experiments.

3.2.5.1 Primers

Primers were designed using the Primer3Plus programme (available online) using the parameters: product size range (100-300), minimum primer size (17), optimum primer size (20), maximum primer size (22), minimum melting temperature (57°C), optimum melting temperature (59°C), maximum melting temperature (62°C), minimum GC% (30), optimum GC% (50) and maximum GC% (70). A BLAST search was used to ensure primers targeted only the selected region within the *Campylobacter* genome. Primer sequences were selected from the available lists to minimize primer dimer formation. Primers were obtained from Eurofins and first adjusted to 100 pmol/µl in nuclease free water. 1 in 10 dilutions of these were prepared and stored at -20°C as working stock solutions. A list of primers used in these experiments is provided in **Table 11**.

Table 11. Oligonucleotide primers used in this section.

Primer Name	Sequence (5'→ 3')	Product Size (nucleotides)
ATPase-F	TGGTGCAGGTGTTGGTAAA	201
ATPase-R	CTTGCTCCTGGTGGTTCATT	
GroEL-F	CCAAGAGGACGCAATGTTTT	266
GroEL-R	TCCATACCGCGTTTTACCTC	
rpoA-F	CGAGCTTGCTTTGATGAGTG	109
rpoA-R	TCCCACAGGAAAACCTATGC	
Thiol Perox-F	GCCAGTTACAATGGTGCTGA	195
Thiol Perox-R	CACAAATACGGCACGAGCTA	

Genomic DNA was extracted from overnight cultures of *C. jejuni* strain 81-176 according to the manufacturer's instructions (four replicates) using DNeasy Blood & Tissue Kit (Qiagen). The four selected genes were each amplified using 2× concentrate PCR Master Mix (ThermoFisher) in 23 µl volumes containing 20 µl PCR Master Mix, 1 µl of stock forward primer, 1 µl of stock reverse primer and 1 µl genomic DNA in 0.2 ml PCR tubes (only once). Negative controls were included which used 1 µl of nuclease free water in place of genomic DNA. Tubes were heated to 94°C for 5 minutes then subjected to 30 cycles of 94°C for 30 seconds, 50°C for 2 minutes, 72°C for 1 minute, followed by an extension at 72°C for 10 minutes. The PCR products were visualised on 1% agarose gels (1 g agarose (Sigma) in 100 ml 1× Tris EDTA acetate) incorporating 10,000× GelRed™ Nucleic Acid Gel Stain (Biotium). DNA fragment sizes were estimated by using 1 Kb Plus DNA Ladder (ThermoFisher). In all four cases only one product was seen on the gel at the expected position (see **Table 11** for product sizes) for each primer set, confirming that the design of the primers was correct and that only the selected genes were amplified from *C. jejuni* genomic DNA (data not shown). To check that the products (of the expected size) that had been amplified by the primers was indeed the gene of interest, bands were carefully removed from the gel using a scalpel and DNA extracted from them using QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's guidelines (only once). Extracted DNA was sequenced by Source Bioscience, using the relevant primers and resulting nucleotide sequences analysed using CLC Genomics Workbench (Qiagen). All four sequences showed > 95% homology with the relevant genes in the *C. jejuni* 81-176 genome sequence.

3.2.5.2 *Campylobacter jejuni* Pantoprazole Exposure

C. jejuni strain 81-176 was harvested from overnight MHA + B plate cultures into DMEM to an OD_{600} of 1.6 before being split into equal volumes and added to sterile vented cap 25 cm^2 tissue culture flasks. Pantoprazole was prepared as described in **Section 2.2.2** and was added to one flask to give a final concentration of 1 mg/ml (or 1,000 µg/ml) with sterile water being added to the other flask to act as the no pantoprazole control. Following incubation for 1 hour, half of sample volumes were transferred from the flasks into cooled sterile 15 ml falcon tubes. Flasks were then returned to the VAIN and incubated for a further hour. Falcon tubes were centrifuged at 4,500 × g for 15 minutes at 4°C, supernatant removed, 1 ml of the RNA stabilising reagent RNAprotect (Qiagen) added, vortexed and then stored on ice. At the end of the 2 hour incubation the remaining samples were transferred from the flasks into cooled sterile 15 ml falcon tubes and prepared in the same manner.

3.2.5.3 Ribonucleic Acid Extraction

The 1 ml of RNAprotect was vortexed and added to a 1.2 ml Lysing Matrix B tube (MP) containing 0.1 mm silica beads and mixed vigorously using a FastPrep®-24 machine (MP) for 40 seconds. Tubes were then centrifuged at 13,000 × g for 5 minutes at 4°C. Around 750 µl was removed from the top of the tubes and placed in fresh 1.5 ml sterile Eppendorfs and left at room temperature for 5 minutes. 300 µl chloroform was added and tubes then vortexed. After a further 5 minutes at room temperature, tubes were centrifuged at 13,000 × g for 5 minutes at 4°C. The upper phase of the supernatant was removed and placed again in fresh Eppendorfs. The centrifugation was repeated at 13,000 × g for 5 minutes at 4°C and the upper phase again removed into fresh Eppendorfs. Chilled 100% ethanol was added to tube and then vortexed. Tubes were placed in a -20°C freezer for 1 hour before being centrifuged at 13,000 × g for 15 minutes at 4°C. Supernatant was removed and white RNA pellets washed gently with 75% ethanol. Ethanol was removed and the pellets allowed to air dry at room temperature for 5 minutes. Samples with large RNA pellets were dissolved using 100 µl of nuclease free water (ThermoFisher) and smaller pellets using 80 µl. Tubes were vortexed, left at room temperature for 5 minutes and then vortexed again.

To eliminate residual DNA contamination, which would interfere with the quantitative real-time polymerase chain reaction (qRT-PCR), samples were treated according to the manufacturer's guidelines using Turbo DNA-free™ Kit (Ambion). Total RNA was quantified using a NanoDrop 2000 and the 260/280 nm ratio checked to be above 1.95.

3.2.5.4 Complementary Deoxyribonucleic Acid Synthesis

Samples which were found to have total RNA concentrations below 1000 ng/µl were designated as (low RNA concentration) and a higher volume of extracted RNA was used during first strand cDNA synthesis than was used from samples which had over 1000 ng/µl RNA. Random primers (Invitrogen), 10 mM dNTP mix (Invitrogen), extracted RNA samples (see **Section 3.2.5.3**) and nuclease free water were mixed in the volumes shown in **Table 12** in 0.2 ml PCR tubes. Tubes were heated at 65°C for 5 minutes and then placed on ice for 2 minutes.

Table 12. First strand cDNA synthesis reaction volumes.

	Volume Required (µl)	
	High RNA	Low RNA
Random Primers	1.5	1.5
dNTP Mix	1.0	1.0
Extracted RNA	6.5	10.0
Nuclease Free Water	11.0	7.5
Total	20.0	20.0

SuperScript® III reverse transcriptase (Invitrogen) was used to make cDNA according to the manufacturer's instructions. The concentration of cDNA in the samples was measured using a NanoDrop 2000 and were normalised to a concentration of 50 ng/µl using TE buffer (Ambion). To check that the reverse transcription step had worked the four selected genes were each amplified using 2× concentrate PCR Master Mix in 0.2 ml PCR tubes (only once) as described in **Section 3.2.5.1**. Negative controls were included which used 1 µl of nuclease free water in place of genomic DNA and positive controls which used genomic DNA in place of reverse transcribed cDNA. PCR thermocycling conditions were the same as before and the PCR products were again visualised on a 1% agarose gel. In all four cases, only one product was seen on the gel at the expected position (data not shown). cDNA was then stored at -20°C.

3.2.5.5 Quantitative Real-time Polymerase Chain Reaction

Analyses were conducted using ECO^{TM} Real-Time PCR System (Illumina) according to the manufacturer's specifications with KAPA SYBR® FAST (KAPA Biosystems). No template controls (nuclease free water in place of cDNA) were included in each qRT-PCR analysis to detect nucleic acid contamination and primer dimer formation. No reverse transcriptase controls (extracted RNA in place of cDNA) were also included in each qRT-PCR analysis. qRT-PCR master mixes were mixed according to the volume required

immediately prior to use. Volumes used in a typical experiment are listed in **Table 13** and individual reactions were performed using a total volume of 20 µl (19.5 µl of master mix and 0.5 µl of prepared cDNA). At the end of each qRT-PCR a melt curve was performed from 50-95°C and 10 µl aliquots removed from each well and analysed for the presence of DNA on 1% agarose gels (see **Section 3.2.5.1**). The expected melting temperature of the four gene products was determined using the online Oligo Calc: Oligonucleotide Properties Calculator programme (available at http://biotools.nubic.northwestern.edu/Oligo Calc. html). The primer sets used to detect the transcription levels of ATP synthase F1, GroEL co-chaperonin and thiol peroxidase can be seen in **Table 11** as can the primer set for the internal control gene *rpoA*. The *rpoA* gene has been identified as a suitable internal control for use in experiments investigating the stress response of *C. jejuni* (Ritz *et al*., 2009).

Table 13. qRT-PCR master mix preparation.

Component	Volume (µl)
SYBR® FAST (at 2× concentrate)	180
Forward Primer (at 10 µM)	2
Reverse Primer (at 10 µM)	2
Nuclease Free Water	176

3.2.6 Microarrays

C. jejuni strain 81-176 (at around 1×10^8 CFU/ml) was cultured in 2 ml DMEM with or without pantoprazole (at a final concentration of 2 mg/ml or 2,000 µg/ml) microaerophillicaly for 2 hours. 350 µl of bacterial suspension was then removed and added to 700 µl of RNA protect. RNA was extracted using Qiagen RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. RNA was eluted in 30 µl of molecular grade water (Sigma) and concentrations checked by NanoDrop. Samples from four independent biological replicates were standardised to 2 µg of RNA in 9 µl of molecular grade water. Labelled cDNA was prepared from 700 ng total RNA using Cy3-dCTP (GE Healthcare) and SuperScript II reverse transcriptase with random hexamer primers (Life Technologies – Invitrogen). Labelled cDNA was purified by Qiagen MinElute column, combined with 10× CGH blocking agent and 2× Hi-RPM hybridisation buffer (Agilent) and heated at 95°C for 5 minutes prior to loading onto microarray slides. Slides were then incubated overnight in an Agilent rotating oven at 65°C, 20 rpm. After hybridization, slides were washed for 5 minutes at room temperature with CGH Wash Buffer 1 (Agilent) and for 1 minute at 37°C with CGH Wash Buffer 2 (Agilent) then

scanned immediately (using an Agilent High Resolution Microarray Scanner, at 2 µm resolution, 100% PMT). Scanned images were quantified using Feature Extraction software v 10.7.3.1. Results for microarray experiments are notably representative of four separate biological replicates.

3.2.7 Making the *kdpB* Mutant

3.2.7.1 Recombinant Deoxyribonucleic Acid Methods

All of the work described in the *kdpB* mutant sections below was carried out in the laboratories of the LSHTM. NEBcutter was used to search the nucleotide sequence of the different subunits of Kdp for existing restriction enzyme sites. The *kdpA* and *kdpC* genes were found to have no existing restriction enzyme sites within their sequence, whereas *kdpB* and *kdpD* respectively had sites for BclI+BglII and BclI only within their sequence. KdpB is known to be the largest subunit and forms the functional part of the Kdp and so the *kdpB* gene was selected for insertional mutation.

E. coli XL-2-Blue (Stratagene) was used for recombinant plasmid transformation to make a *C. jejuni* 11168-H *kdpB* insertional mutant. The cloning vectors used were pGEM®-T Easy (Promega) for PCR product cloning and *C. jejuni* 11168-H mutagenesis, and pJMK30, a *Campylobacter* compatible plasmid containing a gene encoding resistance to kanamycin (kan^R; an aminoglycoside 3'-phosphotransferase) for insertional mutagenesis.

Genomic DNA was isolated from *C. jejuni* strain 81116 according to the manufacturer's guidelines (QIAamp DNA Mini Kit; Qiagen). Gene specific forward (*kdpB* GS-F) and reverse (*kdpB* GS-R) primers were obtained from Invitrogen, the sequences of which can be found in **Table 14**. The genome sequence of the *kdpB* gene, which was used to design the primers, can be found in **Section 8.1**. These were used to amplify a fragment within the coding region of the *kdpB* gene. The PCR product was cloned into pGEM®-T Easy (to give construct pI). pI was transformed into *E. coli* XL-2-Blue competent cells and transformants selected on LB agar with ampicillin and kanamycin after 48 hours growth. Transformants were screened for the presence of pI and frozen stocks of *E. coli* containing pI were made using Microbank™ beads and stored at -80°C.

Table 14. Oligonucleotide primers used in this section.

Primer Name	Sequence (5'→ 3')
kdpB GS-F	TAATGCCATAAAAGGAGC
kdpB GS-R	ATTCTAAGTATCCAAGC
kan^R F-out	TGGGTTTCAAGCATTAGTCCATGCAAG
kan^R R-out	GTGGTATGACATTGCCTTCTGCG

Restriction digestion at the unique BglII site allowed a 1.4-kb BamHI restriction fragment from pJMK30, containing the kan^R, to be ligated to pI and give construct pIK. To ensure that the kan^R had inserted in the correct position, the plasmid was analysed by restriction digestion with EcoRI, and by standard PCR with the original template primers (*kdpB* GS-F and *kdpB* GS-R) and a set of primers designed for amplification of the kan^R (kan^R F-out) and reverse kan^R R-out), the sequences of which can be found in **Table 14**. The genome sequence of the kan^R, which was used to design the primers, can be found in **Section 8.2**. To confirm that the kan^R had inserted in the same orientation as the insert and that the possibility of polar effects were minimised, pIK was amplified with combinations of template and kan^R primers, *kdpB* GS-F and kan^R F-out and, *kdpB* GS-R and kan^R R-out. Plasmids containing the correct insertional mutation were used to transform *C. jejuni* 11168-H via electroporation. SOC broth (Sigma) was added to the electroporation cuvette immediately, mixed well and left in the VAIN for 1 hour. Electroporated bacteria were then plated onto MHA + B plates and incubated in the VAIN for 24 hours. *Campylobacter* colonies were harvested into PBS and 200 µl aliquots plated onto MHA + B + kan agar and incubated for 48 hours for isolation of insertional mutants. A number of single colonies were selected to check that the observed kanamycin resistance was in the chromosome. The colonies were analysed using PCR with *kdpB* GS-F and *kdpB* GS-R.

pGEM®-T Easy was used for mutagenesis in *C. jejuni* 11168-H because it has an *E. coli* origin of replication. All known vectors with an *E. coli* origin of replication are unable to replicate in *Campylobacter*, and can therefore be used as suicide vectors (Van Vliet *et al.*, 1998). When mutant constructs of *Campylobacter* DNA in *E. coli* vectors are introduced into *Campylobacter*, a double crossover event occurs which leads to the elimination of vector sequences and the replacement of the wild-type gene with the disrupted copy from the vector (Van Vliet *et al.*, 1998). Therefore, transformation of *C. jejuni* 11168-H with pIK would result in incorporation of the disrupted *kdpB* gene and the loss of pGEM®-T Easy.

3.2.7.2 Polymerase Chain Reaction Conditions

Crude cell lysates were amplified using *Taq* polymerase (Gibco) in 20 µl volumes containing 0.1 µg primers and DNA at 94°C for 1 minute, 25 cycles of 94°C for 45 seconds, 50°C for 45 seconds, 72°C for 2 minutes, followed by an extension at 72°C for 7 minutes. The PCR products were analysed on 1% agarose gels.

3.2.7.3 Minimum Bactericidal Concentration

The MBC was determined at 4 and 24 hours for the *kdpB* mutant and the parent strain using a broth microdilution method as described in **Section 2.2.4** where around 5×10^5 CFU/ml in MHB was mixed with an equal volume of pantoprazole in water. Pantoprazole was tested at final concentrations ranging from 10-0 mg/ml (or 10,000-0 µg/ml).

3.2.8 Replicates and Data Analysis

Unless otherwise stated each assay was conducted in triplicate and was independently repeated at least three times. Results are expressed as means +/- standard deviations (SD; error bars) of replicate experiments. The unpaired Students t test was used to determine statistical significance. A P value of > 0.01 but < 0.05 was considered significant (*****) and a P value of < 0.01 highly significant (******). qRT-PCR data were analysed according to the $2^{\Delta\Delta CT}$ method (Livak & Schmittgen, 2001) with target gene expression normalised to *rpoA* expression. ΔΔCT was calculated as (CT (target gene, no PPI exposure) - CT (reference gene, no PPI exposure)) - (CT (target gene, PPI exposed) - CT (reference gene, PPI exposed)).

3.3 Results

3.3.1 Proteomics 1

Experiments were performed to determine whether exposure to pantoprazole caused significant changes to the *C. jejuni* proteome. Seventeen gel spots were selected from individual 2D gels and the identifications for the proteins following mass spectrometry are listed in **Table 15** together with whether the protein was thought to be up- or down-regulated in response to pantoprazole exposure and the role of the protein. There appeared to be more proteins present on the control gels, than there were for *C. jejuni* exposed to pantoprazole for 2 or 4 hours. Hence most of the proteins identified appeared to be down-regulated in their production following pantoprazole exposure rather than up-regulated (see **Table 15**). Most protein spots were identified as being differentially present following the 4 hour exposure (**Figure 18**). Only one gel spot was excised and identified from the 2 hour exposure experiment and the gel pictures have been omitted.

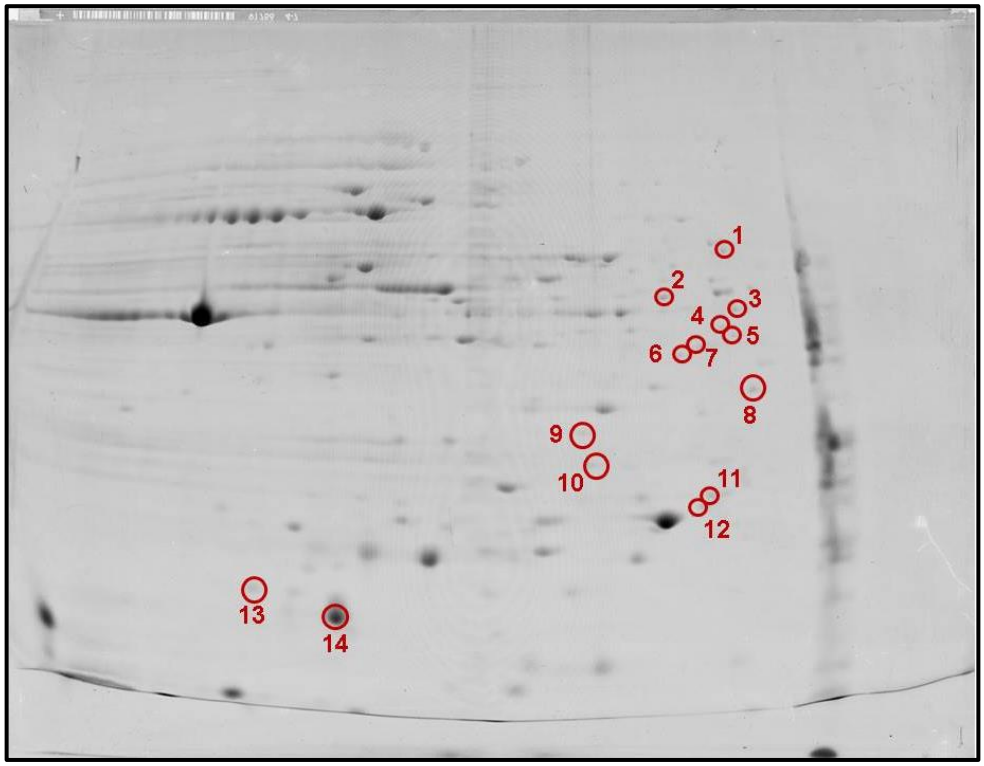
A number of proteins identified as differentially present in pantoprazole exposed gels versus those for the no pantoprazole controls are involved in the oxidative stress response of *C. jejuni* (proteins 6, 7, 11 and 15 in **Table 15**). Of these proteins three appeared to be down-regulated in response to pantoprazole exposure and one up-regulated. The role of oxidative stress in the killing of *C. jejuni* by pantoprazole will be further investigated in **Chapter 4**.

A number of proteins identified as differentially present in pantoprazole exposed gels versus those for the no pantoprazole controls are involved in the synthesis of LPS or the bacterial cell membrane (proteins 8, 10 and 17 in **Table 15**). Of these proteins two appear to be down-regulated in response to pantoprazole exposure and one up-regulated. The effect of pantoprazole exposure on the outer membrane of *C. jejuni* will be further discussed in **Chapter 5**.

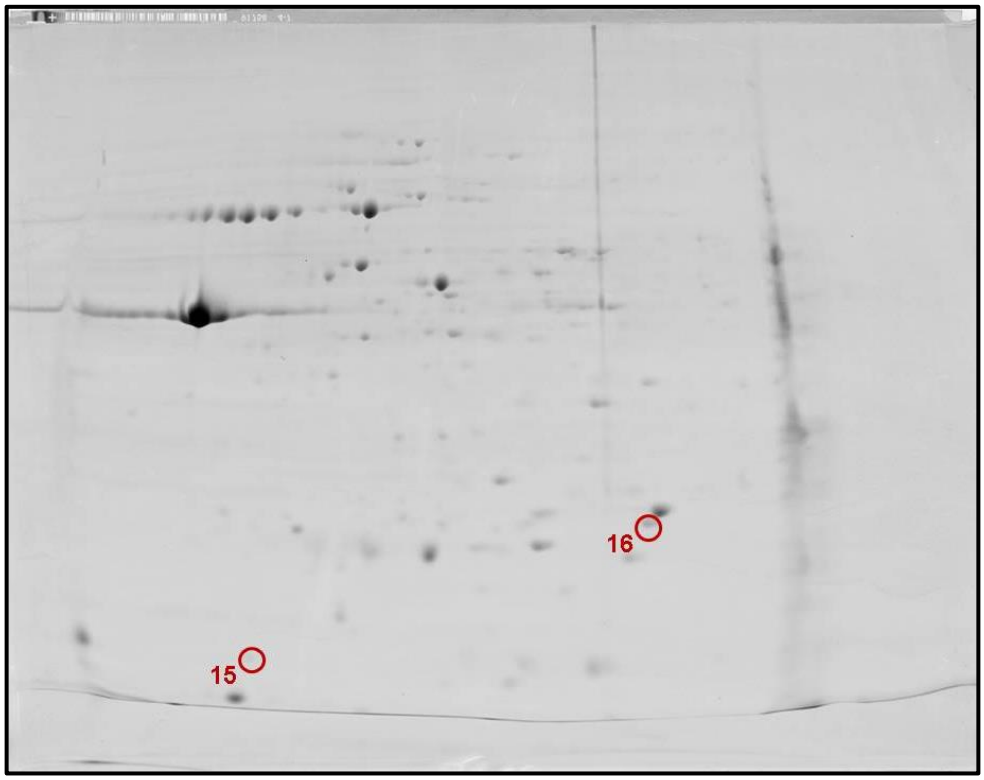
Table 15. List of protein identifications for the 17 gel spots excised from gels in the proteomics 1 experiment.

Gel Spot	Up/ Down	2/4 Hour	Identification	Role
1	Down	4	Phosphate Acetyltransferase	Acetate Metabolism
2	Down	4	3-Oxoacyl-(Acyl-Carrier-Protein) Synthase II	Fatty Acid Synthesis
3	Down	4	OorA	Electron Transport Chain
4	Down	4	2,3,4,5-Tetrahydropyridine-2-Carboxylate N-Succinyltransferase	Lysine Biosynthesis
5	Down	4	Aspartate-Semialdehyde Dehydrogenase	Amino Acid Biosynthesis
6	Down	4	UDP-GlcNAc/Glc 4-Epimerase	Nucleotide Metabolism
7	Down	4	Pyridine Nucleotide-Disulphide Oxidoreductase Family Protein	Oxidoreductase Activity
8	Down	4	ADP-L-Glycero-D-Manno-Heptose-6-Epimerase	LPS Biosynthesis
9	Down	4	Hypothetical Protein CJE0806	Unknown
10	Down	4	3-Deoxy-D-Manno-Octulosonate Cytidylyltransferase	LPS Biosynthesis
11	Down	4	Superoxide Dismutase	Oxidative Stress
12	Down	4	OorC	Electron Transport Chain
13	Down	4	P19 Protein	Iron Transport
14	Down	4	P19 Protein	Iron Transport
15	Up	4	Anti-oxidant AhpCTSA Family Protein	Oxidative Stress
16	Up	4	2-Component Regulator	Unknown
17	Up	2	Peptidoglycan-Associated Lipoprotein Omp18	Antigenic Outer Membrane Protein

*The table indicates whether proteins appeared to be up-regulated or down-regulated, following PPI exposure at 2 mg/ml (or 2,000 µg/ml) for 2 (spot 17) or 4 hours (spots 1-16). Proteins listed in **green** have been identified as possible targets for novel antimicrobials, proteins listed in **red** are thought to be essential for survival and proteins listed in **orange** are thought to be involved in the oxidative stress response.*



a



b

Figure 18. 2D gels from proteomics 1 experiments. *C. jejuni strain 81-176 was grown in MHB in the absence of pantoprazole (a) and in the presence of 2 mg/ml pantoprazole (b) for 4 hours. Spots 1-14 (a) were thought to be missing from the corresponding gel (b) and spots 15 and 16 (b) were thought to be missing from the corresponding gel (a) and were excised for identification using MS.*

3.3.2 Proteomics 2

3.3.2.1 Determining Pantoprazole Exposure Conditions

It was thought that the concentration of pantoprazole used in the proteomics 1 experiments may have been too high. Basic experiments were therefore first performed to determine the pantoprazole concentration and duration of exposure to be used for more rigorous triplicate proteomic analyses. Results in **Table 16** show that there was a 1 log reduction in *C. jejuni* numbers following exposure to 2 mg/ml pantoprazole for 2 hours and a 3 log reduction in *C. jejuni* numbers following exposure to 2 mg/ml pantoprazole for 4 hours (the conditions used in proteomics 1 experiments, see **Section 3.2.3**).

Table 16. Killing of C. jejuni by pantoprazole is both time and concentration dependent.

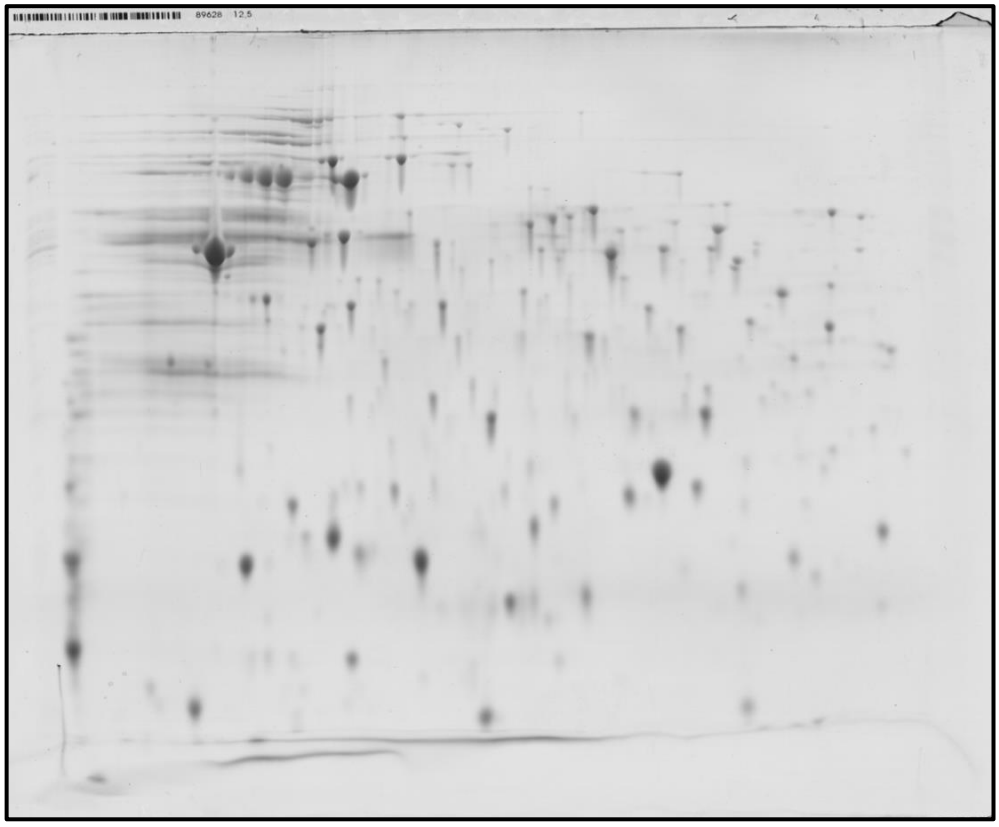
Time (hours)	Pantoprazole Concentration (mg/ml)			
	0.0	**1.0**	**1.5**	**2.0**
1	2.6×10^{9}	2.1×10^{9}	2.7×10^{9}	2.1×10^{9}
2	2.6×10^{9}	1.6×10^{9}	3.0×10^{9}	3.7×10^{8}
3	2.5×10^{9}	2.5×10^{9}	1.9×10^{9}	1.6×10^{7}
4	2.4×10^{9}	1.0×10^{9}	1.5×10^{8}	1.2×10^{6}
5	3.2×10^{9}	1.0×10^{8}	3.0×10^{7}	1.8×10^{4}
6	2.8×10^{9}	1.6×10^{7}	2.8×10^{5}	< 100

C. jejuni strain 81-176 was grown in DMEM in the absence of pantoprazole and in the presence of various pantoprazole concentrations for 6 hours with aliquots being removed, serially diluted and surviving bacteria calculated each hour.

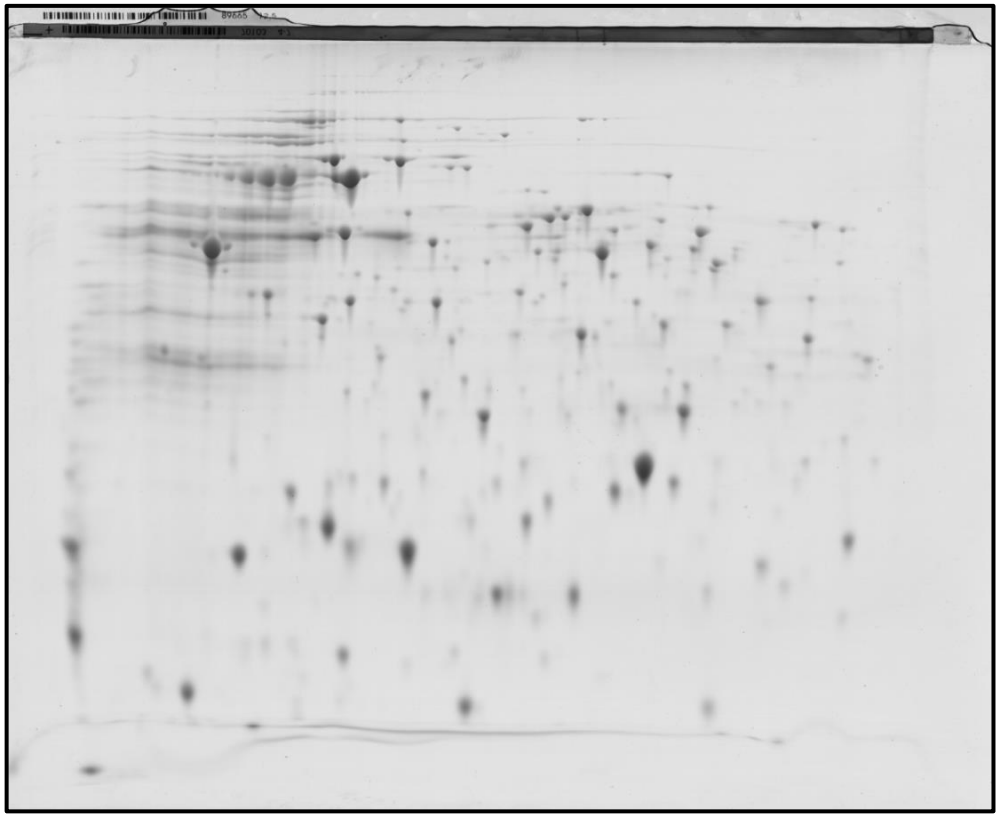
Proteomic analysis was to be used to detect specific changes in the proteome of *C. jejuni* induced by exposure to pantoprazole, but not to detect non-specific changes in the proteome indicative of dying bacteria. The possibility that the lack of proteins detected following exposure to pantoprazole being linked to the overall loss of bacterial cells was also taken into consideration. It was decided that triplicate proteomic analysis would therefore be performed following exposure to 1 mg/ml pantoprazole for 2 hours.

3.3.2.2 Proteomics

Before bacteria were collected for 2D gel analysis, aliquots were serially diluted and CFU/ml in the samples calculated. The average CFU/ml recovered from the no pantoprazole control samples and for the pantoprazole exposed samples were 4.2×10^{9} and 4.5×10^{9} respectively. Hence, as was desired, exposure to pantoprazole at 1 mg/ml (or 1000 µg/ml) for 2 hours did not cause a significant loss of live bacterial cells. Representative examples of 2D gels under both control and pantoprazole exposed conditions are shown in **Figure 19**.



a



b

Figure 19. Examples of 2D gels from proteomics 2 experiments. *C. jejuni strain 81-176 was grown in DMEM in the absence of pantoprazole (a) and in the presence of 1 mg/ml pantoprazole (b) for 2 hours. Gel pictures shown under each test condition are representative examples from triplicate experiments.*

Image Master 2D Platinum software allowed the grouping of triplicate gel pictures for the control samples and grouping of triplicate gel pictures for the pantoprazole exposed samples. The software identified every spot in the series of six pictures and a number of well-defined proteins, which were easily identifiable in all six replicates were highlighted as anchor points, from which the relative positions of all other gel spots were compared (see highlighted proteins **1** and **2** in **green** and **blue** in **Figure 20**). A numerical value was assigned to each spot depending on the size and the intensity of the spot and these values compared between the two different test conditions.

A P value was generated by the software following the comparison for the pantoprazole exposed and control groups. A three dimensional (3D) image was produced of individual gel spots showing relative size and intensity to allow comparison of individual replicates of the experiment for particular proteins (**Figure 21**). Ten proteins were determined to be differentially present under pantoprazole exposed and control conditions (see **Figure 22**) and these were removed from the gels and identified using mass spectrometry. The identifications of the proteins are listed in **Table 17** together with the P values generated by ImageMaster 2D Platinum, whether the protein was up- or down-regulated in response to pantoprazole exposure and the role of the protein.

Table 17. List of protein identifications for the ten gel spots excised from gels in the proteomics 2 experiments.

Gel Spot	P Value	Up/ Down	Identification	Role
1	0.037	Down	NifU Family Protein	Nitrogen Fixation
2	0.042	Down	Acetyl-CoA Carboxylase	Fatty Acid Metabolism
3	0.004	Up	Phosphoribosylformylglycinamidine Synthase II	Purine Metabolism
4	0.004	Up	Thiol Peroxidase	Oxidative Stress
5	0.008	Up	DnaK Chaperone Protein	Chaperone and Oxidative Stress
6	0.008	Up	Nonheme Iron-Containing Ferritin	Iron Storage
7	0.020	Up	GroEL Co-chaperonin	Chaperone and Oxidative Stress
8	0.023	Up	Putative Bacterioferritin	Oxidative Stress and Iron Storage
9	0.033	Up	Nucleoside Diphosphate Kinase	Purine Metabolism
10	0.035	Up	ATP Synthase F1	Proton Pump

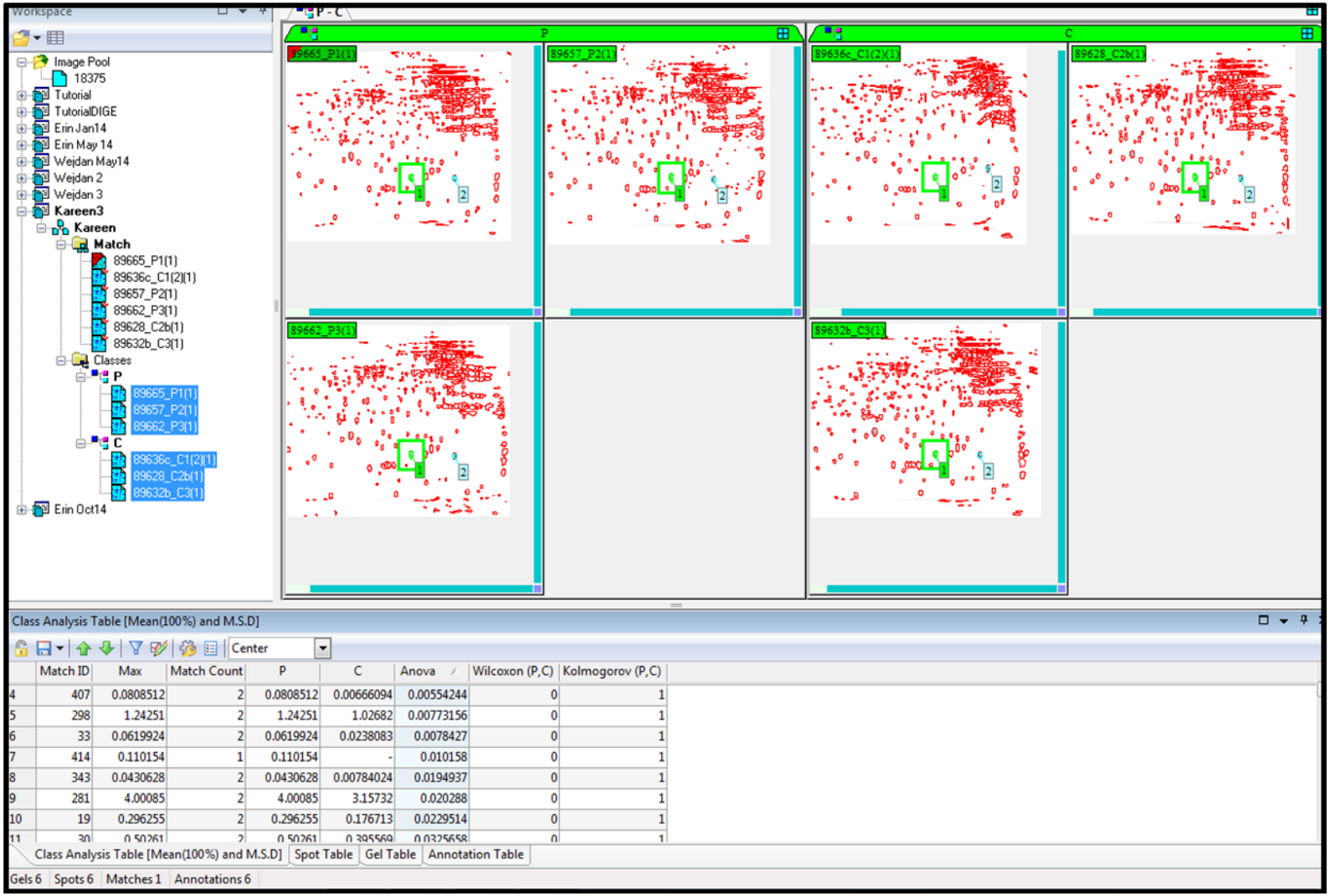
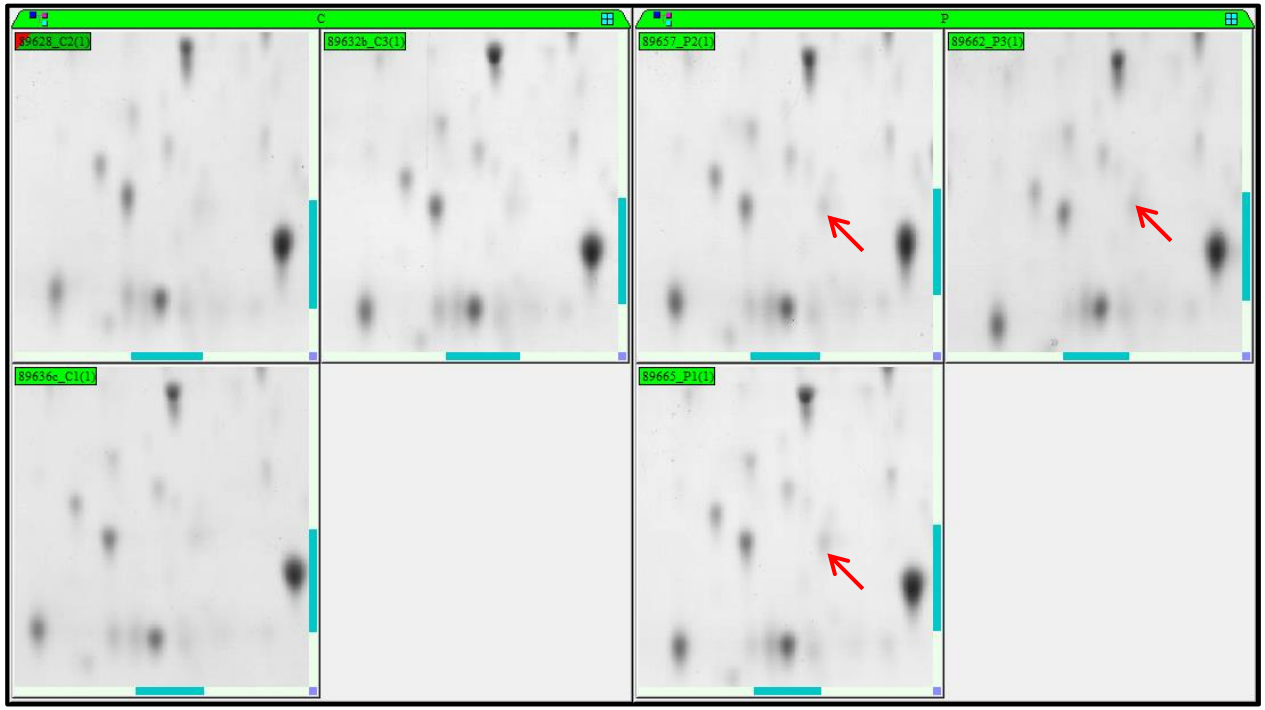
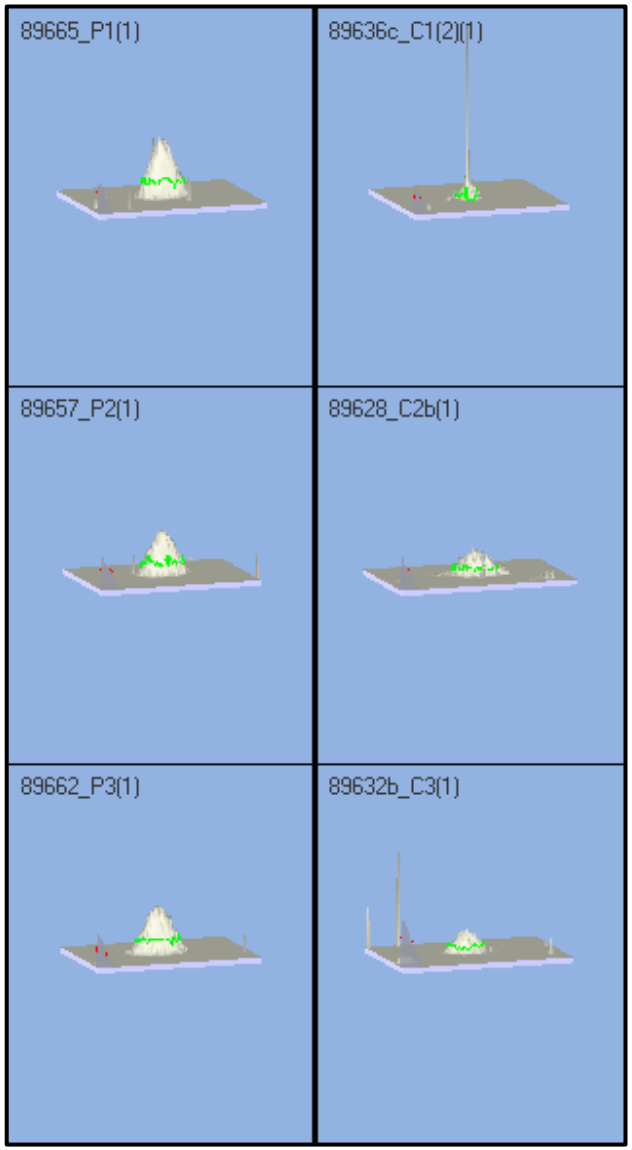


Figure 20. Protein spot analysis using Image Master 2D Platinum software. *In this example protein spots found in triplicate pantoprazole exposed experiments have been compared to the control. The size and intensity of the spots is assessed by the software and each spot is assigned a numerical value. The average values for each spot under the two conditions (pantoprazole exposed (P) and control (C) seen in the table at the bottom of the figure) are compared and a P value assigned. Protein spots that have been identified as significantly different can then be selected for further analysis (see* **Figure 21** *overleaf).*

Figure 21. Individual gel spot analysis. *ImageMaster 2D Platinum software indicates the position of individual proteins (**red** arrows) which have been identified as differentially present between pantoprazole exposed (P1, P2 and P3) and control (C1, C2 and C3) test conditions (a). Spot size and intensity data can be used to produce a 3D image of the protein spots, which shows clearly in this example that the protein is present in greater quantities in the pantoprazole exposed samples than in the control samples (b).*



a



b

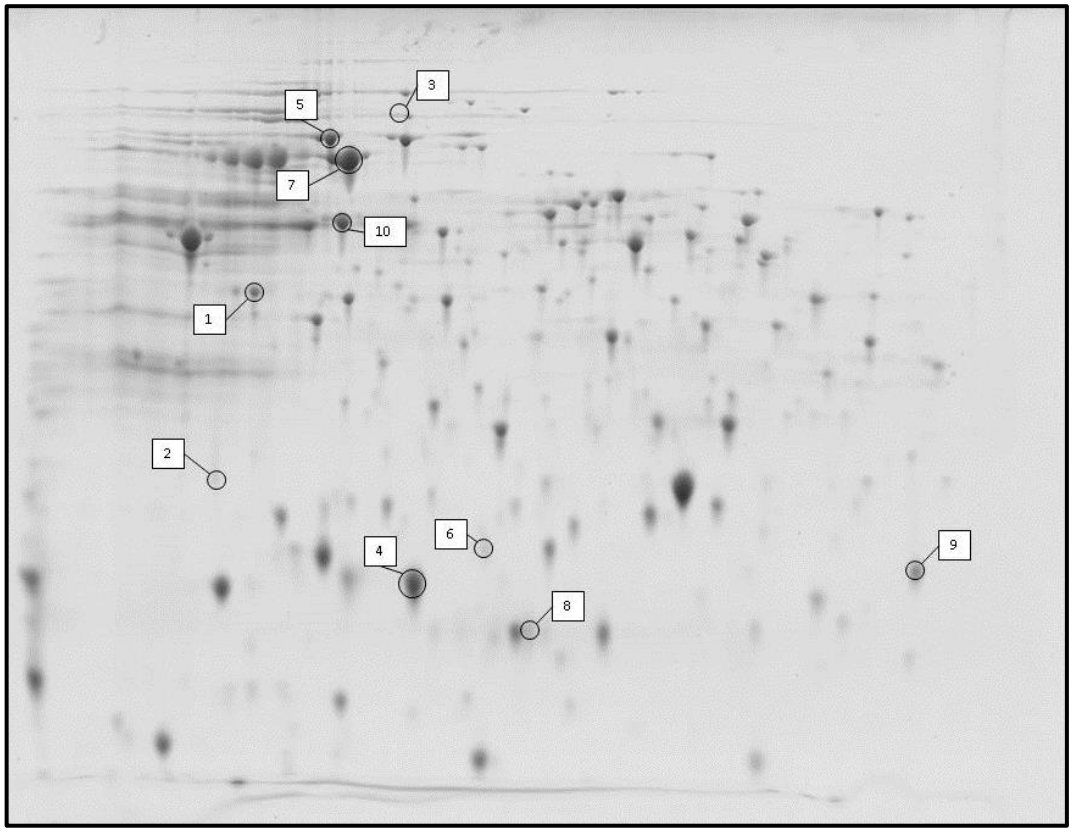


Figure 22. Positions of the ten protein spots identified as differentially present under pantoprazole exposed and control conditions. *The positions of the proteins listed in Table* ***17*** *are shown on a single gel.*

Protein 9 in **Table 17** was up-regulated in response to pantoprazole exposure and is known to be involved in purine metabolism. The metabolite xanthine (also linked to purine metabolism) was also detected in greater than two fold increase (see **Table 28**) following exposure to pantoprazole in metabolomics experiments which are detailed in **Appendix 3**.

In the second round of proteomics experiments, where experiments were performed in triplicate and computer software was used to identify proteins that were differentially present in the gels, four proteins which play roles in the oxidative stress response of *C. jejuni* were identified as being differentially present (proteins 4, 5, 7 and 8 in **Table 17**). All four of these proteins were found to be up-regulated in response to pantoprazole exposure.

A number of proteins were also identified as differentially present in proteomics 1 experiments, under pantoprazole exposed conditions versus the no pantoprazole controls, which were involved in the oxidative stress response of *C. jejuni*. Validation of the differential presence of selected oxidative stress proteins following exposure to pantoprazole was performed using qRT-PCR. qRT-PCR was also used to investigate the

effect of pantoprazole (an inhibitor of the H^+/K^+-ATPase) exposure on the ATP Synthase F1 portion of the *C. jejuni* proton pump (protein 10 in **Table 17**).

3.3.3 Quantitative Real-time Polymerase Chain Reaction

SYBR green is a fluorescent DNA binding dye that emits a fluorescent signal when it binds to double stranded DNA (dsDNA) and hence in qRT-PCR SYBR green can be used as a measure of the amount of primer specific dsDNA products. The *rpoA* gene of *C. jejuni* encodes for the alpha subunit of DNA-directed RNA polymerase was has previously been identified as a highly stable housekeeping gene that can be used to study gene expression variations between different stress conditions (Ritz *et al.*, 2009). Expression of *rpoA* was measured following exposure to 1 mg/ml pantoprazole for 1 or 2 hours and in the absence of pantoprazole exposure (also at 1 or 2 hours) so that the relative expressions of GroEL, ATP synthase F1 and thiol peroxidase could be assessed. Results in **Figure 23** show that in a representative qRT-PCR example, only one DNA product was detected using each of the primer sets listed in **Table 11**. The predicted size of the products (relative to the ladder) also appear to be as expected (see **Table 11** for product sizes) with the rpoA product being the smallest (bottom left of **Figure 23**) and GroEL (top left of **Figure 23**) being the largest.

qRT-PCR was performed in triplicate for each of the triplicate control and pantoprazole exposed samples at both 1 and 2 hours and gel electrophoresis used as described following each PCR. In all other technical replicates of qRT-PCR, only one DNA product was detected for all four tested genes (data not shown).

The presence of only one DNA product following each qRT-PCR was confirmed by analysing melt curve data generated by the Illumina software. In all cases (triplicates of triplicates at both time points) only one peak was detected, with a mean melting temperature that was in the expected range. The melting temperature of a product will depend on the length of the product and the guanine (G) and cytosine (C) content (i.e. a longer product with high G+C content will have a higher melting temperature than a shorter product with low G+C content). A representative example of a melt curve for the ATP synthase F1 gene product can be seen in **Figure 24**.

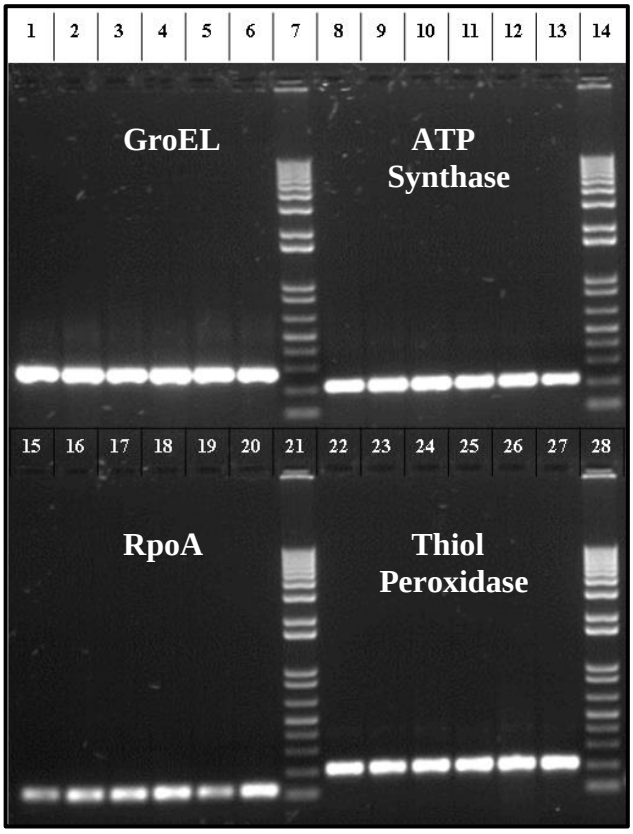


Figure 23. Agarose gel analysis of qRT-PCR products indicate that only one product is formed. *Following qRT-PCR for GroEL (wells 1-6), ATP synthase F1 (wells 8-13), rpoA (wells 15-20) or thiol peroxidase (wells 22-27) aliquots were removed from wells and analysed using gel electrophoresis. Samples were loaded in the order control sample 1, control sample 2, control sample 3, PPI exposed sample 1, PPI exposed sample 2 and PPI exposed sample 3 in all four cases (wells 1-6, wells 8-13, wells 15-20 and wells 22-27). Wells 7, 14, 21 and 28 contain 1 Kb Plus DNA Ladder.*

If primer dimer formation was an issue with the experimental design then a second (smaller peak) would be expected at a lower temperature than the target gene product. This was absent in all analyses (data not shown) and so primer dimer formation was not an issue with the qRT-PCR experiments presented here. Hence the only dsDNA found in the qRT-PCR was the primer specific product and the fluorescence signal emitted by SYBR green could therefore be used as an accurate measure of the amount of starting material.

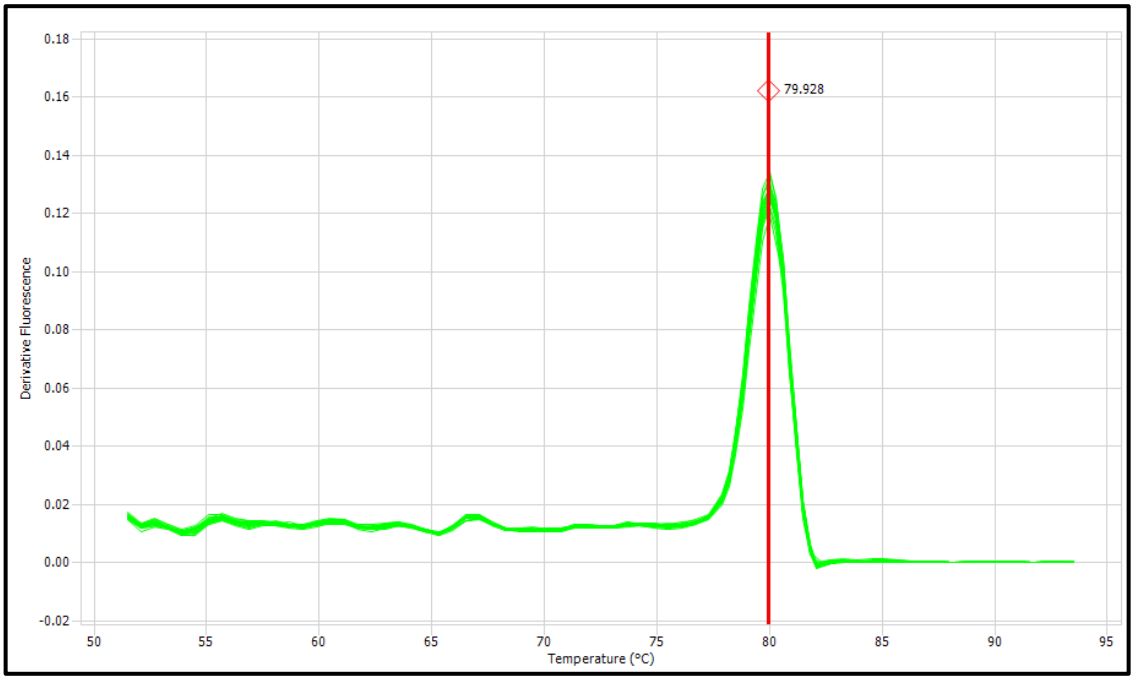


Figure 24. A representative example of melt curve analysis following qRT-PCR for ATP synthase F1 shows that only one product is formed. *Picture acquired using Illumina software.*

Because ImageMaster 2D Platinum software indicated that there was significant and highly significant changes to the proteome of *C. jejuni* strain 81-176 following exposure to 1 mg/ml (or 1000 µg/ml) pantoprazole for 2 hours, qRT-PCR was performed to analyse the relative expression of selected genes following exposure to the same pantoprazole concentration for 1 and 2 hours. The results of the qRT-PCR experiments are listed in **Table 18**. After only 1 hour of pantoprazole exposure thiol peroxidase was expressed 1.54 fold higher than for the no pantoprazole exposed control. The expression of GroEL and the ATP synthase F1 genes was similar under both pantoprazole exposed and control conditions (relative expression values near 1) in the 1 hour exposure experiment. However, following extended exposure to pantoprazole the expression of the two oxidative stress related genes (GroEL and thiol peroxidase) were over four fold higher than in the no pantoprazole exposed control. Up-regulation of the expression of the ATP synthase F1 gene was also seen following 2 hours exposure to 1 mg/ml pantoprazole, but to a much lower degree than that seen for thiol peroxidase and GroEL (a 1.5 fold increase versus around a four fold increase).

Table 18. Results of qRT-PC show that all three selected genes were expressed at a higher level following 2 hours exposure to pantoprazole.

Gene	Relative Expression Following Pantoprazole Exposure	
	1 Hour	2 Hours
ATP Synthase F1	0.94	1.49
GroEL	1.01	4.73
Thiol Peroxidase	1.54	4.02

3.3.4 Microarrays

Microarray experiments were performed to identify any changes in gene expression following exposure to 2 mg/ml (or 2,000 µg/ml) pantoprazole for 2 hours. The only gene which was found to be highly significant in its overexpression in response to exposure to pantoprazole for 2 hours was the gene *Cj0561c*. *Cj0561c* encodes for a putative periplasmic protein or membrane transporter in *C. jejuni*, which is repressed by the known repressor CmeR (Guo *et al.*, 2008, Dzieciol *et al.*, 2011). CmeR is best known as the repressor for the multi-drug efflux pump CmeABC which is involved in resistance to macrolides, fluoroquinolones and bile salts in *C. jejuni*. For this reason, the raw data for all three components of the CmeABC pump, the repressor CmeR and *Cj0561c* were analysed in greater detail.

The average values for the microarray experiments under pantoprazole exposed and control conditions are shown in **Table 19**. No significant difference was found in the expression of *cmeR*, *cmeB* or *cmeC* and yet a significant difference (P = 0.03) was seen in the expression of *cmeA* and a highly significant difference (P = 0.00001) seen in the expression of *Cj0561c*. No other genes were found to be expressed significantly or highly significantly differently in the microarrays. The expression of *cmeA* was around two fold higher following exposure to pantoprazole and the expression of *Cj0561c* was around six fold higher. The genes for the CmeABC pump are arranged in a single operon with the repressor *cmeR* positioned just upstream and *cmeA*, *cmeB* and *cmeC* are transcribed together (see **Figure 25**). When the raw data for the expression of all three genes together is compared for pantoprazole exposed and control conditions, no significant difference in the expression of CmeABC is evident (P = 0.07).

The bile salts cholate and taurocholate are known to block the binding of the repressor protein CmeR to the promotor regions of CmeABC and Cj0561c (see **Figure 25**) and so in the presence of bile, both CmeABC and Cj0561c are up-regulated (Guo *et al.*, 2008, Dzieciol *et al.*, 2011).

Table 19. Statistical analysis of microarray results for selected related genes.

Gene Number	Gene Name	Role	Average Control	Average PPI Exposed	P Value	Significance
Cj0365c	*cmeC*	Multidrug Efflux Pump	11,256	12,792	0.33	NS
Cj0366c	*cmeB*		15,764	15,091	0.80	NS
Cj0367c	*cmeA*		12,932	22,122	0.03	*
Cj0368c	*cmeR*	Repressor	9,228	6,054	0.14	NS
Cj0561c	-	Unknown	8,581	48,091	0.00	**

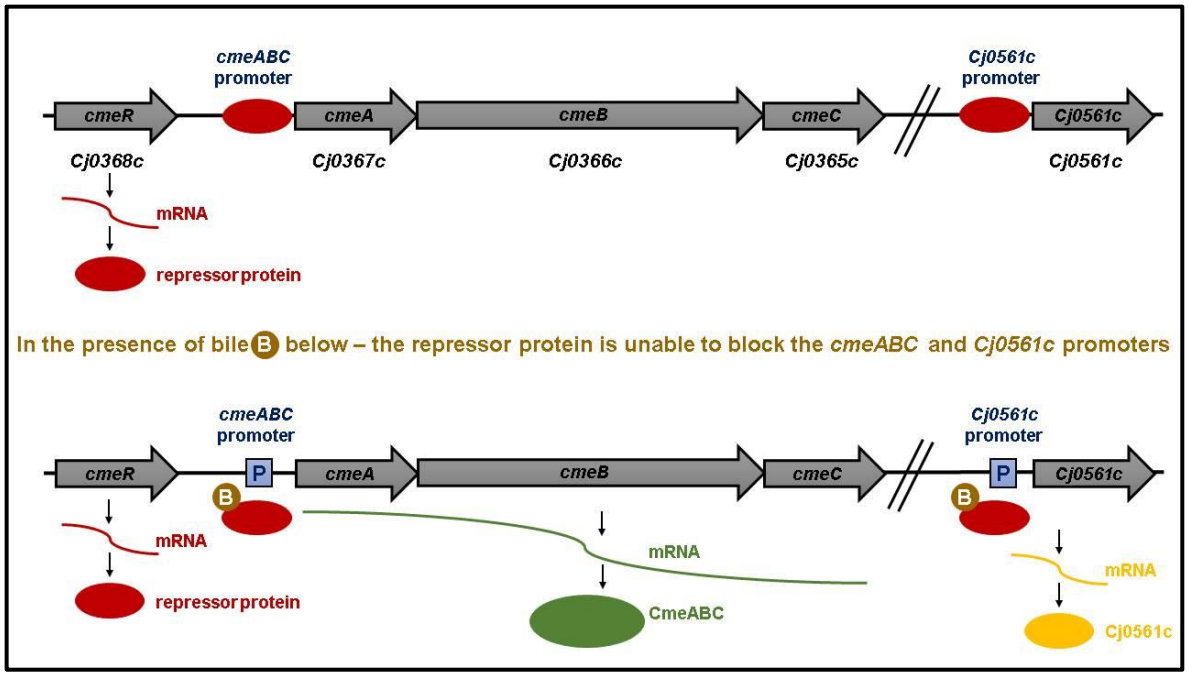


Figure 25. Bile stops the repression of both CmeABC and Cj0561c by interfering with the binding of the CmeR protein to promoter regions. *The repressor cmeR can be transcribed into mRNA and translated into a* ***protein*** *which acts as a repressor by blocking the binding of RNA polymerase to the respective upstream promoters (**P**). As such CmeR is capable of repressing the local CmeABC genes as well as the downstream Cj0561c gene.* ***Bile*** *inhibits the binding of cmeR to both the promoters and results in up-regulation of both CmeABC and Cj0561c expression.*

3.3.5 *kdpB* Mutant Experiments

The H^+/K^+-ATPase of parietal cells that is the target of PPIs is a P-type ATPase as is the bacterial Kdp ATPase. The possibility that the *kdp* of *C. jejuni* was a target for PPI activity was investigated by making an insertional mutant. Mutation of the *kdpB* gene caused no growth defect in *C. jejuni* (data not shown). There was no significant difference found in the susceptibility to pantoprazole killing between the *kdpB* mutant and the parent strain (**Table 20**). The MBC at 24 hours was the same for both strains and the difference

at 4 hours found to be not significant. Hence, mutation of the *kdpB* gene does not significantly alter susceptibility to pantoprazole in *C. jejuni*.

Table 20. Mutation of the kdpB gene causes no significant change in the pantoprazole MBC.

Time	**Mean Pantoprazole MBC (mg/ml) +/- SD**		**P Value**
	***C. jejuni* Strain**		
	11168-H	***kdpB* Mutant**	
4 Hours	2.5 +/- 1.4	3.5 +/- 1.27	0.051
24 Hours	1.25 +/- 0.87	1.25 +/- 0.87	1.000

3.4 Discussion

3.4.1 Proteomics 1

Discussion of the results for the proteomics 1 experiments has been omitted, as these experiments were not validated by the use of biological replicates or confirmed with additional work.

3.4.2 Proteomics 2

3.4.2.1 Determining Pantoprazole Exposure Conditions

Experiments were carried out only once in triplicate but results were in support of other observations (see **Sections 2.3.2**, **2.3.3** and **3.4.1)**. Killing by pantoprazole is both time and concentration dependent. The pantoprazole concentration chosen for triplicate proteomic analysis (1 mg/ml or 1,000 µg/ml) for a time of 2 hours was selected to ensure that there was no significant loss of viable bacteria in pantoprazole exposed samples. This was indeed found to be the case, as serial dilutions performed on samples following the pantoprazole exposure had a similar number of CFU/ml in both control samples and in the PPI exposed samples (see **Section 3.4.2.2**).

3.4.2.2 Proteomics

The predicted isolelectric points of most *C. jejuni* proteins lies between 4 and 11 and when using proteomics to detect changes in the *C. jejuni* response to bile exposure, most of the proteins found to be differentially present fell within the pH 4-7 range (Fox *et al.*, 2007). Hence gel strips in the range pH 4-7 were selected for use in this study and did successfully identify ten proteins which were differentially present under control conditions compared to pantoprazole exposed conditions. None of the proteins identified as differentially present in proteomics 1 experiments were identified as differentially present in proteomics 2 experiments. Results from proteomics 2 experiments are more robust than proteomics 1 because they were conducted in triplicate and gel images were analysed for differences using computer software (proteomics 1 experiments were carried out only once per pantoprazole exposure time and gels were assessed visually for differences).

One of the proteins shown to be up-regulated in response to pantoprazole exposure was the ATP synthase F1 subunit, which forms part of an ATPase that is involved with regulating the proton motive force of *C. jejuni* (Fox *et al*., 2007). It is possible that this ATPase is the target (or one of the targets) of PPIs in *C. jejuni*. If pantoprazole binds to and inhibits the ATP synthase (as it does for the H^+/K^+-ATPase of parietal cells) then the bacterium may be

attempting to make more ATP synthases to maintain the proton motive force (in the same way as stomach acid release resumes only on assembly of new proton pumps). The up-regulation of the ATP synthase F1 subunit of the *C. jejuni* ATPase was confirmed using qRT-PCR.

Bacteria have two different classes of proteins which are used for iron storage: ferritin and bacterioferritin. Both have a non-haem iron core, but bacterioferritins have an additional protohaem. Iron is an essential nutrient for all living organisms, lack of iron delays bacterial growth and so iron storage can be extremely advantageous. In response to pantoprazole exposure two proteins were found to be up-regulated which are used by *C. jejuni* for iron storage (proteins 6 and 8 in **Table 17**). The stress induced by *C. jejuni* during pantoprazole exposure may cause the bacterium to attempt to store more iron. Oxidative stress related proteins were identified in both proteomics 1 and proteomics 2 experiments. For this reason, two proteins involved in the oxidative stress response of *C. jejuni* were selected for gene expression analysis using qRT-PCR.

3.4.3 Quantitative Real-time Polymerase Chain Reaction

qRT-PCR is a very sensitive process which can easily be hindered by DNA contamination, technical error (the volumes used are very small) and primer dimer formation. The analysis of PCR products on agarose gels and the employment of additional melt curves following qRT-PCR indicated that no DNA contamination occurred and that primer dimers did not interfere with the data obtained. In qRT-PCR the cycle threshold (CT) is the number of cycles required for the accumulation of the fluorescence (due to the production of dsDNA in the case of SYBR green used in these experiments) to cross the threshold of background fluorescence. So, the lower the CT value, the higher the concentration of starting material in the original sample. Following exposure for 2 hours to (1 mg/ml or 1,000 µg/ml) pantoprazole expression of the GroEL heat shock protein gene was found to be almost 5 times greater than in the control, expression of the thiol peroxidase gene was around 4 times greater and expression of the ATP synthase F1 gene was around 1.5× greater (**Table 18**). These data confirm that protein spots identified by Image Master 2D Platinum as being differentially present were indeed altered in response to pantoprazole exposure.

In a study investigating the *C. jejuni* response to ox-bile exposure GroEL, the ATP synthase F1 subunit and a ferritin were all found to be up-regulated using 2D proteomic analysis (Fox *et al.*, 2007). This is interesting in light of microarray data which indicate the increased expression of the *Cj0561c* and *cmeA* genes (see below and **Figure 25**).

3.4.4 Microarrays

Cj0561c was the only gene which was highly significant (around a six fold increase) in its up-regulation when *C. jejuni* strain 81-176 was exposed to pantoprazole at 2 mg/ml (or 2,000 µg/ml) for 2 hours and *cmeA* was the only gene to be significantly up-regulated (around a two fold increase). As discussed previously (see **Section 3.3.2.1**), these conditions would have resulted in a loss of live bacteria in the pantoprazole exposed samples compared to the no pantoprazole control.

CmeA is a periplasmic membrane fusion protein, CmeB is an inner membrane efflux transporter and CmeC is an outer membrane channel forming protein (Lin *et al.*, 2003). Together they form the CmeABC pump which is involved in the extrusion of some conventional antibiotics, bile salts, detergents, dyes and heavy metals (Akiba *et al.*, 2006). Bile resistance is hugely important for enteric pathogens like *C. jejuni* as they need to survive in the intestines where bile is present. CmeR is known to be the repressor for the multi-drug efflux pump CmeABC and of the downstream gene *Cj0561c* (Guo *et al.*, 2008). Cj0561c is a putative periplasmic protein the function of which is unknown.

It has been shown that in the presence of bile, CmeR becomes inactivated in *C. jejuni* (see **Figure 25**) and expression of both CmeABC and *Cj0561c* becomes up-regulated (Guo *et al.*, 2008, Dzieciol *et al.*, 2011). So bile salts are therefore both extruded via CmeABC and bile salts also act to block the repression of CmeABC so that CmeABC expression can be increased and the extrusion capability of *C. jejuni* maximised.

Taurocholate is a bile salt that has been shown to effectively block the binding of CmeR to the promoter regions of CmeABC and *Cj0561c* (Shen *et al.*, 2011). Shen *et al* also demonstrated that in the presence of salicylate the CmeA, CmeB, CmeC and Cj0561c proteins appeared to be up-regulated (in varying amounts) even though there was no significant change in the expression of CmeR. Salicylate is one of the main metabolites of the drug aspirin and the authors proposed that their results showed that salicylate also interfered with the binding of CmeR to the promoter regions. In a study by Dzieciol *et al* it was shown that bile salts induced more effectively the *Cj0561c* gene than they did induce the CmeABC pump (Dzieciol *et al.*, 2011). Their results were similar to those shown here (see **Table 19**) in response to pantoprazole exposure and suggest perhaps that pantoprazole is both extruded via the CmeABC pump and that pantoprazole acts to block repression by CmeR. This theory is supported by the observation that no statistical difference was found in the expression of CmeR (see **Table 19**).

If pantoprazole interferes with the binding of CmeR to the promoter region of *Cj0561c* (perhaps in a manner similar to bile or salicylate) and presumably therefore also of CmeABC, then why do we not see increases in the expression of all three subunits *cmeA*, *cmeB* and *cmeC*? What we have actually shown is that there is a statistically significant increase in *cmeA* expression, no statistically significant change in *cmeB* expression (rather the average values for the pantoprazole exposed samples is lower than that of the controls) and that there is a small and statistically not significant increase in *cmeC* expression (**Table *19***). The inconsistencies in these results could be explained by the expected loss of viable *C. jejuni* in the pantoprazole exposed samples compared to the control. The effect of pantoprazole exposure on CmeR, CmeABC and Cj0561c is therefore unclear and the role of the CmeABC pump in response to exposure to pantoprazole will be further investigated in **Chapter 5**.

3.4.5 *kdpB* Mutant Experiments

The Kdp is best characterised in *E.coli* and is a P-type ATPase (as is the H^+/K^+-ATPase of parietal cells that is the target of PPIs). The Kdp-ATPase is involved in maintaining bacterial turgor pressure and in pH homeostasis (Altendorf *et al.*, 1998). The Kdp of *E. coli* has three membrane bound subunits which are KdpA, KdpB and KdpC. Expression of the *kdpABC* operon is controlled by the inner membrane bound protein KdpD and the soluble cytoplasmic protein KdpE (Walderhaug *et al.*, 1992). The *kdpA* gene encodes for the transmembrane subunit, the *kdpB* gene encodes for the catalytic ATPase subunit (which is the largest subunit) and *kdpC* encodes for an inner membrane protein. Kdp is induced under low potassium conditions and is repressed under high potassium conditions, it is not essential for survival.

KdpB is the largest subunit of the KdpABC ATPase and is the functional protein. As stated in **Section 3.2.7.1**, the KdpB of *C. jejuni* was found on examination to contain two existing restriction enzyme sites within its sequence, both of which leave sticky ends, making insertional mutation a simpler process (no need to first create a restriction enzyme site). Hence the *kdpB* gene was selected for insertional mutagenesis. Pseudogenes are genomic DNA sequences which are similar to the sequences of functional genes, but are themselves functionless. Many strains of *C. jejuni* have only pseudogenes or truncated open reading frames of the *kdp* operon but the *kdpB* gene appears to encode for a functional protein (Cameron *et al.*, 2012).

However, following successful formation of the deficient mutant, further study and research provided information that may have accounted for the mutant demonstrating no

differential susceptibility to pantoprazole. According to Hofreuter *et al C. jejuni* strain 81-176 has functional *kdpA*, *kdpB* and *kdpC* genes which encode for a potassium-transporting ATPase and *C. jejuni* strains RM1221 and 11168 have only pseudogenes (Hofreuter *et al.*, 2006). Yet Cameron *et al* used *C. jejuni* 81-176 in their study and state that the potassium-transporting ATPase system they belong to is "degenerate" and that the *kdp* genes are pseudogenes. *C. jejuni* strain 11168-H was selected for mutation, as this is the strain that is routinely used at the LSHTM for gene mutation and other laboratory experiments. On inspection of the nucleotide sequence, the *kdpB* gene appeared to be functional, but whether or not the Kdp ATPase is functional (as stated by Hofreuter *et al*) or "degenerate" (as stated by Cameron *et al*), the *kdpB* gene of 11168-H is likely to be a pseudogene, as is the case for 11168. Not only that, but the *kdpB* gene of *C. jejuni* contains an upstream homopolymeric tract (which is usually associated with hyper-variable or phase-variable gene expression) and so is likely to be unreliably transcribed even under normal culture conditions (Cameron *et al.*, 2012).

3.5 Summary and Conclusions

Proteomics 1 experiments were carried out only once (rather than in triplicate) at each pantoprazole exposure time point and differences in the size and intensity of protein spots assessed visually. The reliability of these results is therefore uncertain, but a number of proteins involved in LPS and bacterial membrane synthesis and proteins involved in the oxidative stress response of *C. jejuni* were identified as being differentially present under pantoprazole exposed and control conditions. Proteomics 2 experiments were carried out in triplicate, differentially present proteins were identified using computer software and results are therefore more reliable. Multiple proteins involved in the oxidative stress response of *C. jejuni* were again identified as being differentially present.

The up-regulation of two oxidative stress proteins (thiol peroxidase and GroEL) in response to pantoprazole exposure was confirmed using qRT-PCR, as was the increase in the ATP synthase F1 subunit. Only two genes were identified as being differentially expressed in response to pantoprazole exposure. These were the *Cj0561c* gene and *cmeA* gene; both were up-regulated following pantoprazole exposure. Mutation of the *kdpB* gene of *C. jejuni* resulted in no significant change to pantoprazole susceptibility and Kdp was thought to be an target of PPIs in *C. jejuni*.

Chapter 4

Role of Oxidative Stress in the Killing of *Campylobacter jejuni* by Pantoprazole

4 INTRODUCTION

4.1.1 Reactive Oxygen Species and Oxidative Stress

During aerobic metabolism, the production of reactive oxygen species (ROS) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^\bullet$) is unavoidable. As oxygen undergoes consecutive univalent reductions in order to be converted to water, all three of these ROS are produced. ROS are able to damage bacterial DNA, lipids and proteins and so must be detoxified by bacteria. Bacteria have various methods of dealing with this so called "oxidative stress", including DNA repair mechanisms and various oxidative stress response enzymes. If the levels of ROS exceed the organisms' ability to detoxify them, then cell death can result, as levels of damage to lipids, proteins and DNA becomes unsalvageable.

The response to oxidative stress is further complicated by the fact that ROS are not only generated during the reduction of oxygen, but also during the detoxification of other ROS as well (see **Figure 26**). For example, SOD enzymes can be used to remove toxic superoxide, but in doing so they contribute to oxidative stress by producing additional hydrogen peroxide. Hydrogen peroxide however can similarly be detoxified, by the activity of catalase or peroxidase enzymes (see **Figure 26**).

Of these three aforementioned ROS then, the hydroxyl radical is the most potent oxidising agent, as no enzyme exists which detoxifies it. Hydroxyl radicals are highly reactive and have a half-life of only nanoseconds. In the presence of intracellular iron, hydrogen peroxide reacts to form hydroxyl radicals in a reaction that is known as the Fenton reaction (see **Figure 26**). This reaction can occur easily in organisms which lack a catalase enzyme and also before hydrogen peroxide has been detoxified by catalase, in organisms which do have the enzyme.

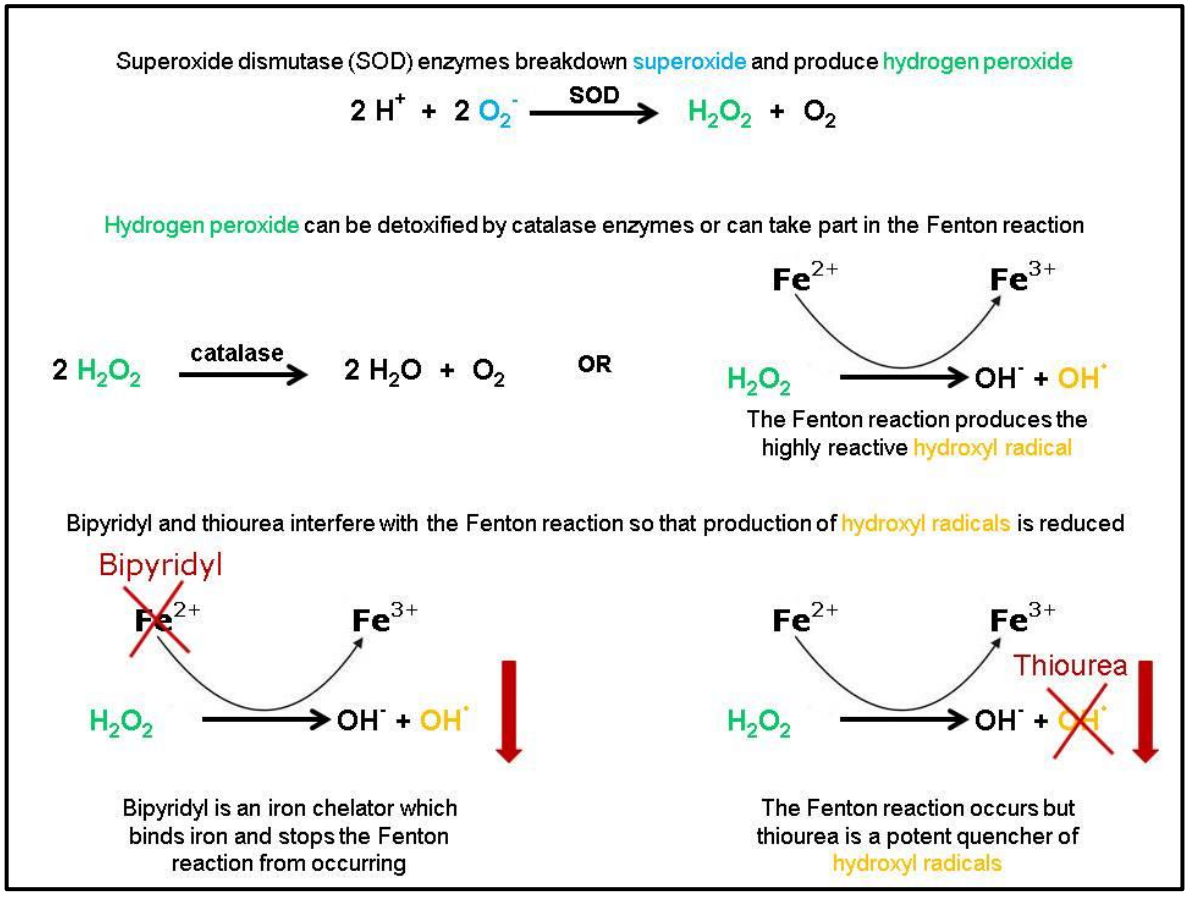


Figure 26. Oxidative stress and the roles of different oxidative stress inhibitors. *Superoxide dismutase (SOD) enzymes can detoxify superoxide and produce hydrogen peroxide. Catalase enzymes are capable of breaking down hydrogen peroxide into water and oxygen. Hydrogen peroxide can also participate in the Fenton reaction in the presence of free iron and be broken down, forming the toxic hydroxyl radical. The oxidative stress inhibitors bipyridyl and thiourea can be used to subvert the damage caused by hydroxyl radicals produced via the Fenton reaction at different stages of the reaction.*

4.1.2 Hydroxyl Radical Contributes to Cell Death

In a study which aimed to determine the role of iron in bacterial susceptibility to the host defence of neutrophil attack, the observation was made that higher levels of intrinsic iron increased the susceptibility of *S. aureus* to killing by hydrogen peroxide (Repine *et al.*, 1981). *S. aureus* cultures (following overnight incubation in broth with or without added iron) were exposed to varying concentrations of hydrogen peroxide for 60 minutes before viable counts were performed. The bacteria which had been incubated overnight in the presence of extra iron, had higher intrinsic levels of iron and were susceptible to killing by lower concentrations of hydrogen peroxide than the no added iron control. The authors highlighted that the Fenton reaction was the likely mechanism behind the results and used the potent hydroxyl radical quencher thiourea to reduce the number of hydroxyl radicals

produced via the Fenton reaction that were then free to damage DNA, lipids and proteins. In so doing, they increased the bacterial survival rates on exposure to hydrogen peroxide.

In 1988 it was reported that the addition of bipyridyl, 5 minutes prior to hydrogen peroxide exposure, increased the survival rates of *E. coli* compared to the no bipyridyl control (Imlay *et al.*, 1988). Bipyridyl is a potent chelator of ferrous iron (Fe^{2+}) and can be used *in vitro* to block the Fenton reaction from occurring, resulting in lower levels of hydroxyl radicals and less cell damage (see **Figure 26**). The addition of thiourea *in vitro* does not stop the Fenton reaction from occurring, but it does provide an effective means of quenching the hydroxyl radicals formed, before they are able to cause damage to essential bacterial components.

Thiourea and bipyridyl are therefore known as potent inhibitors of oxidative stress, each with an independent mechanism of action that can be used to manipulate the destructive potential of hydroxyl radicals formed via the Fenton reaction. Use of either (or both) of these two agents results in less intracellular damage to bacteria as a result of hydroxyl radicals, which in turn causes an increase in bacterial survival.

4.1.3 Oxidative Stress and Antibiotic Killing

Antibiotics usually fall into one of two categories, those which inhibit active replication or growth of bacteria (bacteriostatic) and those which kill > 99.9% of a bacterial population (bactericidal). Bacteriostatic agents often target ribosome function and bactericidal agents often target one of DNA replication/repair mechanisms, protein synthesis or cell wall synthesis.

In 2007 a common mechanism of cell death, for three different classes of bactericidal antibiotics (aminoglycosides, β-lactams, and quinolones) was reported, that involved the generation of hydroxyl radicals via the Fenton reaction (Kohanski *et al.*, 2007). Both thiourea and bipyridyl were used by the authors to increase the survival of *E. coli* exposed to kanamycin (an aminoglycoside which targets protein synthesis), ampicillin (a β-lactam which targets cell wall synthesis) and norfloxacin (a quinolone which targets DNA replication). The bacteriostatic antibiotics chloramphenicol, erythromycin, rifamycin, spectinomycin and tetracycline were also tested and these were found not to generate hydroxyl radicals and were therefore unaffected by the addition of either thiourea or bipyridyl. This study was the first to argue that bactericidal agents, in addition to affecting their own specific targets, also contribute to cell death by inducing the production of toxic levels of hydroxyl radicals via the Fenton reaction (see **Figure 27**).

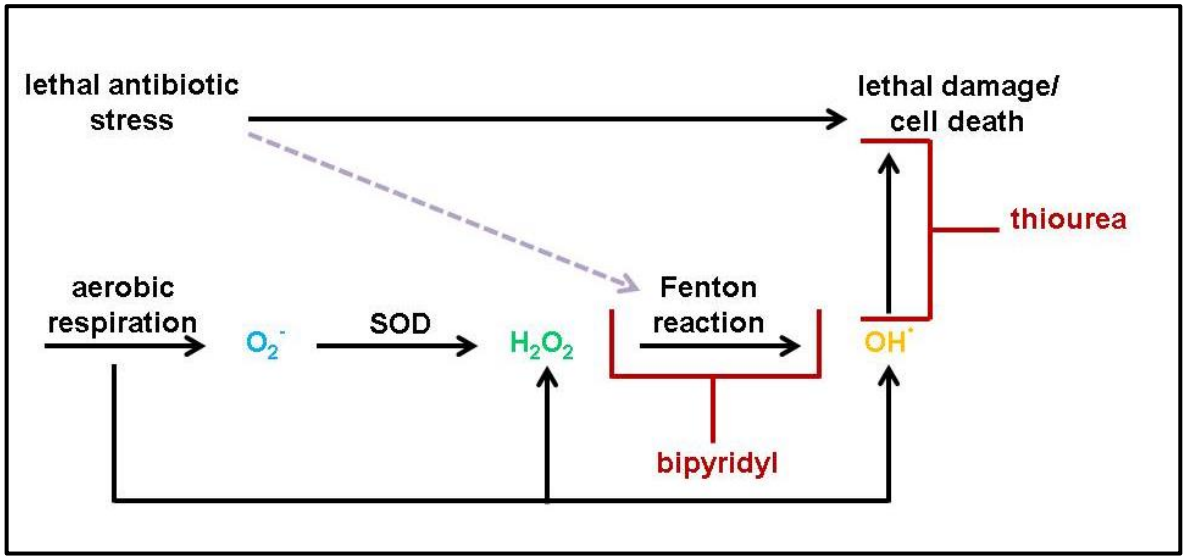


Figure 27. Hydroxyl radicals contribute to cell death. *Kohanski et al proposed that bactericidal antibiotics caused cell death via their own specific targets and also by inducing the production of hydroxyl radicals via the Fenton reaction. The contribution to cell death due to the induction of oxidative stress can be inhibited, at different stages, by* ***bipyridyl*** *and* ***thiourea****.*

Later other authors investigated the role of hydroxyl radical formation in the killing of bacteria by conventional antibiotics. In 2009 it was reported that an *E. coli* mutant lacking SOD enzymes was more resistant to killing by norfloxacin than the control (Wang & Zhao, 2009). In the absence of extrinsically added hydrogen peroxide, hydrogen peroxide is generated mainly via the activity of SOD enzymes. Hence, if the SOD activity is impaired, then the levels of hydrogen peroxide available to participate in the Fenton reaction would be expected to be lower and hence, the amount of hydroxyl radicals also lower. It was proposed by Wang and Zhao that this was indeed the reason that SOD deficient mutants were less susceptible to killing by norfloxacin. In further support of their argument, it was reported that an *E. coli* mutant lacking catalase activity (and hence having more hydrogen peroxide free to participate in the Fenton reaction) was more susceptible to killing by norfloxacin than the control.

The role of hydroxyl radical formation in the antibiotic killing of bacteria other than *E. coli* has also been investigated. The bactericidal class of antibiotics polymixins (small cationic peptides that target the cell membrane) were investigated using *E. coli* and *Acinetobacter baumannii* (*A. baumannii*) strains which were susceptible to and strains which were resistant to polymixins. The authors reported that hydroxyl radical levels only increased in the susceptible strains and that whereas bipyridyl and thiourea could be used to increase bacterial survival in these susceptible strains on exposure to polymixins, the oxidative

stress inhibitors had no effect on the resistant strains (Sampson *et al.*, 2012). Thiourea was used to show that killing of *Mycobacterium tuberculosis* by various antibiotic combinations resulted in the production of ROS (Grant *et al.*, 2012). Thiourea and bipyridyl in combination were also used to show that killing of *S. aureus* by oxacillin, daptomycin and moxifloxacin was enhanced by oxidative stress (Liu *et al.*, 2012).

Other bactericidal antibiotics have been shown to induce the stress response of *E. coli* that in turn inhibits the TCA cycle, thereby inhibiting NADH production and affecting the electron transport chain. Disrupting the electron transport chain can promote the production of superoxide, which is a precursor of the Fenton reaction (Sampson *et al.*, 2012).

4.1.4 Oxidative Stress in *Campylobacter jejuni*

C. jejuni is an obligate microaerophile as it uses oxygen as a terminal electron acceptor, but it is also extremely sensitive to high concentrations of oxygen. It grows best at 5-10% oxygen (the percentage of oxygen present in atmospheric air is around 20%). It has been established that addition of antioxidants (such as catalase) to growth media, enhances the growth of *C. jejuni* and suggested that *C. jejuni* might therefore be more susceptible to damage by free radicals than aero-tolerant bacteria (Kaakoush *et al.*, 2007). This suggestion seems reasonable as *C. jejuni* has only a single catalase enzyme (KatA) and a single SOD (SodB); in contrast the Gram negative enteric pathogen *E. coli* has three SODs and two catalase enzymes.

Gentamicin is an aminoglycoside that can be used to treat severe/systemic *Campylobacter* infections (Suy *et al.*, 2013) and ciprofloxacin is a fluoroquinolone that can be used in the treatment of severe campylobacteriosis (Joint-Formulary-Committee., 2015). Gentamicin is a broad-spectrum bactericidal agent which binds irreversibly to 30S ribosomal subunits and thereby inhibits protein synthesis. Ciprofloxacin is also a broad-spectrum bactericidal agent but inhibits DNA gyrase and thereby inhibits DNA synthesis. These two conventional antibiotics would therefore be excellent candidates for testing the contribution that hydroxyl radicals make to the killing of *C. jejuni*.

4.1.5 Chapter Aims

We have shown in **Chapter 2** that at certain concentrations, PPIs are able to kill *C. jejuni in vitro*. Experiments presented in this chapter were performed to investigate the role of oxidative stress in the killing of *C. jejuni* by selected conventional antibiotics which are relevant to the treatment of campylobacteriosis and belong to classes that reportedly induce

hydroxyl radical production in other bacterial species. The role of oxidative stress in the killing of *C. jejuni* by pantoprazole was also investigated using similar methods.

4.2 Materials and Methods

4.2.1 Bacterial Strains and Culture Conditions

The *C. jejuni* strains used in this chapter of the study are listed in **Table 21**. All strains were stored at -80°C, revived, cultured and incubated as detailed in **Section 2.2.1**. Strains from overnight growths were used in all individual experiments.

Table 21. Bacterial strains used in this chapter.

Strain	Features	Origin/Reference
C. jejuni 11168-H	Hypermotile derivative of strain 11168	(Karlyshev *et al.*, 2002)
C. jejuni 81-176	Human clinically isolated strain	(Korlath *et al.*, 1985)

4.2.2 Antibiotics, Oxidative Stress Inhibitors and Pantoprazole

A 2 mg/ml stock solution of gentamicin 10 mg/ml solution (Sigma) was prepared by diluting with sterile water and stored in the fridge. A stock solution of ciprofloxacin (Sigma) was prepared by dissolving in 1% acetic acid to give a 2 mg/ml solution. This was then diluted with sterile water to a final concentration of 150 µg/ml, was wrapped in foil and stored in the fridge. A 5 mM stock solution of 2,2'-bipyridyl (bipyridyl; Sigma) was prepared by dissolving in boiling water and a one molar stock solution of thiourea (BDH Laboratory Supplies) was prepared by dissolving in water. Both of these were stored at room temperature. All stock solutions were sterilised using a 0.2 µm filter (Corning) and further diluted when required in sterile water. Pantoprazole sodium hydrate was prepared as described in **Section 2.2.2**. Control cultures were performed routinely to ensure the sterility of water, oxidative stress inhibitors, PPI, the highest and the lowest dilution of all antibiotics used in each experiment.

4.2.3 Thiourea and Bipyridyl Tolerance

A bipyridyl and thiourea concentration that did not critically affect the survival of *C. jejuni* was determined. Three ml MHB was added to 7 ml plastic bijous with loosened caps and allowed to equilibrate in the VAIN for 4 hours. Then bipyridyl (final concentrations ranging from 500-50 µM), thiourea (final concentrations ranging from 150-37.5 mM) or water for oxidative stress inhibitor free controls was added to individual flasks. A bacterial suspension was prepared by harvesting colonies from an overnight culture on MHA + B of *C. jejuni* 11168-H and correcting the OD_{600} in MHB to 0.2. The flasks were inoculated with 400 µl each, mixed gently and incubated in the VAIN. At 24 hours the OD_{600} was

determined (once for each replicate of the experiment) and serial dilutions performed in PBS to determine CFU/ml.

4.2.4 Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

The MIC (for gentamicin and ciprofloxacin) and the MBC (for pantoprazole) were determined using the broth microdilution method described in **Section 2.2.4**. The antibiotics were tested at concentrations ranging from 20-0.02 µg/ml and pantoprazole at concentrations ranging from 10-0.02 mg/ml (or 10,000-20 µg/ml) with sterile water being used for additional no drug controls. The microtitre plates were covered with a sterile lid and wrapped in foil (to protect ciprofloxacin from light) before being incubated in the VAIN for 24 hours. OD_{595} was measured using a Labsystems Ascent Multiscan plate reader.

4.2.5 Oxidative Stress Inhibition

A 1:100 dilution in fresh MHB of bacterial suspensions corrected to OD_{600} of 0.2 in MHB were prepared from plate cultures. *C. jejuni* strains 11168-H and 81-176 were exposed to ½ MIC ciprofloxacin (0.05 and 0.04 µg/ml respectively), ½ MIC gentamicin (0.25 and 0.15 µg/ml respectively) or sub-lethal pantoprazole (250 µg/ml) in the presence and absence of bipyridyl at 75 µM and thiourea at 50 mM. 96 well microtitre plates were used to test in triplicate each antibiotic, each antibiotic in the presence of either bipyridyl or thiourea, pantoprazole alone, pantoprazole in the presence of either bipyridyl or thiourea, each antibiotic in the presence of pantoprazole and each antibiotic in the presence of pantoprazole and either bipyridyl or thiourea. Positive controls were included which were free from any antibiotic, pantoprazole or oxidative stress inhibitors. Plates were covered and incubated as described previously and OD_{595} nm determined at 24 hours.

4.2.6 Replicates and Data Analysis

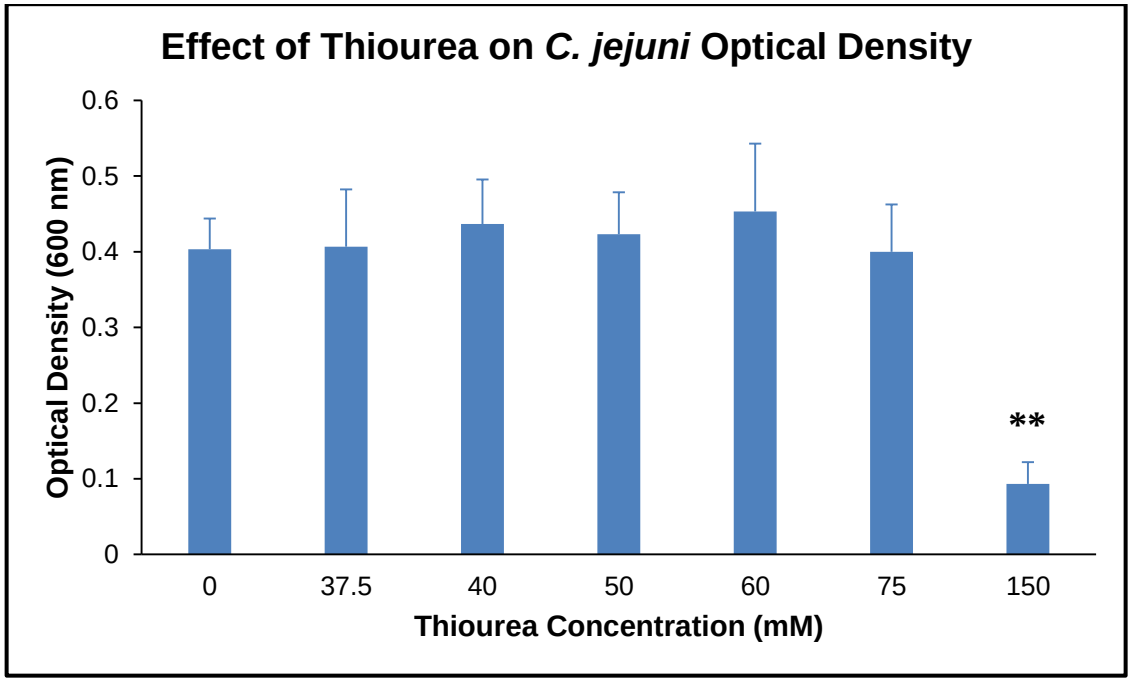
Unless otherwise stated each assay was conducted in triplicate and was independently repeated at least three times. Results are expressed as means +/- standard deviations (error bars) of replicate experiments. The unpaired Students t test was used to determine statistical significance. A P value of > 0.01 but < 0.05 was considered significant (*****) and a P value of < 0.01 highly significant (******).

4.3 Results

4.3.1 Thiourea and Bipyridyl Tolerance

Thiourea has been used as an inhibitor of oxidative stress in various bacteria by other authors at concentrations of 50 and 100 mM (Liu *et al.*, 2012), 100 mM (Wang & Zhao, 2009), 300 mM (Sampson *et al.*, 2012), but most commonly at 150 mM (Kohanski *et al.*, 2007, Grant *et al.*, 2012, Keren *et al.*, 2013). Experiments were first performed to find a thiourea concentration that did not critically affect the survival of *C. jejuni*. Results in **Figure 28** show that *C. jejuni* was unable to tolerate 150 mM thiourea and a final concentration of 50 mM was used for further work in this study.

Bipyridyl has been used as an inhibitor of oxidative stress by other authors at concentrations of 250 µM (Wang & Zhao, 2009), 500 µM (Kohanski *et al.*, 2007), 600 µM (Sampson *et al.*, 2012) and at 500 and 750 µM (Liu *et al.*, 2012). Results in **Figure 29** show that *C. jejuni* was unable to tolerate concentrations of bipyridyl above 150 µM and a final concentration of 75 µM was selected for further work in this study. Results in **Figures 28** and **29** also show that a reduction in OD is indicative of a corresponding reduction in surviving *C. jejuni* CFU/ml.



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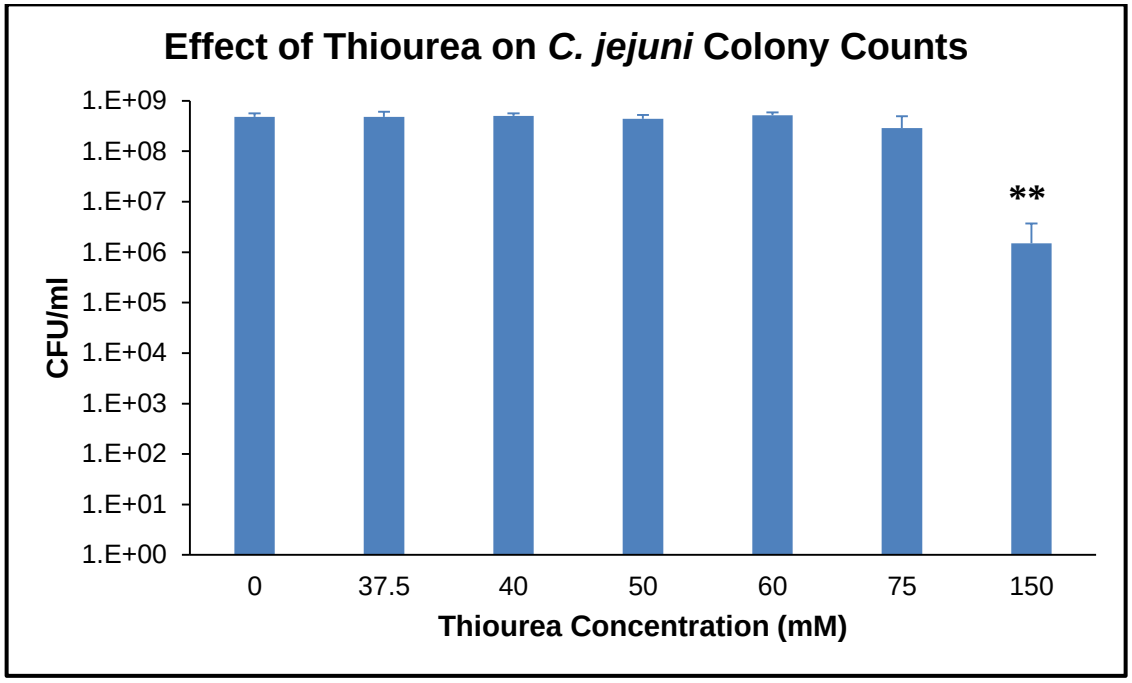
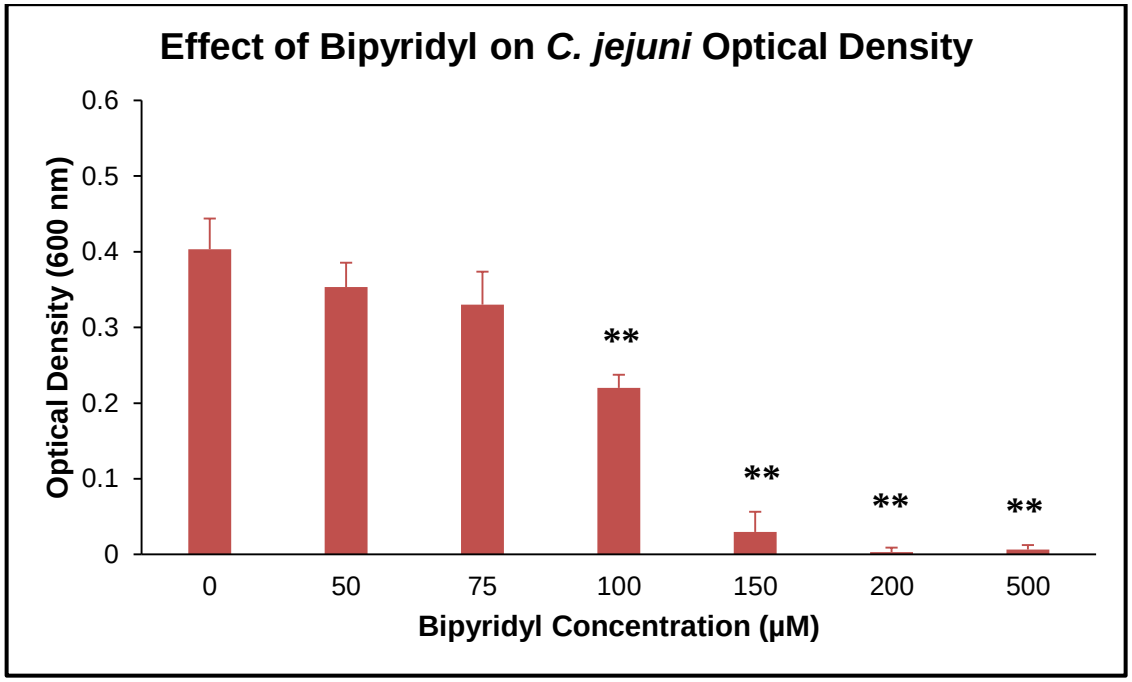


b

Figure 28. Effect of the oxidative stress inhibitor thiourea on the survival of C. jejuni. *C. jejuni strain 11168-H in MHB was exposed to a range of thiourea concentrations and incubated in the VAIN for 24 hours before OD was determined at 600 nm (a) and serial dilutions performed to calculate CFU/ml (b). Levels of significance, as indicated by* ** *(P value < 0.01) relate to the individual test conditions compared to the no thiourea control.*



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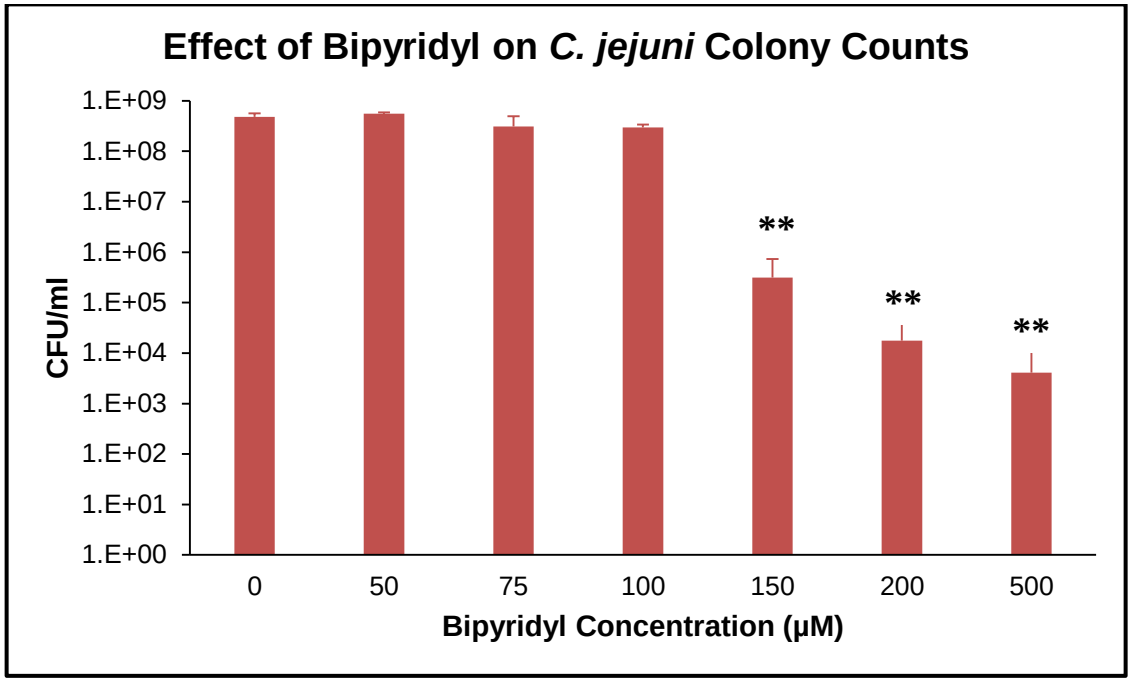


b

Figure 29. Effect of the oxidative stress inhibitor bipyridyl on the survival of C. jejuni. *C. jejuni strain 11168-H in MHB was exposed to a range of bipyridyl concentrations and incubated in the VAIN for 24 hours before OD was determined at 600 nm (a) and serial dilutions performed to calculate CFU/ml (b). Levels of significance, as indicated by* ** *(P value < 0.01) relate to the individual test conditions compared to the no bipyridyl control.*

4.3.2 Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

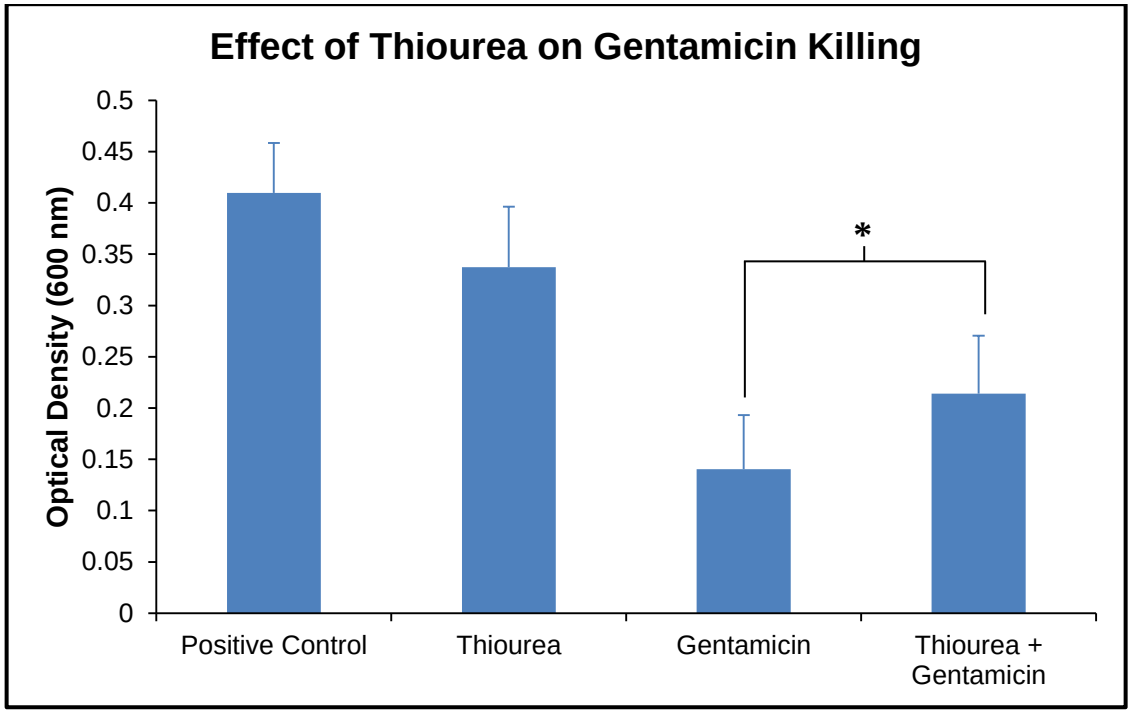
Because oxidative stress inhibition experiments were to be carried out at ½ MIC gentamicin and ½ MIC ciprofloxacin, the MIC for both of these antibiotics was first determined for *C. jejuni* strain 81-176. As ciprofloxacin was dissolved in acetic acid, the acetic acid concentration in serial dilutions which was itself sufficient to inhibit/kill *C. jejuni* was also determined. *C. jejuni* was found to tolerate final concentrations of ≤ 0.01% acetic acid. Preparation of ciprofloxacin stock solution (150 µg/ml) in the manner described in **Section 4.2.2** results in a final acetic acid concentration of 0.075%. Ciprofloxacin was then tested at a maximum final concentration of 20 µg/ml and this ensures that the residual acetic acid concentration was low enough to have no effect on *C. jejuni* survival. The MICs for ciprofloxacin were found to be 0.1 and 0.08 µg/ml respectively for strains 11168-H and 81-176 and for gentamicin were 0.5 and 0.3 µg/ml respectively for the two strains. As previously described (see **Section 2.3.2**) an MIC for pantoprazole could not be accurately determined but the MBC for both 11168-H and 81-176 was found to be 1 mg/ml (or 1,000 µg/ml).

The MIC is often ½ of the measured MBC (Sjostrom *et al.*, 1997). Gentamicin and ciprofloxacin were to be used at ½ MIC and so pantoprazole was used at a concentration of 250 µg/ml in these experiments.

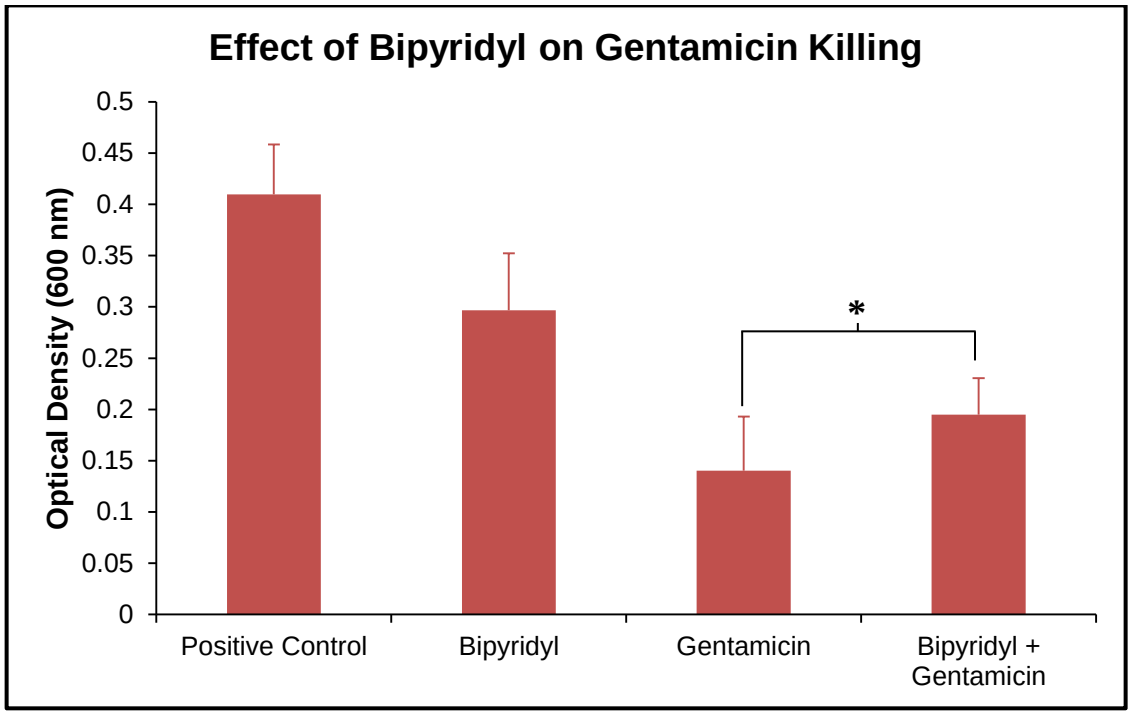
4.3.3 Oxidative Stress Inhibition

4.3.3.1 Gentamicin

Gentamicin is an aminoglycoside that can be used to treat severe or systemic *Campylobacter* infections (Quinn *et al.*, 2007) and killing by aminoglycosides has previously been shown to be inhibited by thiourea and bipyridyl (Kohanski *et al.*, 2007, Wang & Zhao, 2009). *C. jejuni strain 81-176* was exposed to ½ MIC gentamicin in the absence and presence of either thiourea or bipyridyl to investigate whether the production of hydroxyl radicals via the Fenton reaction plays a role in the killing of *C. jejuni* by gentamicin. Both thiourea ($P = 0.011$) and bipyridyl ($P = 0.027$) significantly increased *C. jejuni* survival (**Figure 30**) as demonstrated by an increase in OD, suggesting that killing of *C. jejuni* by gentamicin is partly mediated by the production of hydroxyl radicals. This shows that using similar methods to those used by others with *E. coli* the killing of *C. jejuni* by gentamicin induces hydroxyl radical production (as is true for *E. coli*).



a



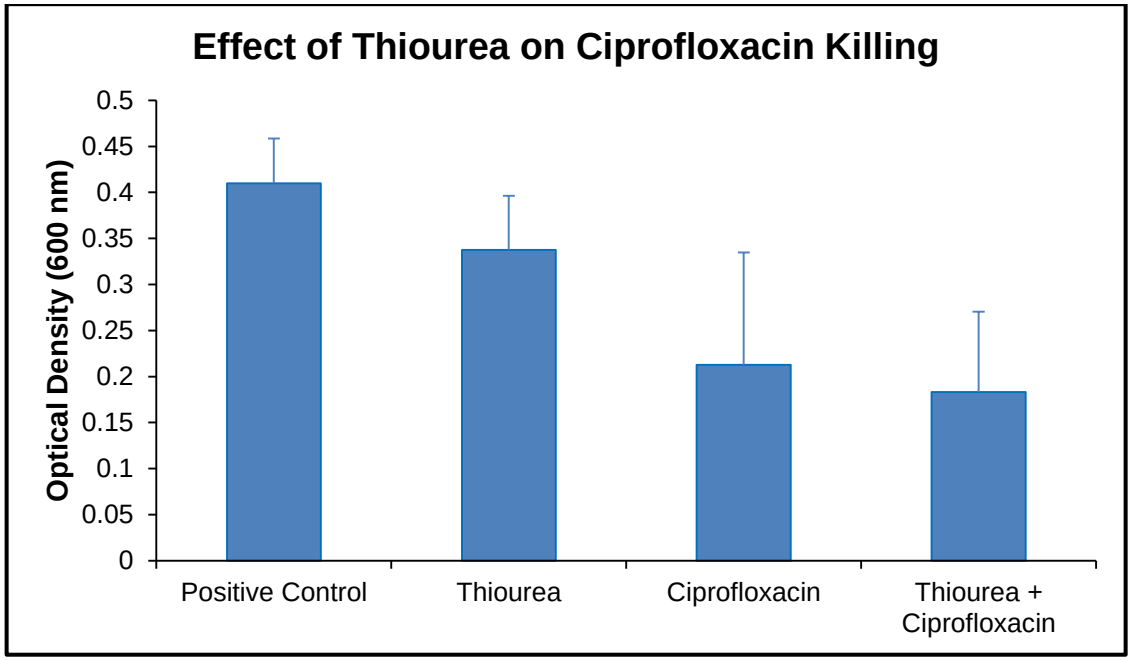
b

Figure 30. Both thiourea and bipyridyl protect C. jejuni from killing by gentamicin. *C. jejuni strain 81-176 in MHB positive control was grown in the absence of any gentamicin, thiourea or bipyridyl. C. jejuni was also grown in the presence of 0.15 µg/ml gentamicin for 24 hours before measuring* OD_{600}*. Thiourea (a) and bipyridyl (b) were used as inhibitors of oxidative stress at final concentrations of 50 mM and 75 µM respectively. Addition of the oxidative stress inhibitors thiourea or bipyridyl results in a significant increase in bacterial survival, compared to the gentamicin only control, as indicated by* ***** *(P value > 0.01 but < 0.05).*

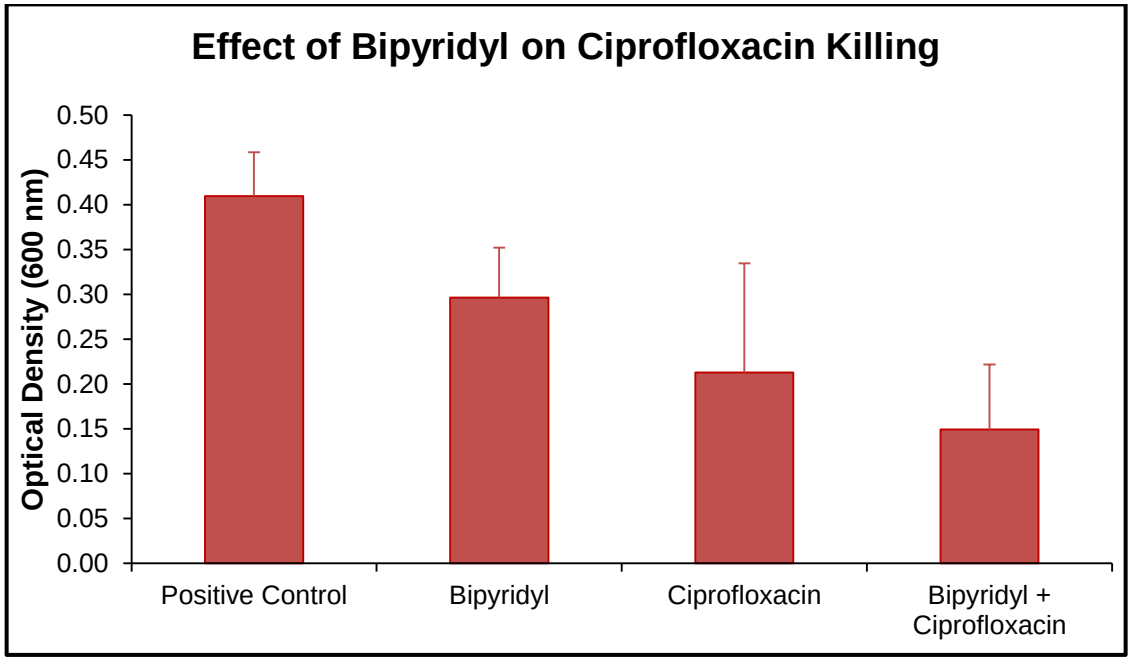
4.3.3.2 Ciprofloxacin

Ciprofloxacin is a fluoroquinolone that can be used to treat *Campylobacter* infections (Quinn *et al.*, 2007). It is also the drug of choice when treating gastroenteritis of unknown aetiology (Zilbauer *et al.*, 2008) and killing by quinolones has previously been shown to be inhibited by thiourea and bipyridyl (Kohanski *et al.*, 2007, Wang & Zhao, 2009, Grant *et al.*, 2012, Liu *et al.*, 2012).

No increase in OD at 24 hours was observed for *C. jejuni* strain 81-176 exposed to ½ MIC ciprofloxacin in the presence of either thiourea (**Figure 31a**) or bipyridyl (**Figure 31b**). The same pattern was also observed using 11168-H (data not shown) suggesting that killing of *Campylobacter* by ciprofloxacin is not supplemented by the production of hydroxyl radicals.



a



b

Figure 31. Neither thiourea nor bipyridyl protect C. jejuni from killing by ciprofloxacin. *C. jejuni strain 81-176 in MHB positive control was grown in the absence of any gentamicin, thiourea or bipyridyl. C. jejuni was also grown in the presence of 0.04 µg/ml ciprofloxacin for 24 hours before measuring OD_{600}. Thiourea (a) and bipyridyl (b) were used as inhibitors of oxidative stress at final concentrations of 50 mM and 75 µM respectively. Addition of the oxidative stress inhibitors thiourea or bipyridyl does not result in any significant increase in bacterial survival, compared to the ciprofloxacin only control.*

4.3.3.3 Pantoprazole

Thiourea and bipyridyl were used at the same concentrations used in gentamicin and ciprofloxacin experiments to investigate whether inhibition of oxidative stress could increase the survival of *C. jejuni* exposed to sub-lethal pantoprazole. No increase in

survival was seen with either inhibitor in the presence of pantoprazole, rather the OD at 24 hours was reduced in both cases (last two bars on the far right of **Figure 32**). This suggests that killing of *C. jejuni* by pantoprazole is not mediated by the production of hydroxyl radicals. Sub-lethal levels of pantoprazole in combination with sub-MIC thiourea or bipyridyl results in reduced bacterial survival. Co-exposure to thiourea and pantoprazole caused a highly significant reduction in bacterial survival compared to thiourea alone (P = 0.0001) or compared to pantoprazole alone (P = 0.0001). Co-exposure to bipyridyl and pantoprazole caused a highly significant reduction in bacterial survival compared to bipyridyl alone (P = 0.0002) or compared to pantoprazole alone (P = 0.0001).

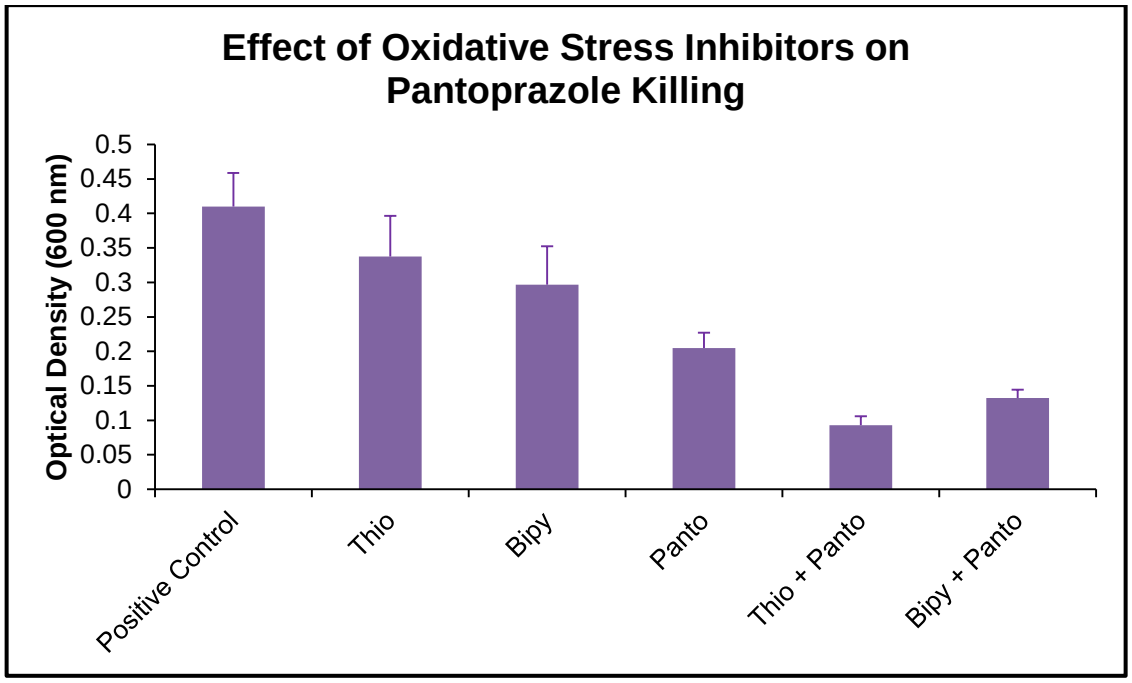


Figure 32. Neither thiourea nor bipyridyl protect C. jejuni from killing by pantoprazole. *C. jejuni strain 11168-H in MHB positive control was grown in the absence of thiourea (Thio), bipyridyl (Bipy) or pantoprazole (Panto). C. jejuni was also grown in the presence of thiourea, bipyridyl or pantoprazole at final concentrations of 50 mM, 75 µM or 250 µg/ml respectively for 24 hours before measuring* OD_{600}*. Thiourea and bipyridyl were used as inhibitors of oxidative stress, in the presence of pantoprazole at the concentrations stated above.*

4.4 Discussion

4.4.1 Thiourea and Bipyridyl Tolerance

The oxidative stress inhibitors thiourea and bipyridyl have been used in other studies at a variety of concentrations with various Gram negative and Gram positive organisms. Some studies state that both thiourea and bipyridyl inhibit bacterial growth (Kohanski *et al.*, 2007, Wang & Zhao, 2009, Liu *et al.*, 2012, Sampson *et al.*, 2012), other studies use only one of the inhibitors and agree that growth inhibition occurs (Cole *et al.*, 2006, Keren *et al.*, 2013), whereas others state that no growth inhibition occurs (Repine *et al.*, 1981, Olekhnovich *et al.*, 2014). The discrepancy even exists between studies using the same bacterial species, for example *S. aureus* is reportedly not inhibited by thiourea in one study (Olekhnovich *et al.*, 2014) and *S. aureus* is reportedly inhibited by both thiourea and bipyridyl in another (Liu *et al.*, 2012). Where studies report that thiourea and/or bipyridyl affect bacterial growth, then a concentration which is below the MIC is often used. In some cases a concentration of ½ MIC is suggested (Wang & Zhao, 2009).

Thiourea has been used most often at a concentration of 150 mM in other studies (Kohanski *et al.*, 2007, Grant *et al.*, 2012, Keren *et al.*, 2013) and the lowest concentration reportedly used was 50 mM (Liu *et al.*, 2012). Bipyridyl has been used in other studies at concentrations as low at 250 µM (Wang & Zhao, 2009) and as high as 750 µM (Liu *et al.*, 2012). Results in **Figures 28** and **29** show that both thiourea and bipyridyl have a dose dependent inhibitory effect on *C. jejuni*. This is in support of another study which reports that bipyridyl affects the survival of *C. jejuni* (Cole *et al.*, 2006).

C. jejuni growth was highly significantly inhibited by 150 mM thiourea and so a concentration of 50 mM was used in further experiments, as this was the lowest reported concentration used by others and 50 mM thiourea did not significantly affect *C. jejuni* growth. *C. jejuni* growth was highly significantly inhibited by concentrations as low as 100 µM bipyridyl and so a concentration that was lower than those reportedly used by others had to be used in further *C. jejuni* experiments. *C. jejuni* appears to be highly sensitive to these two agents (particularly to the iron chelator bipyridyl). As a consequence, bipyridyl was used in these experiments at a concentration much lower than those used in other studies. It has been suggested that using thiourea and bipyridyl at low concentrations would lead to incomplete protection against killing due to incomplete prevention of hydroxyl radical accumulation (Wang & Zhao, 2009). This was likely a factor in the experiments presented in this chapter and it is possible that oxidative stress

could not be inhibited to the same degree for *C. jejuni* as was achieved by other authors using the chemical inhibitors thiourea and bipyridyl at higher concentrations.

The possibility that exposure to pantoprazole increases the permeability of the outer membrane of *C. jejuni* is discussed in further detail in **Chapter 5**. This possibility however may account for the increased susceptibility to other agents when *C. jejuni* is exposed to them in combination with pantoprazole. It is possible that in the presence of pantoprazole, increased membrane permeability causes a higher intracellular concentration at a set extracellular concentration to develop.

4.4.2 Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

Whilst investigating the anti-*Helicobacter* activity of a PPI, Sjostrom *et al* reported that the bactericidal activity was more pronounced over time and following acid activation, suggesting that one or more of the sulfenamide derivatives was responsible for the anti-bacterial activity (Sjostrom *et al.*, 1997). Killing of *C. jejuni* by pantoprazole has been shown to be both concentration and time dependent (see **Table 8**) and the anti-*Campylobacter* activity of pantoprazole is probably also due to one or more of the sulfenamide derivatives. Hence an exposure time of 24 hours was selected for investigation of the anti-*Campylobacter* activity of pantoprazole and for the conventional antimicrobials ciprofloxacin and gentamicin. The MIC of ciprofloxacin was found to be similar for the two strains of *C. jejuni* tested, as was the MIC of gentamicin. These were notably in the µg/ml range (as is common for conventional antibiotics). The pantoprazole concentration which is required to kill is therefore notably much higher (in the mg/ml range).

4.4.3 Oxidative Stress Inhibition

4.4.3.1 Gentamicin and Ciprofloxacin

Aminoglycosides and quinolones have been shown by others to induce hydroxyl radical accumulation and the aminoglycoside gentamicin and the quinolone ciprofloxacin are both antibiotics that can be used in the treatment of *Campylobacter* infections. Hence ciprofloxacin and gentamicin were selected for use in these experiments. Experiments were performed using either thiourea (a quencher of hydroxyl radicals) or bipyridyl (an iron chelator) as inhibitors of oxidative stress, to investigate if hydroxyl radical accumulation contributed to the killing of *C. jejuni* by these two antibiotics.

As has been reported by others, using other bacterial genera such as *E. coli* (Kohanski *et al.*, 2007, Wang & Zhao, 2009, Foti *et al.*, 2012) and *A. baumannii* (Sampson *et al.*, 2012), killing of *C. jejuni* by gentamicin was found to be mediated by the production of hydroxyl radicals. Killing of *C. jejuni* by ciprofloxacin was found not to be mediated by the production of hydroxyl radicals. In one of the key studies relevant to this work, addition of bipyridyl caused an increase in bacterial survival (compared to the antibiotic alone) of around 4 log for an aminoglycoside but only of 2 log for a quinolone (Kohanski *et al.*, 2007). This suggests that hydroxyl radicals contributed more to the killing of *E. coli* by aminoglycosides than to killing by quinolones. This, in combination with the lower concentrations of bipyridyl and thiourea that could be used in *C. jejuni* experiments may account for why the two antibiotic classes did not equally induce hydroxyl radical production.

It is also worth noting that in many other studies, shorter time points were used and antibiotics added at much higher concentrations than were employed here (sometimes as high at 10× the MIC (Grant *et al.*, 2012)). In a study which reportedly dismissed the findings of Kohanski *et al*, the killing of *E. coli* by a quinolone was shown to be significantly decreased, on the addition of thiourea, at some concentrations of norfloxacin, but not at other norfloxacin concentrations (Keren *et al.*, 2013).

4.4.3.2 Pantoprazole

The killing of *C. jejuni* by pantoprazole was shown not to be mediated by the production of hydroxyl radicals. Instead of demonstrating an increase in bacterial survival (as would be expected if killing was mediated by hydroxyl radical production) a highly significant decrease in *C. jejuni* survival was found on exposure to sub-lethal levels of pantoprazole and thiourea and on exposure to sub-lethal levels of pantoprazole and bipyridyl.

It has been suggested that published works on oxidative stress and hydroxyl radical killing, that have made use of the iron chelator bipyridyl, may have overlooked effects on other bacterial processes that are dependent on iron and that the presence of bipyridyl does more than just block hydroxyl radical production via the Fenton reaction (Liu *et al.*, 2012).

Results in **Figure 32** show that the killing of *C. jejuni* by pantoprazole can be enhanced if the *C. jejuni* is exposed to pantoprazole and thiourea, even when the concentrations of the individual agents are sub-lethal. The same is true for pantoprazole in combination with bipyridyl. Thiourea is a hydroxyl radical quencher and bipyridyl is a chelator of ferrous iron and yet the presence of either chemical increases the anti-*Campylobacter* activity of

pantoprazole. This may be due to *C. jejuni* being unable to successfully extrude via efflux two compounds which are identified as potential toxins, thereby increasing the susceptibility to the anti-*Campylobacter* activity of pantoprazole. The role of efflux in response to *C. jejuni* exposure to pantoprazole will be further investigated in **Chapter 5**.

4.5 Summary and Conclusions

Development of therapeutic agents requires the identification of unique target sites that are essential to the pathogen (Smith *et al.*, 1999). In recent years, the development rate of new antibiotics has slowed considerably, whilst resistance rates have continued to increase. The effectiveness of our current arsenal of antibiotics therefore is diminishing and it may prove useful and cost effective to investigate means of potentiating the efficacy of currently used antibiotics. This may include methods of inducing bacterial oxidative stress or supressing the bacterial protective responses to oxidative stress (Belenky & Collins, 2011).

The killing of *C. jejuni* by gentamicin was shown to be mediated by the production of hydroxyl radicals and the induction of oxidative stress in the bacterium. The killing of *C. jejuni* by ciprofloxacin and pantoprazole however was shown not to be mediated by the production of hydroxyl radicals. The co-exposure to sub-lethal levels of ciprofloxacin (a conventional antibiotic) and bipyridyl (an iron chelator which inhibits *C. jejuni* growth in a dose dependent manner) was shown to cause a significant reduction in *C. jejuni* growth (compared to the antibiotic alone). The same was true for *C. jejuni* co-exposed to sub-lethal levels of pantoprazole and bipyridyl and for *C. jejuni* co-exposed to sub-lethal levels of pantoprazole and thiourea. The effect of co-exposure to pantoprazole and conventional antibiotics will be further investigated in **Chapter 5**.

Chapter 5

Effect of Pantoprazole on the Susceptibility of *Campylobacter jejuni* to Conventional Antibiotics

5 INTRODUCTION

5.1 Antibiotic Resistance

Antibiotic resistance is a growing problem worldwide and as such poses an important risk to public health. Factors contributing to the problem are many and varied but increased resistance may be linked to increased use of antibiotics in animal husbandry and veterinary practice where they are used both as growth promoters and as therapeutic agents. This activity can cause selective pressure on organisms, which then develop resistance and resistant organisms can be transferred to humans and then possibly go on to cause difficult to treat infections.

New anti-bacterial agents could be developed either by modifying the chemical structures of existing antibiotics, using high throughput chemical screening methods or by identifying new bacterial targets that are essential for growth/replication and can be chemically inhibited. Yet the numbers of new antibiotics being discovered and brought to market has slowed dramatically in recent years.

5.1.1 Resistance Mechanisms

Resistance to antibiotics can develop in a number of different ways. Genetic material such as plasmids can be transferred horizontally or vertically; latent genetic elements such as transposons may become activated and resistance may result from DNA mutagenesis in the host genome. Mobile genetic elements such as plasmids may encode for a number of different resistance mechanisms and therefore have the ability to transfer resistance to multiple agents at the same time.

5.1.2 Antibiotic Resistance in *Campylobacter*

Campylobacteriosis is most commonly a self-limiting infection that does not require treatment with antibiotics. However, in severe, systemic, recurring infections or infections in immunocompromised individuals, antibiotic treatment may be required. Fluoroquinolones like ciprofloxacin and macrolides like erythromycin are drugs of choice in the treatment of *C. jejuni* infection. It is however known that antibiotic resistance is rising in *Campylobacter* spp. and this is of concern due to the sheer number of infections caused worldwide. Infections caused by resistant strains of *Campylobacter* are linked with longer duration of symptoms, increased healthcare costs and higher risk of systemic or serious illness (Helms *et al.,* 2005). Developing antibiotic resistance in *Campylobacter* spp. poses a significant risk to public health with up to 70% of *C. coli* isolates and around

12% of *C. jejuni* isolates now being reportedly resistant to erythromycin (Quinn *et al.*, 2007)

The horizontal transfer of both plasmid and chromosomal DNA occurs in *C. jejuni* both *in vitro* and during chick colonization, which indicates that natural transformation could have an important role in genome plasticity and in the spread of new factors such as antibiotic resistance, even in the absence of selective pressure (Avrain *et al.*, 2004).

5.1.2.1 The CmeABC of *Campylobacter*

Although there are as many as 13 putative efflux transporters encoded for in the genome of *C. jejuni* strain 11168, CmeABC (*Campylobacter* multidrug efflux) is the most important and best characterised efflux pump of *C. jejuni* (Su *et al.*, 2014). CmeABC was first identified in *C. jejuni* as a multidrug efflux pump in 2002 (Lin *et al.*, 2002, Pumbwe & Piddock, 2002) and is a resistance-nodulation-cell division (RND) type efflux pump. RND efflux pumps are found in many Gram negative organisms and they move compounds from the cytoplasm, to the outside of the cell, by moving protons inside the cell. CmeABC is tripartite, chromosomally encoded and extrudes various dyes, detergents, bile salts and antibiotics in an energy-dependent process (Quinn *et al.*, 2007).

The *cmeA* gene encodes for a periplasmic membrane fusion protein, *cmeB* for an energy-dependent inner membrane efflux transporter and *cmeC* for an outer membrane channel forming protein. CmeABC is regulated by the Tet-R like local repressor *cmeR* (see **Figure 33**). Insertional mutagenesis of *cmeR* (see **Figure 33**) has been shown to cause overexpression of both *Cj0561c* and *cmeB* (Guo *et al.*, 2008). Overexpression of CmeABC confers increased resistance to agents which can be more effectively extruded by CmeABC (Shen *et al.*, 2011).

Conversely, insertional mutagenesis of the largest subunit (CmeB) of the CmeABC tripartite pump confers increased susceptibility to selected agents, because they could not be extruded from the cell to the same extent as in the wild-type. A *cmeB* mutant was shown by Lin *et al* to be more sensitive to various bile acids and a range of structurally diverse conventional antibiotics including rifampicin, ciprofloxacin, erythromycin and gentamicin (Lin *et al.*, 2002). These antibiotics have been listed according to the impact of the mutation on susceptibility i.e. the largest fold difference in susceptibility between the *cmeB* mutant and the wild-type was seen for rifampicin and the lowest for gentamicin. Pumbwe and Piddock made a kanamycin insertion mutant of *cmeB* in *C. jejuni* strain

11168 and also showed that the mutant was more susceptible to various agents, including ciprofloxacin, erythromycin, detergents and dyes (Pumbwe & Piddock, 2002).

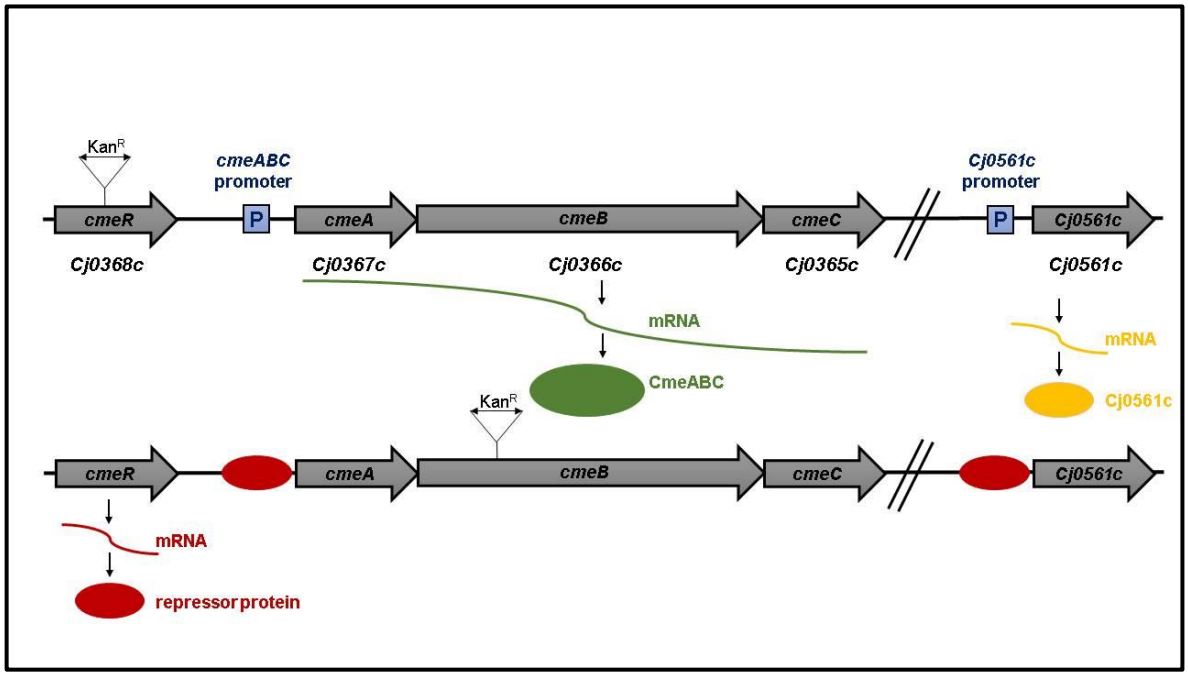


Figure 33. The cmeR gene controls expression of both CmeABC and Cj0561c. *The repressor cmeR can be transcribed into mRNA and translated into a protein which is capable of repressing the local CmeABC genes, as well as the downstream Cj0561c gene, by binding to their upstream promoters (**P**). A cmeB mutant has been used in this study which has had a kanamycin resistance cassette (kan^R) inserted into the cmeB gene. The cmeR gene can also be mutated causing overexpression of CmeABC and Cj0561c.*

Results in **Section 3.3.4** showed that *Cj0561c* was the only gene highly significantly up-regulated (around ten fold increase) when *C. jejuni* strain 81-176 was exposed to pantoprazole at 2 mg/ml (2,000 µg/ml) for 2 hours. It is known that expression of *Cj0561c* is controlled by the repressor CmeR (Guo *et al.*, 2008) and that CmeR also regulates the expression of CmeABC (see **Figure 33**). The microarray results also showed that there was a small (only two fold) increase in the expression of CmeABC following exposure to pantoprazole.

It has been shown in *C. jejuni*, that in the presence of bile, CmeR is unable to bind to and repress the expression of both *CmeABC* and *Cj0561c* (see **Figure 25**) and so both of these products are up-regulated in the presence of bile (Guo *et al.*, 2008, Dzieciol *et al.*, 2011). The statistically significant up-regulation of *Cj0561c* and the two fold increase in CmeABC expression seen in response to pantoprazole exposure suggests either that pantoprazole acts in a similar manner to bile (i.e. actively preventing the binding of CmeR

to the CmeABC and *Cj0561c* promotors) or that the *C. jejuni* is actively down-regulating (perhaps as a result of identifying the pantoprazole as a potential toxic molecule which should be extruded) the expression of the CmeR protein, thereby up-regulating the expression of CmeABC and *Cj0561c*.

5.1.2.2 Fluoroquinolone Resistance in *Campylobacter*

As discussed in **Section 5.1.2.1** the CmeABC pump contributes to fluoroquinolone resistance in *Campylobacter*. Fluoroquinolone resistance can also result following target modification (mutations to GyrA) (Luo *et al.*, 2003). The *gyrA* gene encodes for the A subunit of DNA gyrase (a key enzyme involved in DNA replication and transcription). A single point mutation in the quinolone resistance-determining region of *gyrA* is sufficient to significantly increase the resistance of *Campylobacter* to fluoroquinolones (Luo *et al.*, 2005). A T86I substitution in GyrA confers high-level resistance to fluoroquinolones, while T86K, A70T, or D90N substitutions are associated with moderate resistance to fluoroquinolones (Payot *et al.*, 2006). Strains with GyrA mutations conferring fluoroquinolone resistance must retain a functional CmeABC pump as without CmeABC, GyrA mutants are unable to maintain the resistance phenotype (Yan *et al.*, 2006).

5.1.2.3 Macrolide Resistance in *Campylobacter*

As discussed in **Section 5.1.2.1** the CmeABC pump also contributes to macrolide resistance in *Campylobacter*. However high-level macrolide resistance in *Campylobacter* is associated with an A2075G mutation in the peptidyl transferase region in domain V of the 23S ribosomal ribonucleic acid (rRNA) gene, which is the target of macrolides (Hannula & Hanninen, 2008).

5.1.2.4 Tetracycline Resistance in *Campylobacter*

Tetracycline is a broad-spectrum inhibitor of protein synthesis and is a bacteriostatic agent. Resistance to tetracycline usually results from the acquisition of the plasmid known as pTet in *Campylobacter* spp. *C. jejuni* strain 81-176 is known to contain pTet and is therefore resistant to tetracycline, whereas *C. jejuni* strain 11168-O does not contain the plasmid and is susceptible to tetracycline (Hofreuter *et al.*, 2006). Both of these strains are used extensively in the experimental work detailed in this thesis. pTet encodes for the *tetO* gene which offers ribosomal protection to *C. jejuni* and renders it unsusceptible to the activity of tetracyclines. There is also evidence to suggest that the CmeABC pump also contributes to tetracycline resistance in *Campylobacter* as *cmeB* mutants have been shown to be more susceptible to tetracycline (Lin *et al.*, 2002, Pumbwe & Piddock, 2002).

5.1.3 Rifampicin

Rifampicin is a semi-synthetic derivative of rifamycin (a fermentation product of *Streptomyces mediterranei*) which inhibits RNA polymerase, thereby inhibiting RNA synthesis (Drapeau *et al.*, 2010). It is bactericidal, but unlike vancomycin, it has good bioavailability when taken orally (Joint-Formulary-Committee., 2015). It has a broad-spectrum of activity inclusive of some Gram positive and Gram negative pathogens although it is not recommended for use in single therapy because of rapid emergence of high-level resistance *in vivo* and *in vitro* (Zavascki *et al.*, 2013).

5.1.4 Vancomycin

Vancomycin is a structurally unique glycopeptide antibiotic that is poorly absorbed when taken orally and is therefore more commonly administered intravenously. It is effective against Gram positive bacteria and is commonly used in the treatment of *S. aureus* and *C. difficile* infections (Joint-Formulary-Committee., 2015). Vancomycin blocks cell wall synthesis by binding to peptidoglycan precursors and as such is a bactericidal agent. Although the cell walls of both Gram negative and Gram positive organisms contain peptidoglycan, vancomycin is selective for Gram positive organisms because its large molecular weight and complex structure prevent it from penetrating the outer membrane of Gram negative organisms (Neu & Gootz, 1996). In essence, vancomycin would successfully interfere with the cell wall synthesis of Gram negative organisms if vancomycin could gain access to the cell cytoplasm through the bacterial membrane. Vancomycin has been used in experiments described in this thesis to investigate the ability of pantoprazole to induce susceptibility of a Gram negative bacterium to the bactericidal action of the drug, by interfering with the permeability of the outer membrane.

5.1.5 Chapter Aims

Broth microdilution MIC and MBC methods, similar to those used in earlier chapters, were extensively employed to investigate if *in vitro* exposure to pantoprazole affected *C. jejuni* susceptibility to selected conventional antibiotics, which could be prescribed in the treatment of campylobacteriosis. *C. jejuni* susceptibility (in the presence of pantoprazole) to selected conventional antibiotics, which would not be used to treat campylobacteriosis, was also investigated. Selected experiments were also performed using a *cmeB* insertional mutant which was a very kind gift from Sherif Abouelhadid at the LSHTM. The susceptibility of the *cmeB* mutant to conventional antibiotics, to pantoprazole and to conventional antibiotics in the presence of additional pantoprazole was assessed.

5.2 Materials and Methods

5.2.1 Bacterial Strains and Culture Conditions

The *C. jejuni* strains used in this chapter of the study are listed in **Table 22**. All strains were stored at -80°C, revived, cultured and incubated as detailed in **Section 2.2.1**. Strains from overnight growths were used in all individual experiments.

Table 22. Bacterial strains used in this chapter.

Strain	Features	Origin/Reference
C. jejuni 11168-H	Hypermotile derivative of strain 11168	(Karlyshev *et al.*, 2002)
C. jejuni 11168-H *cmeB* mutant	kan^R inserted into the B subunit gene of the Cme pump	Sherif Abouelhadid, LSHTM
C. jejuni 81-176	Human clinically isolated strain	(Korlath *et al.*, 1985)

5.2.1.1 *cmeB* Mutant

The same kan^R used in **Section 3.2.7** was also used to make an insertional mutant in the *cmeB* gene of *C. jejuni* 11168-H using a method similar to that previously described for the *kdpB* mutant. Briefly, a 1 kb region of the *cmeB* gene (encoding a single BclI site) was amplified using PCR and the amplicon cloned into pJET to form construct pI. BclI was used to cut the plasmid and the kan^R (which had been retrieved from pJMK30 using BamHI) was inserted to form construct pIK. pIK was introduced into *C. jejuni* in the same way as for the *kdpB* mutant and selected colonies checked for the presence of the kan^R insert using PCR. The *C. jejuni cmeB* mutant was transported on a Transwab® Amies Charcoal swab (Medical Wire and Equipment) from the LSHTM, inoculated onto MHA + B + kan agar immediately upon arrival and incubated for 24 hours. A well isolated single colony was selected and subcultured to a fresh MHA + B + kan agar plate and the overnight culture used to make a Microbank™ bead stock for storage at -80°C. Forward primer with the sequence (5′-GACGTAATGAAGGAGAGCCA-3′) and reverse primer with the sequence (5′-CTGATCCACTCCAAGCTATG-3′) were used to check that the size of the product included the 1.4 kb kan^R.

5.2.2 Antibiotics and Pantoprazole

Pantoprazole sodium hydrate was prepared as described in **Section 2.2.2**. A 400 µg/ml stock solution of gentamicin 10 mg/ml solution (Sigma) was prepared by diluting with sterile water. A stock solution of ciprofloxacin (Sigma) was prepared by dissolving in

1% acetic acid to give a 2 mg/ml solution. This was then diluted with sterile water to a final concentration of 400 µg/ml and was wrapped in foil to protect it from the light. Erythromycin (Sigma) was dissolved in 10% ethanol to 2 mg/ml and the concentration then adjusted to 400 µg/ml using sterile water, to prepare the stock solution. Rifampicin (Sigma) was dissolved in DMSO to 25 mg/ml and the concentration then adjusted to 4 mg/ml using sterile water for the stock solution. Vancomycin (Sigma) was dissolved in water to 2 mg/ml for the stock solution.

All antibiotic stock solutions were sterilised using a 0.2 µm filter (Corning), stored in the fridge and further diluted when required in sterile water. Control cultures were performed routinely on MHA incubated aerobically to ensure the sterility of water, pantoprazole, the highest and the lowest dilution of all antibiotics used in each experiment.

5.2.3 *cmeB* Mutant Experiments

5.2.3.1 Susceptibility of *cmeB* Mutant to Conventional Antibiotics

The *cmeB* insertional mutant and the parent strain 11168-H were exposed to halving dilutions of the bactericidal agents' gentamicin and rifampicin and to the bacteriostatic agent erythromycin in a broth microdilution method similar to those described previously, with one notable exception. Antibiotics were serially diluted once per experiment and equal volumes removed into two fresh sterile 96 well microtitre plates (one for each strain). An equal volume of sterile water was added to all wells and double the volume of bacterial strains in MHB was added to all wells. Gentamicin and erythromycin solutions were used at 400 µg/ml and rifampicin adjusted to 2 mg/ml. Antibiotic free positive controls were also included. Gentamicin and erythromycin were tested at final concentrations of 100-0 µg/ml and rifampicin at 500-0 µg/ml. 96 well microtitre plates were incubated for 24 hours before reading the OD_{600} to help determine the MIC. MIC was also assessed visually using a light box. MBC was determined by spotting 10 µl aliquots onto MHA + B plates and incubating plates for 48 hours. Aliquots were also removed from the two wells either side of the MIC and these serially diluted in PBS and dilutions plated on MHA + B to calculate surviving CFU/ml.

5.2.3.2 *cmeB* Mutant Pantoprazole Minimum Bactericidal Concentration

A broth microdilution MBC experiment as detailed in **Section 2.2.4** was performed using overnight growths of C. *jejuni* 11168-H and the *cmeB* mutant harvested into MHB. Pantoprazole was tested at final concentrations ranging 1,000-0 µg/ml with sterile water being used for the no pantoprazole control.

5.2.3.3 Effect of Additional 100 µg/ml Pantoprazole on *cmeB* Mutant Antibiotic Susceptibility

400 µg/ml stock solutions of gentamicin, ciprofloxacin and erythromycin were adjusted to 25 µg/ml and 4 mg/ml rifampicin adjusted to 125 µg/ml. Halving dilutions of each of these four antibiotics were made in sterile 96 well microtitre plates. 50 µl of each dilution was removed into two rows of a fresh 96 well microtitre plate. 50 µl of water was added to one row and 50 µl pantoprazole (at 400 µg/ml) added to the other row. The *cmeB* mutant of *C. jejuni* 11168-H was grown overnight on MHA + B + kan agar. Colonies were harvested into MHB to an OD_{600} of 0.2 and a 1 in 100 dilution made from this into fresh MHB. This was equal to around 3.5×10^6 CFU/ml. 100 µl of the prepared bacterial suspension was added to each well, mixed gently, the 96 well microtitre plate wrapped in foil and incubated for 24 hours. Positive controls with no antibiotics and no pantoprazole and controls in the presence of pantoprazole alone (final concentration 100 µg/ml) were also performed. As in **Section 5.2.3.1** MIC was assessed visually using a light box by reading the OD_{600} 10 µl aliquots were spotted onto MHA + B plates to determine MBC and serial dilutions performed on aliquots removed from selected wells to calculate surviving CFU/ml.

5.2.3.4 *cmeB* Mutant Pantoprazole Minimum Inhibitory Concentration

A broth microdilution MIC experiment was performed as described in **Section 2.2.4**. Pantoprazole was tested at final concentrations ranging 500-0 µg/ml with sterile water being used for the no pantoprazole control.

5.2.3.5 Effect of 10 µg/ml Pantoprazole on *cmeB* Mutant Antibiotic Susceptibility

The method described in **Section 5.2.3.3** was used, with pantoprazole solution made to 40 µg/ml and so tested at a final concentration of 10 µg/ml. Rifampicin was not tested in combination with pantoprazole in this manner, only gentamicin, ciprofloxacin and erythromycin.

5.2.4 Effect of Pantoprazole on Wild-type *Campylobacter jejuni* Antibiotic Susceptibility

A 1:100 dilution in fresh MHB of bacterial suspension corrected to OD_{600} of 0.2 in MHB was prepared from overnight plate cultures of *C. jejuni* strain 81-176. Bacteria were exposed to sub-lethal pantoprazole (250 µg/ml), ½ MIC gentamicin (0.15 µg/ml), to a combination of the two (gentamicin at 0.15 µg/ml and pantoprazole at 250 µg/ml), ½ MIC ciprofloxacin (0.04 µg/ml) and a combination of the two (ciprofloxacin at 0.04 µg/ml and

pantoprazole at 250 µg/ml) in 96 well microtitre plates. A positive control was included which was free from any gentamicin, ciprofloxacin or pantoprazole. Plates were incubated for 24 hours before OD_{595} was determined and 50 µl aliquots removed from wells and plated over the entire surface of MHA + B plates. Plates were then incubated for 48 hours before being examined for the growth of *Campylobacter*.

An Erythromycin MIC experiment was performed using *C. jejuni* strains 81-176 and 11168-H in a manner similar to that described in **Section 5.2.3.1**. Erythromycin was serially diluted once and split between two fresh 96 well plates. An equal volume of water was added to one plate (to act as the erythromycin alone exposed control) and pantoprazole added to the other at a final concentration of 250 µg/ml.

In a similar manner *C. jejuni* strain 81-176 was exposed to rifampicin at 500, 250 and 125 µg/ml with and without the addition of sub-lethal pantoprazole (250 µg/ml) for 24 hours before 50 µl aliquots were removed and plated onto MHA + B plates. Plates were then incubated for 48 hours before being examined for the growth of *Campylobacter*.

In a similar manner *C. jejuni* strains 81-176 and 11168-H were exposed to vancomycin at 500, 250 and 125 and 62.5 µg/ml with and without the addition of sub-lethal pantoprazole (at 100 or 250 µg/ml) for 24 hours before 10 µl aliquots were removed and spotted onto MHA + B plates. Plates were then incubated for 48 hours before being examined for the growth of *Campylobacter*.

5.2.5 Replicates and Data Analysis

Unless otherwise stated each assay was conducted in triplicate and was independently repeated at least three times. Results are expressed as means +/- standard deviations (error bars) of replicate experiments. The unpaired Students t test was used to determine statistical significance. A P value of > 0.01 but < 0.05 was considered significant (*****) and a P value of < 0.01 highly significant (******).

5.3 Results

5.3.1 *cmeB* Mutant Experiments

Initial experiments were performed using the *C. jejuni cmeB* insertional mutant from the LSHTM and the parent strain 11168-H to determine if there was a difference in susceptibility to selected conventional antibiotics relevant to the treatment of *C. jejuni* infections.

5.3.1.1 Susceptibility of *cmeB* Mutant to Conventional Antibiotics

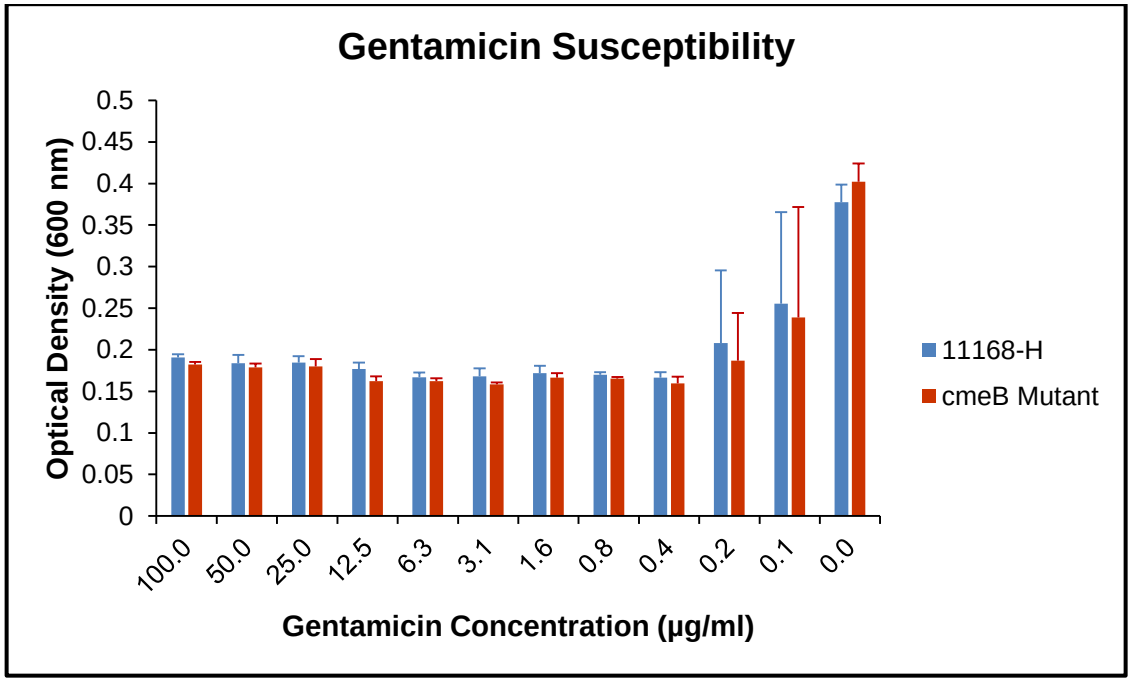
5.3.1.1.1 Gentamicin and Erythromycin

Results in **Figure 34a** show that the *cmeB* mutant and the parent strain were similarly susceptible to gentamicin. At all concentrations of gentamicin tested, the P values comparing the OD for the *cmeB* mutant and those for the parent strain 11168-H were > 0.05, indicating that there was no statistically significant difference in gentamicin susceptibility between the two strains. The MICs for gentamicin can be found in **Table 23** where the mean MIC for triplicate experiments was 0.2 µg/ml, for both strains and the range was also the same for both strains.

Table 23. The MICs of gentamicin and erythromycin for the parent strain 11168-H and the cmeB mutant.

C. *jejuni* Strain	Antibiotic MIC (µg/ml)			
	Gentamicin		Erythromycin	
	Mean +/- SD	Range	Mean +/- SD	Range
11168-H	0.20 +/- 0.15	0.40-0.10	1.60 +/- 0.00	1.60-1.60
***cmeB* Mutant**	0.20 +/- 0.17	0.40-0.10	0.20 +/- 0.00	0.20-0.20

Results in **Figure 34b** however, show that the *cmeB* mutant was more susceptible to erythromycin than the parent strain. There was a significant difference in OD results for 11168-H exposed to 1.6 µg/ml erythromycin versus 0.8 µg/ml (P = 0.0223) indicating that the MIC for 11168-H was 1.6 µg/ml erythromycin. The erythromycin MIC for the *cmeB* mutant however was found to be 0.2 µg/ml (P value for 0.2 µg/ml erythromycin versus 0.1 µg/ml = 0.0001). The difference in susceptibility to erythromycin was clear and consistent in all three replicates of the experiment (see standard deviations and ranges listed in **Table 23**).



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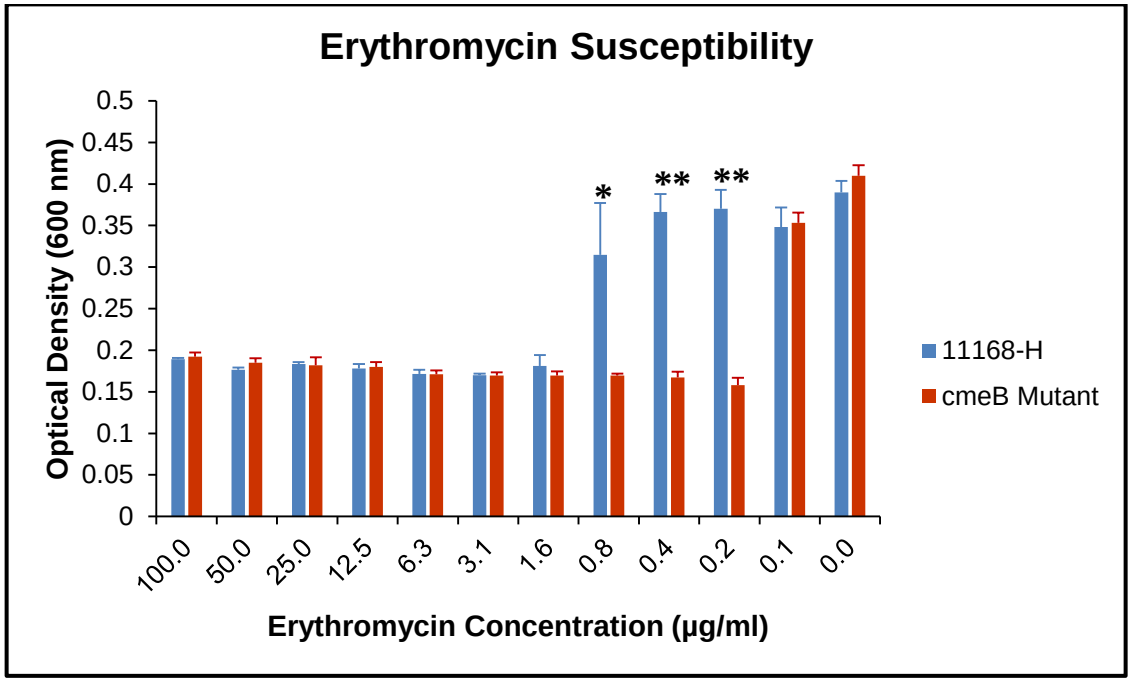


b

Figure 34. The cmeB mutant of C. jejuni displays differential susceptibility (compared to the parent strain) to erythromycin but not gentamicin. *C. jejuni strain 11168-H and the cmeB insertion mutant were exposed to varying concentrations of gentamicin (a) or erythromycin (b) for 24 hours before MIC was determined by measuring OD_{600}. Levels of significance, as indicated by* ***** *(P value > 0.01 but < 0.05) or* ****** *(P value < 0.01) compared the bacterial survival at a given antibiotic concentration between the two strains.*

The differential susceptibility of the *cmeB* mutant (versus the parent strain) to erythromycin was also confirmed by comparing the ODs for the two strains at concentrations < MIC of the parent strain but ≥ the MIC of the *cmeB* mutant (e.g. 0.8, 0.4

and 0.2 µg/ml erythromycin). The P values for these comparisons were 0.0159, 0.0001 and 0.0001 respectively (see **Figure 34b**).

Aliquots were removed from selected wells of microtitre plates following antibiotic exposures and serially diluted to calculate surviving CFU/ml. Following exposure to 0.4 µg/ml erythromycin, an average of 1.3×10^7 CFU/ml were recovered for the parent strain 11168-H, compared to < 100 CFU/ml for the *cmeB* mutant. Similarly, following exposure to 0.2 µg/ml erythromycin, an average of 1.8×10^7 CFU/ml were recovered for the parent strain compared to 1.1×10^5 CFU/ml for the *cmeB* mutant. Culture results therefore support the OD results shown in **Figure 34b** and the *cmeB* mutant is indeed significantly more susceptible to the action of erythromycin than the parent strain.

5.3.1.1.2 Rifampicin

C. *jejuni* is usually inherently resistant to rifampicin, as a result of the CmeABC pump (Lin *et al.*, 2002, Hannula & Hanninen, 2008). However, because Lin *et al* reported that mutation of *cmeB* conferred susceptibility to rifampicin (Lin *et al.*, 2002) further experiments were performed to assess parent strain and *cmeB* mutant susceptibility to rifampicin.

The *cmeB* mutant was found to be more susceptible to rifampicin than the parent strain (**Table 24**). Significant and highly significant differences in the susceptibility of the *cmeB* mutant (versus the parent strain) to rifampicin were found by comparing the ODs for both strains at 31.3, 15.6, 7.8, 3.9, 2.0, 1.0 and 0.5 µg/ml rifampicin (see **Figure 35**).

Table 24. The MIC of rifampicin for the parent strain 11168-H and the cmeB mutant.

C. *jejuni* Strain	**Rifampicin MIC (µg/ml)**	
	Mean +/- SD	**Range**
11168-H	52.1 +/- 18.1	62.5-31.3
***cmeB* Mutant**	0.8 +/- 0.3	1.0-0.5

Wild-type resistance to rifampicin was reflected in the MBC results, as the MBC for the parent strain was found to be > 500 µg/ml. However the MBC for the *cmeB* mutant was found to be 15.6 µg/ml. Following exposure to 31.3 µg/ml rifampicin, an average of 1.1×10^8 CFU/ml were recovered for 11168-H, compared to < 100 CFU/ml for the *cmeB* mutant. Similarly, following exposure to 15.6 µg/ml rifampicin, an average of 5.6×10^8 CFU/ml were recovered for the parent strain compared to < 100 CFU/ml for the *cmeB* mutant. Differential susceptibility was still evident at much lower concentrations of

rifampicin. A 2 log difference in survival was seen following exposure to 0.5 µg/ml rifampicin, with 1.3×10^9 CFU/ml of 11168-H being recovered versus 3.4×10^7 CFU/ml of the *cmeB* mutant.

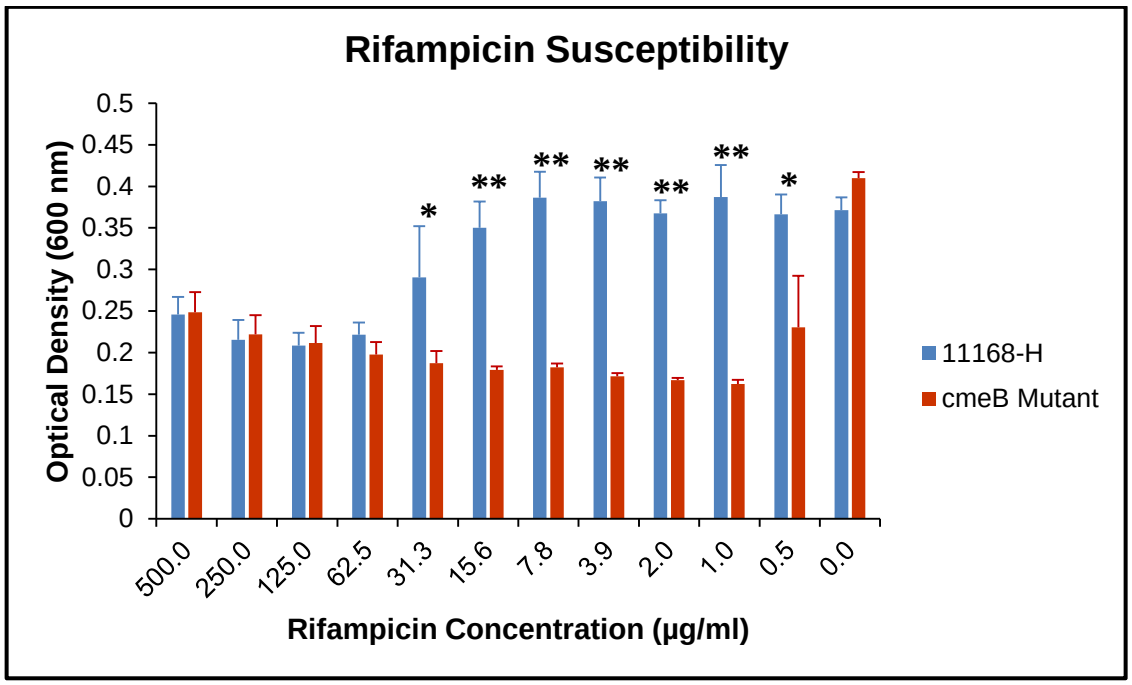


Figure 35. The cmeB mutant of C. jejuni displays differential susceptibility (compared to the parent strain) to rifampicin. *C. jejuni strain 11168-H and the cmeB insertion mutant were exposed to varying concentrations of rifampicin for 24 hours before MIC was determined by measuring OD_{600}. Levels of significance, as indicated by* ***** *(P value > 0.01 but < 0.05) or* ****** *(P value < 0.01) compared the bacterial survival at a given antibiotic concentration between the two strains.*

Culture results therefore support the OD results shown in **Figure 35** and the *cmeB* mutant used in this study is indeed more susceptible to the action of rifampicin than the parent strain. Results for the no antibiotic positive controls (seen in the last set of **blue** and **red** bars in **Figure 34** and **Figure 35**) show that the *cmeB* mutation caused no growth defect in the strain, as there was no significant difference seen in the ODs for the mutant versus the parent strain for the no antibiotic positive controls in the experiments.

5.3.1.2 *cmeB* Mutant Pantoprazole Minimum Bactericidal Concentration

An MBC experiment similar to the MBC experiments used previously for wild-type strains (see **Section 2.2.4**) was used to assess the susceptibility to pantoprazole of the *cmeB* mutant versus the parent strain. The MBC at 24 hours for the parent strain 11168-H was found to be 1 mg/ml (or 1,000 µg/ml), which is similar to those previously reported in **Table 8** for wild-type strains with functional CmeABC pumps. However the MBC for the *cmeB* mutant was found to be four times lower, at 250 µg/ml and so the *cmeB* mutant is

demonstrably more susceptible to the antimicrobial activity of pantoprazole than the *C. jejuni* parent strain.

5.3.1.3 Effect of Additional 100 µg/ml Pantoprazole on *cmeB* Mutant Antibiotic Susceptibility

Because the *cmeB* mutant was more susceptible to some conventional antibiotics and to pantoprazole than the parent strain, experiments were performed exposing the mutant and parent strain to combinations of pantoprazole and selected conventional antibiotics. Pantoprazole was added at a fixed final concentration of 100 µg/ml (sub-lethal for both the mutant and parent strain) and antibiotics serially diluted in doubling dilutions.

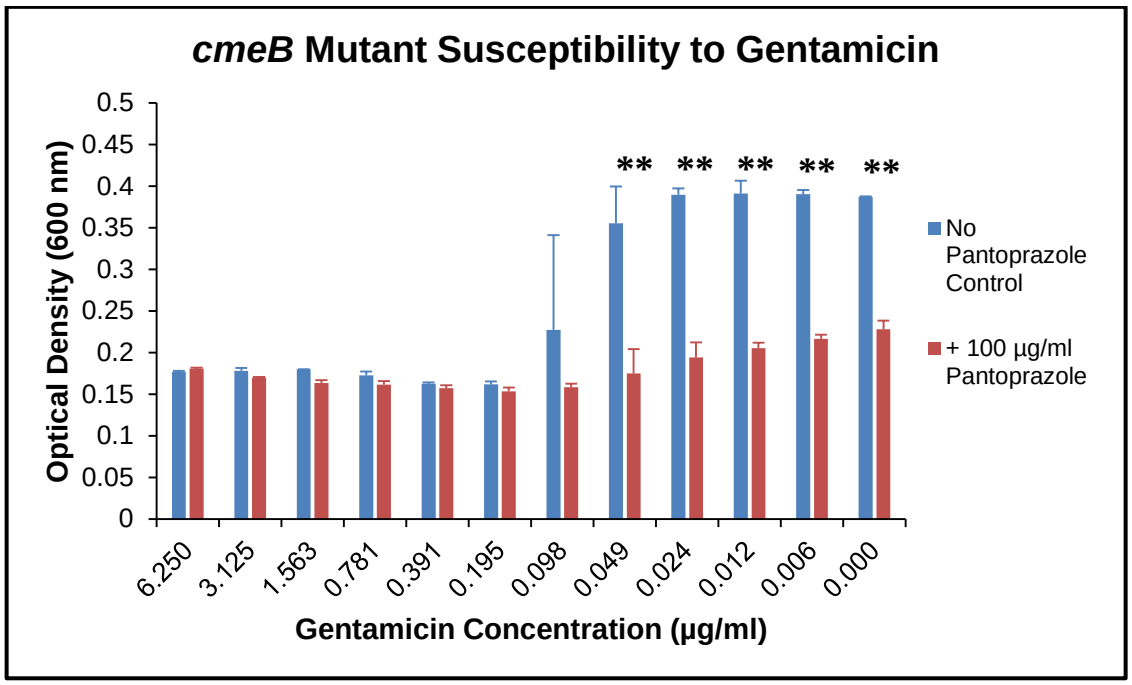
For both ciprofloxacin and rifampicin, the MIC was the same in all three replicates of the experiment (0.024 and 0.97 µg/ml respectively, see **Table 25**) and clearly defined MICs can therefore be seen in **Figure 36b** and **d**. Comparing the ODs at one concentration (0.024 and 0.97 µg/ml respectively for ciprofloxacin and rifampicin) with the ODs achieved for the next lowest antibiotic dilution (0.012 and 0.48 µg/ml respectively for ciprofloxacin and rifampicin) gives highly significant results for both agents. The MICs for gentamicin and erythromycin were found to be 0.195 µg/ml in some replicates of the experiment and 0.098 µg/ml in others, which is reflected in the standard deviations shown in **Table 25** and the large error bars seen in **Figure 36a** and **c** for 0.098 µg/ml antibiotic.

Table 25. Addition of 100 µg/ml pantoprazole inhibits the growth of the cmeB mutant and hinders determining an MIC for pantoprazole + selected conventional antibiotics.

Antibiotic	*cmeB* Mutant Mean MIC (µg/ml) +/- SD	
	Antibiotic Alone	With 100 µg/ml Pantoprazole
Gentamicin	0.13 +/- 0.06	< 0.006
Ciprofloxacin	0.02 +/- 0.00	< 0.006
Erythromycin	0.13 +/- 0.06	< 0.006
Rifampicin	0.97 +/- 0.00	< 0.030

In **Section 5.3.1.2** the MBC of pantoprazole for the *cmeB* mutant was found to be 250 µg/ml and so a sub-lethal concentration of 100 µg/ml was selected for use in these conventional antibiotic and PPI co-exposure experiments. However it is evident that there was a highly significant difference between the ODs for the no antibiotic controls (with and without the presence of 100 µg/ml pantoprazole) as seen in the last set of **blue** and **red**

bars on the far right of all of the graphs in **Figure 36**. This indicates that the presence of pantoprazole alone, at a concentration of 100 µg/ml is highly significant in its ability to inhibit the growth of the *cmeB* mutant.



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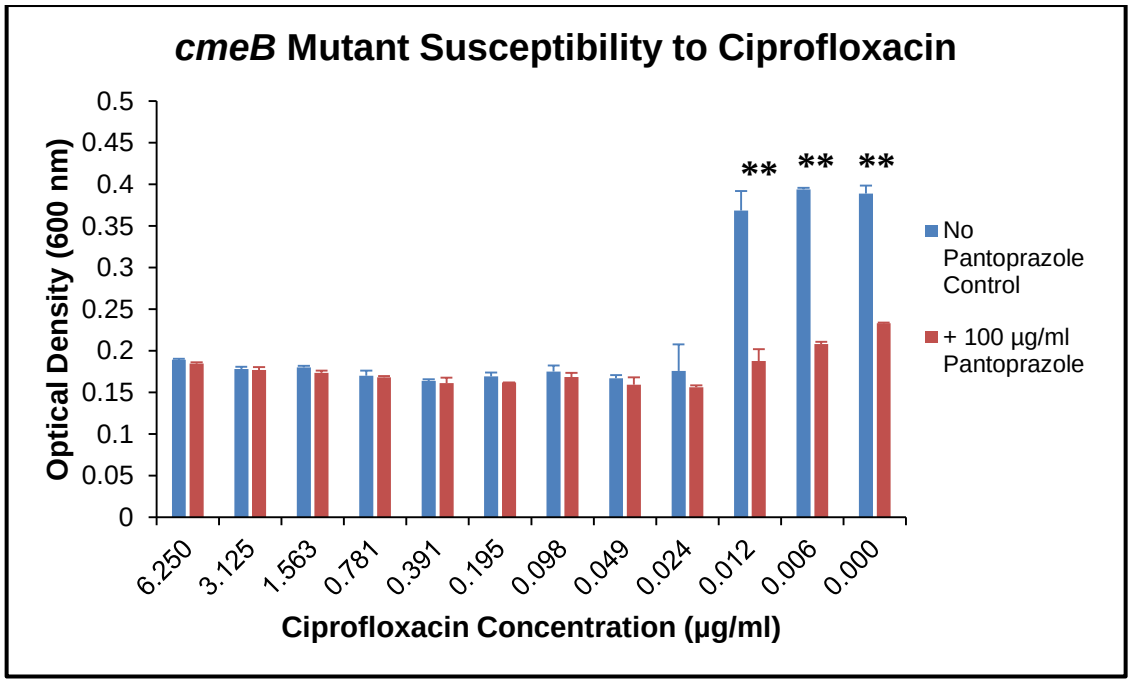

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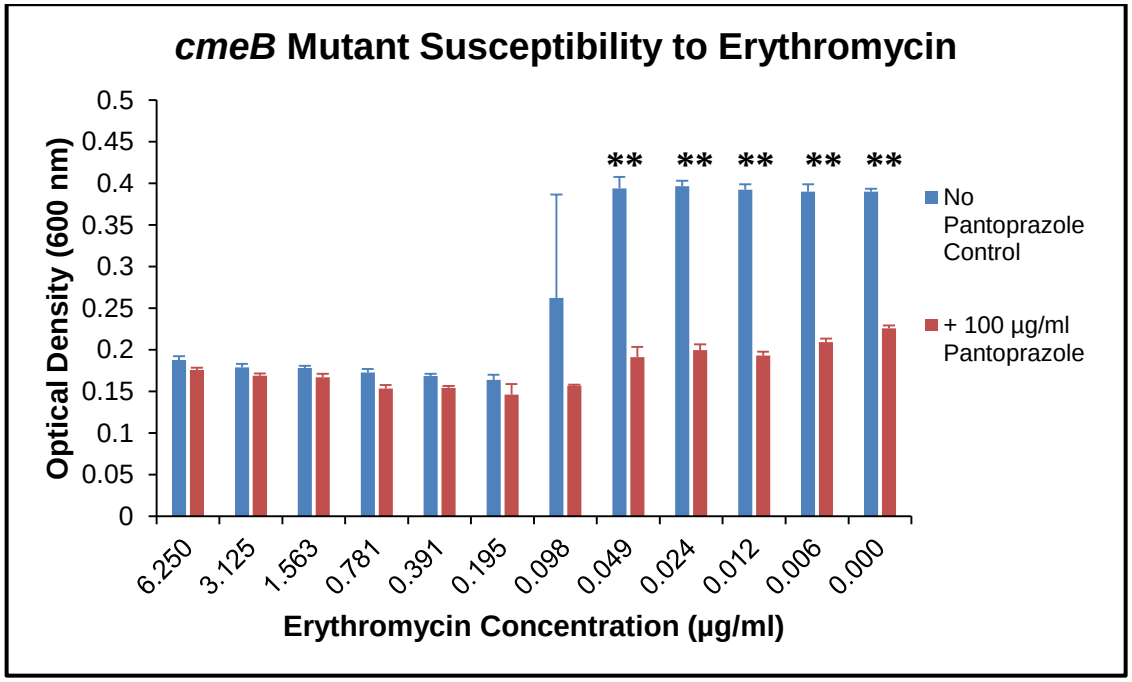

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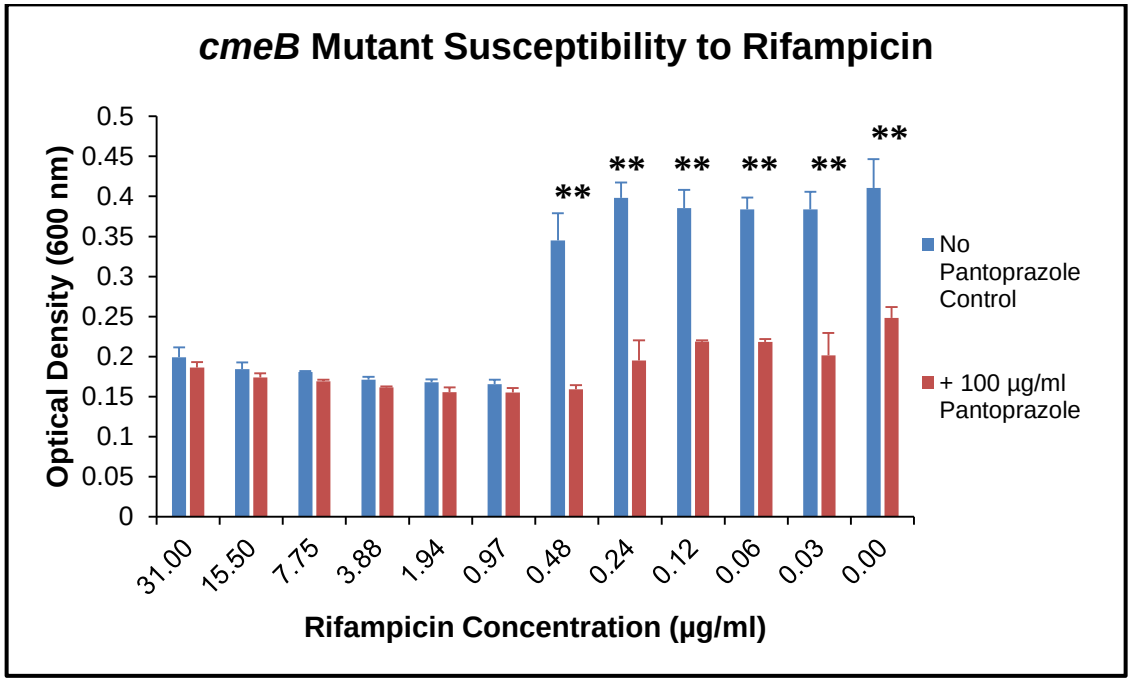


d

Figure 36. Presence of sub-lethal 100 µg/ml pantoprazole significantly affects growth of the cmeB mutant. *An MIC experiment was performed using a broth microdilution method before OD600 was measured. The C. jejuni cmeB mutant was exposed to various concentrations of gentamicin (a), ciprofloxacin (b), erythromycin (c) or rifampicin (d) in the absence of pantoprazole (blue bars) and in the presence of pantoprazole (red bars). Levels of significance, as indicated by* ** *(P value < 0.01) compared the bacterial survival at a given antibiotic concentration between the no pantoprazole control and with additional 100 µg/ml pantoprazole.*

5.3.1.4 *cmeB* Mutant Pantoprazole Minimum Inhibitory Concentration

Because the *cmeB* mutant was found to be inhibited (see **Figure 36**) by concentrations of pantoprazole lower than the bactericidal concentration (see **Section 5.3.1.2**) a standard

broth microdilution MIC experiment, using the *cmeB* mutant, was performed for pantoprazole. The ODs achieved following exposure to each pantoprazole concentration were compared to the values obtained for the concentration one dilution lower (see **Figure *37*** for concentrations along the X axis). The only highly significant difference was seen when comparing the ODs achieved following exposure to 125 µg/ml pantoprazole with those achieved following exposure to 63 µg/ml ($P = 0.00001$). No significant differences ($P > 0.01$ but ≤ 0.05) were found across the range. The pantoprazole MIC for the *cmeB* mutant was therefore found to be 125 µg/ml in these experiments. We have already shown that 100 µg/ml pantoprazole significantly inhibits the growth of the *cmeB* mutant (**Figure 36**) and so it is likely that the pantoprazole concentration which is sufficiently low enough to allow the *cmeB* mutant to grow lies somewhere between 63 and 100 µg/ml.

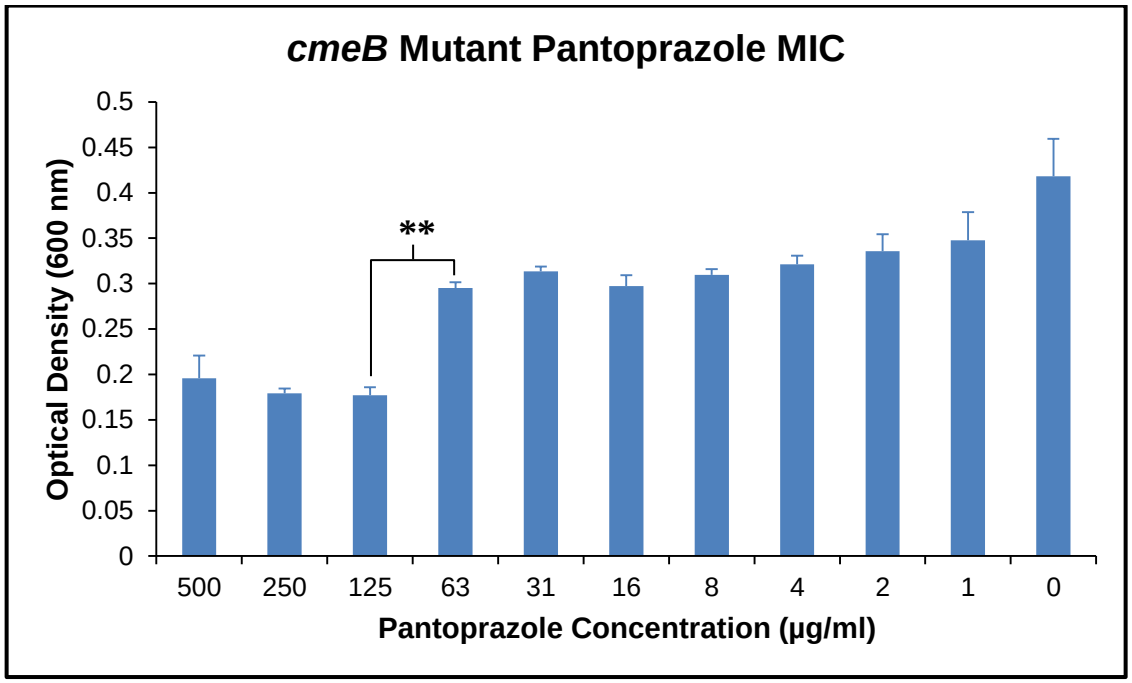


Figure 37. Standard broth microdilution can be used to determine the pantoprazole MIC for the cmeB mutant. *The cmeB mutant in MHB was exposed to varying concentrations of pantoprazole for 24 hours before MIC was determined by measuring OD_{600}. Level of significance, as indicated by* ** *(P value < 0.01) compared the bacterial survival at a given concentration with survival at the next lowest dilution.*

5.3.1.5 Effect of 10 µg/ml Pantoprazole on *cmeB* Mutant Antibiotic Susceptibility

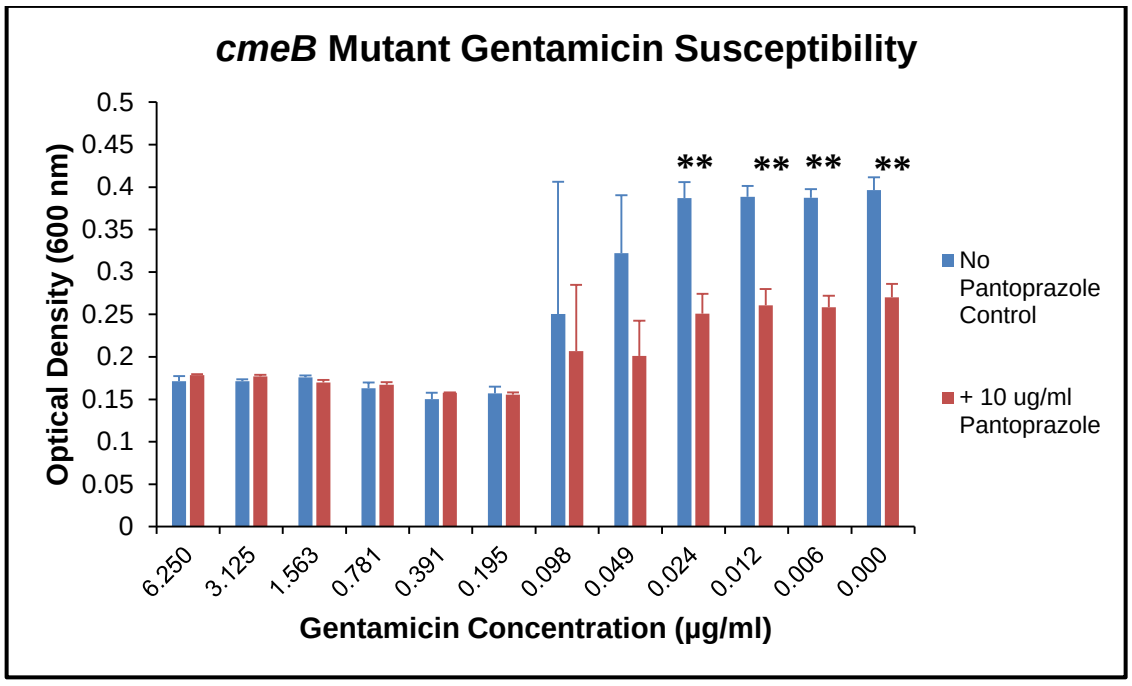
Because growth of the *cmeB* mutant was inhibited by much lower concentrations of pantoprazole than wild-type *C. jejuni*, experiments were performed exposing the *cmeB* mutant to selected conventional antibiotics in combination with 10 µg/ml pantoprazole (in a manner similar to that used in **Section 5.3.1.3**). Again, a clear MIC was defined in all

three replicates of the experiment for ciprofloxacin (see **Figure 38b**) and the MICs for gentamicin and erythromycin again varied between two subsequent dilutions (0.195 or 0.098 µg/ml). This contributed to the higher standard deviations shown in **Table 26** and the large error bars seen in **Figure 38a** and **c** for 0.098 µg/ml gentamicin and erythromycin respectively.

However there was a highly significant difference between the ODs for the no antibiotic controls (with and without the presence of 10 µg/ml pantoprazole) as seen in the last set of **blue** and **red** bars on the far right of the graphs in **Figure 38a** and **c**. A significant difference in the OD achieved following exposure to 10 µg/ml pantoprazole alone can also be seen in **Figure 38b**. This indicates that the presence of pantoprazole alone, at a concentration of 10 µg/ml is still significantly able to inhibit growth of the *cmeB* mutant.

Table 26. Addition of 10 µg/ml pantoprazole still inhibits the growth of the cmeB mutant and hinders determining an MIC for pantoprazole + conventional antibiotics.

Antibiotic	*cmeB* Mutant Mean MIC (µg/ml) +/- SD	
	Antibiotic Alone	With 10 µg/ml Pantoprazole
Gentamicin	0.16 +/- 0.06	< 0.006
Ciprofloxacin	0.02 +/- 0.00	< 0.006
Erythromycin	0.13 +/- 0.06	< 0.006



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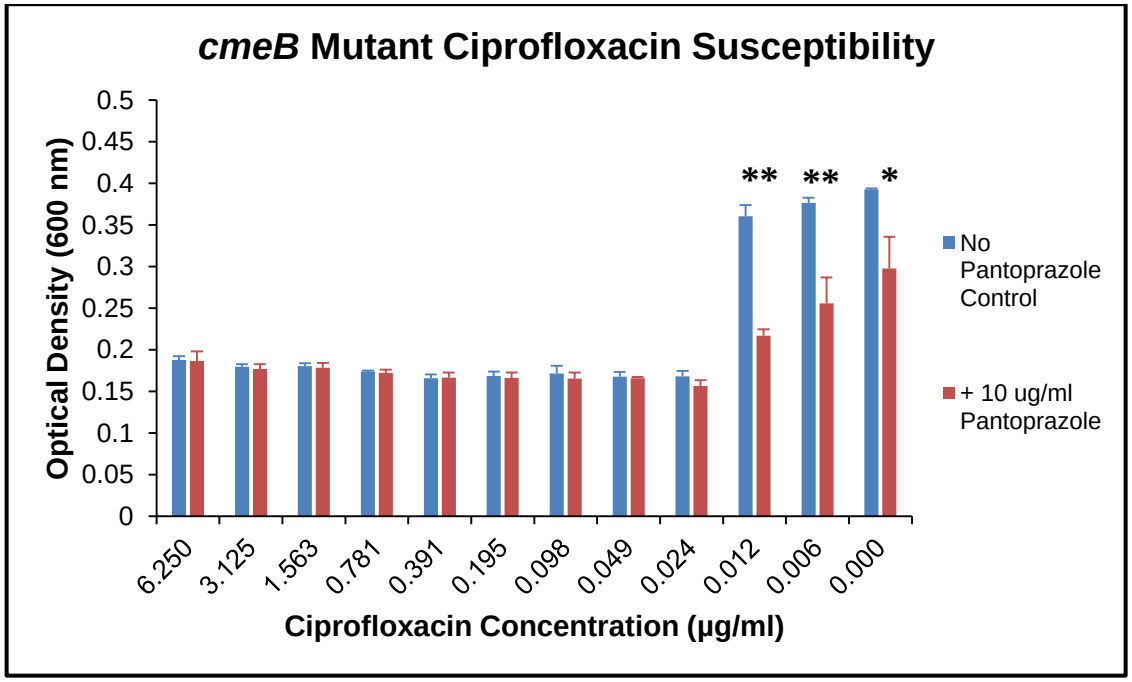

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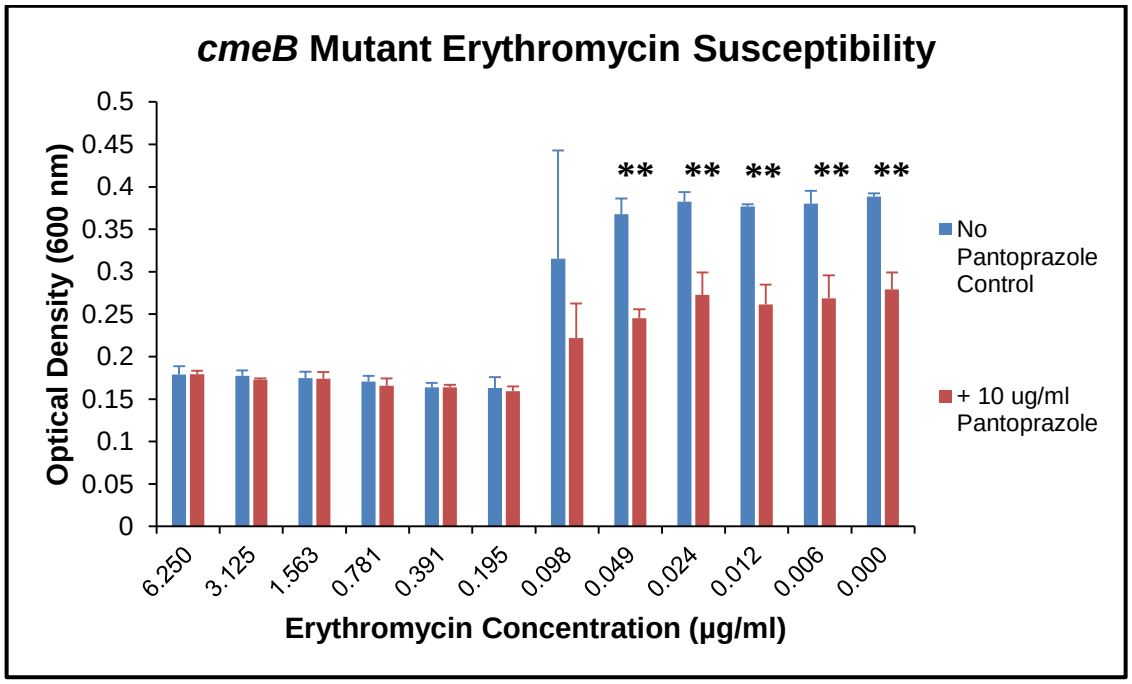


c

Figure 38. Presence of 10 µg/ml pantoprazole inhibits cmeB mutant growth. *An MIC experiment was performed using a broth microdilution method before OD_{600} was measured. The C. jejuni cmeB mutant was exposed to various concentrations of gentamicin (a), ciprofloxacin (b) or erythromycin (c) in the absence of pantoprazole (**blue** bars) and in the presence of 10 µg/ml pantoprazole (**red** bars). Levels of significance, as indicated by * (P value > 0.01 but < 0.05) or ** (P value < 0.01) compared the bacterial survival at a given antibiotic concentration between the no pantoprazole control and with additional 10 µg/ml.*

5.3.1.6 2 µg/ml Pantoprazole Significantly Inhibits *cmeB* Mutant Growth

Because 10 µg/ml pantoprazole was found to be highly significant (**Figure 38a** and **c**) or significant (**Figure 38b**) in its inhibition of the *cmeB* mutant growth, data from the pantoprazole MIC experiment in **Section 5.3.1.4** was re-examined. Results in **Figure** *37* show that the ODs achieved following exposure to ≤ 63 µg/ml pantoprazole were higher (all ODs above 0.3) than those achieved at higher concentrations of pantoprazole (at 500, 250 and 125 µg/ml pantoprazole all ODs were below 0.2). So at pantoprazole concentrations of ≤ 63 µg/ml, the *cmeB* mutant is able to grow. However, when the ODs for each individual concentration ≤ 63 µg/ml pantoprazole are compared to the ODs for the no pantoprazole positive control, highly significant and significant reductions in OD were found between 63 and 2 µg/ml pantoprazole (see **Table 27**). Hence concentrations of pantoprazole ≥ 2 µg/ml significantly inhibit the growth of the *cmeB* mutant.

Table 27. The ODs achieved following exposure to various pantoprazole concentrations have been compared to those achieved for the no pantoprazole positive control.

	Pantoprazole Concentration (µg/ml) for which Data have been Compared to the no Pantoprazole Control						
	63	**31**	**16**	**8**	**4**	**2**	**1**
P value	0.002	0.004	0.002	0.003	0.007	0.027	0.071
Significance Level	**	**	**	**	**	*	NS

Highly significant (**) and significant (*) were assigned for P values of < 0.01 and > 0.01 but < 0.05 *respectively. P values > 0.05 were designated not significant (NS).*

5.3.2 Effect of Pantoprazole on Wild-type *Campylobacter jejuni* Antibiotic Susceptibility

5.3.2.1 Gentamicin, Ciprofloxacin and Erythromycin

Because the *cmeB* mutant displayed such pronounced susceptibility to pantoprazole and the CmeABC pump has been shown to be involved in the extrusion of conventional antibiotics (Guo *et al.*, 2008) experiments were performed combining sub-lethal pantoprazole with sub-MIC gentamicin and sub-MIC ciprofloxacin using wild-type *C. jejuni*. *C. jejuni* strains 81-176 and 11168-H, have functioning CmeABC pumps and were the strains used in these experiments.

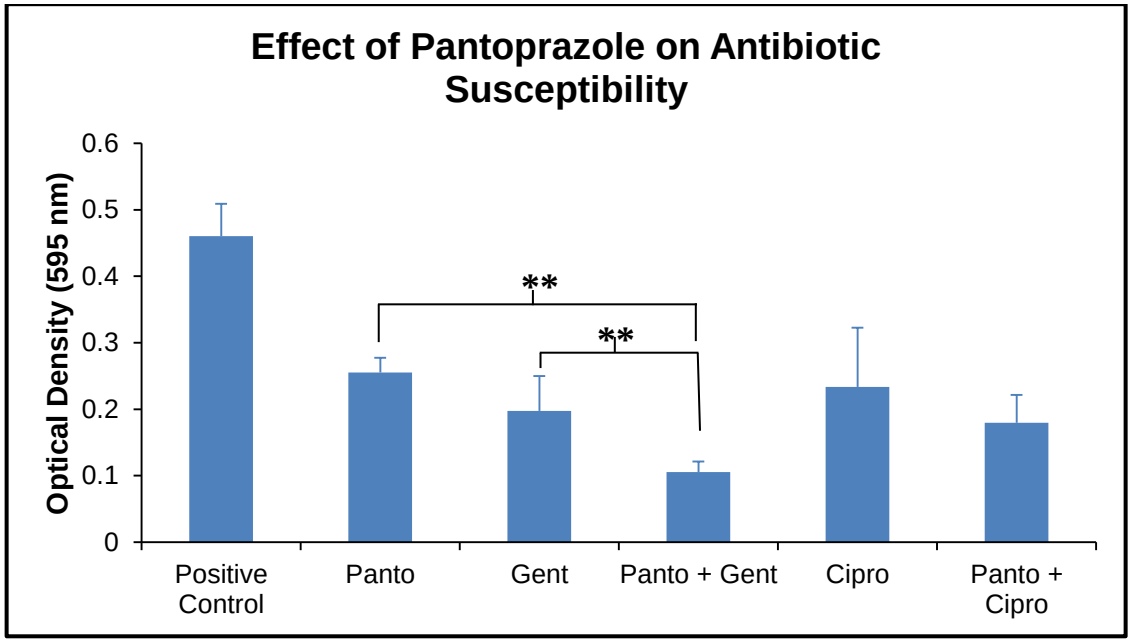
A highly significant decrease in the OD for *C. jejuni* exposed to pantoprazole and gentamicin in combination (compared to both pantoprazole alone and gentamicin alone) was seen (**Figure 39a**). A highly significant decrease in the OD for *C. jejuni* exposed to

pantoprazole and ciprofloxacin in combination was also seen, but in comparison to pantoprazole alone only (not compared to ciprofloxacin alone).

To confirm that the highly significant reduction in OD, which was seen when *C. jejuni* was exposed to gentamicin in the presence of pantoprazole, corresponded to a reduction in surviving bacteria, aliquots were removed from wells and plated onto non-selective agar (**Figure 39b-d**). A confluent growth of *C. jejuni* was recovered (**Figure 39b**) following exposure to 250 µg/ml pantoprazole alone, which is therefore clearly sub-lethal. A semi-confluent growth of *C. jejuni* was recovered following exposure to sub-MIC gentamicin (**Figure 39c**) yet only a few colonies were cultured (**Figure 39d**) following co-exposure to both agents at the same concentrations used in **Figure 39b** and **c**.

In a similar manner the highly significant reduction in OD, which was seen when *C. jejuni* exposed to ciprofloxacin in the presence of pantoprazole was also confirmed to correspond to a reduction in surviving bacteria (pictures not shown). An average of 7.5×10^6 CFU/ml were recovered following exposure to ciprofloxacin (0.125 µg/ml) alone, compared to 1.4×10^5 CFU/ml recovered following exposure to 0.125 µg/ml ciprofloxacin with additional 250 µg/ml pantoprazole.

The MIC of erythromycin for *C. jejuni* strain 81-176 consistently showed a one fold reduction (on addition of 250 µg/ml pantoprazole versus erythromycin only control). An average of 3.6×10^3 CFU/ml of *C. jejuni* strain 81-176 were recovered following 24 hour exposure to 1 µg/ml erythromycin alone versus < 100 CFU/ml recovered following 24 hour exposure to 1 µg/ml erythromycin in the presence of additional 250 µg/ml pantoprazole. *C. jejuni* strain 11168-H also demonstrated the same one fold reduction in erythromycin MIC when 250 µg/ml pantoprazole was added.



a

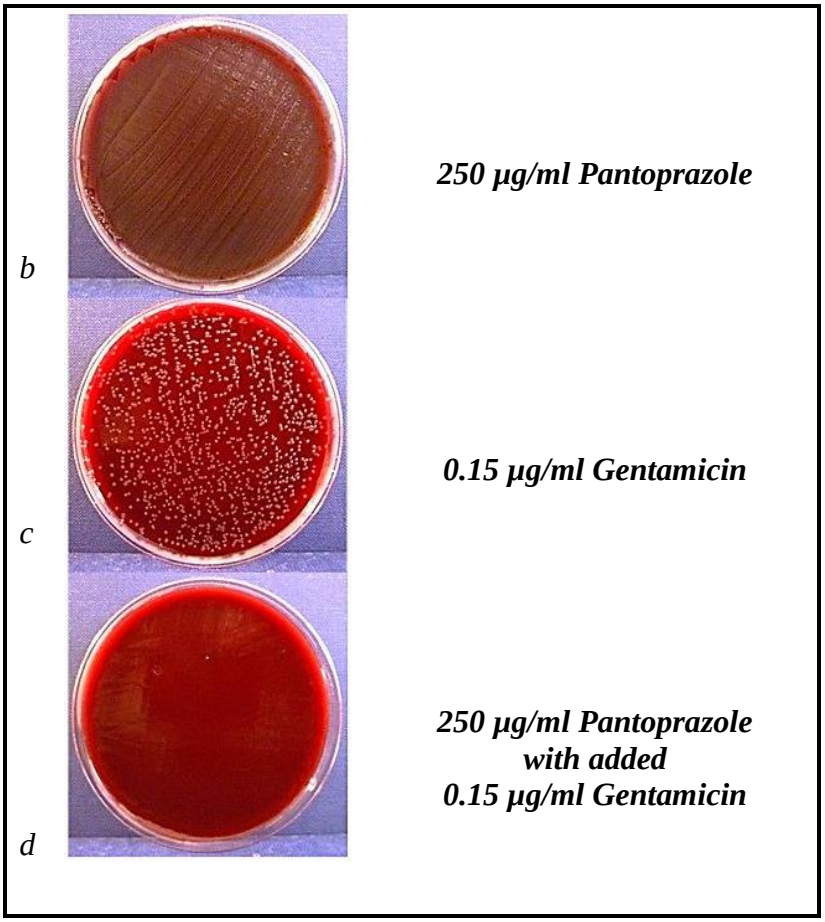


Figure 39. Pantoprazole enhances killing of C. jejuni by gentamicin and ciprofloxacin. *C. jejuni strain 81-176 was exposed to pantoprazole (Panto) at 250 µg/ml, gentamicin (Gent) at 0.15 µg/ml, a combination of both (Panto + Gent), ciprofloxacin (Cipro) at 0.04 µg/ml and a combination of both (Panto + Cipro) for 24 hours before measuring* OD_{595} *(a). 50 µl was aliquoted and spread onto MHA + B plates and plates incubated for 48 hours following the exposures to pantoprazole (b), gentamicin (c) or a combination of both (Panto + Gent; d). Levels of significance, as indicated by* ** *(P value < 0.01) showed that significantly less bacteria survive exposure to pantoprazole and gentamicin in combination than survive either exposure to gentamicin alone or pantoprazole alone.*

5.3.2.2 Rifampicin

Because the *cmeB* mutant displayed such pronounced susceptibility to pantoprazole and the CmeABC pump is known to be involved in resistance to rifampicin (Lin *et al.*, 2002), experiments were performed combining sub-lethal pantoprazole with rifampicin using wild-type *C. jejuni*.

C. jejuni strain 81-176 displayed a dose depended response to killing by rifampicin (see the series of three images on the left of **Figure 40**) but this was at concentrations notably much higher than those required for antibiotics to which *C. jejuni* would be considered susceptible to. For example, a similar number of colonies can be cultured from a 50 µl aliquot following *C. jejuni* 81-176 exposure to 0.15 µg/ml gentamicin as can be cultured following *C. jejuni* 81-176 exposure to 250 µg/ml rifampicin (see **Figures 39c** and **40b** respectively).

C. jejuni 81-176 exposed to rifampicin in the presence of additional 250 µg/ml pantoprazole is however killed more effectively than *C. jejuni* exposed to rifampicin alone (**Figure 40**), although 250 µg/ml pantoprazole is itself sub-lethal to *C. jejuni* 81-176 (see **Figure 39b**).

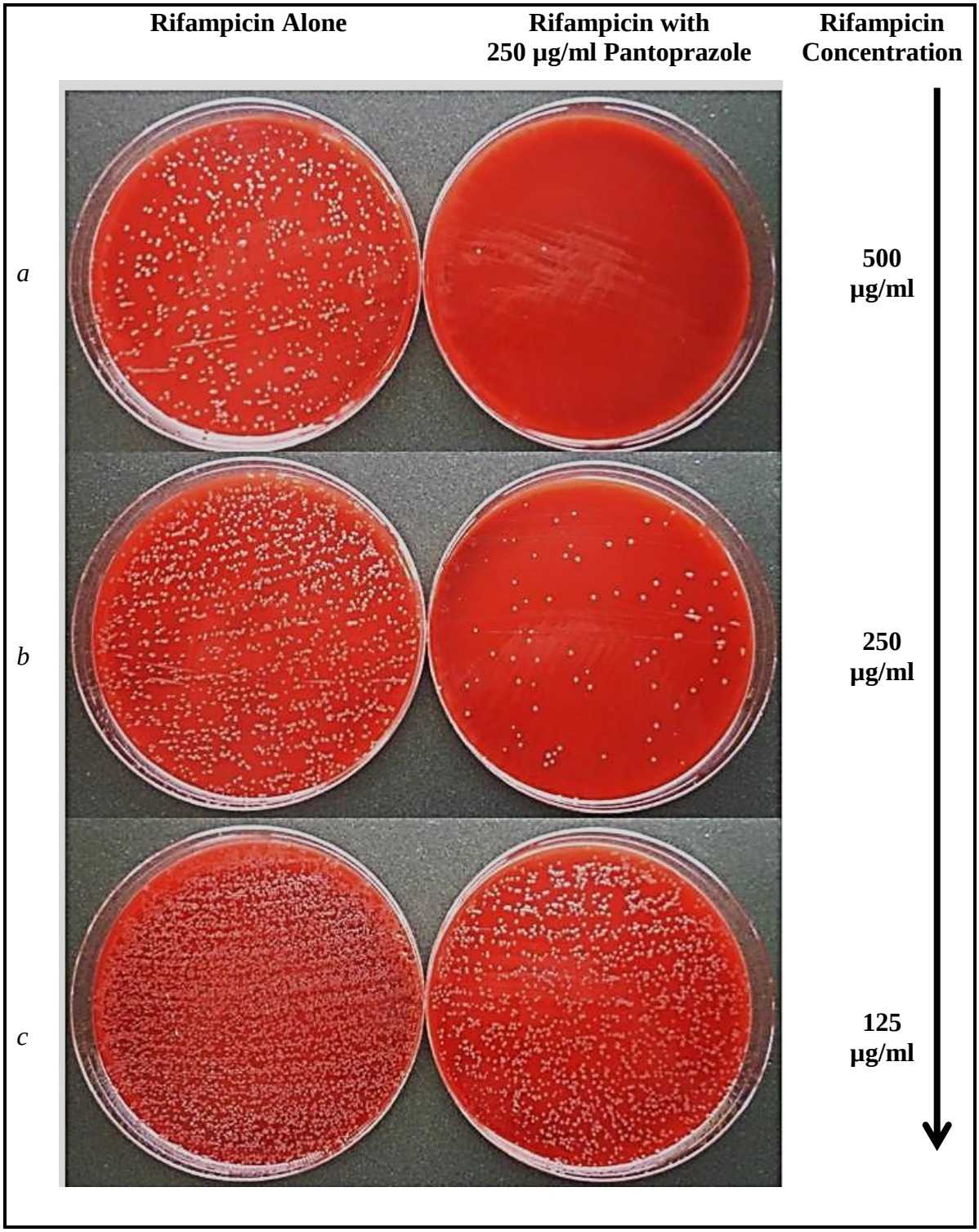


Figure 40. Presence of a sub-lethal pantoprazole concentration enhances rifampicin killing. *C. jejuni strain 81-176 in MHB was exposed to rifampicin at 500 (a), 250 (b) or 125 (c) µg/ml and rifampicin alone and with added 250 µg/ml pantoprazole for 24 hours before 50 µl aliquots were spread onto MHA + B plates. Plates were incubated for 48 hours before being examined for the growth of Campylobacter.*

5.3.2.3 Vancomycin

Vancomycin is a bactericidal agent which inhibits cell wall synthesis in Gram positive bacteria and would therefore not be expected to be effective against the Gram negative

organism *C. jejuni*. The vancomycin MBC for both strains of *C. jejuni* tested was found to be > 500 µg/ml and these results were therefore as expected. Although vancomycin (up to a maximum concentration of 500 µg/ml) was unable to kill *C. jejuni in vitro*, results in **Figure 41** suggest that vancomycin is capable of inhibiting *C. jejuni* growth. A highly significant increase in the OD achieved following *C. jejuni* exposure to 125 µg/ml vancomycin (compared to the OD achieved following exposure to 250 µg/ml vancomycin) was seen in the absence of pantoprazole (**blue** bars). The vancomycin MIC for *C. jejuni* strain 81-176 was therefore found to be 250 µg/ml. Again, this concentration is notably much higher than those required for conventional antibiotics to which *C. jejuni* would be considered susceptible to.

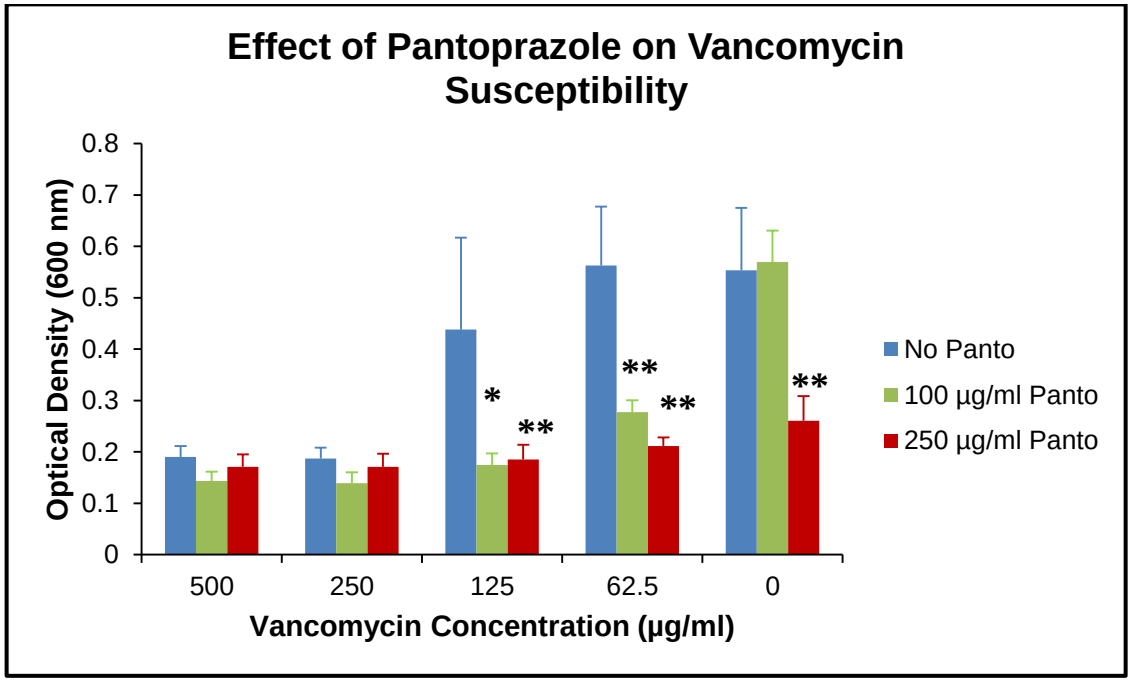


Figure 41. Presence of pantoprazole significantly increases susceptibility to vancomycin. *C. jejuni strain 81-176 in MHB was exposed to varying concentrations of vancomycin (with and without the presence of 100 µg/ml (**green** bars) or 250 µg/ml pantoprazole (panto) (**red** bars)) for 24 hours before OD_{600} was measured. Levels of significance, as indicated by* ***** *(P value > 0.01 but < 0.05) or* ****** *(P value < 0.01) relate to the individual test conditions compared to the no PPI control.*

At vancomycin concentrations below the MIC, significant and highly significant decreases in OD were found with the addition of either 100 or 250 µg/ml pantoprazole (**Figure 41**). Of particular interest was that a significant decrease in OD was seen at 125 µg/ml vancomycin when 100 µg/ml pantoprazole was also present (when compared to the no vancomycin control for 100 µg/ml pantoprazole) and that a highly significant decrease in OD was seen at 62.5 µg/ml vancomycin when 100 µg/ml pantoprazole was also present

(again when compared to the no vancomycin control for 100 µg/ml pantoprazole). Vancomycin in the presence of 100 µg/ml pantoprazole therefore inhibits the growth of *C. jejuni*; even though the presence of 100 µg/ml pantoprazole does not itself significantly inhibit *C. jejuni* growth (see the last set of **blue** and **green** bars on the far right of **Figure 41**).

The presence of 250 µg/ml pantoprazole itself was highly significant in its ability to inhibit the growth of *C. jejuni* (see the last set of **blue** and **red** bars on the far right of **Figure 41**) even though this concentration is known to be below the cidal level (see **Table 8** and **Figure 39b**).

The MBC of vancomycin alone was > 500 µg/ml and in the presence of additional 100 µg/ml pantoprazole the MBC remained unchanged at > 500 µg/ml. However in the presence of a confirmed sub-MBC concentration of 250 µg/ml pantoprazole, the vancomycin MBC was found to be 250 µg/ml in 12 replicates and 500 µg/ml in three replicates. The median MBC of vancomycin (in the presence of 250 µg/ml pantoprazole) was therefore 300 µg/ml.

5.4 Discussion

5.4.1 *cmeB* Mutant Experiments

5.4.1.1 Susceptibility of *cmeB* Mutant to Conventional Antibiotics

Results in **Table 23** and **Figure 34a** show that there was no significant difference in the *cmeB* mutant susceptibility to gentamicin when compared to that of the parent strain. Previously it has been reported that mutation of *cmeB* leads to a two fold increase in susceptibility to gentamicin (Lin *et al.*, 2002) but these authors mutated the *cmeB* of *C. jejuni* strain 81-176 and not 11168-H, as was the strains used in this study. Both *C. jejuni* strains 11168 and 81-176 have functional CmeABC pumps. It is however worth noting that in their study, the authors reported that the *cmeB* mutant showed fold differences in the range of 256-2 fold for a variety of agents and therefore the reported increase in susceptibility to gentamicin was very small. Also noteworthy is that although the authors used broth microdilution in MHB (as was used in this study), they calculated MIC following two days incubation at 42°C (and not following one day incubation at 37°C as was used in this study). This may account for the discrepancy in *cmeB* mutant gentamicin susceptibility results. The *cmeB* mutant used by Pumbwe and Piddock was made using kan^R inserted into strain 11168, but they unfortunately did not report on gentamicin susceptibility (Pumbwe & Piddock, 2002).

Following exposure to 0.4 µg/ml erythromycin, 1.3×10^7 CFU/ml of 11168-H were recovered, compared to the *cmeB* mutant which had no live colonies recovered. Erythromycin is a bacteriostatic agent and the parent strain 11168-H is able to withstand exposure to 0.4 µg/ml and actively grow in its presence to give a 2 log increase in CFU/ml from the initial inoculum. Similarly, following exposure to 0.2 µg/ml erythromycin, the *cmeB* mutant demonstrated a 2 log reduction in survival rate compared to that of the parent strain. Both Lin *et al* and Pumbwe and Piddock reported that their individual *cmeB* mutants displayed an increased susceptibility to erythromycin. The *cmeB* mutant used in this study also showed an increased susceptibility to erythromycin (see **Figure 34b**) and is therefore in support of these two previous works.

The CmeABC pump must therefore play a role in the extrusion of erythromycin in wild-type strains of *C. jejuni*. The CmeABC pump is the most important mechanism of energy-dependent efflux in wild-type *C. jejuni* and disruption of this pump clearly has the ability to increase susceptibility to conventional antibiotics that may be used in the treatment of infections caused by *C. jejuni*.

It has been previously shown that insertional mutation of *cmeB* renders the *C. jejuni* mutant susceptible to rifampicin, a bactericidal antibiotic which *C. jejuni* is usually inherently resistant to (Lin *et al.*, 2002). Experiments were therefore performed using the *cmeB* mutant from the LSHTM to investigate if disruption of the CmeABC pump could truly induce rifampicin susceptibility. Results in **Table 24** and **Figure 35** show that the *cmeB* mutant was indeed more susceptible to rifampicin than the parent strain and these results are in support of Lin *et al*.

The CmeABC pump must therefore also be involved in the extrusion of rifampicin in wild-type strains of *C. jejuni*. Disruption of this pump clearly has the ability to induce susceptibility to conventional antibiotics that may otherwise not normally be used in the treatment of infections caused by *C. jejuni*.

5.4.1.2 *cmeB* Mutant Pantoprazole Minimum Bactericidal Concentration

The MBC for the *cmeB* mutant was only ¼ of the MBC for the parent strain (250 µg/ml compared to 1,000 µg/ml) and so the *cmeB* mutant is clearly more susceptible to the antimicrobial activity of pantoprazole than the parent strain. This perhaps suggests that the CmeABC pump is involved in the extrusion of pantoprazole in wild-type *C. jejuni*. To our knowledge, this is the first report that disruption of the CmeABC pump confers increased susceptibility to agents from the PPI family.

5.4.1.3 Effect of Additional 100 µg/ml Pantoprazole on *cmeB* Mutant Antibiotic Susceptibility

The experiments failed to determine if the *cmeB* mutant was differentially susceptible to conventional antibiotics in the presence of additional 100 µg/ml pantoprazole because 100 µg/ml pantoprazole was shown to be highly significant in its ability to inhibit the growth of the *cmeB* mutant (**Figure 36**). This result was unexpected as 100 µg/ml is less than ½ of the pantoprazole MBC for the *cmeB* mutant and it has been stated that the MIC is often ½ of the measured MBC (Sjostrom *et al.*, 1997). Because significant growth inhibition of the *cmeB* mutant was apparent at a concentration less than ½ of the pantoprazole MBC and that the *cmeB* mutant was susceptible to lower concentrations of pantoprazole than wild-type *C. jejuni*, a standard broth microdilution MIC experiment was performed.

5.4.1.4 *cmeB* Mutant Pantoprazole Minimum Inhibitory Concentration

A pantoprazole MIC could not be accurately determined for wild-type strains of *C. jejuni* because the inhibitory concentration appeared to be close to concentrations of pantoprazole

where yellow benzimidazole intermediates made visual and automated MIC determination difficult (see **Figure 8**). The *cmeB* mutant was however shown to be inhibited by lower concentrations of pantoprazole than the parent strain and this concentration was low enough that an MIC could be determined using OD readings. The pantoprazole MIC for the *cmeB* mutant was shown to be 125 µg/ml (**Figure 37**). At concentrations of pantoprazole ≤ 500 µg/ml there were no issues with yellow colour development or PPI solubility that made visual MIC determination difficult and affected the OD of solutions used in earlier experiments (see **Section 2.3.2**).

The *cmeB* mutant is demonstrably more susceptible to killing by pantoprazole than the parent strain and other wild-type strains (see **Table 8** and **Section 5.3.1.2**). Hence it is clear that the CmeABC pump is not only important for the extrusion of detergents, dyes, bile salts and conventional antibiotics, but also important in the extrusion of the PPI pantoprazole.

5.4.1.5 Effect of 10 µg/ml Pantoprazole on *cmeB* Mutant Antibiotic Susceptibility

Experiments failed to determine if the *cmeB* mutant was differentially susceptible to selected conventional antibiotics in the presence of additional 10 µg/ml pantoprazole because 10 µg/ml pantoprazole was shown to be highly significant in two experiments and significant in one set of experiments in its ability to inhibit the growth of the *cmeB* mutant (**Figure 38**). For this reason, the experiment was not performed using rifampicin (as was the case in **Section 5.3.1.3** where 100 µg/ml pantoprazole was utilised).

Re-examination of the pantoprazole MIC data shown in **Figure 37** showed that growth of the *cmeB* mutant was highly significantly and significantly inhibited by concentrations as low as 2 µg/ml pantoprazole (see **Table 27**).

5.4.2 Effect of Pantoprazole on Wild-type *Campylobacter jejuni* Antibiotic Susceptibility

5.4.2.1 Gentamicin, Ciprofloxacin and Erythromycin

The ODs of cultures exposed to sub-MIC gentamicin in the presence of additional 250 µg/ml pantoprazole were shown to be highly significant in their reduction, when compared to those for gentamicin alone or pantoprazole alone (**Figure 39a**). The proton motive force is important for the uptake of gentamicin into bacterial cells. The highly significant increase in bacterial killing on exposure to both gentamicin and pantoprazole concurrently suggest that pantoprazole does not adversely affect the proton motive force of

C. jejuni. Culture results confirmed that the reduction in OD corresponded to a reduction in recoverable *C. jejuni* (**Figure 39d**). Combinations of OD and culture results also showed that the same was true for ciprofloxacin and erythromycin. These three agents can be prescribed in the treatment of campylobacteriosis and we have shown that antibiotic killing can be improved in the presence of sub-lethal concentrations of pantoprazole.

The ability of PPIs to contribute to growth inhibition or killing by conventional antibiotics has previously been reported for organisms unrelated to *C. jejuni*, e.g. in *S. aureus* (Aeschlimann *et al.*, 1999, Vidaillac *et al.*, 2007) and for *P. aeruginosa* and *S. aureus* (Singh *et al.*, 2012). Enhanced activity of conventional antibiotics in the presence of PPIs has also previously been reported for the related organism *H. pylori* (Bamba *et al.*, 1997, Midolo *et al.*, 1997, Trautmann *et al.*, 1999, Tanaka *et al.*, 2002). *H. pylori* eradication triple therapy regimes often combine two antibiotics (e.g. metronidazole, clarithromycin or tetracycline) prescribed alongside a PPI (Mills *et al.*, 2004). We know that by making the stomach environment less acidic, sensitivity to conventional antibiotics can be increased (Sachs *et al.*, 1995). We also know that PPIs exhibit a direct anti-bacterial effect on *H. pylori* and so the benefits of treating *H. pylori* infections with an antibiotic and PPI combination are multifactorial (Spengler *et al.*, 2004).

5.4.2.2 Rifampicin

Results in **Figure 40** show that resistance to rifampicin can be lessened by exposing wild-type *C. jejuni* to rifampicin in the presence of additional 250 µg/ml pantoprazole. The numbers of CFU/ml recovered following co-exposure to pantoprazole and rifampicin are lower than those recovered following exposure to pantoprazole alone (see **Figure 39b**) and are also lower than those recovered following exposure to rifampicin alone (**Figure 40**).

It has been previously reported that inherent wild-type *C. jejuni* resistance to rifampicin can be overcome by the mutation of *cmeB* (Lin *et al.*, 2002) and results by these authors have been confirmed in this study (see **Table 24** and **Figure 35**). This study has provided evidence that *C. jejuni* growth can be inhibited by pantoprazole (**Figure 10**), that (at concentrations higher than inhibitory concentrations) pantoprazole is bactericidal (**Table 8**) and that the CmeABC pump must be involved in the extrusion of pantoprazole (**Figure 38**). Perhaps in wild-type *C. jejuni* exposed to two bactericidal agents which are extruded via CmeABC pump, the pumps are unable to extrude the agents as quickly, the agents are able to exert their bactericidal effect more easily and therefore bacterial killing is more pronounced on exposure to both agents. This may also account for the increased

susceptibility seen in **Chapter 4** when *C. jejuni* was exposed to thiourea and pantoprazole concurrently and also bipyridyl and pantoprazole concurrently.

5.4.2.3 Vancomycin

Vancomycin (up to a maximum concentration of 500 µg/ml) was unable to kill *C. jejuni in vitro* and this was expected, as the large size and complex structure of vancomycin makes it unable to penetrate the cell membrane of Gram negative bacteria. However results in **Figure 41** suggest that vancomycin is capable of inhibiting *C. jejuni* growth. This result was unexpected but may be a result of prolonged exposure (24 hours) to high concentrations of vancomycin (100s of µg/ml). Vancomycin is a bactericidal agent and would therefore not be expected to cause growth inhibition, with no subsequent killing, in a susceptible strain. It is however clear in this instance that exposure to vancomycin alone is insufficient to kill *C. jejuni* even though vancomycin is a bactericidal agent.

However results indicate that in the presence of additional 100 µg/ml pantoprazole, vancomycin is significantly better at inhibiting the growth of *C. jejuni* (**Figure 41 green** bars) compared to vancomycin alone or pantoprazole alone. In the presence of 250 µg/ml pantoprazole we see *C. jejuni* growth inhibition (but not killing) as a result of the pantoprazole activity. Killing of *C. jejuni* occurs following exposure to 500 µg/ml vancomycin and 250 µg/ml pantoprazole concurrently and this killing must be a result of vancomycin activity as 250 µg/ml pantoprazole is sub-lethal. We suggest that exposure to sub-lethal concentrations of pantoprazole causes damage to the outer membrane of *C. jejuni,* allowing some of the vancomycin entry to kill *C. jejuni*.

5.5 Summary and Conclusions

It would be hugely beneficial to return to the golden age of antibiotic development with new families of antibiotics being discovered and novel targets being identified and inhibited successfully. It would also be desirable to develop selective target antibiotics so infectious bacteria can be eliminated without harming bacteria that make up the natural flora.

Broth microdilution MIC and MBC methods, similar to those used in earlier chapters were extensively employed in the experiments detailed in this chapter. However with the notable exception that in these experiments only one serial dilution was made per antibiotic agent utilised. Equal volumes of these dilutions were then removed into duplicate wells of fresh 96 well microtitre plates and supplemented with equal volumes of sterile water before adding twice the volume of bacterial suspension in broth. This results in a 1 in 4

dilution of the antibiotic agent (which can easily be adjusted for by changing the concentration of the stock solution) but still finally uses equal volumes of bacterial suspension in broth and diluent. In previous chapters two separate serial dilutions were performed and one set of dilutions used for one condition and the second set of dilutions for the other. This previously used method introduces more risk of error compared to the adapted method used in this chapter. The adapted method benefits from a reduced risk of error and therefore less chance of large standard deviations or error bars.

We have shown that the *cmeB* mutant is four times more susceptible to killing by pantoprazole than the parent strain (MBC of 250 µg/ml versus. 1,000 µg/ml respectively). We have also shown that, at concentrations of pantoprazole well below the cidal level, growth of the *cmeB* mutant is significantly inhibited (down to 2 µg/ml pantoprazole).

Wild-type *C. jejuni* is demonstrably more effectively killed by conventional antibiotics, in combination with pantoprazole, that are relevant to the treatment of campylobacteriosis (gentamicin, ciprofloxacin and erythromycin). In addition, antibiotics not usually used in the treatment of campylobacteriosis are enhanced in their inhibitory and cidal activity when used in combination with pantoprazole. Our results lead us to conclude that pantoprazole damages the outer cell membrane of *C. jejuni*, making it more permeable to large molecules such as vancomycin, which usually cannot access the Gram negative cell wall.

Chapter 6

General Discussion

6 GENERAL DISCUSSION

The benzimidazoles are a diverse group of chemicals that are easily modified and the activities of the different chemical structures are far reaching. Benzimidazole derivatives have previously been shown to have anti-parasitic, anti-cancer, anti-viral and anti-fungal activities (Kazimierczuk *et al.*, 2002, Andrzejewska *et al.*, 2004, Navarrete-Vazquez *et al.*, 2006). The PPI family are the most commonly used drugs belonging to the benzimidazole group and the anti-*Helicobacter* activity of PPIs has long since been established. PPIs are also known to be extremely safe for use in humans (even at very high doses) and are available as oral agents.

This project aimed to investigate the effect of direct exposure of *C. jejuni* to PPIs. Results similar to those reported by others using other bacterial genera were obtained. Exposure to PPI affects *C. jejuni* growth/survival, motility, morphology, biofilm formation and invasion potential of cultured epithelial cells. Proteomics identified a number of proteins as being differentially present under pantoprazole exposed and control conditions that were involved in the oxidative stress response of *C. jejuni*. The up-regulation of two oxidative stress proteins (thiol peroxidase and GroEL) in response to pantoprazole exposure was confirmed using qRT-PCR, as was the increase in the ATP synthase F1 subunit. Only two genes (*Cj0561c* and *cmeA*) were identified as being differentially expressed in response to pantoprazole exposure using microarrays. Both were up-regulated in response to pantoprazole and the importance of the CmeABC pump in the susceptibility of *C. jejuni* to pantoprazole has been here demonstrated.

Bacterial transport mechanisms like influx/efflux are used to acquire essential nutrients, maintain the pH gradient across the cytoplasmic membrane as well as to extrude toxic compounds (Kaatz *et al.*, 1993). The *cmeB* mutant was demonstrably more susceptible to killing by pantoprazole than the parent strain and other wild-type strains. Hence it is clear that CmeABC is not only important for the extrusion of detergents, dyes, bile salts and conventional antibiotics, but also important in the extrusion of the PPI pantoprazole. Microarray results showed a significant increase in some of the genes controlled by CmeR but there was no statistically significant decrease seen in the expression of CmeR and so, during exposure to pantoprazole, the repressor of CmeABC and *Cj0561c* is still being produced by *C. jejuni*. It is therefore possible that pantoprazole acts on *C. jejuni* in a manner similar to that of bile, i.e. pantoprazole is identified as a potentially toxic agent, that should be extruded via CmeABC but the pantoprazole itself is also capable of interfering with the activity of the CmeR (see the scenario proposed in **Figure 42**).

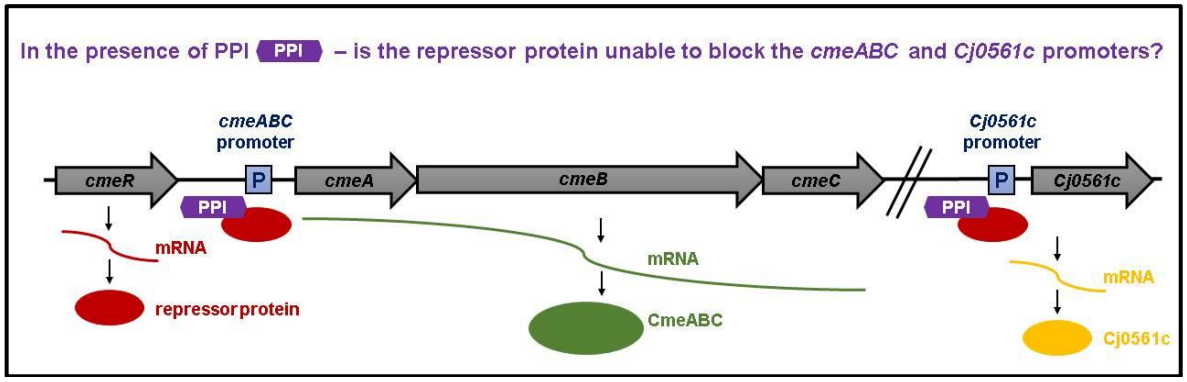


Figure 42. Possible role for pantoprazole in C. jejuni. *In the presence of pantoprazole the cmeR gene is transcribed successfully and the functional protein produced but pantoprazole interferes with the binding of the repressor to the promoters and the Cj0561c gene and the CmeABC genes are successfully transcribed.*

Although the oxidative stress response of *C. jejuni* appeared to be important during pantoprazole exposure, the killing by pantoprazole was shown not to be mediated by the production of hydroxyl radicals. Had more time been available it may have proved insightful to use both thiourea and bipyridyl together in oxidative stress inhibition experiments. The addition of both bipyridyl and thiourea may have meant that any hydroxyl radicals formed via the Fenton reaction (due to incomplete protection by bipyridyl, as a result of using a low concentration) could be scavenged by the thiourea and therefore oxidative stress might better have been inhibited. The use of both agents in combination is quite unusual but in one study, both bipyridyl and thiourea were used to inhibit oxidative stress (Liu *et al.*, 2012).

The identification of the significant up-regulation of the F1 protein subunit of the ATP synthase of *C. jejuni* (which was confirmed using qRT-PCR) may indicate that pantoprazole is binding to and inhibiting this bacterial ATPase, hence the bacterium is attempting to counteract this by producing more functional ATPases. Although the ATP synthase of *E. coli* is known to be non-essential for survival (Santana *et al.*, 1994) the enzyme is important for bacterial energy production, *C. jejuni* flagellar motion and is up-regulated in the *C. jejuni* response to bile (Fox *et al.*, 2007). *H. pylori* has an ATP synthase which has similar homology to the ATP synthase of *C. jejuni* (Slonczewski *et al.*, 2009). Whether the ATP synthase is the (or one of the) targets of PPIs in *C. jejuni* and *H. pylori* requires further investigation.

It is possible that the relatively small genome of the campylobacterales contributes to the "taxonomic boundary" of the anti-bacterial activity of PPIs that was originally proposed by Iwahi *et al*. Whereas the disruption of the ATP synthase of *E. coli* may prove to be non-

lethal (perhaps as a result of a wide range of available alternative enzymes produced by the bacterium from its relatively large genome), disruption of the ATP synthase of *C. jejuni* may be much more problematic (perhaps as a result of a lack of available alternative enzymes which are not encoded for in the relatively small genome).

Bacterial resistance to antibiotics is a growing problem and poses a great risk to public health around the world. The development of new antibiotics is currently scarce, as is the identification of new potential targets for novel antibiotic development. It was hoped that modern proteomic and genomic analysis might identify new bacterial targets for which inhibiting agents could be designed, but this has shown limited success in recent years (Liu *et al.*, 2012). Potentiation of currently used antibiotics has been proposed as a cost-effective option that might be extremely advantageous and may benefit patient care (Belenky & Collins, 2011). This may be achieved by developing small molecules that enhance the efficacy of conventional antibiotics (Liu *et al.*, 2012) or by enhancing additional damage to bacteria by inducing the production of ROS (Brynildsen *et al.*, 2013). Consumer preference is also moving towards natural antibiotics (Castillo *et al.*, 2011) and increasing awareness of the wider implications of disrupting the natural host flora during antibiotic treatment means that targeted (and perhaps pathogen specific) treatments are also being sought.

Campylobacter is the most common cause of bacterial gastroenteritis in Europe and in many other areas of the world. Antibiotic resistance in the genus is on the rise and patients infected with multi-resistant strains have been shown to have a longer duration of illness, are at greater risk of developing invasive disease and have higher associated healthcare costs (Quinn *et al.*, 2007). The CmeC portion of the CmeABC pump was found to be antigenic in chickens and has been proposed that the outer-membrane components of Gram negative multi-drug efflux pumps may be immune targets that can be successfully used as treatment enhancing intervention strategies (Lin *et al.*, 2003). The enhanced killing of *C. jejuni*, in the presence of sub-lethal concentrations of PPI, by conventional antibiotics suggest there may be potential benefits to prescribing PPIs in combination with antibiotics for the treatment of campylobacteriosis.

The *in vitro* antimicrobial activity of pantoprazole against strains of *C. jejuni* has been here demonstrated, as has the detrimental effect on bacterial motility, ability to form a protective biofilm, ability to invade epithelial cells and to enhance susceptibility to and killing by some conventional antibiotics. Data presented herein therefore suggest that exposure to pantoprazole adversely affects a number of factors that might be expected to

result in reduced pathogenicity of the organism. Lack of functional flagella prevents the organism colonising the host and non-motile *C. jejuni* do not cause disease. This may be especially true in those patients taking higher doses of PPIs on empty stomachs. Due to a lack of available information on the subject, the major difficulty in interpreting these results is in estimating the PPI concentrations that might be clinically achievable *in vivo* (see **Table 3**).

In the past it has been suggested that people taking PPIs are at higher risk of developing enteric infections like campylobacteriosis than people not taking PPIs (Tam *et al.*, 2009). In light of the results presented in this study, that may be seen as quite a dichotomy. Yet in a more recent study it has been suggested that the predisposition to GI infections may lie, not directly with the taking of PPIs, but rather as a result of the GI troubles that lead to the prescription of PPIs as a treatment (Brophy *et al.*, 2013). These results suggest that, for some patients taking large doses of PPIs, the residual PPI concentration in the GI tract may be sufficient to reduce the pathogenicity of *C. jejuni* and that for some people, the taking of PPIs may actually be "protective" against *C. jejuni* causing disease in that individual.

The actual mechanism (or mechanisms) responsible for the killing of *C. jejuni* by PPIs is not yet clear, but from our results, a number of potential mechanisms can be speculated upon. On exposure to PPI the morphology of *C. jejuni* changes from spiral to coccal, which has previously been associated with a non-culturable state. The proteomic data gathered suggests several targets involved in bacterial electron transport inside the cell may be inhibited, which have been shown to be lethal when deficient. The ability of vancomycin to exert its killing effect shows that the outer membrane is leaky and hence more permeable to large molecules which are conventionally excluded. This effect must be the result of pantoprazole exposure. In order for *C. jejuni* to be killed by PPIs, the effect must be greater than the ability of the Cme efflux system to extrude the drug. Some, or all, of these mechanisms may act together to exert bactericidal action on *C. jejuni*. Finally, we have successfully demonstrated that the safe for human consumption drugs PPIs can be used to increase the efficacy of currently used conventional antibiotics. We suggest that benzimidazole derivatives may be good candidates for drug repurposing and further development in this field.

7 APPENDIX 1 – MEDIA AND BUFFERS

7.1 Growth Media

All sterilisations were carried out by heating to 121°C for 15 minutes in an autoclave. Media was then cooled before use.

MHA – Mueller-Hinton agar

MHA (Oxoid)	15.2 g
Distilled water	400 ml
25 ml per 90 mm petri dish	

MHA + B – Mueller-Hinton agar with 7% horse blood

MHA (Oxoid)	15.2 g
Distilled water	400 ml
Allowed to cool before adding:	
Defibrinated horse blood (E+O)	28 ml
25 ml per 90 mm petri dish	

SSA – Semi-solid agar 0.4%

MHB (Oxoid)	8.4 g
Agar Bacteriological Agar No. 1 (Oxoid)	1.6 g
Distilled water	400 ml
30 ml per 90 mm petri dish	

STA – Soft top agar 0.8%

MHB (Oxoid)	1.84 g
Agar Bacteriological Agar No. 1 (Oxoid)	0.64 g
Distilled water	80 ml
10 ml per aliquot	

MHB – Mueller-Hinton broth

MHB (Oxoid)	2.1 g
Distilled water	
	100 ml

SOB – Super optimal broth

Yeast extract (Sigma)	0.5 g
Tryptone (Sigma)	2 g
NaCl (Sigma)	58.4 mg
KCl (Sigma)	18.6 mg
$MgSO_4$ (Sigma)	240.8 mg
Distilled water	100 ml

TSB – Tryptic soy broth

TSB (LabM)	3 g
Distilled water	100 ml

7.2 Buffers and Solutions

Ammonium bicarbonate	200 mM stock
Ammonium bicarbonate	7.905 g
Distilled water	500 ml

Diluted 1:2 with distilled water for use at 100 mM and also then mixed with an equal volume of Acetonitrile for 50% acetonitrile/100 mM ammonium bicarbonate

Farmer's reducing agent – FA	
20% Sodium thiosulphate	50 ml
1% Potassium ferricyanide	50 ml

5% Coomassie Blue G-250 (Biorad)*	5× stock
Coomassie Blue G-250	2.5 g
Double-distilled water	50 ml

Coomassie dye stock solution	
Coomassie Blue G-250 5% 5× stock solution	50 ml – see *
85% Phosphoric acid	30 ml
Ammonium sulfate	250 g
Double-distilled water	2.5 l

Coomassie working solution	
Coomassie Blue G-250 5% 5× stock solution	400 ml – see *
Methanol	100 ml

SDS equilibration buffer**	stock solution
1.5 M Tris-Cl at pH 8.8	6.7 ml
7 M Urea	72.07 g
87% Glycerol	69 ml
Sodium doecyl sulfate	4.0 g
Bromophenol blue	2 mg
Double-distilled water	124.3 ml

Equilibration buffer with DDT	
SDS equilibration buffer	10 ml – see **
D-dopachrome tautomerase – DDT	100 mg

Equilibration buffer with iodoacetamide	
SDS equilibration buffer	10 ml – see **
Iodoacetamide	250 mg

Fixative	
10% Acetic acid	300 ml
40% Ethanol	1.2 l
Double-distilled water	1.5 l

Rehydration Buffer
2% CHAPS
7 M Urea
0.3% DTT
2 M Thiourea
1% IPG buffer
Bromophenol blue

8 APPENDIX 2 – NUCLEOTIDE SEQUENCES AND PRIMER DESIGN

8.1 kdpB

ATGTCTAAAAAACAAAATAAACTCATTACAAAAGAAATTTTAAA**TAATGCCA**
TAAAAGGAGCATTTTTAAAATTTGATCCACGCTTTATGGTAAAAAATCCTGTT
ATGTTTATGGTGGAAGTTGGATTGATTCTTACTTTGATTTTAAGTATTTTTCCTA
CTTTGTTTAATGGAAATTCTGATGAAAGAATTTATAACATCTTAATCACTTTTA
TTTTATTTATAACCTTGCTTTTTGCAAATTTTGCAGAAAGTATTGCAGAAGGAA
GGGGTAAAGCCCAAGCAGCTACCTTAAGACAAAGCAAAAAGGATTCTAAAGC
TAGACGCATAAAAAGTGATGGCAGTGAAGAAATGCTTAATTCTAGCGAGTTAA
AAATAGGTGATATAGTTTTAGTTAAAGCAGGTGAACTTATACCTAATGATGGA
GAAATTATAGAAGGTGCTGCAAGTGTTGATGAATCAGCTATTACAGGTGAAAG
TGCTCCTGTTATGCGTGAAGCGGGCGGTGATTTTTCTTCTGTTACAGGTGGGAC
TACGGTTTTAACTGATTTTTTAAAGATTAAAATTTTAGTTGGAGCTGGGGAAAG
TTTTTTAGATAAAA**TGATCA**ATCTTGTAGAAGGTGCTGCGCGTCAAAAAACTC
CAAATGAAATTGCTCTTAATACTCTTTTAATTGTTCTTAGTTTGAGTTTTTTGGT
GGTGGTTGTAAGTTTATATCCTTTTATGCAATTTTTAGGCGTGAGTTTGCCTATT
TCGTGGTTAGTAGCATTGCTTGTATGTCTTATTCCTACAACTATAGGGGGGCTT
TTATCAGCTATAGGAATAGCAGGTATGGATAGGGTAACGCGTTTTAATGTGAT
CGCACTTTCAGGCAAGGCTGTTGAAAGTTGTGGTGATGTTGATACTATGATTTT
GGATAAAACAGGAACGATTACTTTTGGAAACCGTTTGGCAAATGAATTTTATG
AAGTCCAAGGTATAAGTAAAGAAGAAATGATTAAAGCTTGTGTTTTATCCTCT
TTAAAAGATGAAACTCCAGAAGGTAAAAGCATAGTTGCATTGGCTCAAAAAAT
GGGTTATGAATTAGAAGGTAATGATATTAAAGAATTTATCGAATTTAGCGCTC
AAAATAGAATGAGTGGTGTGGATTTACAAGATAATACAAAAATTCGCAAAGGT
GCTTTTGATGCTATAAGAGCTTATATAAGCGAAATGAATGGAAAAATTCCTAG
CGATTTAGAAACTAAGGTAATGGAAATTTCAAATCTTGGTGGCACTCCTTTGGT
AGTGTGTAAAAATGAAAAAATTTTAGGAGTGATTTATCTAAAGGATACAGTAA
AACCAGGACTTAAAGAGCGCTTTGATGAGCTTAGAAAAATGGGCATAAAAAC
TTTAATGTGTACTGGAGACAATCCTTTAACAGCAGCTACTATAGCTAAAGAAG
CAGGGCTTGATGGATTTATAGCAGAATGCAAGCCTGAAGATAAAATAGAAGC
CATTAAAAAAGAACAAGCTCAAGGTAAGATAGTAGCCATGACAGGAGATGGA
ACCAATGATGCTCCGGCTCTTGCACAAGCTGATGTAGGTATAGCTATGAACTC
AGGAACTCAGGCGGCCAAAGAAGCAGCCAATATGAT**AGATCT**TGATTCTAATC
CTACTAAAATTTTAGAAGTGGTTGAAATAGGAAAAGGTTTGCTTATTACTAGA
GGCAGTCTTACAACTTTTAGTATGGCAAATGATATTGCTAAGTATTTTACTATT
TTACCTGCTATGTTTAGTGTGGTTTTACCTCAAATGCAAATTTTAAATATTATGC
ATTTGGCTACCCCGCAAAGCGCTATTTTATCAGCACTTATTTTTAATGCTATTAT
TATACCTTTGCTCATACCTATTGCTATGCGTGGAGTTAAATTTAAGCCTATGAA
AAGTGAGCATTTGCTTTTAAGAAATTTGAGTATTTATGGTTTAGGTGGTATGAT
AGCACCTTTTATAGGGATAAAAATAATTGATATTCCTACA**GCTTGGATACTTAG**
AATTTTAGGAGTGTGA

Figure 43. Sequence of the kdpB gene of C. jejuni 81116. *The nucleotide sequence of the kdpB gene is shown with existing BclI and BglII sites (highlighted in green and blue respectively). Gene specific **forward** (kdpB GS-F) and **reverse** (kdpB GS-R) primers were selected to target the areas highlighted in yellow. The sequences of these were **5'-TAATGCCATAAAAGGAGC-3'** and **5'-ATTCTAAGTATCCAAGC-3'** respectively.*

8.2 Kan^{R}

GATAAACCCAGCGAACCATTTGAGGTGATAGGTAAGATTATACCGAGGTATGA
AAACGAGAATTGGACCTTTACAGAATTACTCTATGAAGCGCCATATTTAAAAA
GCTACCAAGACGAAGAGGATGAAGAGGATGAGGAGGCAGATTGCCTTGAATA
TATTGACAATACTGATAAGATAATATATAATATATCTTTACTACCAAGACGATA
AATGCGTCGGAAAAGTTAAACTGCGAAAAAATTGGAACCGGTACGCTTATATA
GAAGATATCGCCGTATGTAAGGATTTCAGGGGGCAAGGCATAGGCAGCGCGC
TTATCAATATATCTATAGAATGGGCAAAGCATAAAAA**CTTGCATGGACTAAT**
GCTTGAAACCCAGGACAATAACCTTATAGCTTGTAAATTCTATCATAATTGTG
GTTTCAAAATCGGCTCCGTCGATACTATGTTATACGCCAACTTTGAAAACAACT
TTGAAAAAGCTGTTTTCTGGTATTTAAGGTTTTAGAATGCAAGGAACAGTGAA
TTGGAGTTCGTCTTGTTATAATTAGCTTCTTGGGGTATCTTTAAATACTGTAGA
AAAGAGGAAGGAAATAATAAATGGCTAAAATGAGAATATCACCGGAATTGAA
AAAACTGATCGAAAAATACCGCTGCGTAAAAGATACGGAAGGAATGTCTCCT
GCTAAGGTATATAAGCTGGTGGGAGAAAATGAAAACCTATATTTAAAAATGAC
GGACAGCCGGTATAAAGGGACCACCTATGATGTGGAACGGGAAAAGGACATG
ATGCTATGGCTGGAAGGAAAGCTGCCTGTTCCAAAGGTCCTGCACTTTGAACG
GCATGATGGCTGGAGCAATCTGCTCATGAGTGAGGCCGATGGCGTCCTTTGCT
CGGAAGAGTATGAAGATGAACAAAGCCCTGAAAAGATTATCGAGCTGTATGC
GGAGTGCATCAGGCTCTTTCACTCCATCGACATATCGGATTGTCCCTATACGAA
TAGCTTAGACAGCCGCTTAGCCGAATTGGATTACTTACTGAATAACGATCTGG
CCGATGTGGATTGCGAAAACTGGGAAGAAGACACTCCATTTAAAGATCCGCGC
GAGCTGTATGATTTTTTAAAGACGGAAAAGCCCGAAGAGGAACTTGTCTTTTC
CCACGGCGACCTGGGAGACAGCAACATCTTTGTGAAAGATGGCAAAGTAAGT
GGCTTTATTGATCTTGGGAGAAGCGGCAGGGCGGACAA**GTGGTATGACATTG**
CCTTCTGCGTCCGGTCGATCAGGGAGGATATCGGGGAAGAACAGTATGTCGA
GCTATTTTTTGACTTACTGGGGATCAAGCCTGATTGGGAGAAAATAAAATATT
ATATTTTACTGGATGAATTGTTTTAGTACCTAGATTTAGATGTCTAAAAAGCTT

Figure 44. Sequence of the kanamycin resistance cassette found in pJMK30. *The nucleotide sequence of the kanamycin resistance cassette encodes for an aminoglycoside 3'-phosphotransferase (aph-3) which can be excised using BamHI to give sticky ends. Gene specific* ***forward*** *(kan^{R} F-out) and* ***reverse*** *(kan^{R} R-out) primers were selected to target the areas highlighted in yellow. The sequences of these were* ***5'-TGGGTTTCAAGCATTAGTCCATGCAAG-3'*** *and* ***5'-GTGGTATGACATTGCCTTCTG CG-3'*** *respectively.*

9 APPENDIX 3 – METABOLOMICS

9.1 Materials and Methods

A suspension of *C. jejuni* 81-176 in MHB was prepared from 48 hour plate cultures and corrected to an OD_{595} of 1.5. One ml of this was added to each of two vented cap 75 cm^2 tissue culture flasks (Corning). 37 ml of fresh sterile MHB was added to each flask and 2 ml of sterile water added to one flask to act as the PPI free control and 2 ml of pantoprazole in water was added to the other (to give a final pantoprazole concentration of 2 mg/ml or 2,000 µg/ml). Flasks were mixed well and incubated for 4 hours before plunging flasks into ice buckets, mixing regularly for 5 minutes. Cooled 40 ml samples were then transferred from tissue culture flasks into cooled sterile 50 ml falcon tubes (Corning) and left on ice for a further 5 minutes. Tubes were then centrifuged at 1,000 × g for 10 minutes at 4°C. A large proportion of supernatant was removed, leaving around 1 ml in which to resuspend pellets. This was then transferred to 1.5 ml sterile Eppendorfs and centrifuged again at 2,500 × g for 5 minutes at 4°C. All of the supernatant was then removed and the pellets re-suspended in 200 µl of iced chloroform/methanol/water mixture (ratio 1:3:1). Eppendorfs were then vortexed at 4°C for 1 hour before being centrifuged at 13,000 × g for 3 minutes at 4°C. 180 µl supernatant was then removed into fresh Eppendorfs and stored at -80°C until analysis by LCMS was carried out on three biological replicates.

9.2 Results

Only metabolites with an identity confidence score of between seven and ten (ten being the highest achievable) have been selected for inclusion in **Table 28**. Metabolites with low confidence scores have not been matched with known authentic standards which are included as internal controls before each batch of analysis. The detected mass and retention time of metabolites with low confidence scores is such that the identity cannot be accurately established. All metabolites found in the control samples are given a score of 1.00. Metabolites with fold differences (either higher or lower) for which the P value was found to be significant (≤ 0.05) have been included in the table. Undetected levels are designated as 0.00 but values in other sample sets cannot be statistically compared to undetected levels and so these have been disregarded from statistical analysis. Therefore all metabolites with a detection level of 0.00 in either the control samples or the PPI exposed samples have been omitted. Hence, the chemical structure identity of the PPI is missing from the table, because the score for the control samples was found to be 0.00. Metabolites which were detected in higher levels in the pantoprazole exposed samples are

shown in **blue** and metabolites detected in lower levels in the pantoprazole exposed samples are shown in **orange**.

Table 28. Metabolites with high confidence in their identity, which were detected in significantly different amounts between control and pantoprazole exposed C. jejuni.

Putative Metabolite	Confidence	Related Pathway	Control	PPI Exposed	T Test
Chyrsophanol 8-O-beta-D-glucoside	7	Aromatic polyketides	1.00	97.46	0.01
Cyrysophanol	7	Chyrsophanol biosynthesis	1.00	34.92	0.00
Trp-Gly-His	7	Basic peptide	1.00	14.51	0.02
Pyridoxamine	8	Vitamin B6 metabolism	1.00	11.46	0.02
Solanidine	7	Steroidal glycoalkaloid	1.00	10.38	0.02
Isopyridoxal	7	Vitamin B6 metabolism	1.00	6.59	0.02
L-2,3-Dihydrodipicolinate	8	Lysine biosynthesis	1.00	5.68	0.03
Methyloxaloacetate	8	C5-branched dibasic acid	1.00	5.64	0.02
Cys-Lys-Pro-Pro	7	Basic peptide	1.00	3.77	0.03
3-methoxyanthranilate	8	Tryptophan metabolism	1.00	3.20	0.03
Xanthine	10	Purine metabolism	1.00	2.35	0.03
D-mannose	8	Fructose and mannose	1.00	2.29	0.02
Urocanate	8	Histidine metabolism	1.00	2.09	0.03
But-2-ene-1,2,3-tricarboxylate	8	Propanoate metabolism	1.00	2.04	0.04
Hexadecanoic acid	8	Fatty acid biosynthesis	1.00	1.95	0.03
octanoic acid	7	Fatty acid biosynthesis	1.00	0.38	0.04
Ile-Phe-Thr-Pro	7	Hydrophobic peptide	1.00	0.10	0.02
nonanoic acid	7	Fatty acids and conjugates	1.00	0.05	0.04
chavicol	7	Volatile cinnamoic	1.00	0.04	0.02

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