ANTIBIOTIC & BIOCIDE RESISTANCE IN *SALMONELLA ENTERICA* AND *ESCHERICHIA COLI* O157.

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Summary

Bacterial resistance to antibiotics and biocides is a prevalent problem, which may be exacerbated by the commonplace and often unnecessary inclusion of biocides into domestic products. Addition of antimicrobials to domestic disinfectants has raised concern about promoting microbial resistance and potential cross-resistance to therapeutic antibiotics.

This study investigated the potential for resistance in *Salmonella enterica* serovars Enteritidis, Typhimurium, Virchow and *Escherichia coli* O157 to commonly used biocides, to identify mechanisms underlying resistance and whether these provided cross-resistance to antibiotics. *Salmonella enterica* and *E. coli* O157 strains were serially exposed to sub-inhibitory concentrations of erythromycin (ERY), benzalkonium chloride (BKC), chlorhexidine hydrochloride (CHX) and triclosan (TLN). Once resistance was achieved permeability changes in the outer membrane, including LPS, cell surface charge and hydrophobicity and the presence of an active efflux were investigated as possible resistance candidates. Thin layer chromatography (TLC) and Gas chromatography (GC) were carried out to examine fatty acid and lipid changes in *E. coli* O157 isolates with reduced susceptibility to TLN. Cross-resistance was studied by the Stoke's method and standard microdilution assays.

Examination of the outer membrane proteins and LPS did not reveal any significant changes between parent and resistant strains. The hydrophobicity of the cells increased as the cells were passaged and became less susceptible. An active efflux system was the most likely mechanism of resistance in all strains tested and a *fabI* mutation was associated with *E. coli* O157 resistant to TLN isolates. In all isolates investigated the resistance was stable for over 30 passages in biocide-free media. A high degree of cross-resistance was obtained in TLN-resistant *Escherichia coli* O157 strains, which repeatedly exerted decreased susceptibility to various antimicrobials, including chloramphenicol, erythromycin, imipenem, tetracycline and trimethoprim, as well as to various biocides.

The results of this laboratory-based investigation suggest that it is possible for microorganisms to become resistant to biocides when repeatedly exposed to sublethal concentrations. This may be especially the case in the domestic environment where administration of biocides is poorly controlled. Eventually it could lead to the undesirable situation of resident strains becoming resistant to disinfection and cross-resistant to other antimicrobials.

Keywords: Triclosan, Cross-resistance, *E. coli*, *Salmonella*, biocides, antibiotics.

Στη μνημη του παππου μου Αντωνη

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List of Contents

Chapter 6: Final Discussion

List of Figures

Chapter 3

Chapter 4

Chapter 5

List of Tables

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

ABBREVIATIONS

A1	TLN- Resistant *Escherichia coli* O157 with primer 1
A3	TLN- Resistant *Escherichia coli* O157 with primer 3
ABC	ATP-binding cassette
AMC	Amoxicillin-clavulanic acid
AMX	Amoxicillin
ANOVA	One way analysis of variance
API	Analytical Profile Index
ATCC	American Type Culture Collection
BKC	Benzalkonium chloride
Bp	Base pairs
C	Control
°C	Degrees Centigrade
CCCP	carbonyl cyanide m-chlorophenyl hydrazone
Cfu	Colony forming unit
CHL	Chloramphenicol
CHX	Chlorhexidine hydrochloride
CIP	Ciprofloxacin
CLI	Clindamycin
CS	Colistin sulfate
Da	Daltons
DAEC	Diffuse adhering *E. coli*
DHEC	Diarrhoea associated haemolytic *E. coli*
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
DPG	Diphospatidylglycerol
EDTA	Ethylenediamine-tetracetic acid
EAEC	Enteroaggregative *E. coli*
EaggEC	Enteroaggregative *E. coli*
EHEC	Enterohaemorragic *E. coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
ERY	Erythromycin
ETEC	Enterotoxigenic *E. coli*
FA	Fatty acids
FAME	Fatty acid modifying enzyme
FD	Fusidic acid
GC	Gas chromatography
GEN	Gentamicin
h	Hour
HC	Haemorrhagic colitis
Hep	L-glycero-D-manno-heptopyranose
HUS	Haemolytic ureamic syndrome
H_2O	Water
IPM	Imipenem
kb	kilo-base pairs
KCl	Potassium chloride
kDa	kilodaltons
Kdo	3-deoxy-D-manno-oct-2-ulosonic acid

LPS	Lipopolysaccharide
M	Molecular weight
MATE	Multidrug and toxic compound extrusion family
MFS	Major facilitator family
$MgCl_2$	Magnessium chloride
MIC	Minimum inhibitory concentration
min	Minutes
No.	Number
OD	Optical density
OM	Outer membrane
OMP	Outer membrane protein
P1	Parent *E. coli* O157 with primer 1
P3	Parent *E. coli* O157 with primer 3
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PE	Phosphatidylethanolamine
Pet	Putative efflux transporter family
PG	Phosphatidylglycerol
RAPD	Randomly amplified polymorphic DNA
RIF	Rifampin
RND	Resistance nodulation division family
RT-PCR	Reverse Transcriptase PCR
sd	Sterile distilled
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate protein agarose gel electrophoresis
SMR	Small multidrug resistance protein family
spp	Species
TAE	Tris acetate EDTA
Taq	DNA polymerase from *Thermus aquaticus*
TCL	Triclosan
TE	Tris EDTA
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
TET	Tetracycline
TLC	Thin layer chromatography
TLN	Triclosan
T_m	Melting Temperature
TMP	Trimethoprim
Tris	Tris [hydroxylmethyl] aminomethane
TTP	Thrombotic thrombocytopenic purpura
VAN	Vancomycin
VTEC	Verocytoxin *E. coli*
UPEC	Uropathogenic *E. coli*
UV	Ultra violet
v/v	volume by volume
w/v	weight by volume
23	second passage of TLN-resistant *E. coli* O157

1. Introduction

A century ago bacteria had a significant impact on daily life as routine infections could be life threatening and improperly stored foods could poison the unsuspecting consumer. However, following the discovery and clinical application of antibacterials, the morbidity and mortality caused from bacterial infections was considerably reduced. Today though, public health is facing a new 'challenge' by the alarming increase in bacterial resistance to most of our existing antibacterial agents. It is therefore essential to identify the factors affecting mechanisms of bacterial resistance so that steps can be taken to reduce the increasing burden of resistance.

1.1 *Salmonella enterica* species and strains

The genus *Salmonella* was so named by Lignieres in honour of Daniel Elmer Salmon, a pathologist, who was director of the Bureau of Animal Industry in Washington D.C, where the organism *Salmonella cholerae-suis* was discovered by his assistant Theobald Smith in 1885 (Candy and Stephen, 1989).

Salmonellae are Gram-negative, non-sporulating rods and are chiefly motile with peritrichous flagella. Environmental sources of the organism include water, soil, insects, factory surfaces, kitchen surfaces, animal faeces, raw meats, raw poultry, and seafood. Routes of transmission are most commonly attributed to contamination of foods by food handlers. Some foods also have an inherent *Salmonella* presence; eggs and chicken meat are often contaminated with *Salmonella* from the animal itself and thus proper cooking and prevention of cross contamination is vital with such foods. Uncooked eggs used in foods such as custards, cream cakes, and meringues are often

associated with *Salmonella* infection in addition to meat and meat products, milk and dairy products. A recognised route of transmission is also directly from animals, including cattle and domestic pets. In addition, intestinal disease associated with *Salmonella* is acquired not only by the consumption of contaminated foods but also by hand to mouth transfer, either directly or indirectly via contaminated surfaces and utensils used in the preparation of food (Barker *et al.,* 2003). In order to reduce infection rates appropriate education on food safety and handling in the home and in industry is essential.

The nomenclature for the genus *Salmonella* has evolved through a serotype system devised by Kauffmann and White. There are currently 2,463 known serotypes (serovars) of *Salmonella* classified on the basis of three antigens. These include the O antigen found on the cell wall lipopolysaccharides (LPS), the H antigen on the flagella and the Vi antigen on the outer polysaccharide layer - found primarily in *Salm.* Typhi strains (Brenner *et al.*, 2000; Madigan *et al.*, 1997). Each serotype was initially considered a separate species and named after their host location of first isolation or disease symptoms; for instance *Salmonella cholera-suis* acquired its name because it caused cholera-like symptoms in swine (Edwards *et al.,* 2002). However, the current taxonomical position recognises that these names were wrongly considered as species names (Euzeby, 1999) and the genus *Salmonella* contains only two species; *Salmonella enterica* and *Salmonella bongori*, each of which contains multiple serotypes (Table 1) (Brenner *et al.*, 2000).

More than 60% of all *Salmonella* serotypes identified and 99% of those responsible for disease in warm blooded animals are members of subspecies I, whereas other

subspecies including subspecies IIIa (Arizona) in addition to *Salm.* Bongori are associated with disease in cold-blooded hosts such as reptiles and snakes. Despite the genetic similarity among *Salmonella* serovars, they differ in their disease causing spectrum and host range (Chan *et al.*, 2003; Porwollik and McClelland, 2003).

Table 1.1: *Salmonella* nomenclature in use at CDC, 2000.

(Adapted from Brenner *et al.*, 2000)

Taxonomic position	Nomenclature
Genus (italics)	*Salmonella*
Species (italics)	• *enterica*, which includes subspecies I, II, III, IIIb, IV and VI • *bongori* (formerly subspecies V)
Serotype (capitalised not italised)	• The 1st time a serotype is mentioned in the text; the name should be preceded by the word 'serotype' or 'ser'. • Serotypes are named in subspecies I and designated by antigenic formulae in subspecies II to VI and *S. bongori*. • Members of subspecies II, IV and VI and *S. bongori* retain their names if named before 1966.

According to eurosurveillance the incidence of salmonellosis has decreased substantially across the European Union declining from 100, 267 in 1997 to 73, 006 in 2001 (O'Brien and de Valk, 2003). *Salm.* Enteritidis reduced by over 50% between 1997 and 2000 in England and Wales, probably as a result of poultry flocks vaccination.

Among the most frequently implicated foodborne disease serotypes in the UK are *Salmonella* Enteritidis, Typhimurium and Virchow and the main method of spread is through contaminated food. *Salmonella* Enteritidis and *Salm.* Virchow are commonly associated with poultry and poultry products, whereas *Salm.* Typhimurium is normally associated with cattle and pigs, but also with poultry and occasionally sheep (Threlfall, 2002). These strains are discussed in more detail below.

1.1.1 *Salmonella* Typhimurium

Salmonella enterica serovar Typhimurium (hereafter called *Salm.* Typhimurium) is among one of the most researched human pathogens due to its relatively easy genetic manipulation and a reproducible, cheap animal infection model. Serovar Typhimurium causes gastrointestinal disease in a wide variety of animals and yet is responsible for a typhoid-like disease in the mouse (Chan *et al.,* 2003).

Salmonella Typhimurium are Gram–negative rods, measuring 0.5 by 1 to 1.5μm, occurring singly. The organism is motile by means of peritrichous flagella (Salle, 1973). *Salmonella* Typhimurium is an invasive organism, which is capable of penetrating into the cytoplasm of an animal cell. Recent observations suggest that the host cell is the major contributor to the phase of internalisation which may be no different from phagocytosis. The latter consists of a succession of phases, which include recognition of the bacterium by the phagocyte, and finally the internalisation of the bacterium (Slater *et al.*, 1983).

Strains of *Salmonella* that are resistant to antimicrobial agents have become a worldwide health problem. More specifically, *Salm.* Typhimurium (DT104) appeared to be resistant

to ampicillin, chloramphenicol, streptomycin, sulphonamides, macrolides and tetracycline and has become a major cause of infection in humans and animals in the United States, and Europe; especially the UK (Wall *et al.*, 1994; Glynn *et al.*, 1998). In addition, it was recently demonstrated that *Salm.*Typhimurium DT104 posed a major health concern due to its apparent enhanced ability to acquire multiple antibiotic resistance genes and its putative hypervirulent phenotype. It is currently unclear if multiresistant *Salm.* Typhimurium are more or less pathogenic than non-resistant counterparts although studies have revealed that the ability to cause disease is not increased but is rather mildly attenuated for certain isolates of multiresistant *Salm.* Typhimurium (Carlson *et al.*, 1999a; Carlson *et al.*, 1999b).

In 2000 both resistance and multiple resistance was most common in *Salm.* Typhimurium with 77% of isolates expressing drug-resistance and 51% multi-resistance (Threlfall *et al.*, 2003). In support, Helms and his colleagues concluded that resistance in *Salm.* Typhimurium was associated with increased mortality and this could be a result from the use of antimicrobial drugs in food production (Helms *et al.*, 2002).

1.1.2 *Salmonella* Enteritidis

Salmonella Enteritidis was first recognised as a cause of foodborne enteritis by Gaertner in 1888. It causes food poisoning in man and in wild and domestic animals and is also associated with poultry and game birds. Many infections caused by *Salm.* Enteritidis have arisen from the consumption of raw or undercooked eggs (Old, 1990) and according to Ward *et al,* (2000) many large outbreaks have been linked to eggs rather than poultry meat. In support, Rahman *et al.* (1997) proposed that *Salm.*

Enteritidis is a virulent human pathogen which in the USA is contracted primarily through consumption of contaminated eggs.

Most studies on the incidence of *Salmonella* infections undertaken since the early 1080s have shown that *Salm.* Enteritidis and *Salm.* Typhimurium are the most frequently isolated serovars in Europe and the USA. In the last decade, European surveillance has observed a declining trend in the incidence of *Salm.* Enteritidis in Western Europe between the years 1993 and 1995, however this was followed by an increase in 1996 into 1998 (Marimon, 2003). Even though the incidence of *Salm.* Enteritidis is decreasing; human salmonellosis caused by those strains is still a matter of great concern. The emergence of antimicrobial resistance particularly to nalidixic acid and ampicillin is a highly important factor that contributes to this issue, as both antimicrobials are used in first-line therapy (Soto *et al.*, 2003). Excess mortality associated with drug resistance in zoonotic *Salmonella* is relatively rare and may not occur until months after initial diagnosis. A number of factors, including chronic and malignant diseases may contribute to death from salmonellosis (Helms *et al.*, 2002). The use of antimicrobial drugs in food animals might well be the predominant aetiology of *Salmonella* resistant strains, which are being transmitted to humans through contaminated food (Davis *et al.,* 1999; Helms *et al.,* 2002; Gupta *et al.*, 2003).

1.1.3 *Salmonella* Virchow

Generally, *Salm.* Virchow is considered to be one of the less invasive non-typhoidal *Salmonellae* species, however a number of reports have revealed the opposite (Bitsori *et al.*, 2001). There are a few reports of cardiac disease and considerable skin lesions

resulting from infection with *Salmonella* spp, most of them involving *Salm.* Virchow (Garcia *et al.*, 1995; Neuwirth, *et al.*, 1999). There is also at least one report implicating *Salm.* Virchow as responsible for a case of meningitis (Johnson *et al.*, 2000).

Over the last decade strains of non-typhoidal *Salm. enterica* with multiple drug resistance profiles have been distributed widely in many European countries. For instance, in England and Wales resistance was common in *Salm.* Virchow, with over 35% of the isolates being multi-resistant. Fifty three percent of the isolates exhibited resistance to nalidixic acid coupled with decreased susceptibility to ciprofloxacin. This is of particular concern not only because of the invasive potential of this organism but for the fact that ciprofloxacin is the first-line drug of choice in such infections (Threlfall, 2002; Threlfall *et al.,* 2003). It has been suggested that the increasing incidence of resistance to ciprofloxacin not only in *Salm.* Virchow but also in *Salm.* Enteritidis and *Salm.* Hadar might be as a consequence of the use of fluoroquinolone antibiotics in poultry since 1993 (Threlfall, 2002) and could result in treatment failure when this antibiotic is used for patients with invasive illness (Threlfall *et al.,* 1997).

1.2 Clinical Features

Salmonellae are ubiquitous human and animal pathogens and are one of the most commonly isolated bacteria from patients in the UK second only to *Campylobacter* spp. The clinical features of *Salmonella enterica* infections are quite different. *Salmonella* Typhimurium, *Salm.* Enteritidis and *Salm.* Virchow are foodborne pathogens, which cause gastroenteritis in humans (Figure 1.1). The major predominant feature of gastroenteritis is diarrhoea - which is watery and may be

severe and sometimes bloody. The incubation period depends on the dose of bacteria consumed, but usually begins 6 to 48 hours after ingestion of contaminated food or beverage. The illness usually lasts 4 to 7 days and most people recover without any treatment, however, some patient groups have more severe illness that may require hospitalisation and antibiotic therapy. These people include the elderly, infants and those with impaired immune systems (Hwang, 1999).

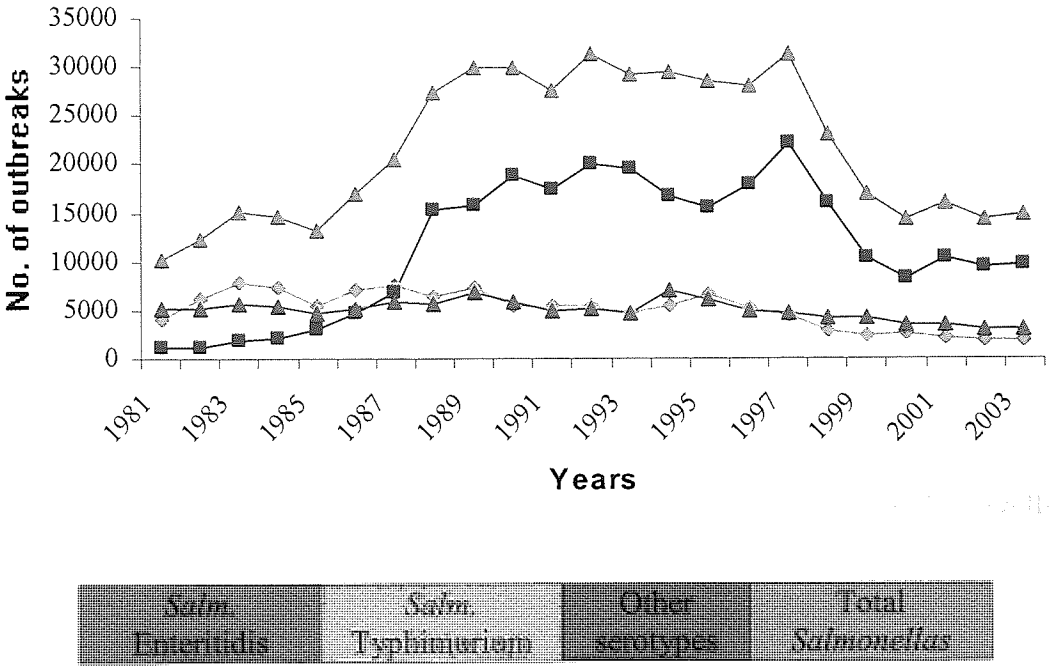


Figure 1.1: *Salmonella* cases in humans between 1981 and 2003.

Adapted from: Health Protection Agency, 2004; URL: http://www.hpa.org.uk

1.3 *Escherichia coli*

Escherichia coli is a Gram-negative, non-spore forming, facultative anaerobe. It is motile by the presence of peritrichous flagella. It is a mesophile, grows at neutral pH ranges and is found as a normal gut commensal of warm-blooded animals. It is also found naturally in soil and water. *Escherichia coli* was discovered in the human colon in 1885 by the German bacteriologist Theodor Escherich and is one of the most

extensively studied microorganisms. Like most mucosal strains, *E. coli* can be said to follow a requisite strategy of infection including colonisation of a mucosal site, evasion of host defences and finally multiplication and host damage. Once colonisation is established, the pathogenetic strategies of diarrhoeagenic *E. coli* show evidence of a notable variety. *Escherichia coli* can cause diarrhoea by a) enterotoxin production (ETEC & EAEC), b) invasion (EIEC) and, or c) adherence with membrane signalling (EPEC & EHEC) (Nataro & Kaper, 1998).

All diarrhoeagenic *E. coli* strains were initially termed enteropathogenic *E. coli*, however as more was discovered about their pathogenic mechanisms they were grouped accordingly (Clarke, 2001). *Escherichia coli* strains that cause human diarrhoea of varying severity have been divided into 6 major categories:

Enterotoxigenic *E. coli* (ETEC) infections are mostly associated with travelling, infection and adults. Enteroinvasive *E. coli* (EIEC) cause very bloody diarrhoea and symptoms similar to that of *Shigella* infection, Enteropathogenic *E. coli* (EPEC) cause infantile diarrhoea but have less significance in adults, Enteroaggregative *E. coli* (EAEC or EAggEC) and diarrhoea associated haemolytic *E. coli* or diffuse adhering *E. coli* or cell detaching *E. coli* (DHEC or DAEC) are mostly associated with persistent diarrhoea in infants older than 24 months of age (Beinke *et al.*, 1998). *Escherichia coli* responsible for urinary tract infections are known as uropathogenic *E. coli* (UPEC) (Clarke, 2001; Groisman, 2001), and finally, Enterohaemorrhagic *E. coli* (EHEC), causes very bloody diarrhoea and can produce toxin. The EHEC group is further subdivided into those that produce toxin and those that do not. Toxin producing EHEC are termed Verocytotoxin *E. coli* (VTEC) named because of the

irreversible cytotoxic effects on vero (African green monkey) cells. Verocytotoxin *E. coli* are also known as *Shigella* toxin *E. coli* (STEC) because of the toxins similarity to the *Shigella* toxin. The VTEC group is of major significance because of the severe infection that it causes.

Recent research suggests the emergence of high level resistance to ciprofloxacin in *E. coli* from cases of invasive disease which is of major concern as isolates are already resistant to a wide range of alternative antibiotics (Threlfall *et al.*, 1997). In support, Livermore *et al.* (2000) suggested significantly increasing proportions of isolates resistant to trimethoprim, ciprofloxacin, gentamicin and ampicillin amongst *E. coli* isolated from patients with bacteraemia or meningitis between 1991 and 1997.

1.4 *Escherichia coli* O157

The most common EHEC serotype in the UK is O157:H7 and has been the cause of widespread outbreaks (Paton and Paton, 1998) (Figure 1.2). *Escherichia coli* O157 was first recognised as a cause of illness in 1982 during two outbreaks of severe bloody diarrhoea that were linked to eating undercooked beef from the same fast-food restaurant chains and since then it has caused a number of outbreaks and sporadic cases of diarrhoeal disease (Boyce *et al.*, 1995; Sowers *et al.*, 1996; Mohle-Boetani *et al.*, 2001).

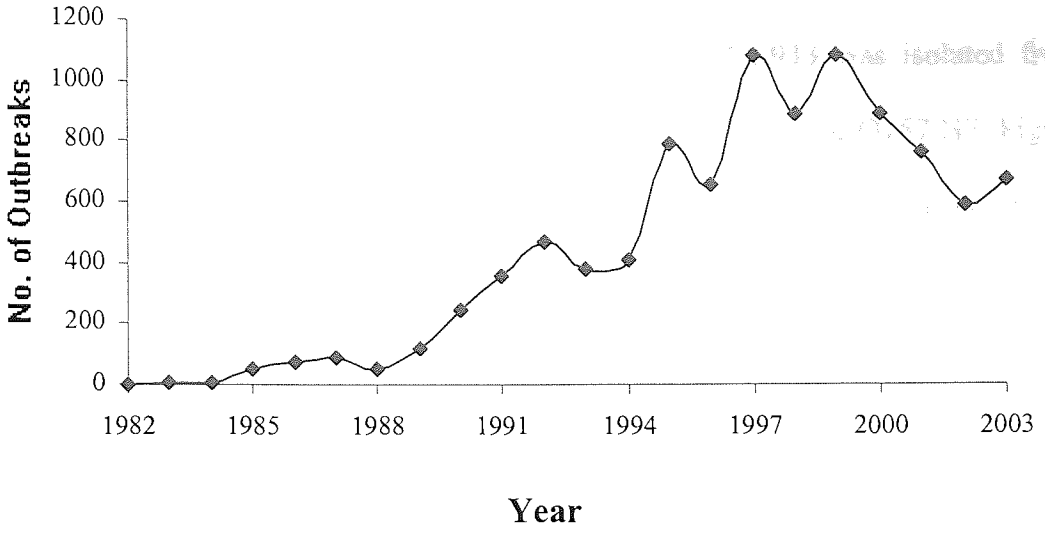


Figure 1.2: *Escherichia coli* O157 cases in England & Wales between 1982 and 2003. Adapted from: Health Protection Agency, 2004; URL: http://www.hpa.org.uk

Escherichia coli O157's natural habitat is probably the gut of cattle where it is non-pathogenic but in humans it adheres to intestinal epithelial cells and produces toxins which can produce both local damage and systemic effects (Cobden, 1998). It can cause a wide range of illnesses from a mild self-limiting diarrhoea to a severe bloody diarrhoea, Haemorrhagic colitis (HC). Typically, HC is characterised by severe abdominal cramps, low-grade or absent fever and occasionally vomiting (Cobden, 1998; Fey *et al.*, 2000). Symptoms usually resolve within two weeks with the exception of haemolytic ureamic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) cases which are discussed in more detail below.

Escherichia coli O157 has acquired numerous characterised and hitherto uncharacterised virulence factors that contribute to its ability to cause disease. *Escherichia coli* O157 is classified as a member of the group of Shiga-toxin producing *E. coli* (STEC). The gene encoding the *E. coli* Shiga-toxin is essentially

indistinguishable from that on the chromosome of *Shigella dysenteriae* type I (Whittam *et al.*, 1993; Wachsmuth, 1997). The strain EDL933 was isolated from Michigan ground beef and has been studied as a reference strain for O157:H7. Figure 1.3 shows the genetic content and organization of the EDL933 genome as compared with the chromosome of the K-12 laboratory strain MG1655 (Perna *et al.*, 2001).

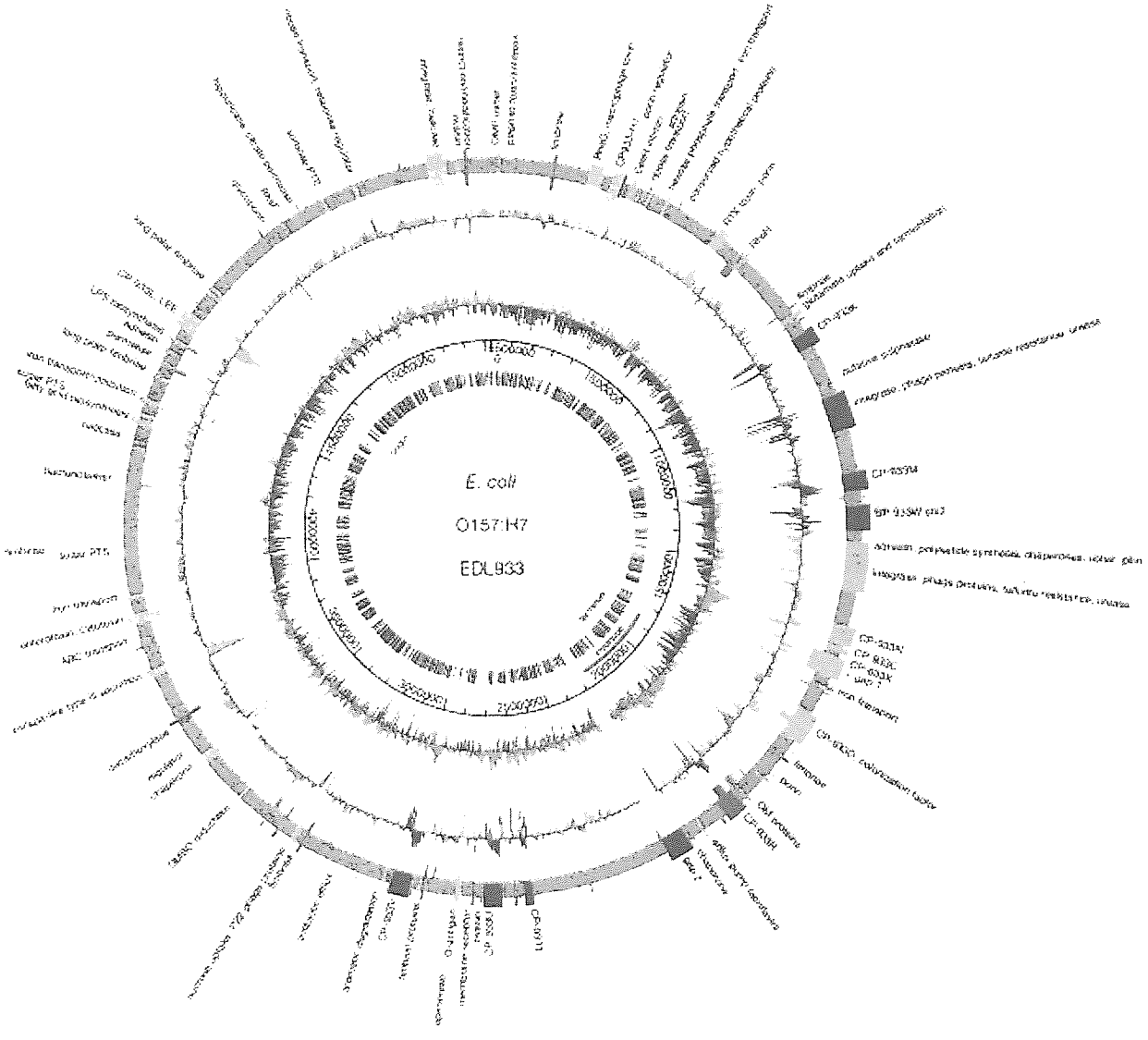


Figure 1.3: Circular genome map of EDL933 compared with MG1655. Outer circle shows the distribution of islands: shared co-linear backbone (blue); position of EDL933-specific sequences (O-islands) (red); MG1655-specific sequences (K-islands) (green); O-islands and K-islands at the same locations in

the backbone (tan); hypervariable (purple). Second circle shows the G+C content calculated for each gene longer than 100 amino acids, plotted around the mean value for the whole genome, colour-coded like outer circle. Third circle shows the GC skew for third-codon position, calculated for each gene longer than 100 amino acids: positive values, lime; negative values, dark green. Fourth circle gives the scale in base pairs. Fifth circle shows the distribution of the highly skewed octamer Chi (GCTGGTGG), where bright blue and purple indicate the two DNA strands. The origin and terminus of replication, the chromosomal inversion and the locations of the sequence gaps are indicated. Figure reproduced from Perna *et al.*, 2003.

1.5 *Escherichia coli* O55

Escherichia coli O55 is a member of the EPEC group of *E. coli*. Both *E. coli* O55 and *E. coli* O157 are considered important pathogenic clones (Nataro & Kaper 1998). It is thought that *E. coli* O55 and *E. coli* O157 are derived from a common genetic ancestor. Molecular genetic studies indicate that *E. coli* O157:H7 evolved from the EPEC progenitor of serotype *E. coli* O55:H7, a non Stx-producing organism associated with cases of infantile diarrhoea, however the timescale for development is unknown (Law, 2000). More specifically, the H7 *fliC* genes of *E. coli* O55:H7 and *E. coli* O157:H7 are almost identical but differ from those strains with other O antigens. Thus, transfer of the *E. coli* O157 O-antigen genes into an O55:H7 strain was proposed as one of the possible events in the origin of *E. coli* O157: H7 (Wang *et al.*, 2003). By looking at genetic differences between *E. coli* O55 strains and *E. coli* O157 strains it may be possible to determine why *E. coli* O157 has such an increased pathogenic advantage (Allen *et al.*, 2001).

1.6 *Escherichia coli* K-12

Escherichia coli K-12 strain was isolated in 1922 at Stanford University from human faeces and was kept under that label as a stock strain in their bacteriology department (Lederberg, 2004). *Escherichia coli* K-12 has long been studied as a model bacterium and was the earliest organism to be suggested as a candidate for whole genome sequencing (Blattner *et al.*, 1997). It is a rod-shaped bacterium, which lives as a harmless inhabitant of the human lower intestine and is widely used in medical and genetic research.

1.7 Clinical Features and Pathogenesis

Strains of *E. coli* O157 colonize the human intestine after surviving passage through the stomach. It is still unclear whether these strains can survive in human stomach acid or are protected by ingested food, however acid tolerance could be considered as a putative pathogenic property of O157 (Chart, 2000). Production of potent Shiga toxins is essential for many of the pathological features as well as the life-threatening sequelae of STEC infections and these are thought to be the aetiology for the principal manifestations of HC and HUS (Paton & Paton, 1998; Law, 2000).

Haemolytic ureamic syndrome is a major cause of renal failure in children. The pathogenesis of HUS probably involves damage to the endothelial cells of small blood vessels in the kidneys and other organs (Karmali *et al.*, 1983). An interval of 5 to 9 days takes place between the onset of diarrhoea and that of HUS. The symptoms of HUS involve diarrhoea, abdominal pain and vomiting associated with no significant fever (Karmali *et al.*, 1983; Moake, 1994). Haemorrhagic colitis and HUS are severe

diseases, which normally require hospitalisation; indeed HUS is often fatal in up to 5% of cases in children under the age of five years (Boerlin *et al.*, 1999).

Thrombotic thrombocytopenic purpura (TTP) has also been reported as a complication of *E. coli* O157 infection in adults (Paton, 1996; Groisman, 2001). In TTP aggregates of platelets obstruct reversibly arterioles and capillaries of various organs and produce ischemia and sometimes infarction. The microcirculation of the brain is involved in at least 50-71% of TTP episodes. It is closely related to HUS, however in HUS the ischemia and infarction are predominantly but not always renal (Moake, 1994).

Most EHEC infections are caused by the consumption of contaminated food, or water; however they may also be acquired by human or animal contact (Benjamin & Datta, 1995). Enterohaemorrhagic *Escherichia coli* produces two types of Shiga-like toxins (SLTs), or verotoxins (VTs), which mediate both the HC and the HUS. *Escherichia coli* Shiga-toxin 1 (Stx1) is almost identical to the Shiga toxin of *Shigella dysenteriae* at the amino acid level and it is not possible to distinguish it from *Shigella* serologically, whereas Shiga –toxin 2 (Stx2) is unrelated to the Shiga toxin of *Shigella* (Boerlin *et al.*, 1999). A single EHEC strain may express Stx1 only, Stx2 only, both toxins and even multiple forms of Stx2 (Nataro & Kaper, 1998).

Apart from the ability to produce the toxin, EHEC may possess accessory virulence factors in relation with the capacity to colonise the gut, such as intimin (Stephan & Untermann, 1999). In addition, although most episodes of HC and HUS are caused by

EHEC strains that belong to serotype O157:H7, other serotypes have also been implicated (Benjamin & Datta, 1995).

1.8 Antibacterial Agents

Antibiotics are chemical substances produced by microorganisms which are used to inhibit or kill other microorganisms. They are natural products that are produced and harvested on an industrial level. More recently laboratories have chemically modified antibiotics to enhance their activity, resulting in so-called semi-synthetic antibiotics. A large array of antibiotics exist but less than 1% have a useful clinical application (Madigan *et al.*, 1997). In bacteria there are four sites: cell wall synthesis, protein synthesis, nucleic acid synthesis and cell membrane function, which 'serve' as targets for antibiotic action. Thus, antibiotics can be classified on the basis of their targets. Some of the classes of antibiotics that act as cell wall synthesis inhibitors include the beta-lactam antibiotics as well as the glycopeptides. The quinolones and the sulphonamides act as inhibitors of nucleic acid synthesis, whereas antibiotics such as polymyxins are inhibitors of cytoplasmic membrane function. Inhibitors of protein synthesis include tetracyclines, chloramphenicol, lincosamines and macrolides, among others (Mims *et al.*, 2001; Madigan *et al.*, 1997).

1.9 Classes of Antibiotics & Modes of Action

In order to investigate any cross-resistance to biocides it is essential to understand the mechanisms of action of antibiotics, as common mechanisms could be the aetiology underlying it. Although to date a plethora of antibiotics exist many share common mechanisms of action.

1.9.1 β –lactam and carbapenems

The β-lactam antimicrobials target the penicillin-binding proteins (PBPs), which catalyse the last steps in cell wall biosynthesis. Inhibition of the PBPs leads to reduction in the cell wall cross-linking and prevents new septum initiation. The most common resistance mechanism to penicillin is inactivation by penicillinases, which in Gram-negative bacteria remain susceptible to inhibitors (Berger-Bächi, 2002). Other mechanisms involve production of inactivating enzymes (β-lactamases), efflux via specific efflux pumps and impaired entry into the bacterial cells (loss of channels – porins that permit the entry of the antibiotics into Gram-negative bacteria). Among Gram-negatives the most important mechanism is the production of β-lactamases (Helfand and Bonomo, 2003).

Amoxicillin represents over 35% of the world's antibiotic prescription. This excessive use could easily lead to the acquisition of bacterial resistance. The use of secondary inhibitors can extend the effective spectrum of β-lactam antibiotics, for instance, the β-lactamase enzyme inhibitor clavulanic acid, which is used in combination with the β-lactam antibiotic amoxicillin, continues to be effective after 20 years of clinical use (Hughes, 2003).

Due to the acquisition of resistance carbapenems were developed, which are differentiated from conventional penicillin by the lack of a sulphur atom in the 5-membered ring and a double bond between positions two and three. They are active against both Gram-positive and Gram-negative bacteria. The most widely known carbapenem is imipenem (Hawkey, 1997). Resistance to imipenem in *Pseudomonas*

aeruginosa has been proposed due to changes in the outer membrane profiles (Buscher *et al.*, 1987).

1.9.2 Aminoglycosides

Aminoglycosides were first discovered in 1943. They are broad spectrum agents that require an energy-dependent membrane transport to gain entry into the cell. Inside the cell they bind with the 16S rRNA of the 30S ribosomal fragment, which causes misreading of mRNA and inhibits translocation (Berger-Bächi, 2002).

Aminoglycosides are considered most active against Gram-negative bacteria however they have demonstratable antistaphylococcal activity when used with a cell-wall active agent (Smith and Jarvis, 1999). Gentamicin (Figure 1.4) is a widely used antimicrobial within this family group.

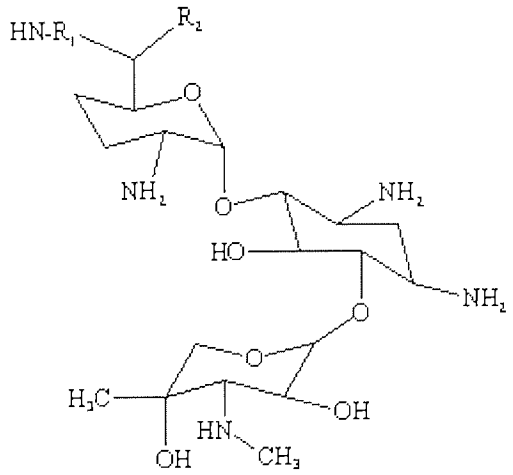


Figure 1.4: Chemical structure of gentamicin, (adapted from Antimicrobial Chemotherapy, 2004;URL:http://www.bmb.leeds.ac.uk/mbiology/ug/ugteach/icu8/antibiotics/protein.html).

1.9.3 Chloramphenicol

Chloramphenicol (Figure 1.5) is a broad-spectrum antibiotic whose activity is primarily bacteriostatic. It binds to the 50S subunit of the ribosome and inhibits bacterial protein synthesis. It rarely causes the potentially lethal complication of aplastic anaemia, which is dose related, and thus, chloramphenicol should be restricted to serious infections (Hugo & Russell, 1999).

Chloramphenicol resistance is a result of an inducible plasmid-borne chloramphenicol acetyltransferase (CAT). When acetylated by CAT, it is unable to bind to the ribosome (Smith and Jarvis, 1999).

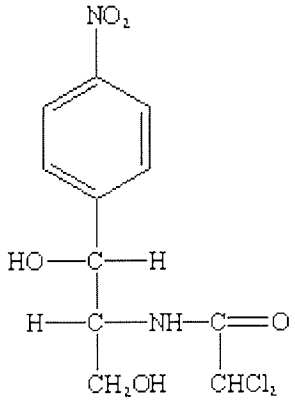


Figure 1.5: Chemical structure of chloramphenicol (adapted from Antimicrobial Chemotherapy, 2004,URL:http://www.bmb.leeds.ac.uk/mbiology/ug/ugteach/icu8/antibiotics/protein.html).

1.9.4 Glycopeptides

Vancomycin has been available since 1958 (Smith and Jarvis, 1999) and is frequently referred to as the drug of 'last resort' against multidrug-resistant Gram-positive

bacteria. It prevents the last stages of cell wall assembly by forming complexes with the terminal D-alanyl-D-alanine of the peptydoglycan precursors, thus preventing the cross-linking reactions catalysed by transpeptidases, transglycosylases and carboxypeptidases (Berger-Bächi, 2002).

Glycopeptide resistance in enterococci was first reported in the 1980s in France and England and since then vancomycin-resistant enterococci (VRE) strains have emerged (Leclercq *et al.*, 1988; Hastings, 1997).

1.9.5 Quinolones

Depending on the bacterial species and the quinolone, either DNA gyrase or topoisomerase IV are the targets of quinolones (Berger-Bächi, 2002). The quinolone targets are different in Gram-negative and Gram-positive bacteria; in Gram-negatives it is the DNA gyrase, whereas in the Gram-positives it is the topoisomerase IV (Ruiz, 2003). They trap the enzyme reaction at the step where the enzyme is bound to the DNA and converted to one in which the DNA strands are cleaved (Berger-Bächi, 2002).

Resistance to fluoroquinolones derives from the emergence of mutations in the genes encoding group II topoisomerases in Gram-negative bacteria and also from the presence of active efflux pump systems in Gram-positive bacteria (Munoz-Bellido *et al.*, 2002). To date though, according to Ruiz (2003), two main mechanisms (chromosomally-mediated) of quinolone resistance exist: 1) alterations in the targets of these and 2) decreased accumulation inside the bacteria due to impermeability of the membrane and, or an overexpression of active efflux pump systems.

Ciprofloxacin is a broad spectrum fluoroquinolone antibacterial agent, which since the 1980s has remained active against Gram-negative bacteria and at a lesser degree against Gram-positives (Davis *et al.*, 1996). According to Johnson and Speller (1997) ciprofloxacin resistance in *E. coli* is increasing, however at a lower rate compared with *Staphylococcus aureus*.

1.9.6 Sulphonamides

Sulphonamides are synthetic antibacterial agents that were first used in 1932. They are antimetabolites that share the same mode of action with trimethoprim. They inhibit tetrahydrofolate synthesis, which is required for thymidine, purine, DNA and some amino acid production (Smith and Jarvis, 1999). They are extremely useful for the treatment of uncomplicated urinary tract infection caused by *Escherichia coli* (Hugo and Russell, 1998).

1.9.7 Trimethoprim

Trimethoprim inhibits tetrahydrofolate synthesis in a similar manner to the sulphonamides. Trimethoprim is a synthetic antibacterial agent, which was first used in England in 1962. Since 1968, trimethoprim was used in combination with sulphonamides, however this stopped in 1970s when it was proposed that *in vivo* the trimethoprim-sulfonamide combinations had no synergism (Huovinen *et al.*, 1995; Smith and Jarvis, 1999).

Decreased susceptibility to trimethoprim results from alterations in the chromosomal dihydrofolate reductase (DHFR) (Thompson, 1997), which in *E. coli* results in overproduction of DHFR (Hugo and Russell, 1998).

1.9.8 Tetracyclines

Tetracyclines (Figure 1.6) were first discovered in 1950. They are broad spectrum antibiotics active against Gram-positive and Gram-negative bacteria as well as against *Mycoplasma, Chlamydia*, *Rickettsia* and Mycobacteria (Bellido *et al.*, 2002). Tetracyclines are protein synthesis inhibitors; they interfere with the 30S ribosomal subunit of the 70S ribosome and inhibit the protein synthesis elongation (Smith and Jarvis, 1999).

Three distinct mechanisms of resistance to tetracyclines have been identified: 1) energy-dependent-efflux, 2) ribosomal protection, whereby tetracyclines no longer bind productively to the bacterial ribosome and 3) chemical alteration of the tetracycline molecule by a reaction that requires oxygen and renders the antibiotic ineffective as a protein synthesis inhibitor (Chopra *et al.*, 1992).

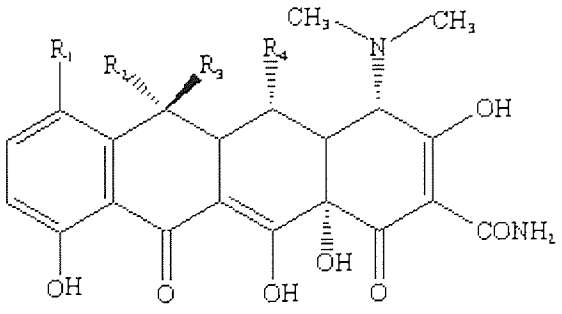


Figure 1.6: Chemical structure of a tetracycline, (adapted from Antimicrobial Chemotherapy,2004,URL:http://www.bmb.leeds.ac.uk/mbiology/ug/ugteach/icu8 /antibiotics/protein.html).

1.9 Macrolides and Erythromycin (ERY)

Macrolide antibiotics contain a macrocyclic (12- to 22-carbon) lactone ring bound to various sugars. Erythromycin, (Figure 1.7), is thought to be the most effective antibiotic from this family which also includes the antibiotics clindamycin, oleandomycin, triacetyloleandomycin, spiramycin and carbomycin among others (Volk *et al.*, 1996).

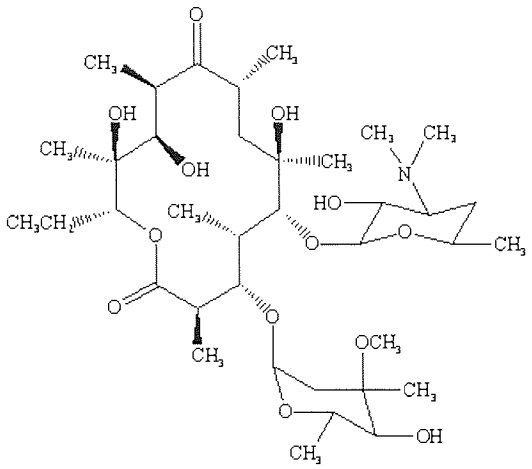


Figure 1.7: The structure of Erythromycin, adapted from: Hugo & Russell, 1998

The molecular mechanisms by which bacteria become resistant to macrolides are various and include drug efflux, drug inactivation, or alterations in the drug target site (Vester & Douthwaite, 2001). Erythromycin, as a macrolide antibiotic, blocks protein synthesis by binding to the 50S subunit of the ribosome and thus has a bacteriostatic and bactericidal effect. Erythromycin is considered to be one of the lesser toxic antibiotics and it is frequently employed in the place of penicillin. Macrolide antibiotics enter eukaryotic cells, whereas β-lactams (penicillin) are essentially confined to the extracellular fluid (Facinelli *et al.*, 2001). The dose of erythromycin permitted daily in an adult ranges between 250-500mg every 6 hours and up to 4g daily in severe infections (BNF, 1996).

Shortly after the introduction of erythromycin into therapy in 1950s, resistance was observed in bacterial pathogens. More worrying was the observation that erythromycin-resistant strains were cross-resistant not only to all other macrolides but also to the chemically unrelated lincosamine and streptogramin B drugs (Vester & Douthwaite, 2001). In addition, research suggests that resistance to erythromycin often emerges during treatment. This is frequently due to methylation of the 23S r-RNA target via a plasmid mediated methylase enzyme. In various bacteria including *Escherichia coli* among others, resistance to erythromycin depends on a mutation in the peptidyl transferase region of 23S rDNA at the position equivalent to A2059.

A low level of effectiveness of erythromycin against a large proportion of isolates has been reported in Australia, Finland, UK and Japan (Carsenti-Etesse *et al.*, 1999). The individual activity of erythromycin in combination with compounds known to modify bacterial resistance to given antibiotics was studied and results revealed that the combination of promethazine as well as the combination of methylene blue and erythromycin produced significant synergistic activity against *E. coli* (Gunics *et al.*, 2000).

1.10 Others

1.10.1. Fusidic acid

Fusidic acid (Figure 1.8) inhibits the 50S ribosomal subunit peptide chain elongation step of protein synthesis by interacting with a complex of elongation factor G (EF-G). Overall, it prevents the release of EF-G-GDP from the ribosome (Smith and Jarvis, 1999; Berger-Bächi, 2002). It is a sodium salt active against Gram-positive bacteria particularly staphylococci and to a lesser degree against streptococci. Gram-negative

bacteria are intrinsically resistant due to the outer membrane they possess (Hugo & Russell, 1999). Fusidic acid resistance during treatment could develop due to decreased affinity for an altered G factor due to chromosomal mutation of the protein synthesis apparatus (Smith and Jarvis, 1999).

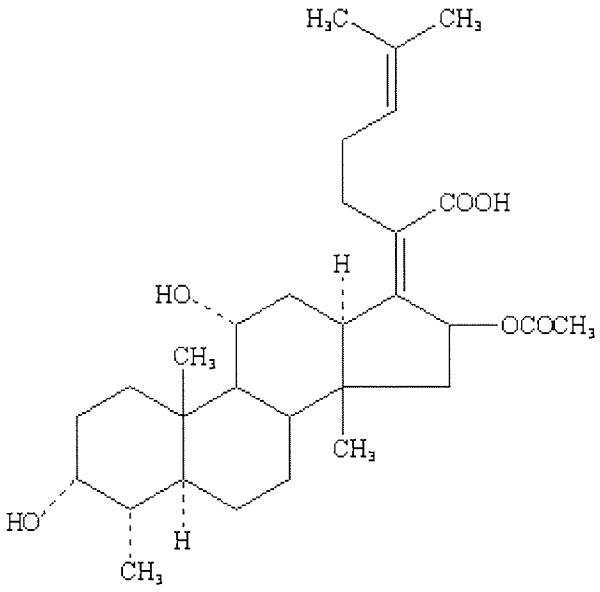


Figure 1.8: Chemical structure of fusidic acid, (adapted from Antimicrobial Chemotherapy,2004;URL:http://www.bmb.leeds.ac.uk/mbiology/ug/ugteach/icu8 /antibiotics/protein.html).

1.10.2 Colistin Sulfate

Colistin sulfate is a polypeptide antimicrobial derived from *Bacillus colistinusis* that has a bactericidal mechanism of action against most Gram-negative organisms. It is used effectively against multidrug-resistant *Pseudomonas aeruginosa* with slow development of resistance (Li *et al.*, 2003), *E. coli Klebsiella* and *Aerobacter*. Since colistin is similar in structure to polymyxin, its mechanism of action is probably similar; penetration into the cytoplasmic membrane where they disrupt membrane integrity, causing leakage of cytoplasmic components (Hugo and Russell, 1998).

1.10.3 Rifampicin

Rifampicin is an antibiotic that inhibits DNA-dependent RNA polymerase, leading to suppression of RNA synthesis. It is bactericidal and has a very broad spectrum of activity against most Gram-positive and Gram-negative organisms. Because of rapid emergence of resistant bacteria use is restricted to the treatment of mycobacterial infections. Resistance might be a result of chromosomal mutations which lead to an altered beta-subunit of DNA-dependent RNA polymerase. It has been used effectively *in vivo* in combination with aminoglycosides, ciprofloxacin, methicillin, ampicillin, penicillin or vancomycin (Smith and Jarvis, 1999).

1.11. Biocides

Numerous biocides exist and are used in both industry and domestic settings where they can be either bacteriostatic or bactericidal. Most biocides have a broad spectrum of activity and act at several target sites. The main groups of biocides include alcohols, aldehydes, anilides, biguanides, phenols, and quaternary ammonium compounds.

Biocides have been employed for centuries and resistance was first described during the 1950s and 1960s. Cationic agents such as quaternary ammonium compounds and chlorhexidine, among others, as well as triclosan have been implicated as the possible causes for the selection and persistence of bacterial strains with low-level antibiotic resistance (Russell, 2002a).

The overall mechanisms of action of biocides suggest that unlike antibiotics for which selective action against specific cell targets is fundamental, they act at one, or several

other sites within the cell wall, membrane or the cytoplasm and the overall damage to these sites results in the bactericidal effect (Beumer *et al.*, 2000; Maillard, 2002). According to Gilbert and McBain, (2003) the actions of biocides are rarely pharmacologically precise and do not usually permit their use as therapeutic agents, in contrast to antibiotics which are generally pharmacologically precise and exert their action at a single physiological target, often an enzyme (i.e. dihydrofolate reductase).

However, there are numerous factors that influence the effectiveness of a biocide. These include whether or not a surface has been sufficiently cleaned prior to disinfection. Many disinfectants are readily inactivated by organic matter and it is vital that proper cleaning precedes the application of the disinfectant. The length of contact time is also a consideration when applying a disinfectant. The 'in-use' concentration is also of major importance in disinfection. At the 'in-use' concentration multiple target sites are affected and death is rapid, however, at low concentrations below that recommended, a biocide may have a sub-inhibitory effect. At these concentrations it is possible that only one of the many target sites is being affected and therefore it is easier for bacteria to alter that particular target site and to become resistant. Promoting alterations in organisms which confer resistance by exposing them to sub-inhibitory levels of biocide may then confer cross-resistance to antibiotics and make treatment of such organisms very difficult. This fact is not commonly recognised by the food industry or in a domestic setting so it is feasible that not as much attention is being paid to ensure in-use concentration and correct contact times are being implemented.

1.11.1 Quaternary compounds and Benzalkonium Chloride (BKC)

Quaternary ammonium compounds (QACs) are among the most useful antiseptics and disinfectants belonging to the group of cationic detergents. They have been used for a variety of clinical purposes such as application to mucous membranes and disinfection of unbroken skin, among others. Quaternary ammonium compounds are also widely used in the food industry and are excellent for hand surface cleaning and deodorisation (McDonell & Russell, 1999; Aase *et al.*, 2000).

Quaternary ammonium compounds are active against cell membranes, with the target site mostly being the inner cytoplasmic membrane. The mode of action has been suggested as adsorption and penetration of the compound into the cell wall, reaction with the membrane (either a lipid or a protein) leading to membrane disorganisation, leakage of intracellular low molecular weight material, degradation of proteins and nucleic acids and cell wall auto-lysis resulting in loss of structural integrity. Quaternary Ammonium Compounds are also believed to interfere with the proton motive force of the cell leading to cell death. It has also been shown in Gram-negative bacteria that the QAC damage to the cell membrane promotes self entry of the biocide into the cell. Quaternary ammonium compounds such as BKC share similar mechanisms of action with chlorhexidine (CHX). These compounds are suggested to produce distortion of the cytoplasmic membrane by reacting with the phospholipids (Lambert, 2002).

Benzalkonium chloride (Figure 1.9) is the product of a nucleophilic substitution reaction of alkyldimethylamine with benzyl chloride (Pernak *et al.*, 1999). Resistance to QACs has, in some instances, been linked to antibiotic resistance. For instance, it

appears that resistance to BKC is readily acquired in some *Pseudomonas aeruginosa* strains following sub-lethal exposure and in mutants resistant to BKC there is cross-resistance with the membrane-active antibiotic polymyxin B (Loughlin *et al.*, 2002).

The most common mechanisms of resistance to disinfectants are impaired uptake or active transporters for pumping disinfectants from the cell. Research also suggests the importance of lipids in the increased resistance of Gram-negative bacteria to QACs (Joynson *et al.*, 2002). In addition, these investigations propose other characteristics that may accompany BKC resistance and cross-resistance which include alteration in outer membrane proteins, surface charge, cell surface hydrophobicity and fatty acid content in the cell surface membranes.

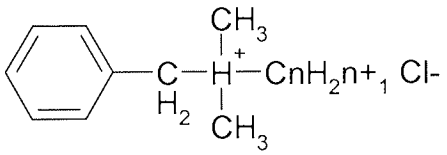


Figure 1.9: The chemical structure of the Benzalkonium chloride.

1.11.2 Biguanides and Chlorhexidine (CHX)

Chlorhexidine (Figure 1.10) has a wide spectrum of antibacterial activity however, due to its cationic nature, its activity is reduced in the presence of soaps and other anionic compounds. Any system containing anions such as phosphate and citrate among others will precipitate chlorhexidine. Chlorhexidine is commonly used in the healthcare environment as a surgical scrub and skin disinfectant as well as in the veterinary field (Fraise, 2002). It is also used to help treat periodontal disease caused by bacteria growing beneath the gum line.

Chlorhexidine is a cationic biguanide, which kills bacteria by membrane damage followed by intracellular coagulation (Fang *et al.*, 2002). Damage to the outer membrane does occur but it is thought to be insufficient to cause cell lysis. The agent passively diffuses into the cell and acts on the inner cytoplasmic membrane causing leakage of cell components. According to Lambert and co-workers (2001) CHX exhibits its mode of action on the cytoplasmic membrane promoting leakage of low molecular components and inhibition of certain membrane bound enzymes and is usually applied in a 0.5 to 4% solution for handwashing. In a clinical environment chlorhexidine concentrations 10- to 50-fold higher than the MICs are used to produce a 99.99% kill within 10 min at 20°C (Fang *et al.*, 2002). High concentrations of chlorhexidine cause coagulation of the intracellular constituents; the cytoplasm becomes congealed and leakage rates decrease. Therefore, the mode of action is biphasic with an initial fast intracellular leakage followed by a decrease in cell leakage as chlorhexidine concentrations inside the cell increase. It has also been suggested that chlorhexidine acts on ATPase, however only at high concentrations, suggesting that the enzyme is not the primary target. Although chlorhexidine collapses the membrane potential it is membrane disruption rather than ATPase inactivation that causes the lethal effects (McDonnell and Russell 1999).

Beumer *et al.* suggested that some strains exhibit resistance to chlorhexidine but these come from situations where there is extensive use of chlorhexidine (Beumer *et al.*, 2000). In general, Gram-negative bacteria are less susceptible to chlorhexidine than Gram-positive bacteria (Fang *et al.*, 2002). The exclusion barrier of Gram-negative bacteria and the permeable cell wall of Gram-positive bacteria, play a significant role in this (Lambert, 2002).

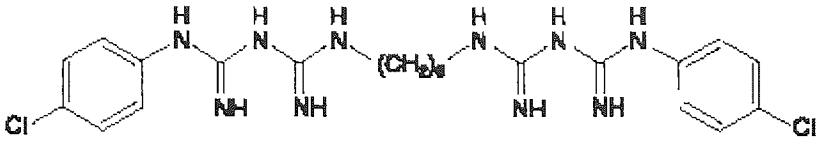


Figure 1.10: Chemical Structure of Chlorhexidine, adapted from: Russell *et al.*, 1982.

1.11.3 Bisphenols and Triclosan (TLN)

Triclosan (2,4,4'-trichloro-2'-hydroxydiphenylether) (Figure 1.11) (Schweizer, 2001) is a bisphenol belonging to a class of compounds that exhibit a broad spectrum of antimicrobial activity. Although according to Gilbert and McBain (2002) triclosan is generally regarded to be considerably more effective against Gram-positive bacteria and it is ineffective against *Pseudomonadaceae.*

Since its introduction in the 1960s, triclosan has become the most potent and widely used member of the 2-hydroxyphenylethers and is used in hand soaps, lotions, toothpastes, and oral rinses as well as in fabrics and plastics (Bhargava and Leonard, 1996; McMurry *et al.*, 1998a; Larkin, 1999; Chuanchuen *et al.*, 2001; Gilbert and McBain, 2002). A recent survey of liquid soaps in the United States revealed that 45% contained antibacterial agents the majority of which included triclosan (Gilbert and McBain, 2002). In addition, it is estimated that between 1992 and 1999 over 700 consumer products with antibacterial properties, the vast majority of them containing triclosan, entered the consumer market (Schweizer, 2001).

Triclosan is a synthetic bisphenol antibacterial agent widely employed for antisepsis and in anti-plaque agents. Its efficacy against Gram-negative bacteria can be

significantly enhanced by formulation effects such as ethylene diamine tetra-acetic acid (EDTA). Research has revealed that in addition to its antibacterial activity, triclosan may also have anti-inflammatory activity (McDonnell & Russell, 1999; Braid & Wale, 2002).

Because triclosan is regarded as a biocide and not as an antibiotic it is thought to have various target sites. The antimicrobial mode of action has not yet been fully determined but one suggestion is that it acts primarily on the cytoplasmic membrane; triclosan is considered to be a non-specific biocide that attacks bacterial membranes (Heath *et al*, 1999). A study with triclosan on *E. coli* demonstrated that at sub-inhibitory concentrations triclosan inhibited the uptake of essential nutrients, at higher concentration release of cellular components was seen, followed by cell death (McDonnell and Russell, 1999). Other research also suggests that triclosan acts on a defined bacterial target in the fatty acid biosynthetic pathway, enoyl acyl carrier protein (ACP) reductase (*fabI*). According to Walsh *et al.* (2003) this could lead to cross-resistance between antibiotics and biocides due to potential similarities in the mode of action. It is also suggested that in general, those drugs that are at present thought to be non-specific biocides, might actually have specific targets (McMurry *et al.*, 1998b; Chuanchuen *et al.*, 2001). On the other hand, McDonnell and Pretzer (1998) proposed that triclosan affects other components as well. If triclosan does act as a specific inhibitor then it is likely that strains with higher levels of resistance will emerge (Suller & Russell, 2000).

Triclosan

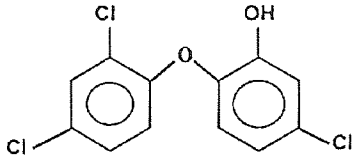


Figure 1.11: Chemical Structure of Triclosan, adapted from: McDonnell & Russell, 1999.

The triclosan concentration found in soap is typically 2500μg/ml and to achieve 90% death rate of *E. coli* the required exposure to 150μg/ml of TLN in soap is for 2 hours at 37°C. In the case of triclosan especially current reports strongly suggest that inappropriate administration could select for a more generalised resistance. This has been demonstrated in a variety of different strains including *Pseudomonas aeruginosa* (Chuanchuen *et al.*, 2001), *Escherichia coli* (McMurry *et al.*, 1998b; Levy 2002a) and *Salmonella enterica* among others. In 1998, McMurry *et al.* (1998a) suggested that triclosan acted on a specific bacterial target rather than as a non-specific biocide, which could facilitate the acquisition of bacterial resistance.

1.12 Resistance & Multidrug Resistance

The acquired ability of a microorganism to grow in the presence of an antibacterial to which the microorganism is usually sensitive is defined as resistance (Madigan *et al.*, 1997). Bacterial resistance to antibiotics has been well-documented for a number of years and poses a threat to human health as currently available antibiotics are rapidly becoming ineffective against common pathogens. Resistance patterns to date have

been blamed on the inappropriate overuse of antibiotics by the healthcare environment, though the use of antibiotics in veterinary medicine and in agricultural feedstuffs has also had a contributing factor (Beumer *et al.*, 2000).

Some organisms are resistant to certain antimicrobials due to their innate metabolic characteristics - intrinsic resistance, whereas others may develop mechanisms to protect themselves – constitutive (Smith and Jarvis 1999). The Minimum Inhibitory Concentrations (MICs) are considered the reference for the measurement of antibacterial resistance; these will be discussed in more detail in chapter 2.

Resistance to multiple antibiotics has been observed in various bacteria such as *Salmonella, Shigella, Klebsiella, Citrobacter, Hafnia, Enterobacter* and *Escherichia coli* (Randall & Woodward, 2001). In fact, a very important feature that characterises *Salm.* Enteritidis, *Salm.* Typhimurium and *Salm.* Virchow has been the possession of plasmid-mediated multiple drug resistance, often with resistance to seven or more antimicrobials (Threlfall, 2002). The intrinsic mechanisms of resistance by those genes that comprise part of the normal genomes of cells are today known as multidrug resistance (Mdr) (George, 1996). Of note in 1994 there was a significant increase in both resistance and multiple drug resistance in the poultry-associated serotype *Salm.* Virchow. The problem of multidrug resistance became common in *Salm.* Typhimurium in the mid-1960s and increased dramatically in the years to come, in contrast to *Salm.* Enteritidis, in which multiple drug resistance is rare (Threlfall, 2002).

1.12.1. Intrinsic Resistance

Bacteria have different intrinsic susceptibilities to varying biocides and antibiotics, which is due, in part, to their varied structure and physiology. The inherent resistance of a cell can change depending on the surrounding environment, however intrinsic properties of cells are mostly attributed to its outer cell layers. In order for a biocide to reach its target and influence its effect it must first traverse the outer layers of the cell. Microbes can acquire resistance by continued exposure to a biocidal agent (Murtough *et al.,* 2001). An organism may be also inherently resistant to a biocide if the permeability of the outer layers is such that the uptake of the biocide is reduced. Innate resistance is due to chromosomal properties that prevent the biocide from having an effect. Due to the structure of the outer cell layers, Gram-negative organisms are more inherently resistant to biocides than Gram-positive organisms. It has been shown that there is a marked difference between the MICs of BKC and TLN of *E. coli* and *S. aureus* however little difference exists between MICs to chlorhexidine (McDonnell and Russell, 1999). The outer membrane of Gram-negative bacteria acts as a barrier and limits the diffusion of many types of biocides. The hydrophobicity of the biocide can play an important role as to whether it can gain entry into the cell. Low molecular weight hydrophilic molecules can easily pass into the cell via porins in the cell wall. Hydrophobic molecules diffuse across the outer membrane. In wild type Gram-negative bacteria, intact LPS prevent the access of hydrophobic molecules to the phospholipid layer and thus prevent entry across the lipid bilayer. Some cationic agents such as QACs can damage the outer cell membrane thus promoting their own self-uptake (Mc Donnell and Russell, 1999). Alternatively, organisms may secrete enzymes which breakdown the biocide and thus inactivate it.

1.12.2 Acquired Resistance

Acquired bacterial resistance mechanisms are mostly caused by either a mutation or the acquisition of new genetic material in the form of plasmids or transposons. The acquisition of new genetic material or mutations leads to changes in the cell that could confer resistance to antimicrobial agents. These changes fall into three board categories: a change in antimicrobial agent target or receptor, a modification or destruction of the antimicrobial agent and finally the removal of the antimicrobial agent from the cell by the use of an efflux pump (Nelson, 2002).

When populations of strains are in an environment in the presence of an antimicrobial agent, any advantageous mutation that occurs will lead to that strain having a competitive advantage. Mutational changes are well documented as means of antibiotic resistance, for example chromosomal mutations may cause changes in metabolic pathways such that the antimicrobial-sensitive step is bypassed, or changes in the expression of the normal target site of the antibiotic may occur. The overproduction of the target enzyme or an efflux pump are also mechanisms by which resistance is acquired due to mutations (Beumer *et al.*, 2000).

Plasmid mediated antibiotic resistance has been demonstrated in a wide variety of strains (Smalla and Sobecky, 2002; Sorum and L'Abee-Lund, 2002; Sherley *et al.*, 2003; Tauch *et al.*, 2003). The acquisition of a plasmid however is a more complex process than the occurrence of a mutation. Discussion as to whether plasmids can encode resistance to biocides has been rife. It was thought that plasmids do not normally encode resistance to biocides (apart from some specific cases including some heavy metals). However, many species resistant to QAC and triclosan have been

found to harbour plasmids (McDonnell and Russell, 1999). Also, there is evidence which suggests that the R124 plasmid in *E. coli* alters the outer membrane protein F (ompF) and subsequently providing resistance to QAC and other agents. Reports have demonstrated that formaldehyde and industrial biocide resistance may occur due to this plasmid. In this latter case a change in cell surface and outer membrane proteins are considered responsible (McDonnell and Russell, 1999).

1.13 Acquisition of Resistance

Disinfectants are designed to be used at "in-use" concentrations, determined as the optimal concentration at which the bactericidal activity is effective. It is possible that inappropriate dilution results in biocide being at sub-inhibitory concentrations. At these levels microorganisms are not targeted effectively and may develop resistance.

If bacteria are continuously exposed to low concentrations of antibiotics or biocides the ones least easily killed survive and reproduce; the population thus becomes dominated by bacteria that are less susceptible to this agent. Once resistance is established it cannot be reversed, however proper use of drugs minimises the flourishing of resistant strains. Although the problem of bacterial resistance is omnipresent in infectious diseases, the scale of the problem varies depending on the antimicrobial, the pathogen and the setting in which transmission occurs (Lipsitch, 2001).

1.14 Stability of antimicrobial resistance

Bacterial resistance is described as the ability of an organism to grow and adapt at high concentrations of antibiotics or biocides. The mechanisms underlying this

phenomenon are several and varied. In order to examine whether or not resistance is due to the continued presence of a selective pressure, it is important to investigate stability of resistance. This can be assessed by passaging bacterial cells in antibiotic/biocide - free media. If the cells that were grown in non-selective broth remain resistant to the same concentrations to which they were previously adapted, then this suggests that another mechanism is responsible for the development of that resistance and not the mere presence of a selective pressure. In addition, if cells develop mechanisms to protect themselves, which places a heavy burden on their biological resources, this could place them at a disadvantage if the organism was in an environment, where its expression was unnecessary. It is also noteworthy that stability in bacterial resistance has a high impact in therapy as reduces the ability to treat and cure certain infections. Preserving the effectiveness of existing therapies is an increasingly urgent consideration.

1.15 Cross-Resistance

Traditionally, biocides have been regarded as distinct from antibiotics due to their lower pharmacological specificity (McBain *et al.*, 2002). However, more recent evidence suggests that the inappropriate use of biocides promotes resistance emergence (Levy, 2002c), and there is concern that this resistance will confer cross-resistance to antibiotics (McDonnell and Russell, 1999). According to Gilbert and McBain (2001) and McBain *et al.* (2002) alterations in biocide susceptibility may be reflected in antibiotic resistance, especially where antibiotic targets are shared with the biocide. If this is true it will prove a major problem as biocides are used widely both in the food industry and in a domestic setting. New trends have seen an increase

in the use of disinfectants in the home and are possibly responsible for the development of further resistance (McDonnell and Russell, 1999).

Exposure to a single drug leads to cross-resistance to many other structurally and functionally unrelated drugs (Poole, 1994). At present, there are comparatively few reports of cross-resistance between biocides and clinically employed antibiotics. For instance Russell (2002a) suggested that following the introduction of triclosan it is possible to select ciprofloxacin resistance in *Pseudomonas aeruginosa.* Microorganisms are infinitely adaptable and have already demonstrated mechanisms of resistance to these biocides. This raises concerns being that these mechanisms may confer cross-resistance to clinically important antibiotics. In this study we investigate the potential link between resistance to biocides and cross-resistance to other antibacterial agents in *Salmonella enterica* serovar Enteritidis, Typhimurium and Virchow and in *Escherichia coli* O157. Given the additional genetic material known to be harboured by *E. coli* O157 compared to ancestral strains (Eisen, 2001), it was also interesting to investigate whether the rapid development of triclosan-resistance observed previously (chapter 2) was *E. coli* O157 specific or a property expressed by other *E. coli* strains. Data is presented on cross-resistance of triclosan-resistant *E. coli* K-12 and the *E. coli* O157 progenitor strain, *E. coli* O55, to a range of antimicrobial agents.

1.16 Means and Mechanisms of Resistance

The precise mechanisms of bacterial resistance to antimicrobial agents remain largely unclear. The mechanisms of antibiotic resistance have been widely studied and elucidated, however biocide resistance has thus far been poorly evaluated in

comparison. The resistance candidates are multiple and diverse, but in general can be collectively characterised as involving efflux, drug inactivation, or alterations in the target site (Vester & Douthwaite. 2001). The latter leaves the antibiotic intact to continue the selection process (Levy 2001; Levy 2002a).

According to Denyer and Stewart (1998) bacterial cells offer the cell wall, the cytoplasmic membrane and the cytoplasm for biocide interaction (Figure 1.12). However, specific mechanisms of resistance include enzymatic inactivation of antibiotics, alterations of the target sites for antimicrobial agents, development of bypass pathways around antimicrobial targets and reductions in the cell wall membrane permeability (Wang *et al.*, 2003).

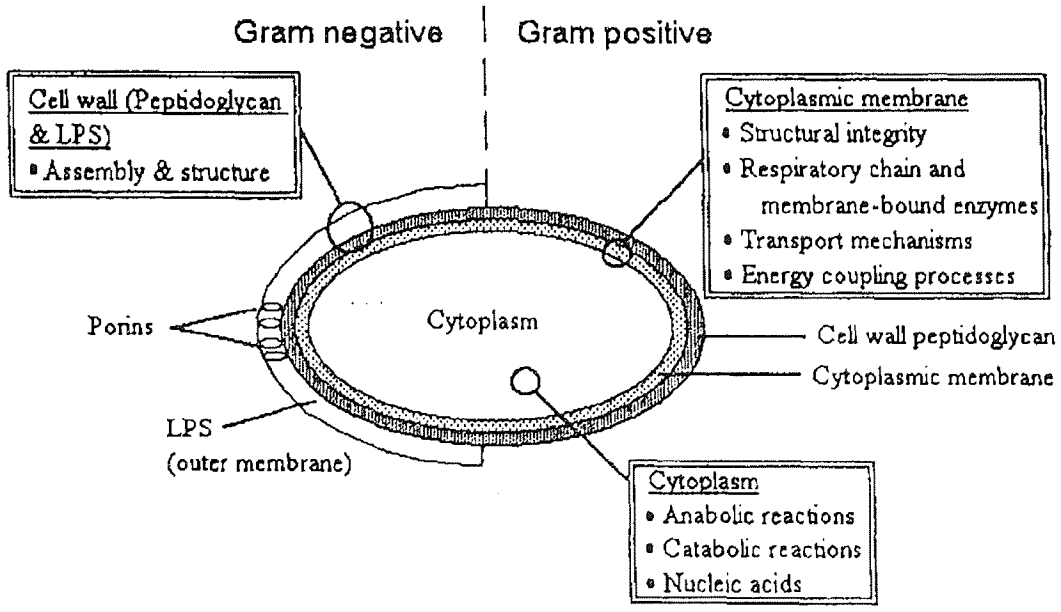


Figure 1.12: Potential targets for biocidal action. Reproduced from Denyer and Stewart, 1998.

1.17 Cell Surface Hydrophobicity

Cell surface hydrophobicity (CSH) is ubiquitously the most studied property in microbial adhesion surfaces and is a vital factor in cellular adhesion mechanisms (van der Mei *et al.,* 1998). Thus, changes in CSH may not only be associated with resistance to antimicrobial agents but also with pathogen virulence. Alterations in cell surface hydrophobicity have been linked to changes in transmembrane penetration of some antimicrobial agents and therefore the CSH may play an important role in resistance (Kobayashi *et al.,* 1991). Increases in CSH have also been shown to correlate with the presence of an additional surface protein (Parker and Munn, 1984), which is of particular interest when studying its potential role in resistance. The determination of CSH measurement depends very much on the method employed and the cellular environment.

The outer surface of microbial cells contain a wide range of chemical compounds which may play a significant role in the adhesion of cells to surfaces. Hydrophophobic / hydrophilic interactions are involved in attachment leading to the development of the concept of cell surface hydrophobicity (CSH) as a measure of the tendency of the cell to attach to a surface. The surfaces of microbial cells are vital to the organisms' survival, since it is via them that bacteria interact with the environment (Pembrey *et al.*, 1999).

1.18 Cell Surface charge

The cell surface charge influences the entire cell polarity and is therefore vital in maintaining the optimal level of cell surface hydrophobicity necessary for cell function (Wilson *et al.,* 2001). Consequently, cell surface charge is of importance in

cellular adhesion and changes may affect virulence factors. All bacterial cells possess a net negative charge, which can be measured by microelectrophoresis in the form of zeta (ζ) potentials. Theoretically, it is the ζ potential that is important in determining the magnitude of the electrostatic repulsive force. The ζ potential is the electrostatic potential at the surface of shear and not at the surface of the particle (Figure 1.13). Zeta potentials are estimated by measuring cellular electrophoretic mobility in an electric field at a fixed temperature, pH and ionic strength of media (Wilson *et al.*, 2001).

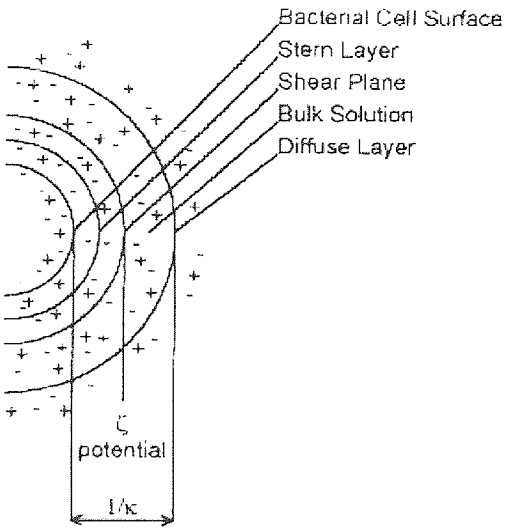


Figure 1.13: The different solvent layers surrounding the bacterial cell, adapted from Wilson *et al.*, 2001.

1.19 Permeability Alterations

1.19.1 Outer membrane proteins (OMPs)

It has been suggested previously that changes in the outer membrane are involved in the elevated intrinsic resistance of Gram-negative bacteria to antimicrobial drugs (Hancock, 1987). The outer membrane is considered to constitute a selective

permeability barrier to the cell exterior; in Gram-negative bacteria the outer membrane prevents toxic compounds from entering the cells. In this regard, the presence of lipopolysaccharide in its outer monolayer is the most significant component (Inouye, 1979; Sedwick, 1996).

The outer membrane is composed of protein, phospholipids and lipopolysaccharides. The outer membrane of *E. coli* K-12 contains matrix proteins (porins), OmpA protein and lipoprotein. The matrix proteins Ia and Ib were found to be present in *E. coli* K-12 and they appear to be coded for by independent genes. Gene expression of both the *ompf* gene (for Ia) and the *ompC* gene (for Ib) is controlled by *ompB* gene. The OmpA protein is known to show an anomalous mobility on SDS gels. Its molecular weight is approximately 30,000. The lipoprotein on the other hand plays an important role in maintaining the integrity of the outer membrane structure and its molecular weight is approximately 7000 (Inouye, 1979).

1.19.2 Lipopolysaccharides

Lipopolysaccharides are the main components of the outer membrane of Gram-negative bacteria. They are essential for the integrity of the outer membrane and for cell viability, making it potential target for the development of therapeutics (Frirdich *et al.*, 2003). Lipopolysaccharides are responsible chiefly for the cell's impermeability characteristics and as a result any alteration in LPS might affect the impermeability barrier (Denyer & Maillard, 2002). Lipopolysaccharides (Figure 1.14) consist of three regions: (i) lipid A, the hydrophobic membrane anchor; (ii) a short core oligosaccharide (core OS); and (iii) a polymer of glycosyl (repeat) units known as O polysaccharide (O-PS), which is divided in the inner and outer core regions, on the

basis of sugar composition. The inner is composed by 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) and L-glycero-D-manno-heptopyranose (Hep), whereas the outer region contains hexose and acetamidohexose sugars (Holst, 1999; Frirdich *et al.*, 2003).

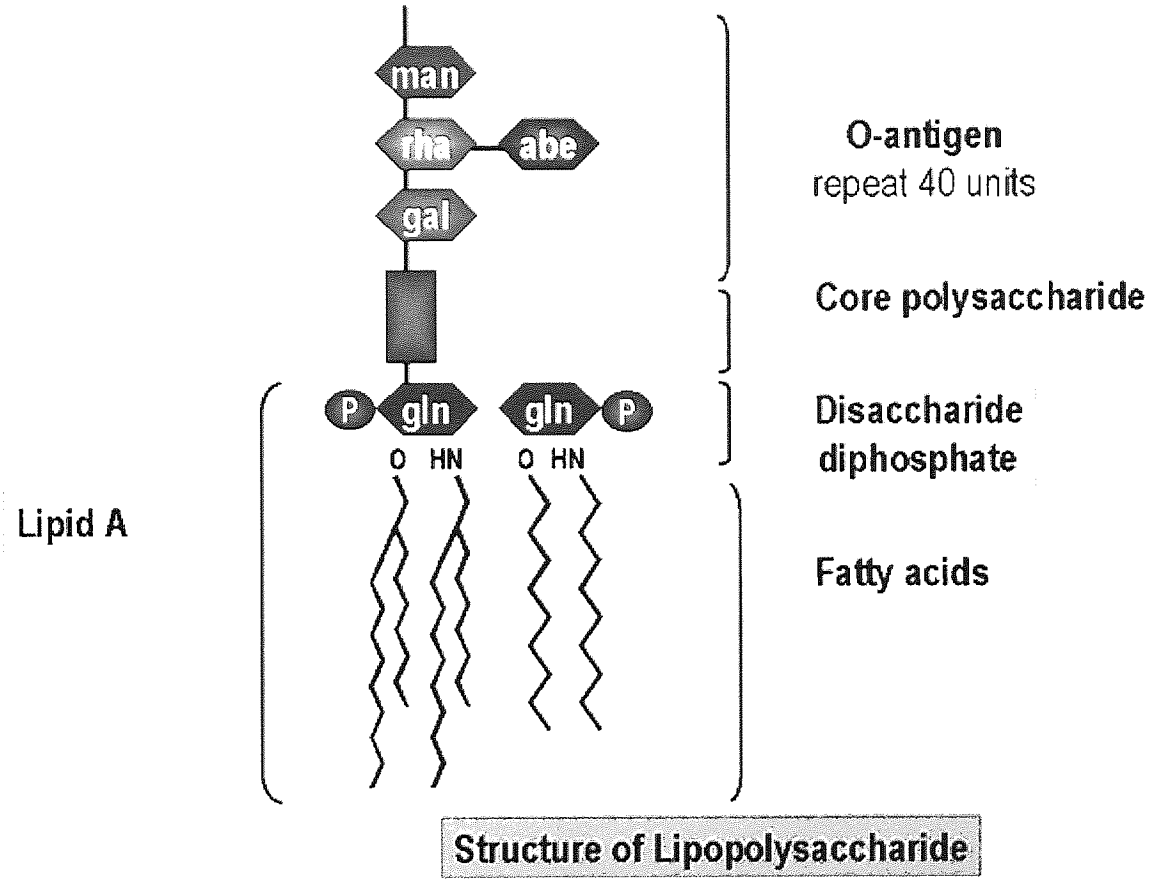


Figure 1.14: The structure of LPS in Gram-negative bacteria, adapted from URL: www.med.sc.rdu:85/fox/cell_envelope.htm (2004).

1.20 Efflux Pumps and Groups of Efflux

Efflux pumps are a well-recognised mechanism of resistance which can be expressed inherently providing intrinsic resistance to the organism. Sometimes resistance occurs due to the increased expression or up-regulation of an existing efflux pump; for example, it has been shown that *E. coli* can become resistant to a number of biocides

due to the over expression of the AcrAB multidrug efflux pump (Nikaido, 1996). Alternatively, genes encoding efflux pumps can be acquired by organisms via the acquisition of new genetic material possibly due to the transfer of plasmids (Jones and Midgley, 1985).

Efflux pumps described to date have been grouped into families. The major facilitator family (MFS), the resistance-nodulation division family (RND) and the small multi drug resistance protein family (SMR) all use proton motive force, pH gradient and electrochemical formation to efflux antibiotic compounds in exchange for protons. The ATP- binding cassette family (ABC) of efflux pumps remove a broad array of chemicals from both prokaryotic and eukaryotic cells and derive their energy for drug transportation from the hydrolysis of ATP. Two families more recently described are the multi drug and toxic compound extrusion family (MATE) and the putative efflux transporter family (PET), but little is known at present about their mechanisms of action (Nelson, 2002).

The intrinsic resistance of Gram-negative bacteria has commonly been attributed to a thick outer membrane decreasing the permeability of the cell to antimicrobial agents. However, it has been suggested that this membrane permeability is not the only factor in the resistance as equilibrium across the membrane is often reached due to the large surface to volume ratio. Therefore, additional mechanisms in the form of efflux pumps may play an important role (Nikaido, 1996).

Gram-negative efflux has been shown to have a different requirement to that of Gram-positive efflux, due to the double structure of the Gram-negative cell wall (Figure 1.15). The tetracycline efflux system, seen in both Gram-negative and Gram-positive organisms pumps molecules across a single cytoplasmic membrane layer. In Gram-negative organisms this means it will pump molecules into the periplasmic space where diffusion back into the cell would be rapid. It is for this reason that efflux pumps of this kind must a have a high throughput and thus are more likely to have a narrower specificity. Gram-negative multidrug efflux pumps that work across both membranes include the AcrAB system of *E. coli* and the MexAB-OprM system of *Pseudomonas aeruginosa* (Nehme *et al.*, 2004). Both of these systems are part of the RND family. Efflux systems of this type are thought to be associated with outer membrane channels and linker proteins causing the fusion of the inner and outer membrane and bypassing the periplasmic space. Often efflux pumps work synergistically with other intrinsic mechanisms of resistance. The AcrAB pumps in *E. coli* are only effective against large lipophilic molecules such as erythromycin that have difficulty penetrating the porin channels. Smaller molecules diffuse rapidly through these channels and therefore the pumps have more difficulty in providing resistance. In the latter cases the membrane permeability provides and an important synergistic mechanism of resistance (Nikaido, 1996).

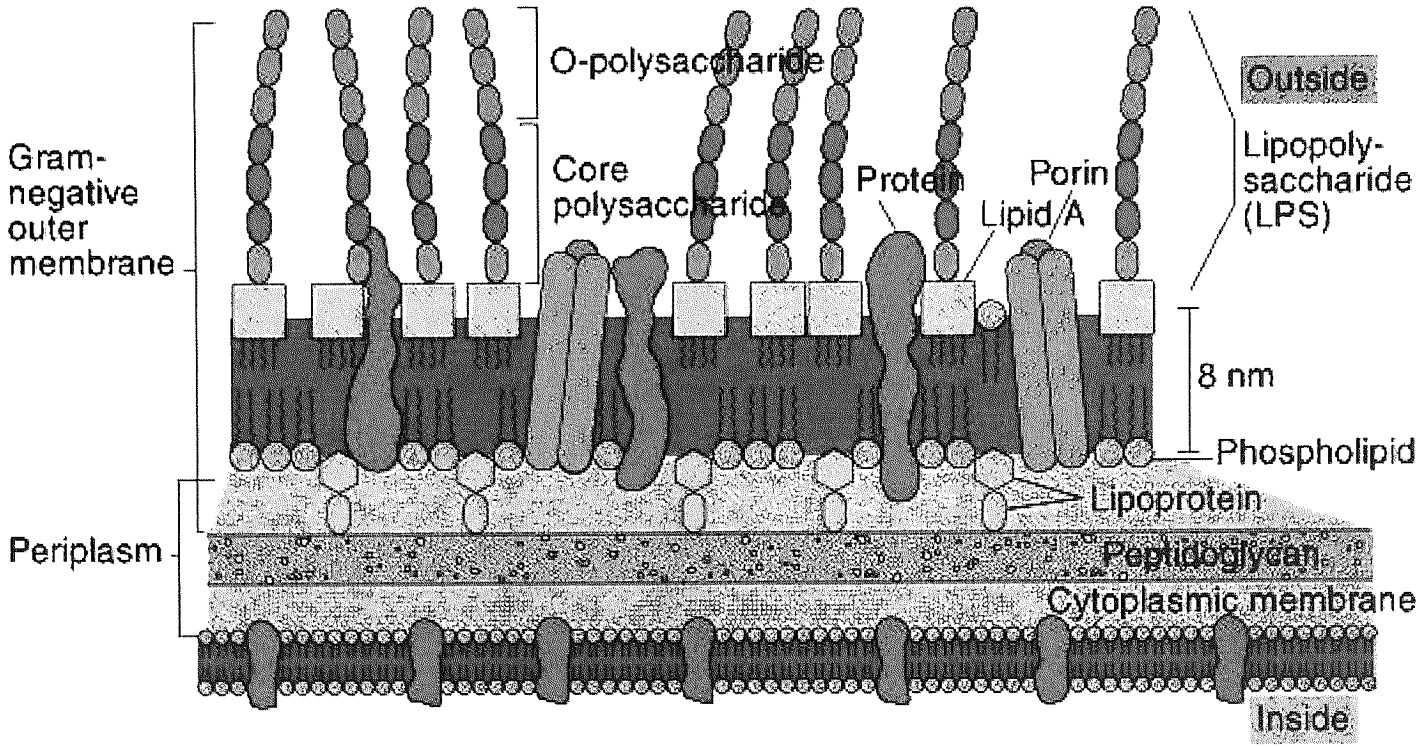


Figure 1.15: Structure of the Gram-negative cell wall including outer and cytoplasmic membrane. Adapted from URL: www.arches.uga.edu/~emilyd/theory.html (2004).

The substrates of the Gram-negative efflux systems are described below. The MFS family has a limited number of substrates; they pump organic cations such as QACs, cationic dyes and fluoroquinolones. The RND transporter family, including the AcrAB and the MexAB-ompM efflux pumps, have a much wider range of specificity and pumps almost all lipophilic and amphiphilic antibiotics, chemotherapeutic agents, metabolic inhibitors, dyes and detergents. The substrates can carry a positive charge, a negative charge or no charge (Nikaido 1998a).

1.21 Fatty acids

Bacteria regulate membrane fluidity by manipulating the relative levels of saturated and unsaturated fatty acids within the phospholipids of their membrane bilayers (Campbell and Cronan, 2001; Heath *et al.*, 2001). Fatty acid synthesis in *E. coli* has been widely studied (Monson and Hayes, 1980) and there has been a remarkable progress in the understanding of the genetics and biochemistry (Marrakchi *et al.*, 2002b). Bacterial fatty acid biosynthesis is carried out by a collection of enzymes each encoded by a separate gene (Heath *et al.*, 1999). There are eight genes (*fab*) involved in fatty acid synthesis in *E. coli* including *fabA, fabB, fabD, fabF, fabG, fabH, fabI* and *fabZ* (Cambell and Cronan, 2001; Heath *et al.*, 2001) The fatty acid biosynthetic pathway in *E. coli* is shown in Figure 1.6.

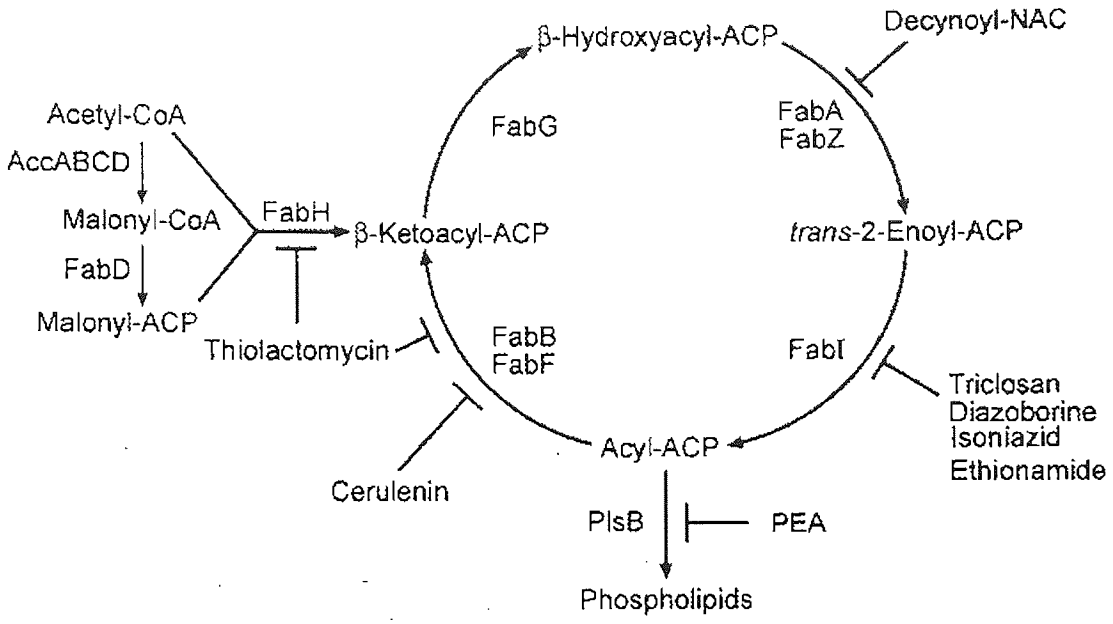


Figure 1.16: Fatty acid biosynthetic pathway. Known inhibitors of the steps are also shown. Adapted from Heath *et al.*, 2001.

It has been suggested that in *E. coli* fatty acid synthesis is tightly coupled to phospholipid synthesis and therefore it could be feasible that fatty acid synthesis is co-ordinately regulated with or by phospholipids synthesis (Jiang and Cronan, 1994). In *E. coli* amino acid deprivation results in the inhibition of a number of metabolic activities including lipid synthesis among others (Podkovyrov and Larson, 1996).

1.22 Lipids

The diversity of lipids signifies a diversity of function; for instance phospholipids play a major structural role in the cytoplasmic and outer membranes. They are amphipatic which makes them ideal permeability barriers for the hydrophilic compounds unable to flow through the hydrophobic fatty acid portion of lipids. In *E.*

coli phospholipids contain mainly saturated fatty acids almost exclusively the 16:0 (Ratledge and Wilkinson, 1988).

Extractable lipids typically contribute 6-9% of the cellular dry weight of *E. coli* with phospholipids being the predominant. Most phospholipids observed include phosphatidylethanolamine (PE), 75-85%; phosphatidylglycerol (PG), 10-20% and diphosphatidylglycerol (DPG), 5-15% (Ratledge and Wilkinson, 1988). Phosphatidylglycerol is believed to play the central role both in metabolism and function of phospholipids in *E. coli* (Shibuya, 1992). The phospholipids of other Enterobacteria and more distantly related Gram-negative organisms are quite similar to those of *E. coli* (Inouye, 1979).

1.23 Aims & Objectives

Biocides are important chemical substances deployed widely in soaps, toothpastes and cleaning products among others. However, the massive increase in biocide use together with the recent findings concerning the mechanism of action of triclosan have led to questioning the suitability of biocides for indiscriminate use in domestic products. This issue is in relation with the current alarming increase in bacterial resistance to them. The mechanisms underlying this reduced susceptibility are so far poorly evaluated and thus this study was undertaken to investigate the possible means and mechanisms underlying resistance in *Salmonella enterica* and *Escherichia coli* O157, for the control of which the efficacy of biocides is vital, and possible cross-resistance to other antimicrobial agents. The contents of each chapter is summarised below.

Chapter 1. The first chapter reviews our current understanding of the microbiology of *Salmonella enterica* serovars Enteritidis, Typhimurium and Virchow and *Escherichia coli*. In addition, the literature concerning the mode of action and the associated resistant mechanisms of antibiotics and biocides is considered.

Chapter 2: The second chapter examines the selection of resistant *Salmonella enterica* and *E. coli* mutant strains through repeated exposure to increasing sub-lethal concentrations of erythromycin, benzalkonium chloride, chlorhexidine and triclosan, as well as the stability of the resistance acquired by those strains. Molecular typing of all bacterial isolates by Random amplification of polymorphic DNA (RAPD) ensured strain continuity throughout passages.

Chapter 3 Cross-resistance of *Salmonella enterica* and *E. coli* strains to other antimicrobials was investigated by a standard broth microdilution assay and by the Stokes' disk susceptibility method. In addition, the rapid development of cross-resistance to triclosan and enhanced cross-resistance to antibiotics in *E. coli* O157 was investigated further and compared with *E. coli* O55 and K-12.

Chapter 4: Candidate resistance mechanisms in *Salmonella enterica* and *E. coli* strains resistant to erythromycin, benzalkonium chloride, chlorhexidine and triclosan were examined. These included possible alterations in the outer membrane including lipopolysaccharides, changes in the cell surface charge and hydrophobicity and the activity of a putative efflux pump system.

Chapter 5: The molecular biology of *fabI* in *E. coli* O157 mutants resistant to triclosan was examined as a possible mechanism of resistance. Alterations in the production of lipids and fatty acids associated with mutations in *fabI* were investigated by thin layer chromatography and gas chromatography. The effect of mutations in fabI on the mode of action of triclosan is illustrated by the use of molecular modelling.

Chapter 6: A final discussion and conclusion from the data generated throughout these studies.

Chapter 2. Selection of resistant strains

2.1 Introduction

The aim of this study was to select for resistant *Salmonella enterica* and *Escherichia coli* strains to erythromycin, benzalkonium chloride, chlorhexidine and triclosan.

2.1.1 Acquisition of antimicrobial resistance and stability

Salmonella enterica and *E. coli* strains were exposed at repeated increasing sub-lethal concentrations of erythromycin, benzalkonium chloride, chlorhexidine and triclosan, in order to promote resistance. Once reduced susceptibility was obtained the strains were passaged to antimicrobial-free media, in order to investigate whether or not they required the presence of a selective pressure to retain their resistance or if they had actually developed mechanisms to protect themselves.

2.1.2 Random Amplification of Polymorphic DNA (RAPD)

In order to confirm strain continuity, RAPD was employed as a molecular tool. The RAPD was first utilised in 1990 by Williams and his colleagues (Hilton *et al.*, 1996). This is a modification of the polymerase chain reaction (PCR) in which one or more primers able to anneal and prime throughout the genome can produce various amplified products characteristic of the template DNA (Hilton *et al.*, 1996). On the other hand, the RAPD method employs short primers of arbitrary sequences to amplify random portions of the sample DNA by PCR (Franklin *et al.*, 1999). According to Hopkins and Hilton, (2001), RAPD has proved to be a useful and maybe favourable technique compared with other typing methods. For instance, it is considered more efficient and discriminatory than ribotyping and quicker and less technically demanding than pulsed field gel electrophoresis (PFGE).

2.1.3 Minimum Inhibitory concentrations

The MICs are considered the 'gold standard' for the determination of the bacterial susceptibility to antimicrobials and are therefore used to judge the performance of all other methods of susceptibility testing (Andrews, 2001). The dilution tests are considered to be the reference methods to determine the MICs; microorganisms are tested for their ability to produce visible growth on a series of agar plates (agar dilution) or in broth (broth microdilution) containing dilutions of an antimicrobial (EUCAST Definitive Document, 2000). The MIC is defined as the lowest concentration of a drug that inhibits the visible growth of an organism following overnight incubation (this is extended for organisms such as anaerobes, which require prolonged incubation for growth). The activity of antimicrobial agents is also quantified as a concentration that leaves no detectable survivors following a specified contact time and is defined as the Minimum Bactericidal Concentration (MBC), generally taken as 99.9% killing (Gilbert and McBain, 2003).

2.1.4 Growth curves

In order to highlight differences in genetic material the use of growth curves can be employed. Growth curves are a way in which the rate of growth of an organism can be measured. They are most commonly employed to highlight differences in growth rates of organisms under different environmental conditions (Nordmann *et al.*, 1994, Duffey *et al.*, 1999; Filali *et al.*, 2000; Viswanathan and Kaur, 2001). However, if the environmental conditions are kept constant and an organism grows more slowly than another, it can be suggested that the slower growing organism possibly has acquired an alteration. The alteration may be the acquisition of extra genetic material and therefore it takes longer to replicate. However, more frequently differences in growth

rate could be attributed to mutations causing differences in structure. Resistance to biocides due to a decreased permeability has been shown to accompany reduced growth rates (Gilleland *et al.,* 1989 and Joynson *et al.,* 2002). This slower growth rate may be attributed to a thickening of the outer cell membrane structure (Gilleland and Murray, 1976). Changes in growth rate may not only highlight structural changes but may be proved to show differences in the regulation of proteins. For example it may be possible that a slow growth rate reflects the expression of outer membrane proteins such as those used in efflux.

2.2 Methods & Materials

2.2.1 Bacterial strains and culture conditions

Salmonella enterica ser. *Enteritidis* was a clinical isolate from Birmingham Heartlands Hospital, UK; *Salm.* Virchow was a food isolate from Campden and Chorleywood Food Research Association, UK and *Salm.* Typhimurium was a reference isolate obtained from the National Collection of Type Cultures (NCTC 74). *Escherichia coli* O157 was a VT-negative strain obtained from the National Collection of Type Cultures (NCTC 12900); *Escherichia coli* O55 was a clinical isolate from Birmingham Heartlands Hospital, Birmingham, UK and *E. coli* K-12 was obtained from the American Type Culture Collection (ATCC 27325). All strains were stored on Microbank beads (Pro-lab Diagnostics, Neston, UK) at -70°C and cultured at 37°C on nutrient agar (Oxoid, Basingstoke, UK) and in nutrient broth (Lab M, Lancashire, UK) where appropriate.

2.2.2 Preparation of Antimicrobial agents

Erythromycin (ERY) was purchased from Sigma, Poole, UK. Biocides benzalkonium chloride (BKC) (Fluka, Buckinghamshire, UK) and chlorhexidine hydrochloride (CHX) (Sigma, Poole, UK) were supplied as laboratory standard powders of known potency and triclosan (TLN) (Aquasept, Oldham, UK) was purchased as a laboratory standard solution. The antimicrobial agents are detailed in Table 2.1. Solutions were filter sterilised using a 0.2μm cellulose syringe filter (Nalgene, Leicester, UK).

Table 2.1: Preparation & Storage of Antimicrobial Agents

Antimicrobial Agents	Stock Concentration (µg/ml)	Preparation	Storage
Erythromycin	4096	10% IMS	Fresh
BKC	4096	*sdH_2O	4°C
Chlorhexidine	4096	sdH_2O	4°C
Triclosan	4096	sdH_2O	4°C

*sd: sterile distilled.

2.2.3 Bacterial Identification – API Tests 20E

The API 20E (BioMerieux, Marcy l' Etoile, France) was performed on parent and resistant strains as per manufacturer's instructions to confirm strain continuity

2.2.4 DNA isolation

DNA was extracted using a standard boiling method. All bacterial isolates were grown at 37°C in 10ml of Nutrient broth for 18-24h. A 1ml volume of culture was transferred to an Eppendorf tube and centrifuged at 9,000g for four min. The supernatant was discarded and the pellet was washed with 1ml sterile distilled water. This was repeated three times. Following the final wash the pellet was resuspended in fresh water and 100µl of the suspension was heated at 100°C for 12 min to inactivate DNases. The cell lysate was stored at -20°C until required.

2.2.5 Random Amplification of Polymorphic DNA (RAPD) fingerprinting

The primer used was 1254 (5'CCGCAGCCAA 3') previously found suitable for *Salmonella enterica* fingerprinting (Hilton *et al.*, 1996). The PCR was performed in a 25µl volume containing 2.5µl of PCR buffer (Table 2.2; 100mmol l^{-1} Tris-HCL, 1.5mmol l^{-1} $MgCl_2$, 25 mmol l^{-1} KCl, pH 8.8) 0.2 µl of 10 mmol l^{-1} dNTPs, 1.2µl of 100pmol primer, 0.25 units of *Taq* DNA polymerase (Promega, M1661), 18.55µl of H_2O and 2µl of DNA template. A DNA-free control was prepared by adding 2µl of sterile distilled water. Amplification was performed in a PTC-100 thermocycler (MJ Reasearch, INC. Waltham, MA, U.S.A) with maximal ramping as follows: one cycle of 4.5 min at 94°C followed by five low strigency cycles comprising 30s at 94°C, two min at 20°C, two min at 72°C and 35 high strigency cycles of 30 sec at 94°C, one min at 32°C and two min at 72°C. The cycling was concluded with five min at 72°C and the reaction products stored at 4°C until required.

Table 2.2:Buffer matrix of opti-prime kit adapted from Schoettlin *et al.*, 1994

10 mmol l^{-1}Tris-HCl	$MgCl_2$	25 mmol l^{-1} KCl	75 mmol l^{-1} KCl
pH 8.3	**1.5 mmol l**	**Buffer 1**	**Buffer 2**
pH 8.3	**3.5 mmol l**	**Buffer 3**	**Buffer 4**
pH 8.8	**1.5 mmol l**	**Buffer 5**	**Buffer 6**
pH 8.8	**3.5 mmol l**	**Buffer 7**	**Buffer 8**
pH 9.2	**1.5 mmol l**	**Buffer 9**	**Buffer 10**
pH 9.2	**3.5 mmol l**	**Buffer 11**	**Buffer 12**

Final reaction concentrations

2.2.6 DNA Analysis

A 15 µl portion of the RAPD reaction product was loaded onto a 2% agarose containing 5µl of 10µg/ml ethidium bromide and electrophoresed in TAE buffer (40mM EDTA and 0.1% ($^{v}/_{v}$) glacial acetic acid) using Bio-Rad Mini Protean II

apparatus (Bio-Rad, Hemel Hemstead, UK) at 100 Volts for one hour. The DNA fragments were visualised by placing the gel on a U.V. transilluminator and the gel recorded using a digital imaging system (Genesnap, SynGene, Cambridge, UK).

2.2.7 Evaluation of Bacterial Concentration-Calibration curves

Calibration curves were prepared for *E. coli* O157 and *Salmonella* Virchow. One colony from overnight nutrient agar plates (Oxoid, Basingstoke, U.K.) was inoculated into 50ml of nutrient broth (Lab M, Lancashire, U.K.) and incubated at 37°C in a shaking water bath at 150g. A 1ml sample was removed from the culture media every half an hour during lag phase. Readings of optical Density (OD) at 600nm were recorded and the samples were diluted and plated out onto nutrient agar at three different concentrations using the Miles & Misra method. Samples were increased to every 15 minutes during log phase and returned to every half an hour during stationary phase. Samples were taken for up to 1.5 hours into stationary growth. The plates were then incubated overnight at 37°C. Colonies were counted for each time point using the most appropriate dilution (plates showing between 15 and 100 colonies per 20μl spot). The number of colony forming units per sample was then calculated and a calibration curve was constructed. From this, the linear regression value R^2 was determined. The calibration curves were repeated until an R^2 value greater than 0.95 was achieved.

2.2.8 Miles & Misra

Six drops of each dilution were delivered into well-dried plates. The droplets (20μl) were then allowed to dry before the plates were inverted for incubation at 37°C for 18-24h (Miles & Misra, 1983).

2.2.9 Growth Curves

Growth curves were prepared in triplicate for each of the twenty strains. One colony from overnight nutrient agar plates (Oxoid, Basingstoke, U.K) was inoculated into 10ml of nutrient broth (Lab M, Lancashire, U.K.). This was grown at 37°C in a shaking water bath at 150g until log phase was reached (OD_{600} ~ 0.4). A 50μl volume of log phase culture was then used to inoculate 50ml of nutrient broth. Optical density readings at 600nm were taken hourly and the colony forming units per ml were determined using the appropriate calibration curve. Samples were read until growth had stopped and two similar OD readings were gained. Growth curves were then plotted using the average cfu/ml and the standard errors gained from the triplicate values.

Growth curves were also prepared automatically with the use of a plate reader (Anthos Reader 2001, Anthos Lactic Instruments, Australia). All strains were grown in triplicate on Nutrient Agar plates (Oxoid, Basingstoke, U.K) at 37°C. One colony from each overnight plate was used to inoculate 10ml of nutrient broth (Lab M, Lancashire, U.K). The 10ml cultures were incubated at 37°C in a shaking water bath at 150g until log phase growth had been reached (OD_{600} ~0.4). A 2μl volume of each log phase culture was then used to inoculate wells of a 97 well micro titre plate containing 200μl of nutrient broth (Lab M, Lancashire, U.K). The plate was then loaded into the plate reader. The plate reader was programmed such that a temperature of 37°C would be maintained, readings would be taken every ten minutes for 8 hours, and shaking would occur between these intervals.

The lag time of parent and resistant strains was interpreted as the time taken for the OD to increase from the baseline by 0.05 OD units and the growth rate constant was calculated by the following formula:

$$Log_{10}N-log_{10}N_0 = (\mu/2.303)\ (t-t_0)$$

Where:

N=Number of bacteria at t

t=Time

N_0=Number of bacteria at t_0

t_0=Time zero

2.2.10 Determining the Minimum Inhibitory Concentration (MIC)

The MIC was determined by using the standard broth microdilution method, which was carried out using a two-fold dilution of each antibacterial agent. One colony of each organism was inoculated into 30ml of Nutrient Broth and grown overnight at 37°C. The antibiotic/biocide stock solution and the antibiotic/biocide dilution range was prepared and approximately $1x10^8$ bacteria were inoculated into 2ml Nutrient Broth containing an additional 2ml of various concentrations of the antibiotic/biocide in Bijoux bottles. The MIC was determined as the lowest concentration of the antibiotic/biocide inhibiting growth.

2.2.11 Acquisition of Resistance

The first tube showing growth below the MIC was selected and used to inoculate increasing concentrations of antibiotic/biocide. This procedure took place daily until a significant increase in the MIC occurred. In addition, the organisms immediately below the MIC were plated on Nutrient Agar in the presence and absence of the same

antibiotic/biocide. Stored cultures were sub-cultured onto fresh media once per month.

2.2.12 Stability of Resistance

The stability of resistance was determined for each resistant strain by repeated subculture of *Salmonella enterica* serovar Enteritidis, Typhimurium, Virchow and *E. coli* O157, O55 and K-12 in nonselective broth. The procedure was repeated every 24 hours for 30 days. The MIC was determined every five days as described in section 2.2.7.

2.2.13 Statistical Analysis

Growth curve data were tested for normality using the Kolmogorov Smirnof test transformed to normality where possible and analysed using repeated measures ANOVA. All analysis was carried out using the StatSoft, Inc. (2001) Statistica (data analysis software system), version 6 Program.

A Two-sample t-test was employed to examine whether there was a significant difference between the growth rate and the length of lag phase between parent and resistant strains. All analysis was carried out using Minitab Statistical software (2002) version 13.

2.3 Results

2.3.1 Bacterial Identification by API 20E

In Figure 2.1, an API test for *Salm.* Typhimurium during exposure to erythromycin is shown. The consistent profile shows strain continuity was maintained throughout.

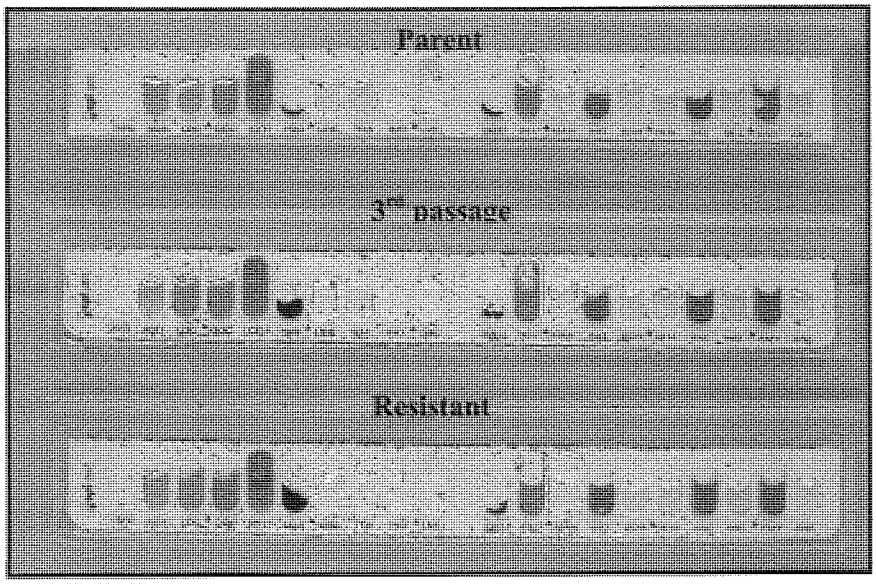


Figure 2.1: An API Test for a *Salm.* Typhimurium strain showing increased MIC to erythromycin following serial passage.

2.3.1.1 RAPD

RAPD profiles obtained from *Salm.* Enteritidis parent (Lane 1) and resistant to ERY (Lane 2), BKC (Lane 3), TLN (Lane 4) strains. Lane M; a 1Kbp molecular weight marker (Bioline, London, UK) and Lane C; No DNA control.

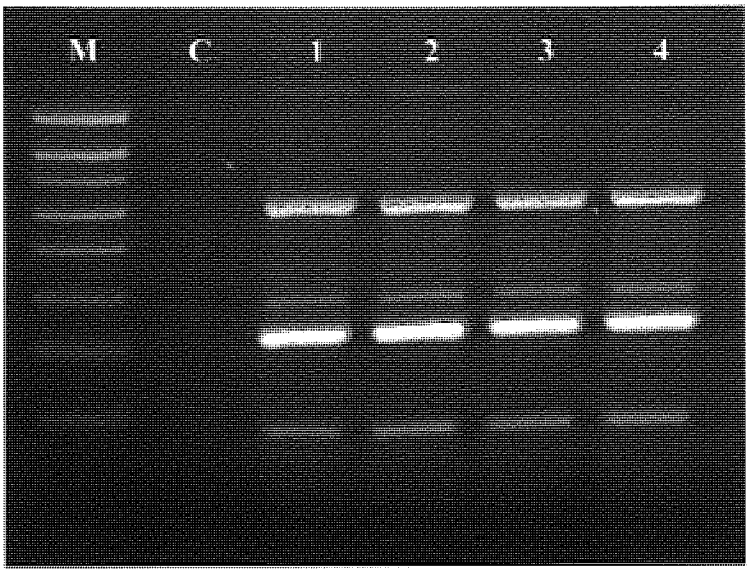


Figure 2.2: RAPD profiles from *Salm.* Enteritidis.

RAPD profiles obtained from *Salm.* Typhimurium parent (Lane 1) and resistant to ERY (Lane 2), BKC (Lane 3), TLN (Lane 4) strains. Lane M; a 1Kbp molecular weight marker (Bioline, London, UK) and Lane C; No DNA control.

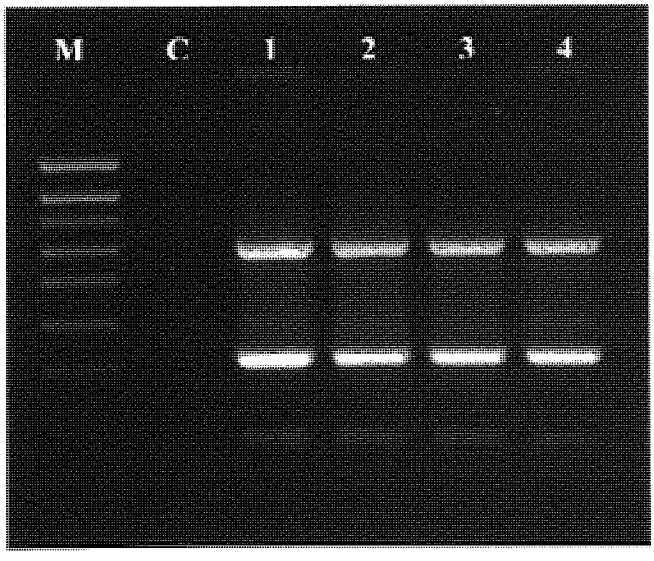


Figure 2.3: RAPD profiles from *Salm.* Typhimurium.

RAPD profiles obtained from *Salm.* Virchow parent (Lane 1) and resistant to ERY (Lane 2), BKC (Lane 3), TLN (Lane 4) strains. Lane M; a 1Kbp molecular weight marker (Bioline, London, UK) and Lane C; No DNA control.

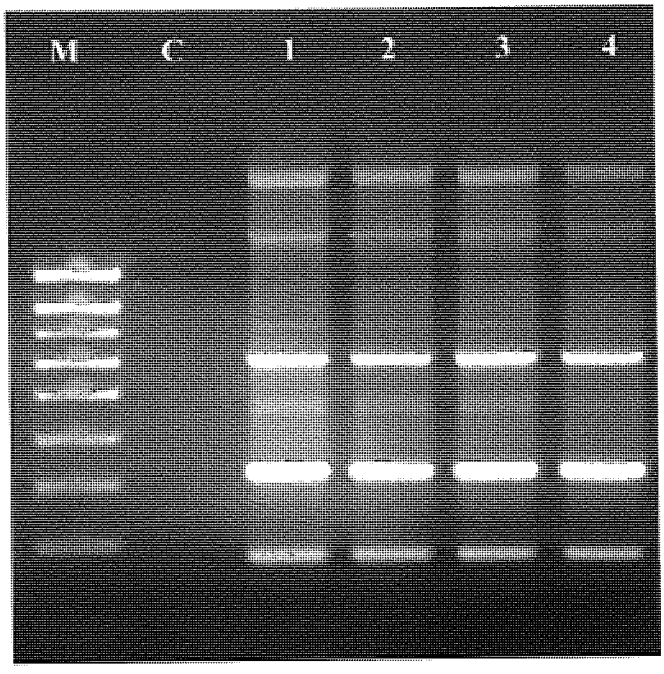


Figure 2.4: RAPD profiles from *Salm.* Virchow.

RAPD profiles obtained from *E. coli* O157 parent (Lane 1) and resistant to ERY (Lane 2), BKC (Lane 3), CHX (Lane 4) and TLN (Lane 5) strains. Lane M; a 1Kbp molecular weight marker (Bioline, London, UK) and Lane C; No DNA control.

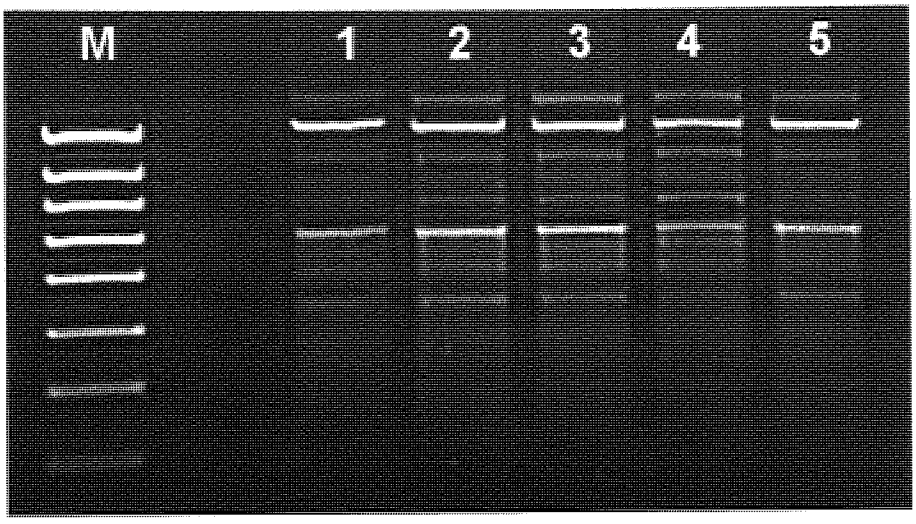


Figure 2.5: RAPD profiles from *E. coli* O157 (12900)

RAPD profiles obtained from *E. coli* O157:H7 (43888) parent (Lane 1) and resistant to TLN (Lane 2) strains. Lane M; a 1Kbp molecular weight marker (Bioline, London, UK) and Lane C; No DNA control.

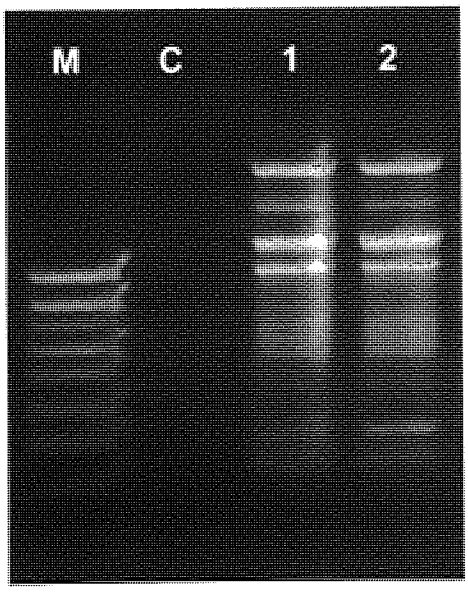


Figure 2.5.1: RAPD profiles from *E. coli* O157:H7 (43888)

RAPD profiles obtained from *E. coli* O55:H7 parent strains (Lane 1) and resistant to TLN (Lane 2). Lane M; a 1Kbp molecular weight marker (Bioline, London, UK) and Lane C; No DNA control.

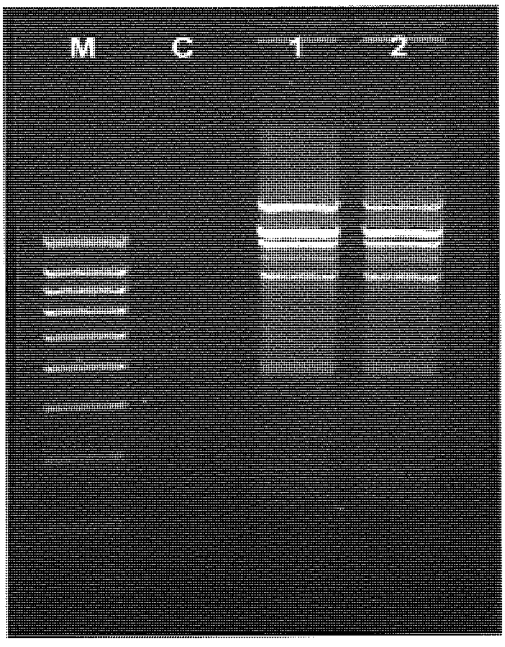


Figure 2.6: RAPD profiles from *E. coli* O55:H7.

RAPD profiles obtained from *E. coli* O55:H29 parent strains (Lane 1) and 1st (Lane 2), 2nd (Lane 3) and final passage (Lane 4) to TLN. Lane M; a 1Kbp molecular weight marker (Bioline, London, UK) and Lane C; No DNA control.

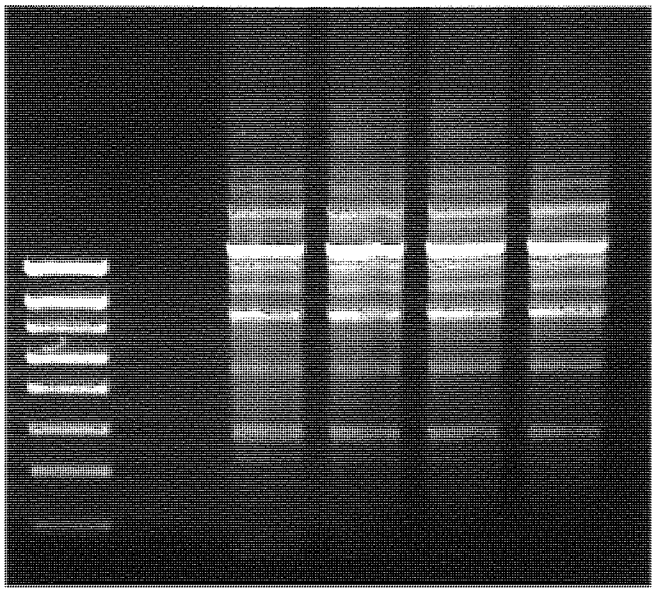


Figure 2.6.1: RAPD profiles from *E. coli* O55:H29.

RAPD profiles obtained from *E. coli* K-12 W3110 parent (Lane 1) and 1st (Lane 2), 2nd (Lane 3) and final passage (Lane 4) to TLN. Lane M; a 1Kbp molecular weight marker (Bioline, London, UK) and Lane C; No DNA control.

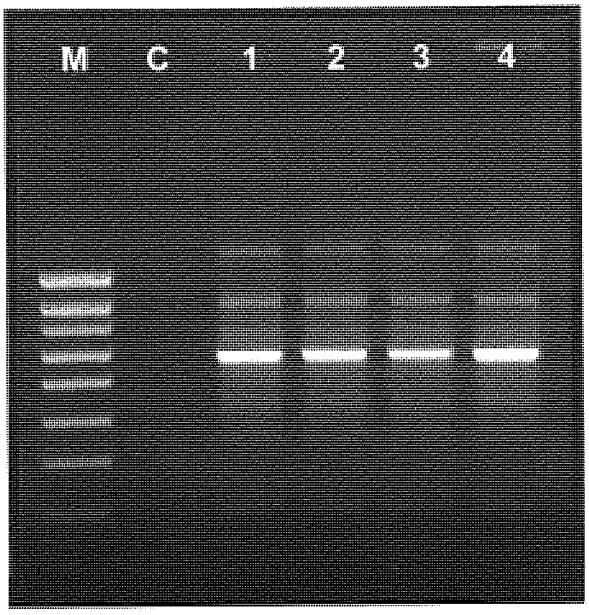


Figure 2.7: RAPD profiles from *E. coli* K-12 W3110

RAPD profiles obtained from *E. coli* K-12 MRE 600 parent (Lane 1) and resistant to TLN (Lane 2) strains. Lane M; a 1Kbp molecular weight marker (Bioline, London, UK) and Lane C; No DNA control.

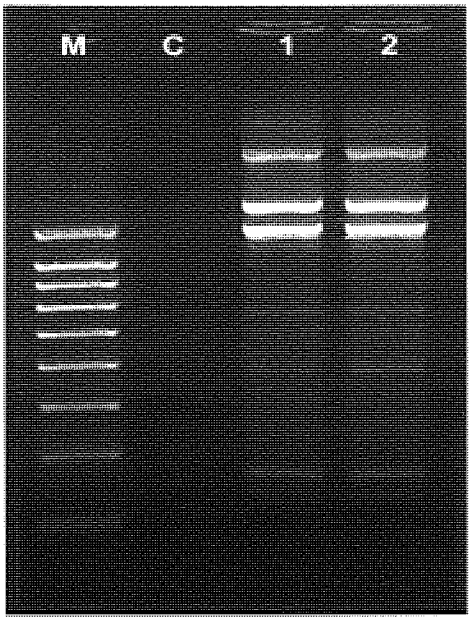


Figure 2.7.1: RAPD profiles from *E. coli* K-12 MRE 600

RAPD profiles obtained from *E. coli* O111:H24 parent (Lane 1) and resistant to TLN (Lane 2) strains. Lane M; a 1Kbp molecular weight marker (Bioline, London, UK) and Lane C; No DNA control.

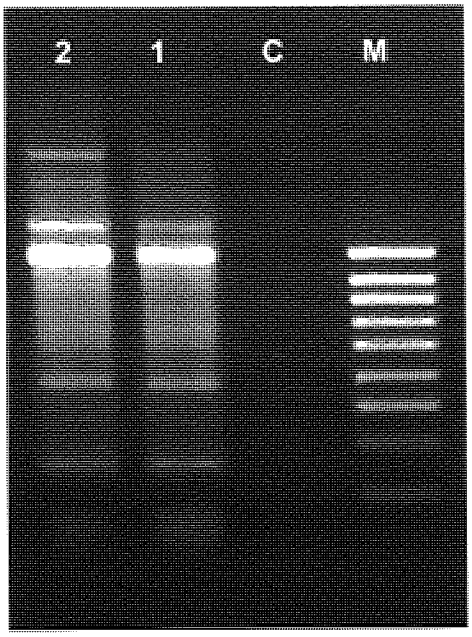


Figure 2.8: RAPD profiles from *E. coli* O111:H24

2.3.2 Evaluation of Bacterial Concentration-Calibration curves

Mean absorbance readings were taken from the diluted washed *Salmonella* Typhimurium at an absorbance of 600nm. The original washed suspension was diluted in 1/4 ratio, in order to give an absorbance reading of 1.0. The diluted (1/4) washed suspension was further serially diluted and mean absorbance readings used to construct a standard calibration graph. In Figure 2.9 the calibration curve of *E. coli* O157 is shown and in Figure 2.10 *Salm.* Virchow. The linear regression value R^2 was determined.

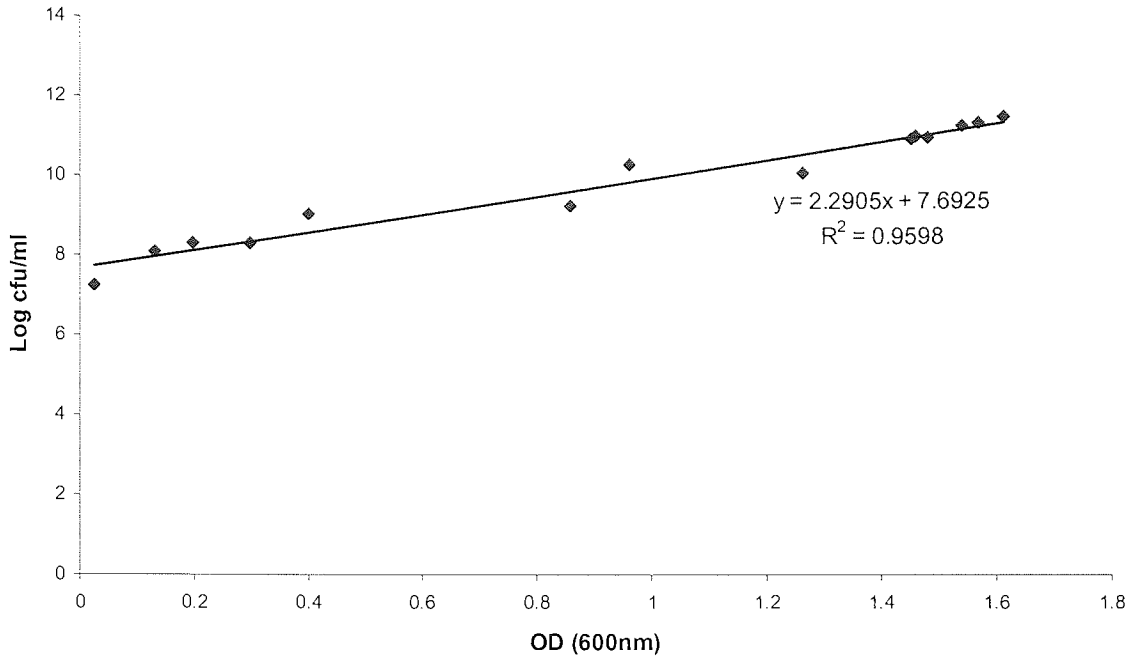


Figure 2.9: A calibration curve of *E. coli* O157.

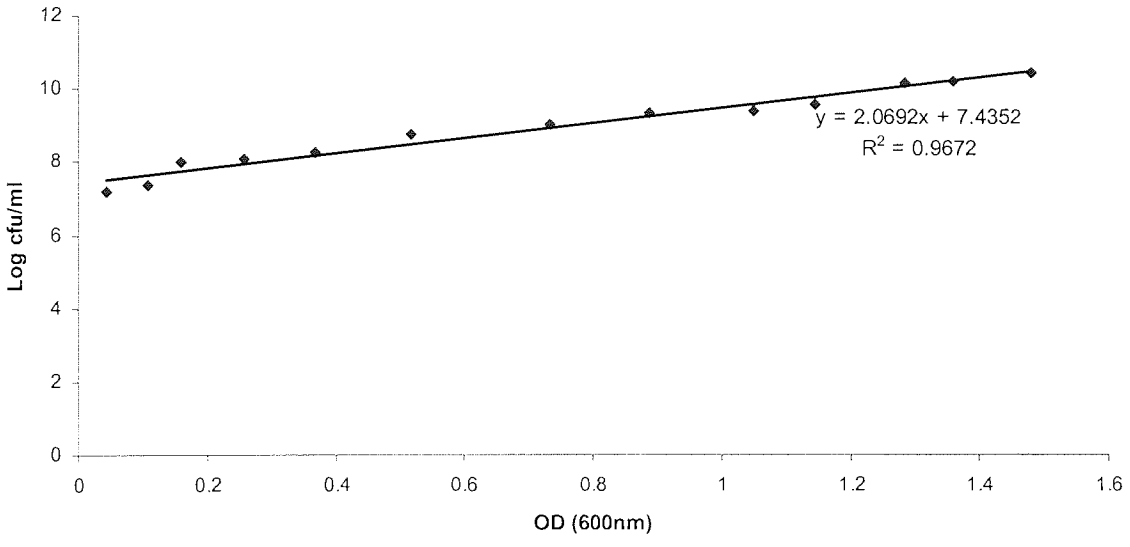


Figure 2.10: A calibration curve of *Salm.* Virchow.

2.3.3 Growth Curves

The graphs for growth curves can be seen in Figures 2.11-2.15. Log colony forming units per ml were calculated from the optical density using calibration curves. The

standard errors are shown in each of the graphs for which it can also be seen that significant differences (p=<0.05) in the growth of parent and some resistant strains exists. In addition, the growth rate and the length of lag phase were calculated for each strain.

In Figure 2.11 the growth curves of *Salm.* Enteritidis parent and resistant strains to ERY, BKC and TLN are shown. Data suggests that the parent strain grew faster than the one resistant to ERY and this reached significance. The length of lag phase of the ERY resistant strain was significantly longer as well. Strains resistant to BKC had a higher growth rate and a longer lag phase, however this did not reach significant levels. TLN-resistant *Salm.* Enteritidis grew significantly quicker compared to the parent as a result of the longer lag phase. The error bars correspond to standard errors.

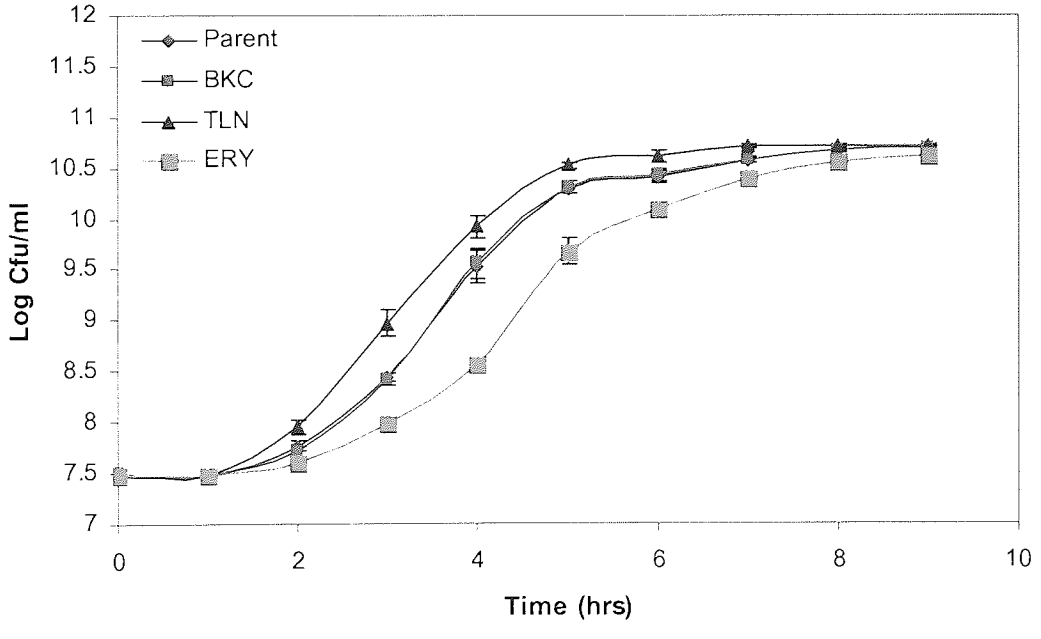


Figure 2.11: Growth curves for *Salm.* Enteritidis parent and resistant strains.

In Figure 2.12 the growth curves of *Salm.* Typhimurium parent and resistant strains to ERY, BKC and TLN are illustrated. In all cases the resistant strains grew faster than the parent which reached significance ($p=<0.05$) in each case. In the cases of ERY and BKC resistant *Salm.* Typhimurium this was a result of their shorter lag phases. TLN-resistant *Salm.* Typhimurium did not have a significantly higher growth rate or a significantly shorter lag phase. The error bars correspond to standard errors.

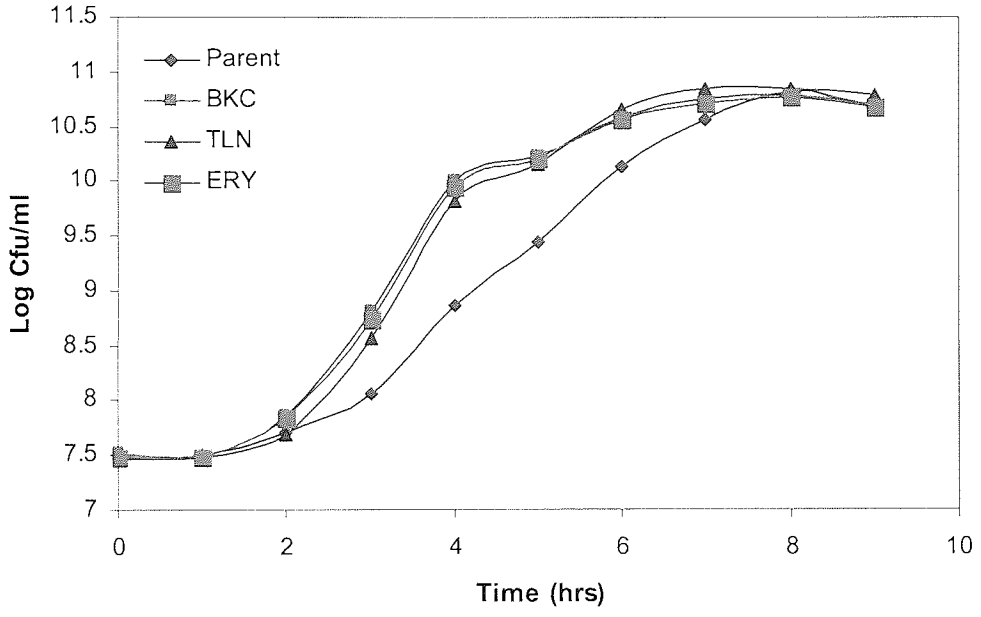


Figure 2.12: Growth curves for *Salm.* Typhimurium parent and resistant strains.

In Figure 2.13 the growth curves of *Salm.* Virchow parent and resistant strains to ERY, BKC, CHX and TLN are shown. Data suggests that strains resistant to CHX and TLN grew faster than the parent whereas those resistant to ERY and BKC grew slower than the parent; however this was significantly different ($p=<0.05$) only in the case of *Salm.* Virchow resistant to ERY, where the lag phase was also significantly longer. The error bars correspond to standard errors.

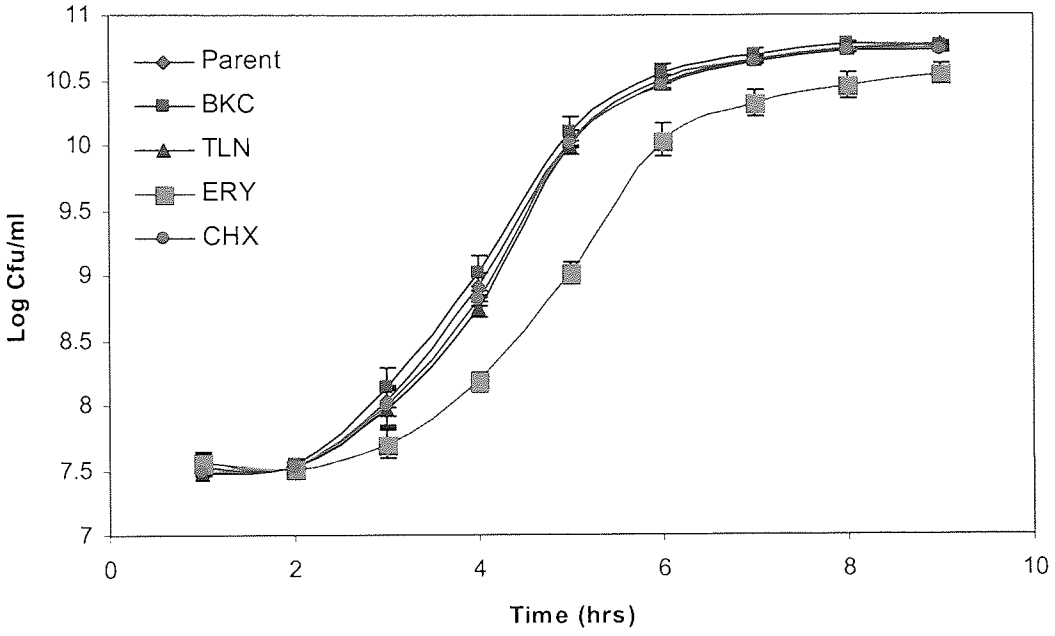


Figure 2.13: Growth curves for *Salm.* Virchow parent and resistant strains.

In Figure 2.14 the growth curves of *E. coli* O157 parent and resistant strains are illustrated. The parent strain grew faster than the strains resistant to ERY, BKC, CHX and TLN. This was not significantly higher ($p=<0.05$) in the case of *E. coli* O157 resistant to BKC, even though the strain had a significantly longer lag phase. As a result of their longer lag phases ERY and CHX resistant strains grew slower than the parent strain. However, in the case of TLN-resistant *E. coli* O157 this was a result of the lower growth rate of the strain and not of its longer lag phase as it did not reach significance. The error bars correspond to standard errors.

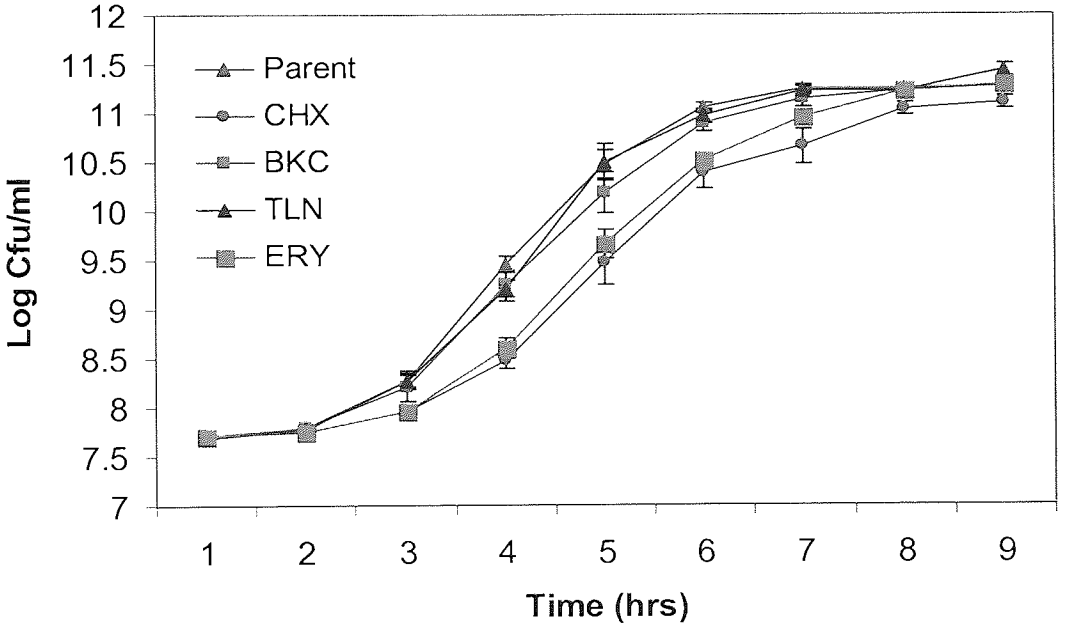


Figure 2.14: Growth curves for *E. coli* O157 parent and resistant strains.

In Figure 2.15 the growth curves of *E. coli* O55 parent and resistant to TLN strains are shown. Even though the TLN resistant strain grew slower than the parent that was not significantly different. The growth rate and the length of the lag phase were not significantly different as well. The errors bars correspond to standard errors.

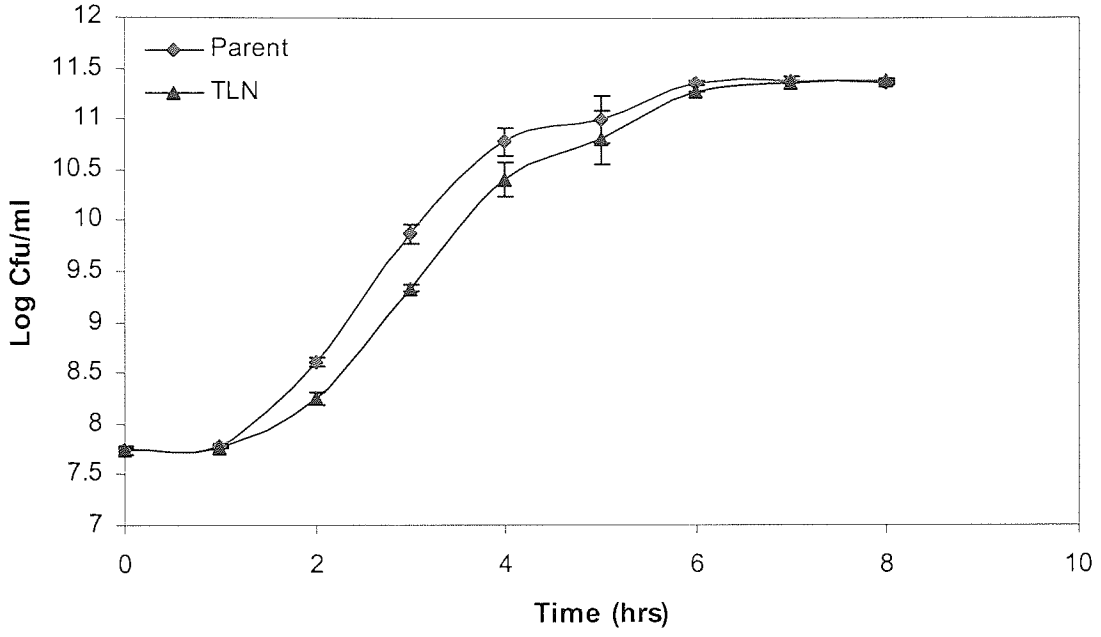


Figure 2.15: Growth curves for *E. coli* O55 parent and resistant strains.

Table 2.3 presents summary of the statistical analysis of growth curves for all strains investigated. A significant difference in the overall growth rate between the parent and resistant is indicated in red. In addition, the growth rates of each strain and the length of their lag phase were calculated. Statistical analysis was also performed for both situations.

Table 2.3: Statistical analysis of differences between parent and resistant strains. Data in red indicates significant differences.

Strains	Overall Growth p- values	Growth rates (min)/ p-values	Length of lag phase (min)/ p-values
Salm. Enteritidis Parent	-	2.91	130
Salm. Enteritidis ERY	0.000000	2.97/0.468	170/0.036
Salm. Enteritidis BKC	0.999	2.920/0.425	135/0.669
Salm. Enteritidis TLN	0.000012	3.08/0.079	110/0.033
Salm. Typhimurium Parent	-	1.58	155
Salm. Typhimurium ERY	0.00002	2.46/0.0785	120/0.035
Salm. Typhimurium BKC	0.000009	2.43/0.25	122/0.043
Salm. Typhimurium TLN	0.000430	2.47/0.77	130/0.093
Salm. Virchow Parent	-	2.72	90
Salm. Virchow ERY	0.000000	3.5/0.105	140/0.02
Salm. Virchow BKC	0.981771	2.66/0.242	75/0.293
Salm. Virchow CHX	0.993226	2.83/0.401	100/0.425
Salm. Virchow TLN	0.794	2.86/0.367	110/0.202
E. coli O157 Parent	-	3.086	58
E. coli O157 ERY	0.000000	2.84/0.1	120/0.007
E. coli O157 BKC	0.387727	3.04/0.411	90.6/0.049
E. coli O157 CHX	0.000000	2.71/0.065	85.3/0.055
E. coli O157 TLN	0.0127	2.75/0.048	67.6/0.426
E. coli O55 Parent	-	2.64	60
E. coli O55 TLN	0.0749	2.85/0.09	85/0.072

2.3.3.1 Automated Growth Curves

Graphs for each *Salmonella enterica* and *E. coli* serotype can be seen in Figures 2.16-2.20. Statistical analysis was not carried out on this data. From the graphs it can be seen that the growth conditions provided by the plate reader do not result in the normal sigmoid curve associated with bacterial growth. The difference between parent and resistant strains using this method is less pronounced, but analysis of the growth curves created do support the results obtained manually.

In Figure 2.16 the automated growth curves of *Salm.* Enteritidis resistant to ERY, BKC and TLN are shown. Data suggests that all resistant strains grew faster than the parent one. Compared with manual growth curves the results do not correlate, where this was the case only in TLN-resistant strains. The errors bars correspond to standard errors.

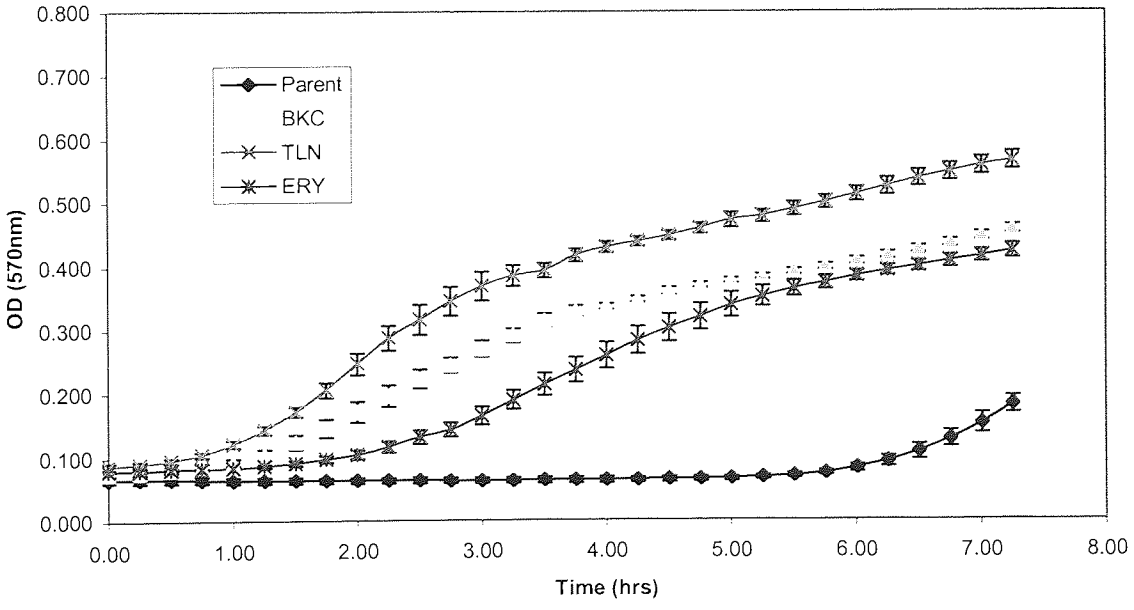


Figure 2.16: Automated growth curves for *Salm.* Enteritidis parent and resistant strains.

In Figure 2.17 the automated growth curves of *Salm.* Typhimurium resistant to ERY, BKC and TLN is shown. According to these data all resistant strains grew faster than the parent. The same was obtained by the manual growth curves. The error bars correspond to standard errors.

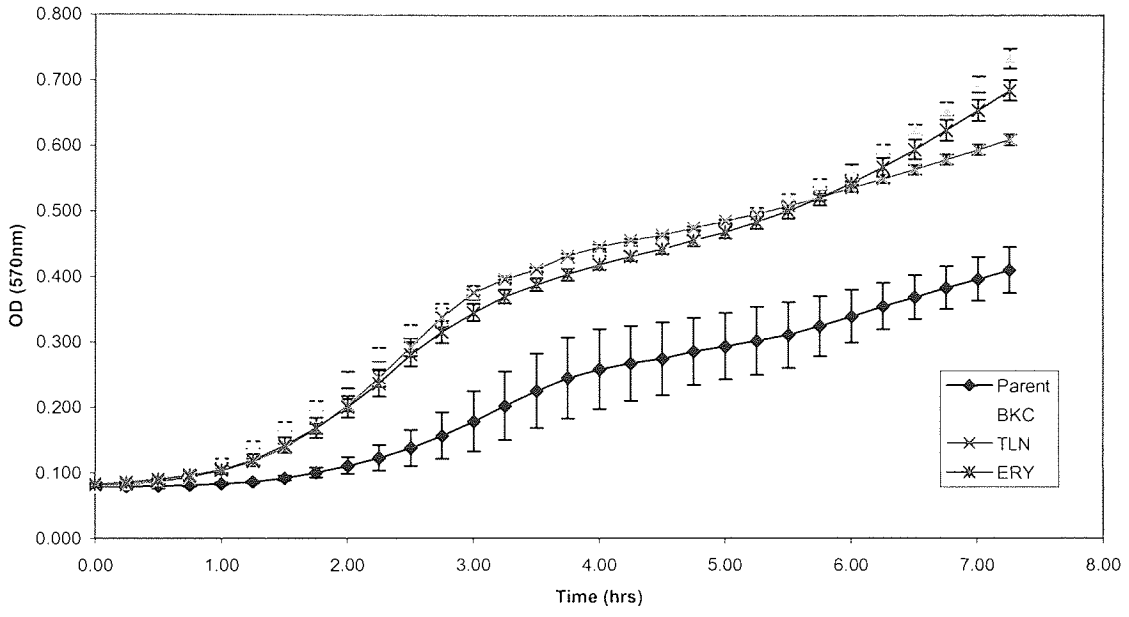


Figure 2.17: Automated growth curves for *Salm.* Typhimurium parent and resistant strains.

In Figure 2.18 the automated growth curves of *Salm.* Virchow resistant to ERY, BKC, CHX and TLN are shown. The data suggests that the parent strain grew faster than the resistant ones, which is not in agreement with the results obtained by the automated growth curves for both *Salm.* Enteritidis and *Salm.* Typhimurium. In addition, it is partly in agreement with the manual growth curves obtained for *Salm.* Virchow as far as it concerns the resistant strains to CHX and TLN. The error bars correspond to standard errors.

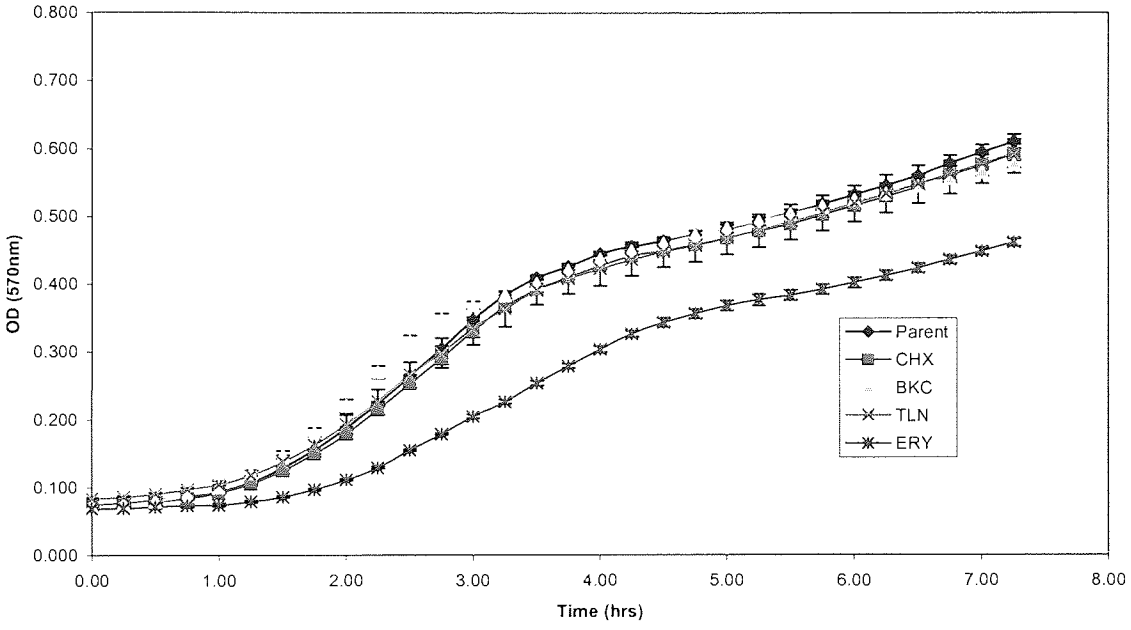


Figure 2.18: Automated growth curves for *Salm.* Virchow parent and resistant strains.

In Figure 2.19 the automated growth curves for *E. coli* O157 resistant to ERY, BKC, CHX and TLN are illustrated. The results suggest that the resistant strains grew slower than the parent. The same was shown by the manual growth curves when *E. coli* O157 was tested. The error bars correspond to standard errors.

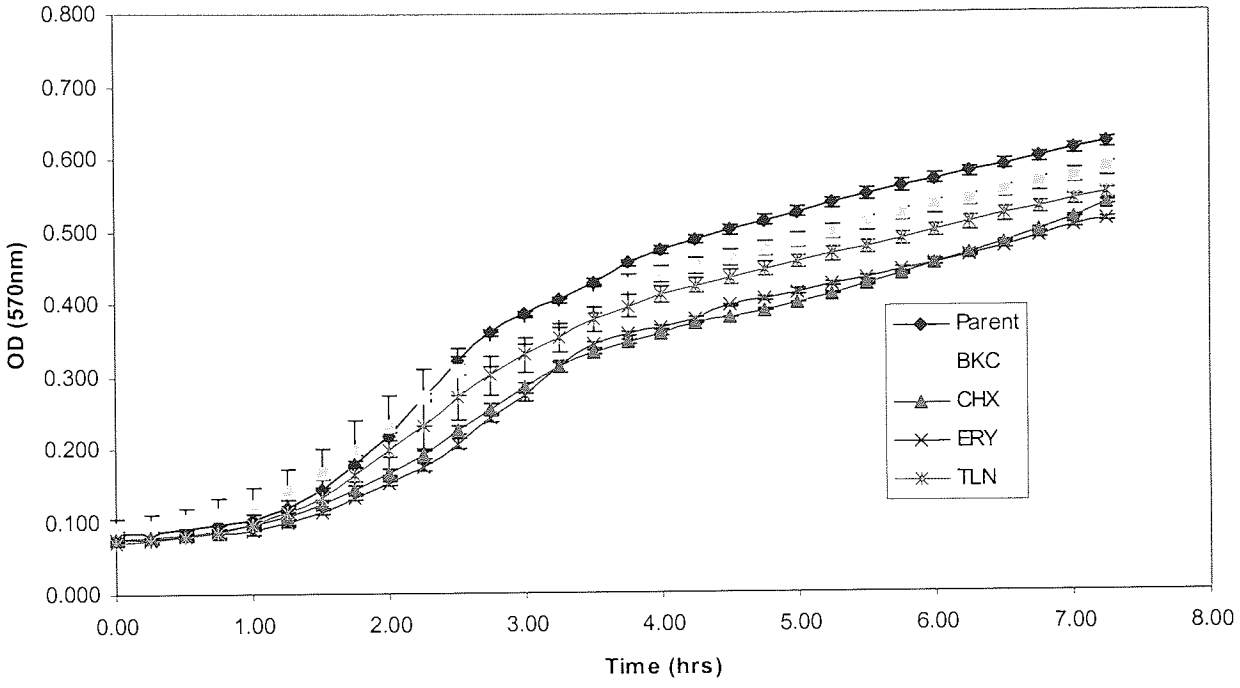


Figure 2.19: Automated growth curves for *E. coli* O157 parent and resistant strains.

In Figure 2.20 the automated growth curves of *E. coli* O55 are shown. Data suggest that the parent strain grew faster than the resistant to TLN strain. This was the case when *E. coli* O55 was tested and the growth curves were performed manually. The error bars correspond to standard errors.

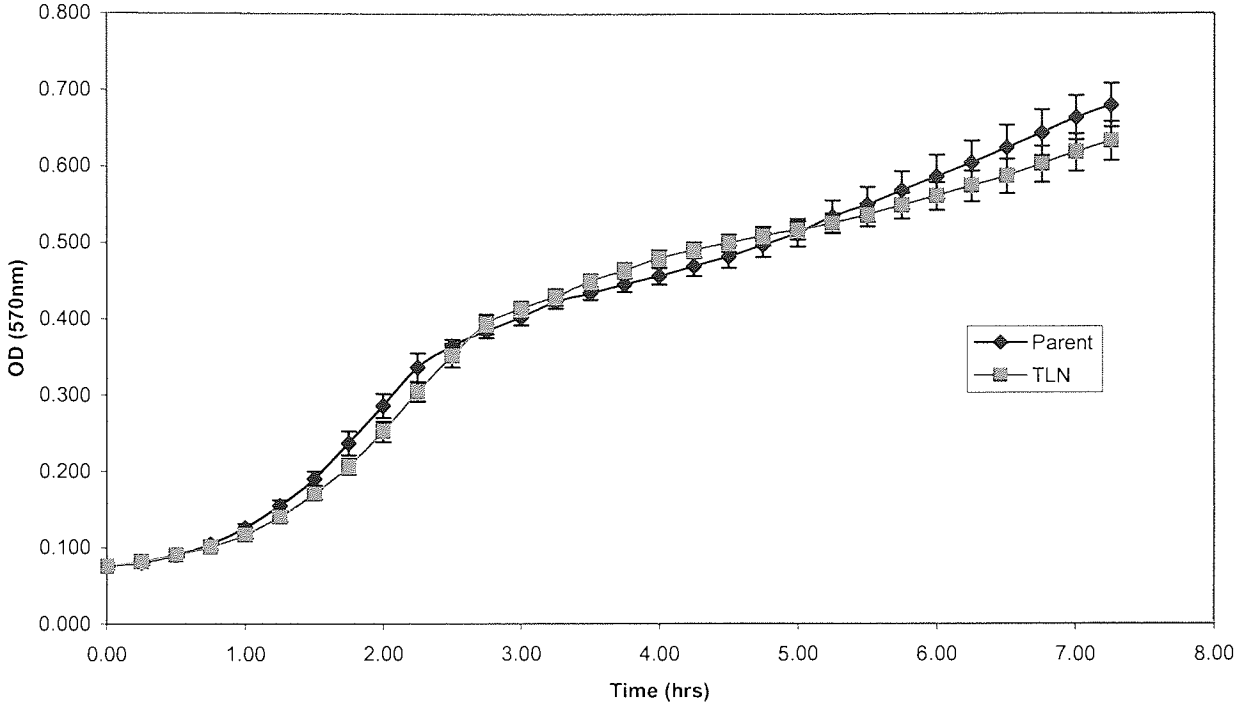


Figure 2.20: Automated growth curves for *E. coli* O55 parent and resistant strains.

2.3.4 Progress of Resistance

The passages of *Salmonella* Enteritidis, Typhimurium, Virchow and *E. coli* O157 to erythromycin and to the biocides tested is illustrated in Figures 2.21-2.24, respectively. All the serotypes of *Salmonella enterica* and *E. coli* O157showed decreased sensitivity to high concentrations of ERY. The concentration of BKC that inhibited *E. coli* O157 was far below that of *Salm.* Enteritidis and *Salm.* Typhimurium, however, *E. coli* O157 rapidly acquired the highest resistance quicker than the rest of the strains investigated in this study. In addition, when *Salm.* Virchow and *E. coli* O157 were exposed to CHX, they repeatedly acquired resistance, even

from their first exposure which was most pronounced in *E. coli* O157. When *E. coli* O157 was exposed to triclosan they were initially sensitive to low levels, however susceptibility was soon lost requiring very high concentrations to inhibit it. This rapid development of high-level resistance was highly reproducible upon repeat experimentation.

In Figure 2.21 the passages of *Salm.* Enteritidis, Typhimurium, Virchow and *E. coli* O157 to erythromycin are illustrated. From the results obtained it is apparent that bacteria quickly reduced susceptibility to high concentrations of erythromycin, especially *Salm.* Enteritidis.

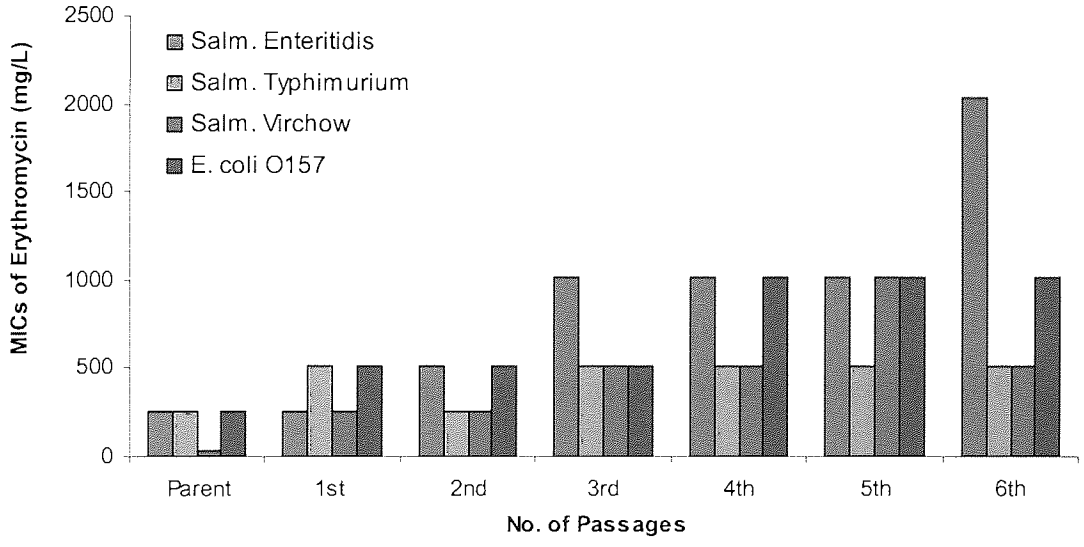


Figure 2.21: The progress of resistance of various bacteria to ERY.

In Figure 2.22 the passages of *Salm.* Enteritidis, Typhimurium, Virchow and *E. coli* O157 to BKC are shown, respectively. Again, results showed that bacteria before exposure had a low MIC especially *E. coli* O157. The data revealed that the concentration of BKC that inhibited *E. coli* O157 was far below that of *Salm.* Enteritidis and *Salm.* Typhimurium, however, *E. coli* O157 rapidly acquired the highest resistance quicker than the rest of the strains in study.

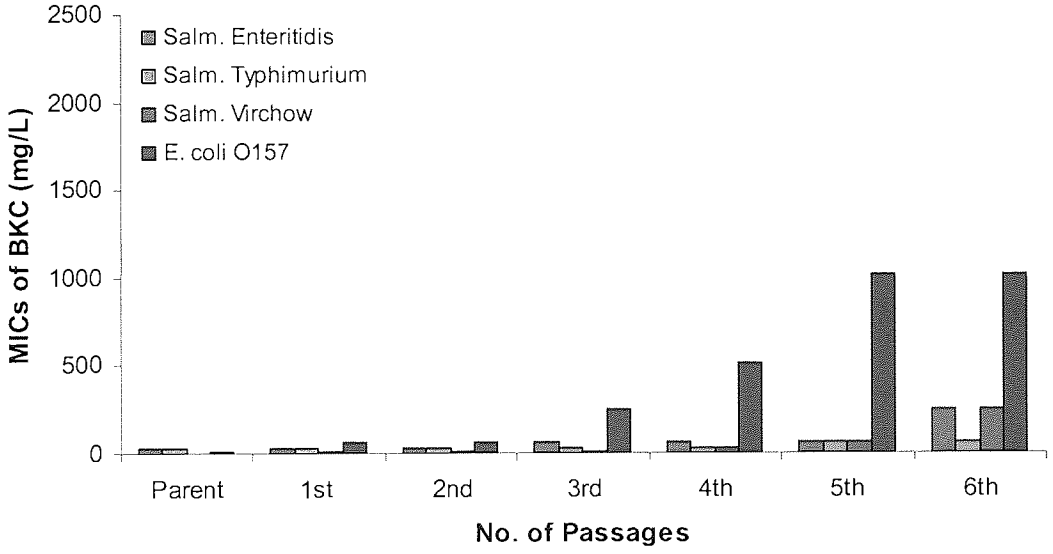


Figure 2.22: The progress of resistance of various bacteria to BKC.

In Figure 2.23 the passages of *Salm.* Virchow and *E. coli* O157 are shown. *Salm.* Enteritidis and *Salm.* Typhimurium have been exposed to chlorhexidine. Results suggested that bacteria even from the first passage reduced susceptibility to CHX, especially *E. coli* O157, which becomes highly resistant.

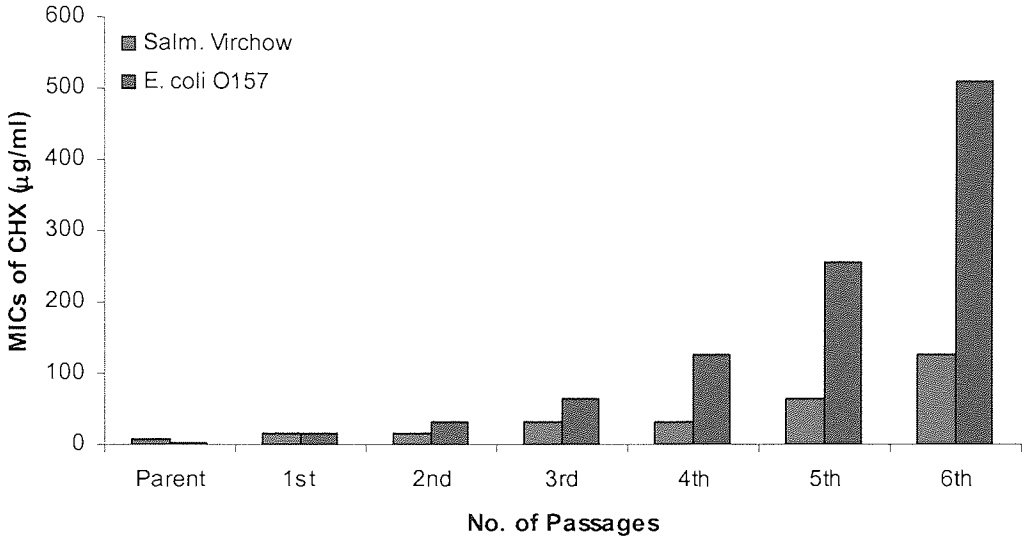


Figure 2.23: The progress of resistance of *Salm.* Virchow and *E. coli* O157 to CHX.

Figure 2.24 illustrates the passages of *Salmonella enterica* serovar Enteritidis, Typhimurium and Virchow in addition to *Escherichia coli* O157, O55 and K-12. This figure shows that all strains acquired increased levels of TLN resistance within less passages as compared with the progress of exposure to antimicrobials other than TLN. *Salmonella* Enteritidis, Typhimurium and Virchow developed resistance within five passages and *Salm.* Virchow was the one that acquired the highest levels of resistance. All *Escherichia coli* strains increased more rapid higher levels of resistance than *Salmonella* in general. *Escherichia coli* O157 became highly resistant within 3 passages, whereas O55 and K-12 acquired resistance within four.

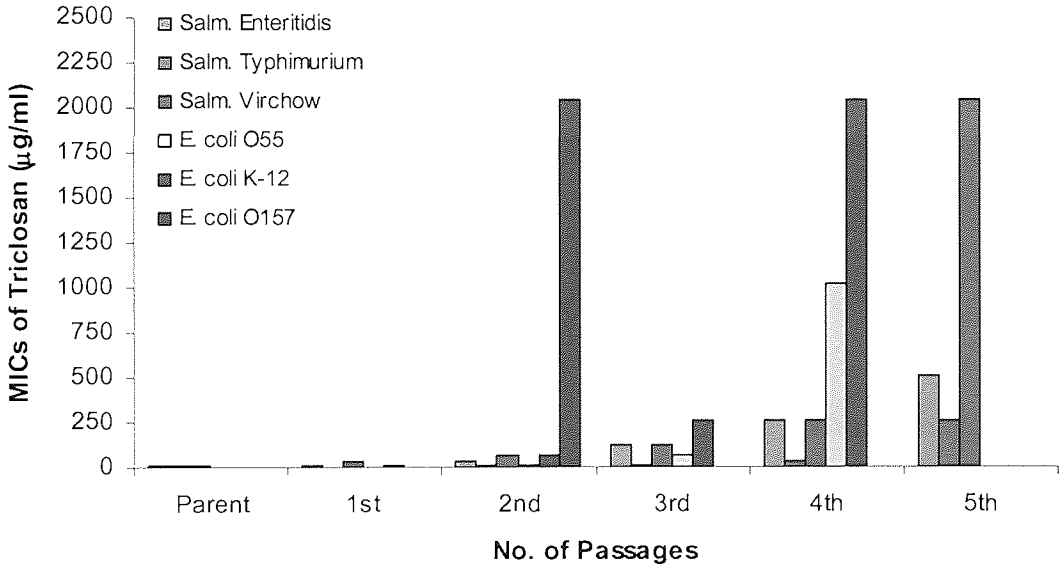


Figure 2.24: The progress of resistance of various bacteria to TLN.

2.3.6 Stability of Resistance

The reduced sensitivity to ERY, BKC, CHX and TLN in *Salm.* Enteritidis, Typhimurium, Virchow and *E. coli* O157 was not lost following more than 30 days of passage in antibiotic / biocide-free media. In the absence of a selective pressure none of the strains returned to the original levels of sensitivity.

Figure 2.25 represents the stability of resistance to erythromycin in resistant cells of *Salm.* Enteritidis, *Salm.* Typhimurium, *Salm.* Virchow and *E. coli* O157. From the results obtained it is apparent that resistance was stable for up to 30 days as none of the strains returned to the initial sensitivity levels of erythromycin.

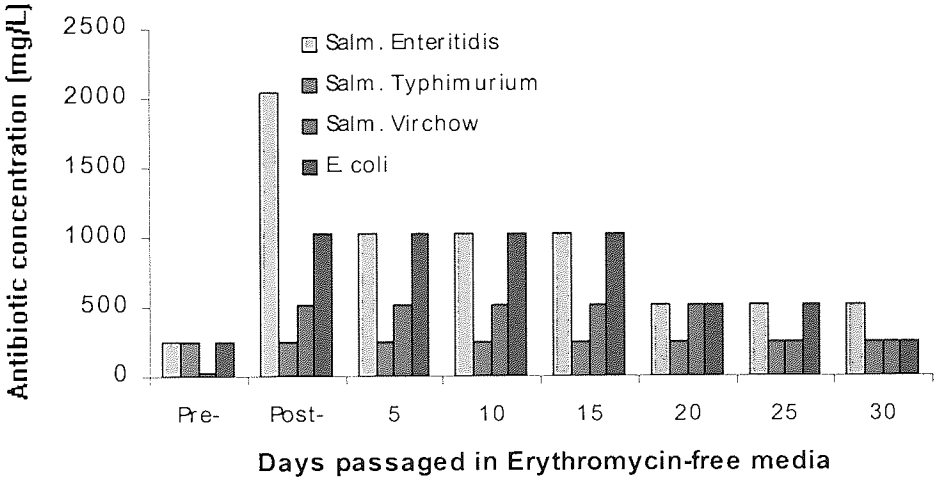


Figure 2.25: Stability of resistance to ERY for test strains.

In Figure 2.26 the MICs of *Salm.* Enteritidis, *Salm.* Typhimurium, *Salm.* Virchow and *E. coli* O157 were recorded when bacteria were serially passaged in BKC-free media. According to the data obtained the bacteria remained stable.

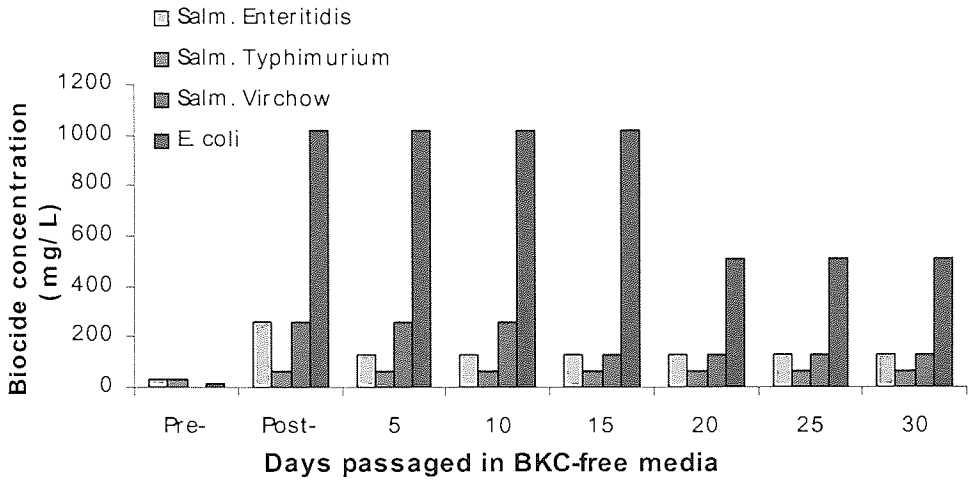


Figure 2.26: Stability of resistance to BKC for test strains.

Figure 2.27 illustrates the stability of resistance to chlorhexidine. In this experiment only cells of *Salm.* Virchow and *E. coli* O157 were exposed to chlorhexidine and acquired resistance, subsequently tested for stability. The results suggest that the resistance was not lost.

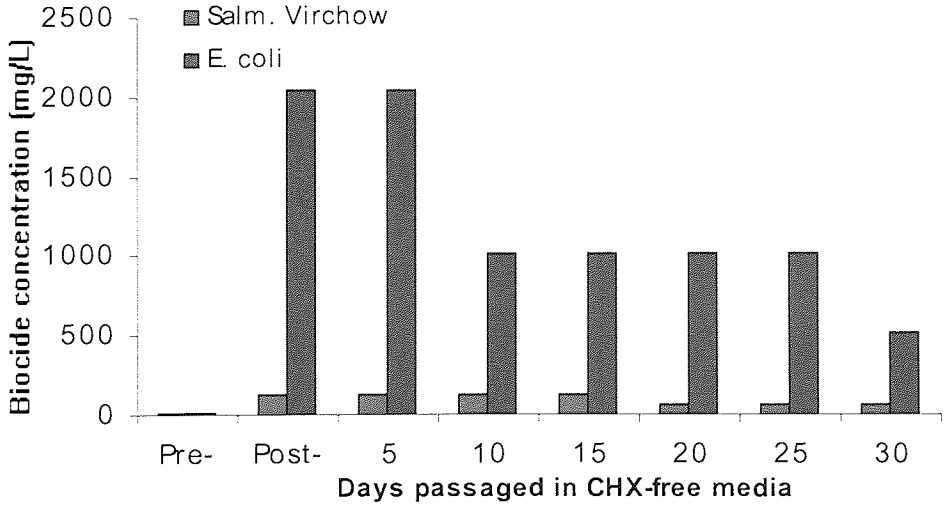


Figure 2.27: Stability of resistance to CHX for test strains

Figure 2.28 demonstrates the MICs of *E. coli* O157 when passaged in TLN-free media. It is apparent that triclosan-resistant mutants remained stable as none of them returned to parent strain levels.

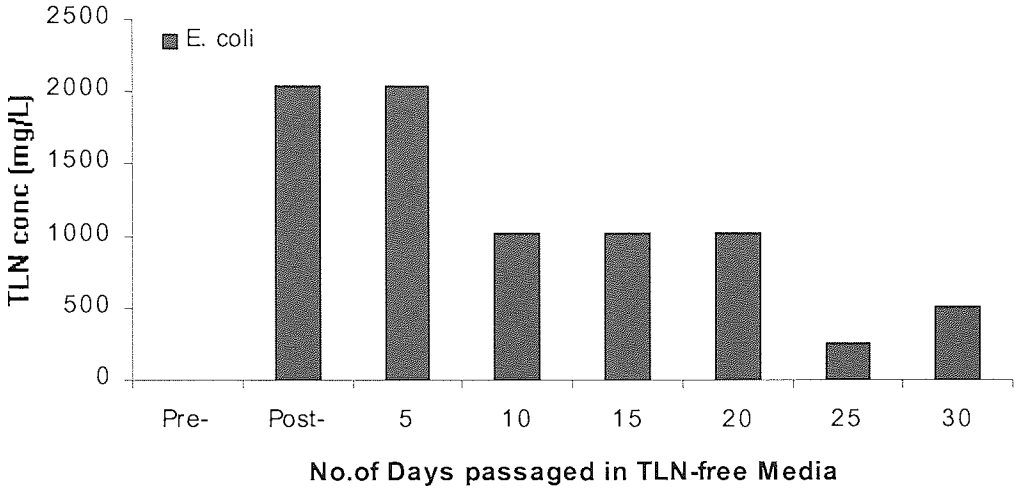


Figure 2.28: Stability of resistance to TLN for *E. coli* O157

2.4 Discussion

2.4.1 Bacterial Identification by API 20E & RAPD

For the biochemical characterisation of *Salmonella enterica* and *E. coli* API tests were employed. The tests followed each passage, to confirm strain continuity. Although relatively expensive and open to subjective interpretation, API profiling provides a rapid confirmation of the continuity of the strains. Due to heterogeneity of resistance of different bacteria it is important to confirm that any increase in resistance observed is a consequence of passage and not substitution of a more competitive contaminant strain.

Random Amplification of Polymorphic DNA was also employed for the genotypic characterisation of the strains. The RAPD is a rapid and relatively inexpensive technique. It was employed either following strain passage, or to compare the genotype of a parent strain with resistant ones to different antimicrobials. The profiles obtained for the parent and resistant strains was identical suggesting that strain continuity had been maintained.

2.4.2 Growth Curves

All data, except that for *Salm.* Enteritidis strains were normally distributed according to the Kolmogorov-Smirnov test. In all serotypes except *Salm.* Typhimurium the resistant strains grew slower than the parent. However, this slower rate was not always statistically significant. It would be expected that the resistant strains would grow more slowly as it has been suggested that slower growth rate indicates structural difference that can confer antimicrobial resistance (Joynson, 2002). It is therefore unusual that the *Salm.* Typhimurium strains have acquired a resistance mechanism

that enables significantly faster growth. The results were repeated four times so are assumed to be reliable. The highest growth is demonstrated by all *Salm.* Typhimurium resistant strains so it is possible that this species has a different mechanism of resistance that is also adding a competitive advantage in terms of growth rate.

All serotypes resistant to ERY were shown to grow at a significantly different rate from the parent. In all cases, except *Salm.* Typhimurium, the resistant strain grew significantly slower. It is suggested that this slower growth rate occurs due to a change in structure, which is conferring resistance to ERY. Investigations into membrane thickness and outer membrane protein regulation may highlight these resistance mechanisms.

In all species resistant to BKC (except *Salm.* Typhimurium) there was no significant difference between the growth of the parent and the resistant strains. Of note, none of the strains had a significantly different growth rate or lag phase with the exception of BKC-resistant *E. coli* O157, which showed to have a significantly longer lag phase. Based on the majority of the data generated it is therefore suggested that the efflux activity associated with BKC is responsible for the resistance and is not hindering the growth rate.

In the two strains resistant to chlorhexidine, *E. coli* showed a significantly slower growth, whereas *Salm.* Virchow did not. It is therefore difficult to determine if chlorhexidine resistance is mediated by a mechanism that affects the growth. However, it is possible that different resistance mechanisms are employed by different

species, and thus the result in *E. coli* is of significant value. The efflux activity found to be associated with chlorhexidine resistance should also be considered further.

Escherichia coli O157 resistant to triclosan was the only triclosan resistant strain to show a statistically slower rate of growth than the parent; in *E. coli* O55 serotype was not significant. It is unusual that the two *E. coli* strains show different growth rates in response to triclosan as they are both of similar heritages, and it would be suggested that both would react to the biocide in a similar way. The difference may be attributed to the fact that the *E. coli* O157 strain has approximately one thousand extra genes that as well as coding virulence may code resistance (Allen *et al.*, 2001). However, it can be seen that the *E. coli* O55 result is approaching significance so it is suggested that similar mechanisms are occurring but that they are more pronounced in the *E. coli* O157 strain. Two of the *Salmonella* serotypes, *Salm.* Typhimurium and *Salm.* Enteritidis, when exposed to triclosan grew significantly faster than the parent. This has been seen for all the *Salm.* Typhimurium strains but for only one of the *Salm.* Enteritidis strain. It is therefore suggested that *Salm.* Enteritidis has developed a competitive advantage in terms of growth as well as resistance to triclosan.

Overall, it has been shown that erythromycin resistance is associated with a slower growth. This is of interest and should be investigated further. Further investigations may include membrane analysis using electron microscopy and electrophoresis of outer membrane proteins. Exposure to benzalkonium chloride has shown to have no significant effect on the growth. Resistance to chlorhexidine and triclosan show no

real patterns in terms of growth and other mechanisms of resistance should be investigated.

The growth curves collected automatically by the plate reader do not show the normal sigmoid curve associated with bacterial growth. It is suggested that after three hours the log phase cannot increase exponentially due to the occurrence of anaerobic growth conditions. The anaerobic conditions would be created due to the small wells of the microtitre plate and thus only limited shaking. Despite this the curves produced automatically do support the manually constructed growth curves and thus further evidence is provided for mechanisms of resistance.

2.4.3 MICs & Acquisition of Resistance

Three functional classes of macrolide resistance mechanisms exist in pathogenic bacteria: those that modify the ribosome, those that modify the antibiotic itself and those that affect the rate of transport of the antibiotic across the cell membrane (Carsenti-Etesse *et al.*, 1999). With ERY it was possible through passage to reach high levels of resistance in all strains tested. Currently it is unknown which mechanisms are contributing to the resistance observed in the strains under study, however it is likely that it is due to the presence of an active efflux (Levy, 2002b).

Reduced susceptibility to biocides is apparently increasing (McDonell & Russell, 1999; Russell 2002a) and the ability to rapidly develop enhanced resistance to BKC in the present investigation is in agreement with other studies (Joynson *et al.*, 2002). Benzalkonium chloride MIC changed from 4μg/ml to 256μg/ml as reduced sensitivity progressed in *Salmonella* Virchow. The MIC of CHX began at 4μg/ml and increased

to 512μg/ml in *E. coli* O157. *Escherichia coli* O157 when exposed to TLN demonstrated an MIC change from 0.25μg/ml to 4μg/ml. It is of interest that the parent strain *E. coli* O157 was sensitive to extremely low concentrations (0.25μg/ml) of TLN at the first exposure, however it acquired resistance rapidly; after the first passage growth at extremely high concentrations (1024μg/ml) was observed. It is particularly concerning the speed and extent to which *E. coli* O157 becomes resistant to BKC and triclosan. Both are commonly used biocide components in a range of domestic disinfectant products, which are often used inappropriately and at sub-inhibitory concentrations (Levy, 2001). For instance, the triclosan concentration usually found in soap is approximately 2500μg/ml (Levy, 2001), however it has been proposed that soap reduces triclosan's efficacy thereby reducing its effective concentration. In this study *E. coli* O157 was found to grow in 1024μg/ml of Aquasept a commercial formulation of triclosan containing amongst others 40% anionic surfactatant and 4% coconut dequalium (both registered pesticides). To what extent the results observed in this study relate to the activity of TLN and how they relate to the other excipients is unknown, however the proposed consequences of the mis-application of TLN-containing products in everyday life remain the same.

2.4.4 Stability of Resistance

Rapid removal of the selective pressure may lead to reversion to sensitivity however it could take longer that the initial process of the acquisition of resistance (Gould, 1999). In the current study, the stability of resistance to ERY, BKC, CHX and TLN was investigated by passage of the resistant cells in non-selective broth. The MIC of biocides required to inhibit growth was tracked as the passages continued over 30 days. When bacterial cells were passaged in ERY-free media, the MIC in all cases did

not return to the wild type levels, thus it can be concluded that stability to ERY is subject to minor variability, but stable. The stability to BKC was also studied in *Salm. enterica* serovars Enteritidis, Typhimurium, Virchow and *E. coli* O157 strains. Acceptable variability was observed in all experiments that were performed, however, as none of the strains returned to initial sensitivity it can be concluded that stability in the resistance to BKC was obtained. Loughlin *et al.* (2002) demonstrated that resistance to BKC was acquired readily when *P. aeruginosa* strains were tested for 30 days and retained in the absence of the disinfectant. Information in the literature regarding stability of resistance of *Salmonella* or *E. coli* O157 to BKC is sparse however the results obtained here support the observations in *Pseudomonas*. The results suggest a degree of stability to both CHX and TLN, however to a lesser extent in the case of TLN. The stability of resistance observed in this study could be a result of the presence of possible separate mutations over several hundred generations leading to the maintenance of the resistant gene (Gould, 1999). Previous resistance to TLN has been shown to decrease in *Staphylococcus aureus*, with subculture in the absence of TLN (Suller & Russell, 2000), which does not entirely correlate with the findings in *E. coli* O157.

2.5 Conclusion

Bacterial resistance to antimicrobial agents is a rapidly growing problem. A number of different factors are potentially involved but a widely accepted contributor is the overuse and abuse of drugs in human and animal medicine; bacteria repeatedly exposed to sub-lethal concentrations of antibacterials rapidly develop decreased susceptibility rendering them ineffective.

The current study focused on the development of resistance through sub-lethal passage in antimicrobials in order to investigate possible mechanisms facilitating resistance. Stable resistance was acquired in all strains investigated, the continuity of which was ensured by API 20E and RAPD characterisation.

Data from analysis of growth curves suggested that erythromycin resistance was associated with a decrease in growth which was demonstrated by all ERY-resistant strains (except *Salm.* Typhimurium). Slower growth was also seen in some strains resistant to CHX, BKC and TLN although no obvious pattern was observed. In most of the cases where resistant strains grew slower than the parent the length of the lag phase was significantly longer with the exception of *E. coli* O157 resistant to TLN in which the lag phase although extended did not reach significance. The slower growth of this strain was found to be attributable to the significantly slower growth rate when compared to the parent strain. To varying degrees a reduced susceptibility to all antimicrobials investigated was observed in all isolates tested. Although laboratory conditions are different to real-life situations this study suggests that the potential for the development of resistance in the environment exists.

Chapter 3. Cross-resistance of *Salmonella enterica* & *Escherichia coli* to antimicrobials.

3. Introduction

Antibiotics and biocides used to be extremely effective in combating bacterial pathogens, however, their current effectiveness is potentially compromised due to sustained misuse. The inclusion of biocides such as BKC, CHX and TLN in particular has become worryingly commonplace in home cleaning and hygiene products, where their contribution to product efficacy is debatable. In-use concentrations are normally substantially higher than MIC values and so clinical failure is less likely to occur as a result of a small increase in the MIC of a biocide (Murtough *et al*, 2001), however there is a high risk in the domestic setting, where the concentration is often poorly controlled.

Bacterial pathogens do not only survive the threat of antibiotics and biocides they may also thrive. More specifically, there has been a persistent increase in the occurrence of antibiotic and biocide resistance in many 'commonplace' bacterial pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VSE) and multidrug-resistant Gram-negative bacteria (Russell, 2002b).

The basic mechanisms of action of antibiotics are generally well documented compared with those of biocides, which are so far poorly understood (Maillard, 2002). However, with respect to Gram-negative bacteria it has been proposed that antibiotics and biocides share common mechanisms of resistance (Tattawasart *et al.*, 1999; Chuanchuen *et al.*, 2001; Poole, 2002; Russell, 2002b). It has become clear that the

up-regulation of multidrug efflux systems are increasingly recognised as resistance determinants (Nikaido *et al.*, 1998; Levy, 2002b), capable of accommodating both antibiotics and biocides (Poole, 2002) and can provide cross-resistance to other drugs (Levy, 2002b).

This chapter describes a study focused on the potential cross-resistance between antibiotics and biocides in *Salmonella enterica* serovar Enteritidis, Typhimurium and Virchow as well as in *Escherichia coli* O157, *E. coli* O55 and *E. coli* K-12.

3.2 Methods & Materials

3.2.1 Bacterial strains

Escherichia coli O157:H7 were VT-negative strains obtained from the National Collection of Type Cultures; NCTC 12900 and NCTC 43888. *Escherichia coli* O55:H7, *E. coli* O55:H29 and *E. coli* O111:H24 were clinical strains from Birmingham Heartlands Hospital, Birmingham, UK, which were isolated from stool specimens. *Escherichia coli* K-12 (W3110) was obtained from the American Type Culture Collection (ATCC 27325) and *E. coli* K-12 (MRE 600) was obtained from National Collection of Industrial and Marine Bacteria (NCIMB 10115). All strains were stored on Microbank beads (Pro-lab Diagnostics, Neston, UK) at -70°C and cultured at 37°C on Nutrient agar (Oxoid, Basingstoke, UK) and in Nutrient broth (Lab M, Lancashire, UK) where appropriate.

3.2.2 Antimicrobial agents

All antimicrobial agent disks and tablets were supplied by Oxoid, Basingstoke, UK and Adatab, Merseyside, UK, respectively, unless otherwise stated. These included amoxicillin (AMX-disc, 25μg/ml; tablet, 32μg/ml), amoxicillin/clavulanic acid (AMC-disc, 30μg/ml; tablet, 32μg/ml), chloramphenicol (CHL-disk, 30μg/ml; tablet, 32μg/ml), ciprofloxacin (CIP-disc, 1μg/ml; tablet, 0.8μg/ml), clindamycin (CLI-disc, 2μg/ml; tablet, 0.1μg/ml), colistin sulfate (CS-disc, 25μg/ml; tablet, 0.8μg/ml), gentamycin (GEN-disc, 10μg/ml; tablet, 0.8 μg/ml), imipenem (IPM-disc, 10μg/ml), rifampicin (RIF-disc, 5μg/ml; tablet, 0.2μg/ml), tetracycline (TET-disc, 10μg/ml; tablet, 32μg/ml) and trimethoprim (TMP-disc,1.25μg/ml; tablet, 0.8μg/ml). Fusidic acid (FD-disc, 10μg/ml; tablet, 32μg/ml) and vancomycin (VAN-disc, 5μg/ml; tablet, 0.4μg/ml) were included as negative controls as they have no activity against the

Enterobacteriaceae. Erythromycin (ERY) was purchased from Sigma, Poole, UK. Biocides benzalkonium chloride (BKC) (Fluka, Buckinghamshire, UK) and chlorhexidine hydrochloride (CHX) (Sigma, Poole, UK) were supplied as laboratory standard powders of known potency and triclosan (TLN) (Aquasept, Oldham, UK) was purchased as a laboratory standard solution. All solutions were filter sterilised using a 0.2μm cellulose syringe filter (Nalgene, Leicester, UK).

3.2.3 Selection of *E. coli* mutants

Selection of resistant mutants by serial passage in sub-lethal concentrations of TLN was described previously in chapter 2. Selection was performed on three independent occasions.

3.2.4 Minimum Inhibitory Concentration (MIC)

The MIC was determined using a standard broth dilution method, (NCCLS, 1997) carried out using a two-fold dilution of each antibacterial agent and was established as the lowest concentration of the antibiotic/biocide inhibiting growth. Refer to Chapter 2; section, 2.2.10.

3.2.5 Cross-Resistance to Antimicrobial Agents & Biocides

Cross-resistance towards various antibiotics and biocides was determined using the Stokes' method (Anon, 1998). Suspensions of the parent *Escherichia coli* and *Salmonella enterica* serotypes were inoculated over the central portion of the surface of separate Mueller-Hinton plates using a rotary plater (Denley Instruments Ltd., Sussex, UK) leaving an outer 1cm ring. Resistant *E. coli* and *Salmonella enterica* strains were inoculated onto the remaining perimeter of the plate and antibiotic discs

placed at the interface between the parent and the resistant strain. Plates were incubated overnight at 37°C and examined for cross-resistance and antibiotic susceptibility by comparing zones of clearance around the disks. An increase in the zone of clearance of the resostant strain compared to the parent strain of >2mm was considered indicative of resistance which was then confirmed by broth micro-dilution assays. Experiments were repeated a minimum of six times.

A range of antimicrobial agents was used, including broad-spectrum antibiotics and those used clinically against *E. coli* (CIP and TMP). In addition VAN and FD were used as control agents as these are not active against *E. coli*, or *Salmonella enterica*. Cross–resistance of TLN resistant strains to BKC and CHX was also determined.

3.3 Results

3.3.1 Selection of *E. coli* mutants

Exposure to TLN for all isolates investigated is shown in Figure 3.1. All trained strains showed an elevated level of resistance to TLN. The MIC of TLN increased from 0.25mg/L to 2048mg/L in *E. coli* K-12 W3110 and from 0.5mg/L to 2048 mg/L in *E. coli* K-12 MRE 600. In *E. coli* O55:H7 the MIC increased from 1mg/L to 2048mg/L, whereas in *E. coli* O55:H29 the MIC increased from 0.25mg/L to 2048mg/L. The MIC of *E. coli* O111:H24 increased from 0.25mg/L to 2048mg/L. Both *E. coli* O157:H7 strains shared similar profiles and demonstrated increased resistance after one sub-lethal exposure to TLN, whereas all other strains tested required supplementary exposures. Molecular fingerprinting of all isolates by RAPD confirmed strain continuity throughout passage (see chapter 2).

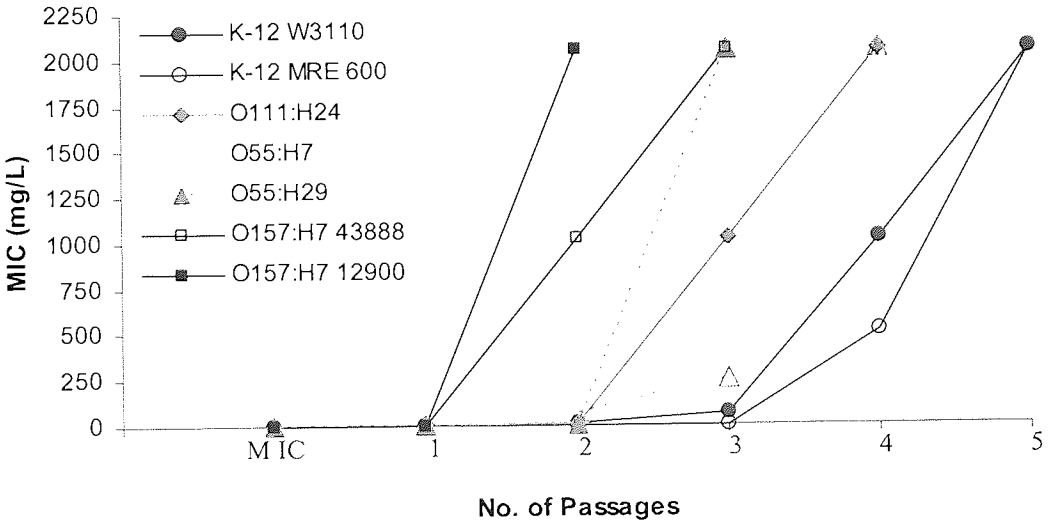


Figure 3.1: Exposure of *E. coli* O157:H7 (12900)*, *E. coli* O157:H7 (43888), *E. coli* O55:H7, *E. coli* O55:H24, *E. coli* K-12 W3110, *E. coli* K-12 MRE 600 and *E. coli* O111:24 to TLN. Both strains of *E. coli* O157 rapidly acquire increased levels of resistance following only one sub-lethal exposure to TLN, whereas other

***E. coli* strains require additional exposure.*Data adapted from chapter 2, Figure 2.2.4.**

3.3.2 Strain Continuity

The RAPD profiles for *E. coli* O55, *E. coli* K-12 and *E. coli* O111:24 confirmed strain continuity throughout passage and are shown in chapter 2, Figures 2.6 and 2.6.1, 2.7 and 2.7.1 and 2.8, respectively.

3.3.3 Cross-Resistance to Antimicrobial Agents & Biocides

Resistance or sensitivity to an antibiotic or biocide was determined by the zone of inhibition (measured in millimetres) around the impregnated disc which was placed at the interface between the parent and resistant strains. For all *E. coli* stains and where a greater than 2mm difference in the zone of clearing was observed for *Salmonella enterica* strains, a broth micro-dilution assay was performed to determine whether a true MIC change had taken place. The only case where the assay was not performed is with IPM. In each case, this was found to represent a minimum of 2 fold increase in concentration for 2-mm zone differences, and a MIC of as much as 9 tube doubling dilution units was found for 11-mm zone differences.

In *E. coli* O157 cross-resistance between antibiotics and biocides occurred frequently. The results are summarised in Tables 3.1 & 3.2 and in Figure 3.2. BKC- resistant strains did exhibit reduced susceptibility to AMC, AMX, CHL, TET, TLN and TMP and TLN-resistant ones showed signs of a higher degree of resistance to the same antibiotics plus ERY and CIP. Cross-resistance between biocides was only observed in one circumstance; specifically when bacteria were selected for resistance to CHX

they consequently demonstrated cross-resistance to TLN. However, cross-resistance between biocides and other antibiotics was demonstrated with resistance to both BKC and TLN. Exposure of *E. coli* O157 to ERY also conferred cross-resistance to several other antibiotics including CHL, CIP, TET and TMP as well as to the biocide TLN. No resistance or sensitivity to VAN and FD was observed as expected. Cross-resistance to antibiotics and biocides in other *E. coli* serotypes was demonstrated only in a minority of cases; triclosan-resistant *E. coli* K-12 demonstrated decreased susceptibility only to CHL from a panel of different antimicrobial agents (Figure 3.3) and triclosan-resistant *E. coli* O55 strains exhibited a decreased sensitivity to just TMP (Figure 3.4).

Cross-resistance to antibiotics and biocides was demonstrated in a majority of cases, but not in *Salm.* Enteritidis. In *Salm.* Enteritidis cross-resistance occurred only between ERY and CHL. *Salmonella* Typhimurium demonstrated cross-resistance between antibiotics and biocides as is apparent with ERY and CHX, as well as between other biocides and specifically CHX. The results for *Salmonella enterica* serovars Enteritidis, Typhimurium and Virchow are shown in Tables 3.3-3.7, where the zones of inhibition and the MICs where possible are determined.

When *Salm.* Virchow was tested there was a high degree of cross-resistance between antibiotics and biocides, (e.g., between ERY and TLN and between ERY and CHX). Generally, cross-resistance was observed between ERY and CHL, ERY and TMP, BKC and AMX, BKC and AMC, BKC and CHL, BKC and IPM, BKC and TLN and CHX and TET, and CHX / TRI. The results are summarised in Tables 3.5 (zones of inhibition) and 3.7 (MICs) and in Figure 3.5.

Table 3.1

MICs (mg/L) & Zones of Inhibition of parent and resistant strains of *E. coli* O157:H7 (12900) resistant to ERY, BKC & CHX, respectively

Strain		MICs & Zones of Inhibition of parent strains/MICs & zone of inhibition of resistant strains[a] with the following antibacterial:													
		AMC	AMX	BKC	CHL	CHX	CIP	CLI	CS	FD	GEN	RIF	TET	TLN	TMP	VAN
ERY-	**(mm)**	12/12	11/11	5/5	**14/7**	4/4	**17/9**	0/0	9/9	0/0	12/12	4/4	10/7	**20/5**	**14/12**	0/0
resistant	**(mg/L)**	16/16	16/16	16/16	**32/128**	16/32	**2/16**	0/0	16/16	0/0	8/8	>256	16/32	**4/64**	**32/128**	0/0
BKC-	**(mm)**	**12/0**	**12/0**	**6/0**	**19/0**	**8/0**	14/14	0/0	10/10	0/0	13/13	5/5	**10/4**	**18/0**	**14/0**	0/0
resistant	**(mg/L)**	**8/256**	**8/128**	**16/1024**	**64/256**	**8/128**	32/32	0/0	16/16	0/0	8/16		**16/64**	**4/128**	**32/>256**	0/0
CHX-	**(mm)**	**12/14**	**12/15**	4/3	14/15	**8/3**	14/14	0/0	9/10	0/0	13/14	5/5	10/10	**19/15**	14/13	0/0
Resistant	**(mg/L)**	**32/16**	**16/4**	8/8	32/32	**8/128**	32/32	0/0	8/16	0/0	16/16	>256	16/16	**4/8**	32/32	0/0

[a] Boldfaced data indicate cross-resistance.

Table 3.2

MICs (mg/L) & Zones of Inhibition of parent and resistant strains of *E. coli* resistant to TLN.

Strain[b]		MICs & Zones of Inhibition of parent strain/MICs & zone of inhibition of resistant to TLN strain with the following antibacterial:														
		AMC	AMX	BKC	CHL	CHX	CIP	CLI	CS	FD	GEN	RIF	TET	TLN	TMP	VAN
E. coli	**(mm)**	6/5	7/7	3/3	**9/6[c]**	7/7	12/10	0/0	8/6	0/0	10/12	0/0	7/6	**14/0**	10/8	0/0
K-12	**(mg/L)**	8/8	8/8	16/16	**16/256**	8/8	4/4	>256	16/16	>256	8/8	256/256	32/32	**0.25/1024**	32/32	>256
E. coli	**(mm)**	12/12	13/13	0/0	10/10	4/4	17/17	0/0	10/10	0/0	15/15	3/3	16/14	**16/0**	**14/10**	0/0
O55:H7	**(mg/L)**	16/8	8/8	16/16	16/8	16/32	2/2	>256	16/16	>256	8/16	>256	32/32	**1/2048**	**32/256**	0/0
E. coli	**(mm)**	**11/0**	**13/0**	**6/0**	**13/5**	**6/0**	14/14	0/0	9/10	0/0	12/12	5/5	17/14	**11/4**	**13/0**	0/0
O157:H7	**(mg/L)**	**4/256**	**32/>256**	**16/>256**	**32/256**	**32/256**	2/2	>256	8/16	>256	16/16	>256	32/>256	**0.25/2048**	**64/>256**	0/0

[b]*Escherichia coli* K-12 (W3110) and *E. coli* O157:H7 (12900) were tested
[c]Boldfaced data indicate cross-resistance

Table 3.3: Zones of Inhibition (mm) of Parent & Resistant strains[d] of *Salm.* Enteritidis, respectively

	AMC	AMX	BKC	CHL	CHX	CIP	CLI	CS	ERY	FD	GEN	IPM	RIF	TET	TLN	TMP	VAN
ERY	15/15	15/15	4/4	**14/11**	10/10	16/16	0/0	10/10	**10/3**	0/0	14/14	16/16	5/5	10/10	13/13	13/13	0/0
BKC	14/14	14/14	**11/6**	14/14	6/6	16/16	0/0	10/10	0/0	0/0	16/16	16/16	4/4	10/11	17/17	10/10	0/0

Table 3.4: Zones of Inhibition (mm) of Parent & Resistant strains of *Salm.* Typhimurium, respectively

	AMC	AMX	BKC	CHL	CHX	CIP	CLI	CS	ERY	FD	GEN	IPM	RIF	TET	TLN	TMP	VAN
ERY	13/15	16/16	4/3	12/11	**14/8**	17/17	0/0	9/9	**10/3**	0/0	16/16	15/15	5/0	7/8	**15/13**	10/10	0/0
BKC	14/14	15/14	**12/1**	15/15	**7/5**	13/15	0/0	9/9	0/0	0/0	13/11	17/16	4/4	6/9	4/3	13/13	0/0

Table 3.5: Zones of Inhibition (mm) of Parent & Resistant strains of *Salm.*Virchow, respectively

	AMC	AMX	BKC	CHL	CHX	CIP	CLI	CS	ERY	FD	GEN	IPM	RIF	TET	TLN	TMP	VAN
ERY	16/16	16/15	0/0	**16/13**	0/0	15/15	0/0	13/13	**8/0**	0/0	14/16	17/17	5/5	11/10	**21/0**	**12/6**	0/0
BKC	**16/0**	**16/1**	**5/0**	**14/2**	**9/4**	0/0	0/0	9/11	4/4	0/0	16/15	**16/12**	5/5	8/8	**17/0**	**14/0**	0/0
CHX	15/15	15/15	0/0	0/0	**10/5**	15/15	0/0	10/10	5/5	0/0	16/15	16/16	5/5	**10/8**	**14/0**	16/16	0/0

[d]Zones of inhibition are measured in millimetres. Boldfaced data indicate cross-resistance.

Table 3.6: MICs (μg/ml) of CHL, CHX & TLN towards Parent & ERY & BKC Resistant strains of *Salm. Enterica* serovars Enteritidis and Typhimurium, respectively

	Salm. Enteritidis	*Salm.* Typhimurium	
	CHL	CHX	TLN
ERY	16/64	16/64	16/4
BKC	-[e]	32/128	-

Table 3.7: MICs (μg/ml) of a panel of antimicrobial agents towards Parent & ERY, BKC & CHX Resistant strains of *Salm. enterica* serovar Virchow, respectively

	AMC	AMX	CHL	CHX	TET	TLN	TMP
ERY	-	-	8/32	-	-	2/128	16/128
BKC	16/256	16/256	16/256	8/128	-	4/256	16/256
CHX	-	-	-	32/128	32/128	16/256	-

[e]Dash (-) indicates that no cross-resistance was observed and thus the MICs were not determined.

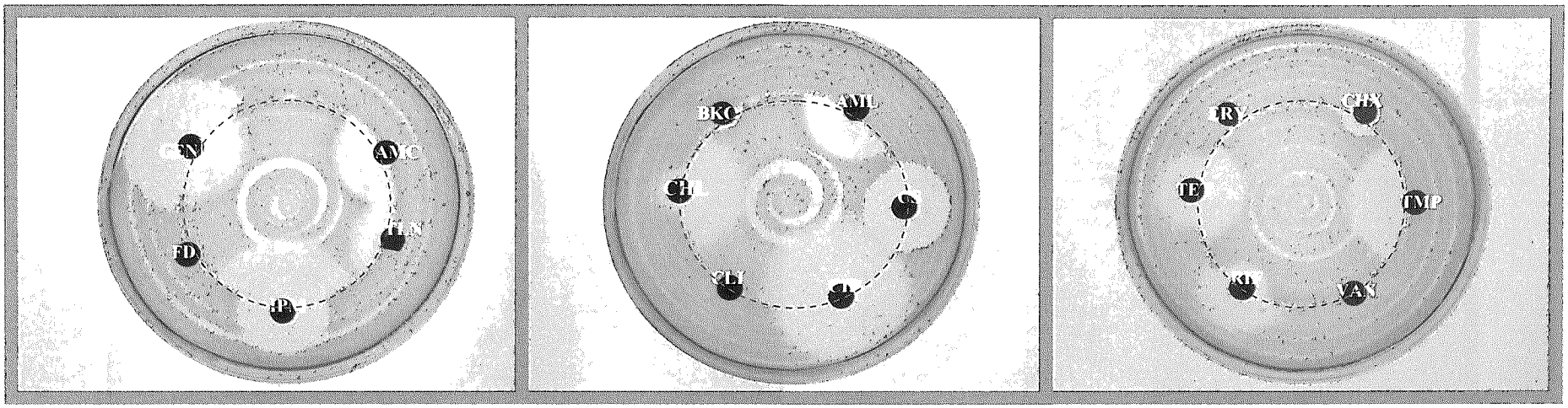


Figure 3.2: Cross-resistance between TLN-resistant *E. coli* O157:H7 (12900) strains and antibacterial agents using Stoke's method. Antibiotic / biocide abbreviations and concentrations are as defined in the section 3.2.2 in the methods section.

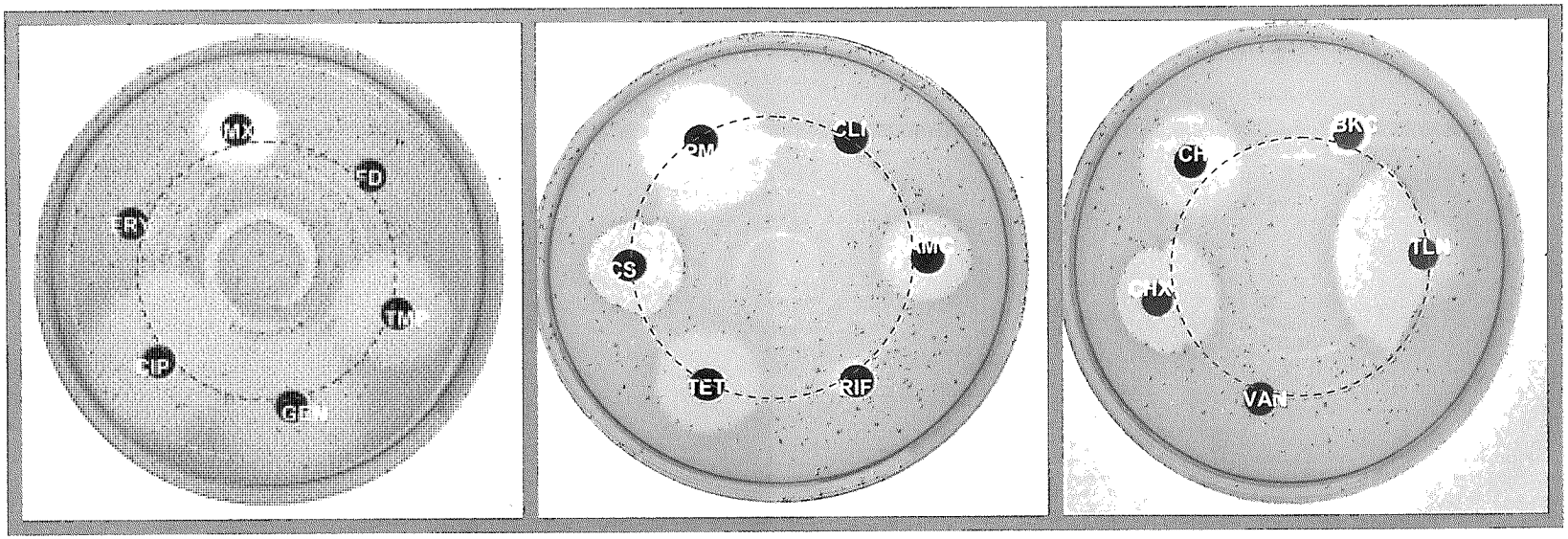


Figure 3.3: Cross-resistance between TLN-resistant *E. coli* K-12 (W3110) strains and antibacterial agents using Stoke's method. Antibiotic / biocide abbreviations and concentration are as defined in section 3.2.2 in the methods section.

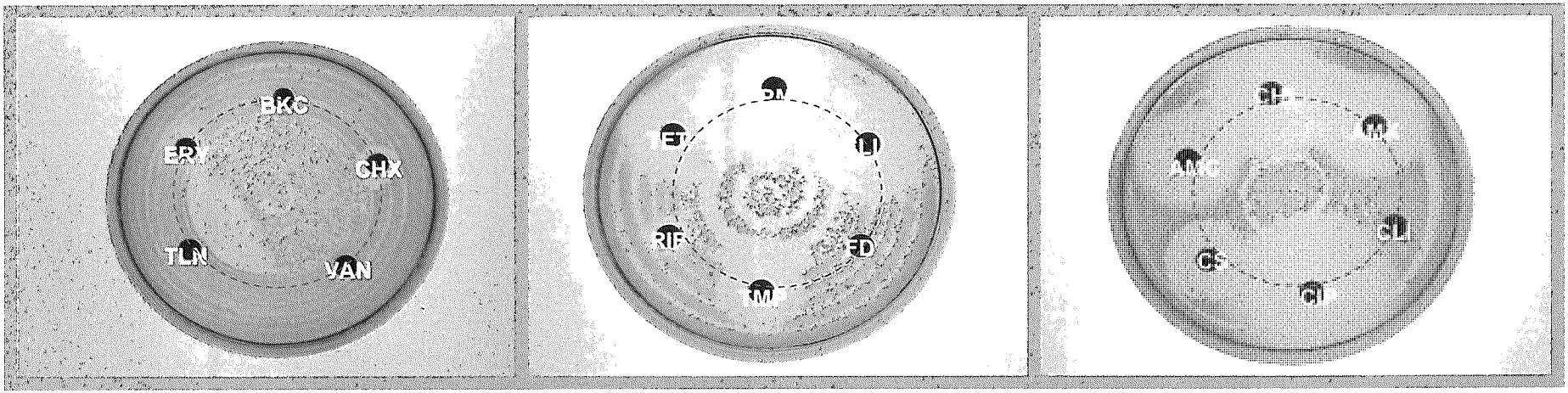


Figure 3.4: Cross-resistance between TLN-resistant *E. coli* O55:H7 strains and antibacterial agents using Stoke's method. Antibiotic / biocide abbreviations and concentration are as defined in the section 3.2.2 in the methods section.

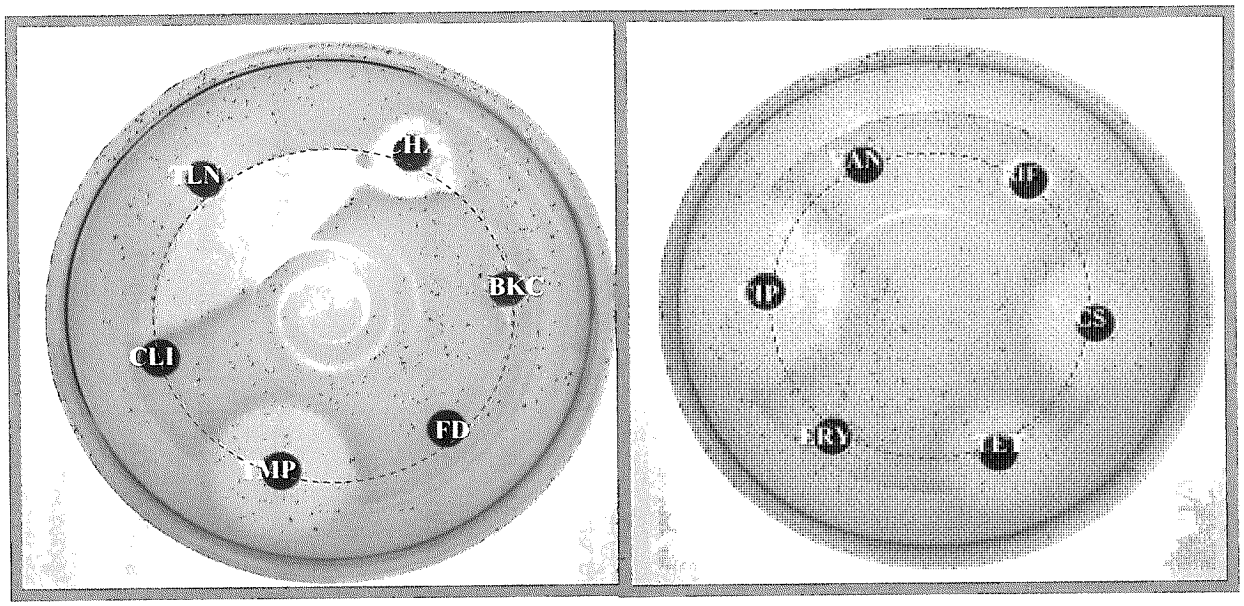


Figure 3.5: Cross-resistance between TLN-resistant *Salm.* Virchow strains and antibacterial agents using Stoke's method. Antibiotic / biocide abbreviations and concentration are as defined in the section 3.2.2 in the methods section.

3.4 Discussion

3.4.1 Resistance to TLN in *E. coli* serotypes

In this study resistance in *E. coli* O55 and *E. coli* K-12 was readily achieved by repeated passage in sub-lethal concentrations of TLN. Exposure to relatively low concentrations of TLN led to a high-level of resistance within four passages for both strains tested. The MIC of TLN increased from 0.25mg/L to 1024mg/L towards *E. coli* K-12, whereas in *E. coli* O55 the MIC increased from 1mg/L to 2048mg/L. In general, the exposure profiles followed by *E. coli* K-12, *E. coli* O55 and *E. coli* O111 share similarities to TLN-resistant *E. coli* O157; all strains were initially extremely sensitive to low concentrations of TLN, in the case of *E. coli* O157 0.25mg/L, prior to any exposure, and subsequently, all acquired high level of resistance following only four sub-lethal exposures. The difference between the profiles obtained in the current study and again compared to that of *E. coli* O157 is the speed with which *E. coli* O157 acquired resistance. It was repeatedly observed that following the first passage *E. coli* O157 became resistant to extremely high concentrations (2048mg/L) of triclosan; however, this was not observed in either *E. coli* K-12, *E. coli* O55, or *E. coli* O111.

This study supports the concerns regarding triclosan resistance since it was proved possible to select laboratory acquired triclosan resistance in *E. coli*. By contrast, according to Gilbert and McBain (2004) the overuse of triclosan has been overstated, since kitchen sink drain studies suggested that antibacterial products containing triclosan failed to select for resistant strains. It may be possible however that resistant strains were colonised at a deeper part of the drain, where the concentration of triclosan was more diluted.

3.4.2 Cross-resistance

Concern about a possible linkage between antibiotic and biocide resistant strains has been expressed and there have been some instances where biocides have been claimed to select for resistant Gram-negative bacteria (Russell *et al.*, 1998). Cross-resistance between different classes of antibacterial agents including quinolones and nalidixic acid, chloramphenicol, trimethoprim and in some cases β-lactam antibiotics is a common phenomenon in Gram-negative bacteria (Sanders *et al.*, 1984; Guttmann *et al.*, 1995). In the present study, both broad – spectrum antimicrobial agents (CLI, TET, RIF, GEN, CHL, IMP, AMX, AMC) and narrow – spectrum antimicrobial agents FD and VAN were employed including those active specifically against *E. coli* and *Salmonella enterica* (TMP and CIP).

3.4.3 Cross-Resistance to Antimicrobial Agents & Biocides in *E. coli* serotypes

Triclosan-resistant *E. coli* O157 strains often showed decreased susceptibility to a range of antimicrobial agents, including CHL, ERY, IPM, TET and TMP, as well as to various biocides. The latter observation has been described previously in *Pseudomonas aeruginosa* by Chuanchuen *et al.* (2001) however, Suller & Russell (2000) demonstrated that TLN-resistant *Staphylococcus aureus* strains exhibit no increase in resistance to ERY, or TET. The fact that a link between TLN and TMP was observed is of particular concern, since this is an antimicrobial agent active against enteric pathogens such as *E. coli* (Andrian *et al.*, 1998; Lee *et al.*, 2001). Proposed mechanisms for TLN resistance and cross-resistance in *E. coli* are the efflux pump AcrAB and mutations in FabI active-site residues (McMurry *et al.*, 1998b). Chuanchuen *et al.* (2001) also proposed that resistance to both antibacterials and antibiotics occurs through efflux systems in *Pseudomonas aeruginosa*.

In a majority of cases, the cross-resistance expressed moved strains from the category of 'sensitive' to that of 'resistant' according to the guidelines on the antimicrobial susceptibility testing (NCCLS, 1997). This was particularly the case when *E. coli* O157 was exposed to BKC; it showed a reduction in the zone of inhibition from 19 to 0 mm in other cases, the level of resistance was not as pronounced; when *E. coli* O157 was exposed to TLN, it moved from 'intermediate sensitive' to 'resistant' to CHL. Although not observed in this study, even a level of cross-resistance below the criteria set by the NCCLS (1997) would still be important, since even a modest change in susceptibility may ultimately confer a growth advantage on a strain.

Cross-resistance of triclosan-resistant *E. coli* to a panel of antibiotics and biocides was investigated in both *E. coli* K-12 and *E. coli* O55. The data generated suggested that there was a degree of cross-resistance in *E. coli* K-12 and *E. coli* O55, however to a lesser extent than that observed in *E. coli* O157. More specifically, *E. coli* K-12 exhibited reduced susceptibility to CHL, whereas *E. coli* O55 demonstrated cross-resistance to TMP, which is important as it is a clinically used drug against *Escherichia coli*. In comparison, TLN-resistant *E. coli* O157 strains repeatedly showed decreased susceptibility to a range of antimicrobial agents, including CIP, ERY, IPM, TET and TMP, as well as to the biocides BKC and CHX. The lack of cross-resistance of the TLN-resistant *E. coli* K-12 and *E. coli* O55 also suggested that the mode of action of TLN is not shared with the other biocides tested; BKC and CHX in these specific strains.

Differences in cross-resistance profiles between *E. coli* O157, *E. coli* K-12 and *E. coli* O55 suggest that strain specific rather than general mechanisms are underlying the resistance observed, some of which may be facilitated by the additional genes *E. coli* O157 is known to possess over *E. coli* K-12; designated 'O'- islands and accounting for an additional 1,387 genes (Eisen, 2001; Perna *et al.*, 2001). It is possible that some product of this additional coding capacity is contributing to the increased resistance observed.

To our knowledge the genome sequence of *E. coli* O55 has not yet been determined, however, it is noteworthy that evolutionary studies have shown that *E. coli* O157 strains are closely related and share a common ancestor with pathogenic *E. coli* O55 strains (Whittam *et al.*, 1998a; Eisen, 2001). *Escherichia coli* O55 is the proposed progenitor of *E. coli* O157 and therefore more likely to share a common genetic background than the more distantly related *E. coli* K-12 (Whittam *et al.*, 1998b). It is interesting, therefore, that *E. coli* O55 showed an adaptation and cross-resistance profile more similar to that of *E. coli* K-12 than *E. coli* O157. This suggests that if the enhanced resistance demonstrated by *E. coli* O157 was obtained by horizontal acquisition of DNA, it is something that has occurred relatively recently.

Another possible explanation for the rapid development resistance is based on the mutator hypothesis, which may account for a speeded up evolutionary process in *E. coli* O157. LeClercq *et al.* (1996) demonstrated that more than 1% of *E. coli* O157 strains had spontaneous rates of mutation 1000-times higher than typical *E. coli* strains. Taken in consideration with work of McMurry *et al.* (1998a) who showed that mutations in the *fabI* leading to amino acid substitutions Gly 93→Val, Met 159→Thr

and Phe 203→Leu conferred resistance to triclosan, it is possible these two mechanisms may contribute to the increased adaptation rate observed in *E. coli* O157. Contrary to this idea, Whittam *et al.* (1998b) found no evidence of a genome-wide increase in the mutation rate in pathogenic *E. coli* compared with *E. coli* K-12, however they could not rule out the effectiveness of the mutator phenotype during short-term evolutionary periods.

The cross-resistance observed could be a result of the presence of active efflux pumps as has been established by the previous work of McMurry *et al.* (1998a), Russell (1999), Denyer & Maillard (2002) and Levy (2002b), among others. According to Schweizer (2001), TLN and antibiotics not only share multidrug efflux systems as common mechanism of resistance but TLN and antibiotics also cause expression of these efflux systems by selecting similar mutations in the respective regulatory loci. This is supported by other studies in *Pseudomonas aeruginosa* (Chuanchuen *et al.*, 2001) in addition to *Escherichia coli* (Levy, 2002b), in which strains repeatedly expressed elevated levels of resistance to a wide range of structurally unrelated antibiotics and this resistance has been shown to result from increased levels of active efflux. For instance, it has been suggested that in *P. aeruginosa* over expression of efflux pumps increased triclosan's MIC more than six fold (Chuanchuen *et al.*, 2001) and in *E. coli* over expression of the AcrAB pump increased triclosan's MIC two fold (Levy, 2002b).

3.4.3 Cross-Resistance to Antimicrobial Agents & Biocides in *Salmonella enterica*

There was good evidence that some types of biocide resistance could provide cross-protection in certain organisms. For *Salm.* Typhimurium and *Salm.* Virchow cross-

resistance between antibacterial agents and biocides was observed, however, for *Salm.* Enteritidis this was not the case, however.

Cross-resistance between antibacterial agents or biocides and TLN was readily achieved in *Salm.* Virchow and in one instance in *Salm.* Typhimurium, between ERY and TLN (Table 3.4). The widespread use of antimicrobial products containing TLN has been suggested as a possible cause of cross-resistance to other antibacterial agents (Braid and Wale, 2002). In *Salm.* Virchow biocide resistance is most probably associated with exposure to CHX, since when exposed to BKC this organism showed an reduced sensitivity to CHX, but when exposed to CHX it did not display increased resistance to BKC. These data confirms the general opinion that biocides act on a multifaceted manner. More specifically, suggests that the targets of BKC and CHX are variable and even though some could be common as suggested by the cross-resistance obtained between BKC and CHX, there are some other targets specific to one of them suggesting that a reciprocal resistance mechanism does not exist between BKC and CHX.

Russell (1998) also reported that CHX-resistant strains of *Pseudomonas stutzeri* showed variable increases in resistance to quaternary ammonium compounds and to triclosan. These also suggest that CHX-resistant strains showed an elevated resistance to many antibiotics including ERY; however this was not observed for the *Salmonella* and *E. coli* O157 strains investigated in this study.

Cross-resistance between biocides and antibiotics and between different biocides has been reported in *Pseudomonas aeruginosa* by Lambert *et al.* (2001) and Murtough *et*

al. (2001), however no reports were found for *E. coli* O157 and *Salmonella*. It has been suggested that the possible linkage between them might be due to common resistance mechanisms (Suller and Russell, 2000) however this hypothesis has never been proven conclusively. The linkage might be also due to a non-specific reduction in the cell permeability, which does not allow chemically unrelated molecules into the resistant cells. This, of course, does not exclude the possibility of the presence of an active efflux (Tattawasart *et al.*, 1999). No obvious correlation could be drawn between the *Salmonella* serotype and resistance to a particular class of antibiotics or group of biocides; however, for particular strain - antibiotic - biocide combinations, strong evidence of cross-resistance was observed.

3.5 Conclusion

Development of resistance to antimicrobial agents and biocides is a particularly worrying problem which is compounded by cross-resistance mechanisms which may exist in certain pathogenic strains. In this study a high degree of cross-resistance to a range of biocides and antibiotics was observed in *Salm.* Virchow and in *E. coli* O157 and to a lesser extent in *Salm.* Typhimurium, *Salm.* Enteritidis, *E. coli* K-12 and *E. coli* O55 when strains were repeatedly exposed to sub-inhibitory concentrations of antimicrobial agents. With the increasing popularity of biocide-containing domestic cleaning products which when used inappropriately may provide sub-lethal exposure, this represents a real risk of the development of resistance and the promotion of cross-resistance to a range of antimicrobial agents. These results add to the growing body of evidence regarding the link between resistance to biocides and antibiotics, especially TLN, likely as a result of its continuous mis- and over-use. In addition, our data suggests that *E. coli* O157 not only possesses an enhanced virulence compared to closely related *E. coli* strains, but also an increased capacity to become resistant to the activity of TLN and other antimicrobial agents.

Chapter 4. Mechanisms of Resistance in *Salmonella enterica* and in *Escherichia coli.*

4. Introduction

This study examined a range of resistance mechanisms in order to understand the exact means by which *Salmonella enterica* and *Escherichia coli* developed reduced susceptibility towards a number of hitherto clinically useful antibiotics and biocides.

4.1.2 Measurement of cell surface hydrophobicity

The measurement of CSH may be determined by methods including water contact angles, the direction of spreading (DoS), hydrophobic interaction chromatography (HIC) and microbial adhesion to hydrocarbons (MATH). The water contact angle method measures the acid-base interactions on the cell surface. These interactions have been shown to be representative of cell surface hydrophobicity (van der Mei *et al.,* 1995; 1998). Hydrophobic Interaction Chromatography (HIC) is a method that separates bio-molecules according to their interactions with hydrophobic ligands attached to an uncharged base matrix. Samples bind in high ionic strengths and are eluted as the ionic strength decreases (Amersham Bioscience, 2003). Microbial adhesion to hydrocarbons (MATH) compares the organism's preference for an aqueous phase or hydrocarbon phase based on the adhesion to a hydrocarbon such as hexadecane (van der Mei *et al.,* 1998).

4.1.3 Bacterial cell surface charge and microelectrophoresis

A number of different methods have been employed to measure the bacterial cell surface charge, however, it should be noted that the cell surface charge cannot be measured directly. In particle vicinity on the other hand, a charge equivalent to that of

the surface, grows, and this surface charge also known as the zeta potential can be measured by particle microelectrophoresis (Smith *et al.*, 1998). The method entails the application of an electric field on the particles dispersed in the cell suspension, which causes the movement of particles towards either the positive or the negative pole of the applied field; the direction they select is used as an indication of the charge they carry. It is essential to measure both the direction and the velocity of particles, in order to calculate the mobility and the zeta potential. According to Wilson and his co-workers the direction and movement is dependent on a variety of factors, which include the ionic concentration, the pH and the temperature of the medium, apart from the field strength and surface charge of the bacterium (Wilson *et al.*, 2001).

4.1.4 Outer membrane proteins & LPS

Alterations in the outer membrane proteins and LPS have been previously linked to changes in resistance to antimicrobial agents (Hancock, 1997). Thus in this study possible permeability changes were investigated by examining the Omps and LPS of *Salmonella enterica* and *E .coli* strains using SDS-PAGE. Following SDS-PAGE examination, the Omps were stained by Coomassie blue, whereas LPS by silver. An alternative test to investigate LPS is by detecting the compound 2-keto-3-deoxyoctonate (KDO) – a component present only in LPS, by a colorimetric assay (Karkhanis *et al.*, 1978).

4.1.5 Efflux pumps

Efflux activity can be observed by employing the use of the fluorescing chemical ethidium bromide. The biochemical nature of efflux systems can be monitored by

observing the kinetics of ethidium accumulation and efflux by the fluorimetric method (Baranova and Neyfakh, 1997 and Aase *et al.*, 2000).

One way of investigating efflux activity is to use chemicals that are known to inhibit the efflux pumps. Reserpine is an indole alkaloid obtained by extraction from the roots of *Rauwolfia serpentin*, which has been shown to inhibit efflux pumps; for example it was shown to reverse the resistance of *Bacillus subtilis* to fluoroquinoles. It has also been shown to reverse the resistance of *Streptococcus pneumoniae* to ethidium bromide and the resistance to fluoroquinolone by *Staphylococcus aureus.* Reserpine inhibits efflux activity by the disruption of the proton motive force so can only be used to test the presence of efflux pumps that operate in this way such as the MFS, SMR and RND families. Other synthetic efflux inhibitors exist including indole derivatives and biphenyl urea which have been shown to be eight fold more potent than reserpine (Nelson, 2002).

Carbonyl cyanide m-chlorophenylhydrazone (CCCP) and tetrachlorosalicylanilide also inhibit efflux pumps. They work as uncouplers, which collapse the proton gradient across the cytoplasmic membrane (Nikaido, 1996). In this study the presence of a putative active efflux pump system was investigated in the presence of both reserpine and CCCP.

4.2. Methods & Materials

4.2.1 MATH Assay

Microbial adhesion to hydrocarbons such as n-Hexadecane as used in this study, is generally considered to be the measure of the organisms' cell surface hydrophobicity.

The organism was grown to stationary phase and cells from the overnight culture were centrifuged at 5,000x g for ten minutes and re-suspended in PUM buffer pH 7.1 to an optical density A_{470nm} of 0.5. A volume of 1.5ml of the adjusted suspension was placed into glass tubes (diameter 1cm, length 7.5cm) containing 300μl of n-Hexadecane. The test tubes were left to stand for 10 minutes at room temperature and then were vortexed at full speed for 45 seconds. Again, they were allowed to settle for ten minutes to allow phase separation. n-Hexadecane phases were carefully removed by pipetting and discarded. The tubes were then placed in the fridge to cooled at 4°C for further ten minutes. Removal of the solid n-Hexadecane from the tubes was conducted at 4°C, using one plastic loop for each. The samples were then transferred into micro-cuvettes and the OD_{470} determined.

The partitioning of the bacterial suspension was expressed as the percentage of cell surface hydrophobicity and was calculated using the following equation:

$$\% \text{ Cell Surface Hydrophobicity} = [(\text{Initial } OD_{470} - \text{Final } OD_{470}) / \text{Initial } OD_{470}] \times 100$$

4.2.2 Microelectrophoresis

Bacterial cells were grown in Nutrient Broth to stationary phase centrifuged at 5,000x g for ten minutes and re-suspended in potassium chloride solution 1mM (KCl) at a concentration of approximately $1x10^7$ cells/ml. The cell suspension was then placed in

an electrophoresis cell of a Zetamaster Particle Electrophoresis Analyser, (Brookhaven Instruments, New York, U.S.A.) and a voltage applied across the cell to measure the ζ potential. When the test was completed the electrodes were rinsed with 70% ethanol.

4.2.3 Preparation and analysis of outer membrane extracts - Sarkosyl method

A volume of 500ml of an overnight bacterial culture was centrifuged at 10,000x g for ten minutes and resuspended in 10ml H_2O. The suspended cells were broken by three passages through a French pressure cell (NIKE, Escilstuna, Sweden) at 5-ton per square inch, or by sonication. Sarkosyl detergent (N- lauroyl sarcosine, Sigma, Poole, UK) was added to the broken cells at a final concentration of 2% ($^{w}/_{v}$). The lysate was then centrifuged for five min at 5,000x g at 5°C to remove any unbroken cells. The outer membrane material was recovered as a pellet and further centrifuged for one hour at 10,000x g at 5°C. The white material around the black pellet was resuspended in sdH_2O. A volume of 1ml of this was transferred in an Eppendorf tube and was centrifuged for 30 minutes at 14, 000x g. The pellet was resuspended in 1.5ml sdH_2O. A volume of 30μl of the outer membrane extract was subjected to SDS-PAGE analysis.

4.2.4 Sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis

The outer membrane proteins isolated in section 4.2.3 were separated by SDS-PAGE. An 11% ($^{w}/_{v}$) separating gel (Table 4.1) was poured against glass until the level reached 1.5cm from top of the front plate and allowed to set. A 5% ($^{w}/_{v}$) stacking gel (Table 4.1) was then poured on the top of the separating gel until it overflowed and a

15 lane comb was inserted 4cm above the stacking gel. Outer membrane proteins were denatured in unequal volume of sample denaturing buffer (5ml 10% ($^{w}/_{v}$) SDS, 2.5ml Tris HCl pH 6.8, 5ml dH_2O, 2.5ml glycerol, 0.25ml 2-mercaptoethanol, 0.01g bromophenol blue) by heating for ten minutes at 100°C. A volume of 10µl sample was loaded into each well of the polyacrylamide gel with one well containing a 5µl of 1kbp pre-stained molecular weight marker (SDS-PAGE pre-stained Broad range marker, Biolabs, New England). The polyacrylamide gel was placed in SDS electrode buffer (25mM Tris, 10% ($^{w}/_{v}$) SDS, 200mM glycine pH 8) and samples were electrophoresed for 50 minutes at 200 volts using Bio-Rad Mini Protean II apparatus (Bio-Rad, Hemel Hempstead, UK).

Table 4.1: Preparation of polyacrylamide gel

	Separating Gel 11% ($^{w}/_{v}$)	**Stacking Gel 5% ($^{w}/_{v}$)**
dH_2O	3.75ml	4ml
1.5M Tris-HCl pH 8.8	3ml	N/A*
0.5 M Tris HCl pH 6.8	N/A	1.875ml
Acrylamide stock I**	2.75ml	N/A/
Acrylamide stock II***	N/A	1.25 ml
10% SDS	0.25ml	75µl
10% ammonium persulfate	35µl	25µl
TEMED (Sigma, Poole, UK)	25µl	20µl

* N/A for not applicable

**Acrylamide stock I: 44% ($^{w}/_{v}$) acrylamide, 0.8 % ($^{w}/_{v}$) Bis (N, N^{1}-methylene-bis-acrylamide (Severn Biotech Ltd., UK)

*** Acrylamide stock II: 30% ($^{w}/_{v}$) acrylamide, 0.8 % ($^{w}/_{v}$) Bis (N, N^{1}-methylene-bis-acrylamide (Severn Biotech Ltd., UK)

4.2.5 Coomassie blue staining

On completion of electrophoresis the gel was placed in Coomasie blue stain (20% ($^{v}/_{v}$ methanol, 10% ($^{v}/_{v}$) acetic acid, 0.1% ($^{w}/_{v}$)) Coomassie brilliant blue (Sigma, Poole, UK) for approximately 1 hour at room temperature with agitation. The Coomassie blue stain was discarded and replaced with destaining solution (20% ($^{v}/_{v}$ methanol, 10% ($^{v}/_{v}$) acetic acid)) for one hour. The destaining solution was replaced until the solution remained clear.

4.2.6 Preparation of LPS

A volume of 1.5ml of whole cells of *Salmonella enterica* or *E. coli* O157 strains were grown in Nutrient Broth and harvested by centrifugation (10,000x g, ten minutes). Cells were suspended in 100µl sdH_2O and 100µl of denaturing buffer added and the samples heated at 100°C for ten minutes. Following cooling, 20µl of sample buffer containing 2.5mg/ml protease K (Sigma, Poole, UK) was added to the samples, which were then heated at 60°C for one hour. The LPS were then run on SDS-PAGE gel (as in section 4.2.4) and stained by silver (section 4.2.7)

4.2.7 Modified silver stain for LPS

Sodium-Dodecyl-Sulfate Polyacrylamide gel Electrophoresis LPS preparations were stained by using a modification of the silver staining method (Fomsgaard *et al.*, 1990). The LPS in the gel were oxidised by immersion in 0.7% periodic acid in 40% ethanol-5% glacial acetic acid at room temperature for 20 minutes. The gel was washed three times with dH_2O for five minutes. It was then stained for ten minutes with freshly prepared staining solution which was prepared as follows; a 4ml volume of concentrated ammonium hydroxide was added to 56ml of 0.1M sodium hydroxide.

Following the addition of 200ml H_2O, 10ml of 20% ($^{w}/_{v}$) silver nitrate (Fisons Scientific equipment, Loughborough, England) was added in drops with stirring. The final volume was adjusted to 300ml with distilled H_2O. The gel was then washed three times with dH_2O for five minutes. The colour was developed by reduction in 200ml of H_2O containing 10mg of citric acid and 0.1ml of 37% formaldehyde. The gel was photographed immediately and the colour reaction was stopped by exposure to 10% acetic acid for 1 minute followed by repeated washings in dH_2O.

4.2.8 Efflux pumps: determination of reserpine and CCCP MICs

A standard broth dilution method specified by the National Committee for Clinical Laboratory Standards (NCCLS, 1997) was used for the MIC determination of reserpine (Sigma, Poole, U.K.) or CCCP (Sigma, Poole, U.K.) using a final inoculum of $1x10^8$ bacteria. The MICs of reserpine or CCCP were determined in duplicate using parent *Salm.* Virchow and *E. coli* O157 strains. The bijoux bottles were incubated at 37°C overnight. The MIC was determined as the lowest concentration of reserpine or CCCP to inhibit growth.

4.2.8.1 The use of reserpine and CCCP in efflux assessment

The parent and resistant strains were tested at the same time and in duplicate. Antimicrobial agents were serially diluted across a series of ten bijoux containing 2ml of double strength nutrient broth (Lab M, Lancashire, U.K.). Concentrations were used such that the MIC for both the parent and resistant strains were covered. A 2ml sample of filter sterilised (Nalgene, Leicester, U.K.) 2μg/ml reserpine, or 50μM CCCP was then added to each bijoux. A 200μl volume of an overnight suspension

(~10^8 cfu/ml) was added to each of the bijoux bottles. A control to test reserpine or CCCP inhibitory activity was carried out using a bijou without antimicrobial agent. The MIC of the resistant strain was also confirmed in the absence of reserpine or CCCP. The bijoux were then gently vortexed and incubated overnight at 37°C. The MIC for the parent and resistant strain in the presence of reserpine or CCCP was recorded. The MIC of the resistant strain in the absence of reserpine or CCCP was confirmed and the control bijou without antimicrobial agent was checked for growth.

4.2.9 Statistical Analysis

All data were checked for normality using the Kolmogorov-Smirnov test. The MATH and microelectrophoresis results were statistically analysed using a single factor analysis of variance (ANOVA) and Fisher LSD post hoc analysis. Growth curve data was analysed using a repeated measures ANOVA. All analysis was carried out using the Statistica Program (StatSoft, 2001, version 6, www.statsoft.com).

4.3 Results

4.3.1 Cell surface charge

All parent *Salmonella enterica* strains were not hydrophobic, however data from selected mutants resistant to ERY, BKC, CHX and TLN suggested that they became increasingly hydrophobic (Table 4.2). This trend reached significance (p= <0.05) for *Salm.* Enteritidis, *Salm.* Typhimurium and *Salm.* Virchow resistant to BKC, as well as for *Salm.* Enteritidis and *Salm.* Typhimurium resistant to ERY. Results were obtained from each of three individual experiments.

Table 4.2: Hydrophobicity (%) of parent & resistant *Salmonella enterica* serotypes.

Strain	% CSH	St. Error ±	Statistical Analysis
***Salm.* Virchow Parent**	0	0	
***Salm.* Virchow BKC**	67.57	0.02	P=0.000000*
***Salm.* Virchow ERY**	32.42	0.09	p=0.256553
***Salm.*Virchow TLN**	6.78	1.87	p=0.185901
***Salm.* Typhimurium Parent**	0	2.52	
***Salm.* Typhimurium BKC**	26.72	0.01	P=0.003508*
***Salm.* Typhimurium ERY**	27.28	0.02	P=0.011292*
***Salm.* Typhimurium TLN**	6.71	0.61	p=0.428552
***Salm.* Enteritidis Parent**	0	1.00	
***Salm.* Enteritidis BKC**	43.61	0.05	P=0.002217*
***Salm.* Enteritidis ERY**	15.15	0.03	P=0.002217*
***Salm.* Enteritidis TLN**	13.77	6.91	p=0.202263

* Significant at p=<0.05

In general, parent stains did not demonstrate a hydrophobic cell surface, compared to resistant strains. There was a significant difference between the CSH of parent and resistant *E. coli* O157 strains to CHX and TLN (p=<0.05), however, this difference did not reach significance for all strains. *Escherichia coli* O157 showed a significant difference in CSH between parent and resistant to CHX and TLN strains (Table 4.3).

Table 4.3: Hydrophobicity (%) of parent & resistant *E. coli* O157.

Strain	% CSH	S. Error ±	Statistical Analysis
O157 Parent	0	0.01	-
O157 BKC	6	2.75	P=0.139292
O157 CHX	9.5	1.70	P=0.033515*
O157 ERY	3.76	1.31	P=0.353416
O157 TLN	28.14	5.01	P=0.000028*
O55 Parent	0	4.12	-
O55 TLN	5.52	1.76	P=0.093011

* Significant at p=<0.05

4.3.2 Cell surface charge

In all cases of *Salmonella enterica* serotypes there was a notable change in the charge between parent and resistant strains. This was demonstrated in all three individual experiments performed for each strain (Table 4.4). An example of raw data collected from the Zetaplus particle electrophoresis analyser can be seen in appendix 1.

Table 4.4: Zeta potentials of parent & resistant *Salmonella enterica serotypes.*

Strain	Zeta Potential	St. Error ±	Statistical Analysis
***Salm.* Virchow Parent**	-6.433	1.52	-
***Salm* Virchow BKC**	-12.7	2.64	P=0.096472
***Salm.* Virchow ERY**	-35.95	3.88	p=0.000006*
***Salm.* Virchow TLN**	-6.33	1.05	P=0.977235
***Salm.*Typhimurium Parent**	-5.28	1.99	-
***Salm.*Typhimurium BKC**	-30.05	5.36	p=0.008186*
***Salm.* Typhimurium ERY**	-13.15	8.26	P=0.298987
***Salm.* Typhimurium TLN**	-3.487	0.169	P=0.809089
***Salm.* Enteritidis Parent**	-25.207	16.18	
***Salm.* Enteritidis BKC**	-26.8	10.85	P=0.914100
***Salm.* Enteritidis ERY**	-31.6	5.22	P=0.666935
***Salm.* Enteritidis TLN**	-15.57	1.7	P=0.519721

* Significant at p=<0.05

Microelectrophoresis revealed that *E. coli* strains carried a negative charge. The CSC of some resistant strains varied significantly for example in the case of strains resistant BKC (p=<0.05) from their corresponding parents, however no strong correlation emerged between CSC and resistance (Table 4.5).

Table 4.5: Zeta potentials of parent & resistant *E. coli* O157.

Strain	Zeta Potential	S. Error ±	Statistical Analysis
O157 Parent	-2.377	0.37	-
O157 BKC	-25.4	2.01	P=0.001628*
O157 CHX	-13.463	8.08	P=0.067516
O157 ERY	-1.3	1.8	P=0.846193
O157 TLN	-1.037	0.5514	P=0.809328
O55 Parent	-7.417	0.856	-
O55 TLN	-4.405	1.536	P=0.039077*

* Significant at p=<0.05

4.3.3 Outer membrane proteins

The OMP profiles and of ERY, BKC, CHX and TLN-resistant strains did not reveal any differences when compared to parent profiles. Figures 4.1, 4.2 and 4.3 demonstrate *Salm.* Virchow parent and resistant to ERY, BKC, CHX and TLN, *Salm.* Typhimurium parent and resistant to ERY, BKC and TLN and *E. coli* O157 parent and resistant to ERY, BKC, CHX and TLN visualized by Coomassie brilliant blue stain, respectively.

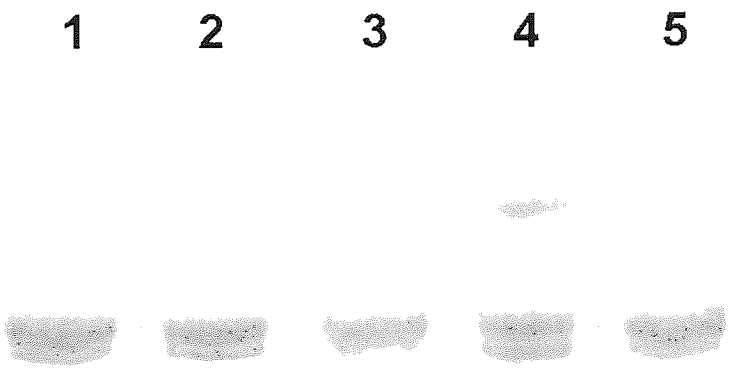


Figure 4.1: OMP profiles of *Salm.* Virchow parent (Lane 1) and resistant to ERY (Lane 2), BKC (Lane 3), CHX (Lane 4) and TLN (Lane 5) strains stained by Coomassie blue. M is a low range pre-stained SDS-PAGE standard 21.4 – 110 kDa.

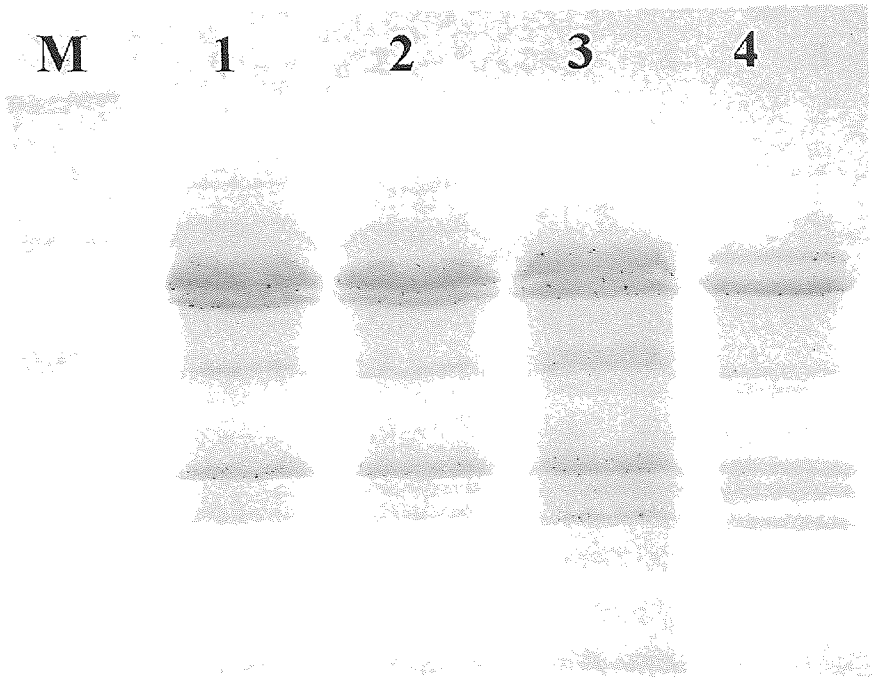


Figure 4.2: OMP profiles of *Salm.* Typhimurium parent (Lane 1) and resistant to ERY (Lane 2), BKC (Lane 3) and TLN (Lane 4) strains stained by Coomassie blue. M is a low range pre-stained SDS-PAGE standard 21.4 – 110 kDa.

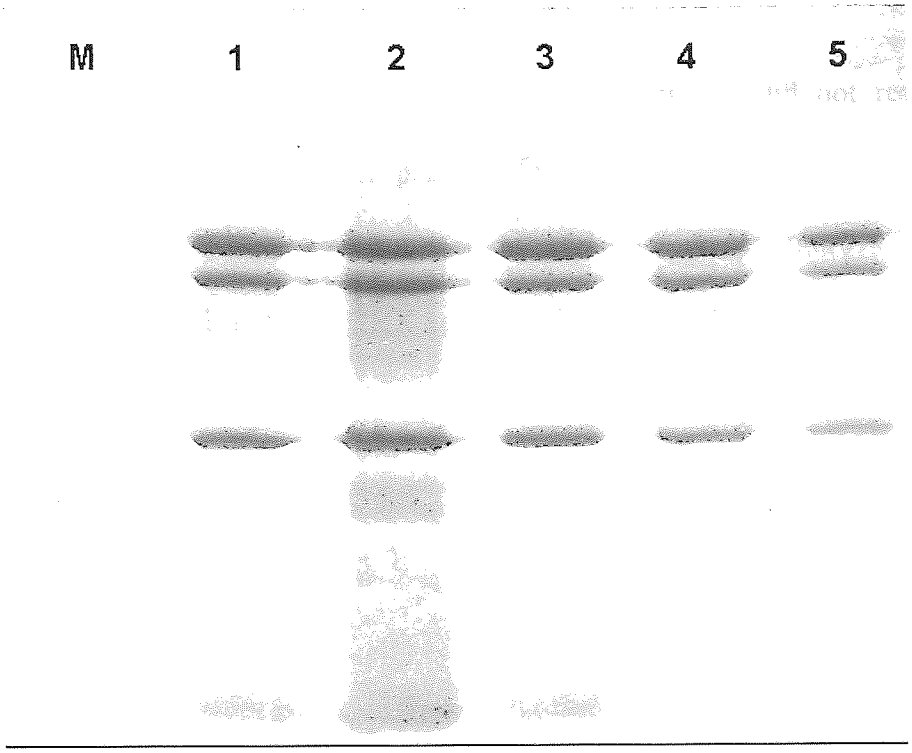


Figure 4.3: Coomassie blue stained OMP profiles from stained parent (Lane 1) and resistant to ERY (Lane 2), BKC (Lane 3), CHX (Lane 4) and TLN (Lane 5) *E. coli* O157 strains. M is a low range pre-stained SDS-PAGE standard 21.4 – 110 kDa.

4.3.4 LPS visualisation

The LPS of the ERY, BKC, CHX and TLN-resistant strains did not reveal any alterations when compared to their parents. Figure 4.4 demonstrates parent and resistant to TLN *Salm.* Virchow visualized by silver, whereas Figure 4.5 reveals parent and resistant to ERY, BKC, CHX and TLN *E. coli* O157 and analysed by silver staining.

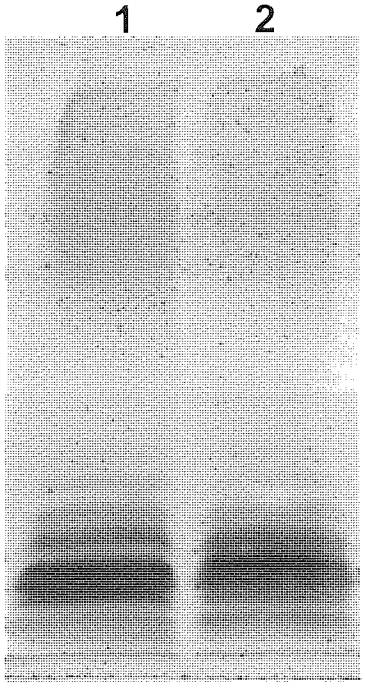


Figure 4.4: LPS profiles of *Salm.* Virchow parent (Lane 1) and resistant to TLN (Lane 2) stained by silver.

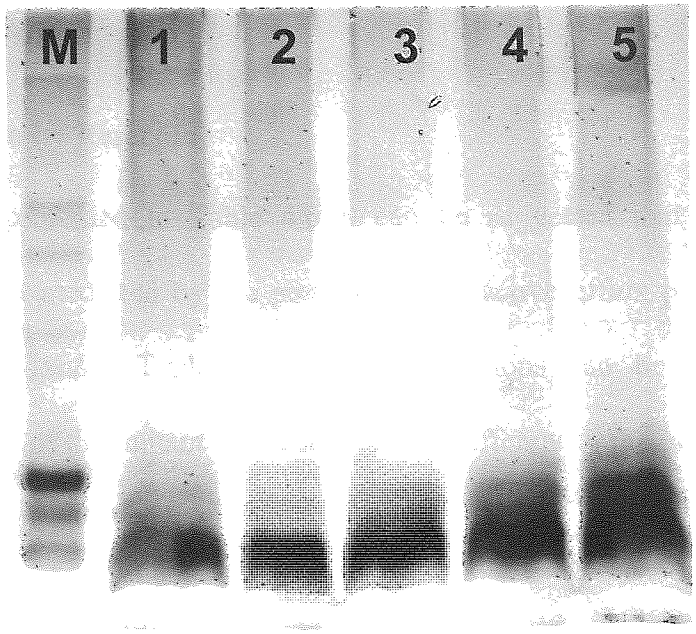


Figure 4.5: Silver stained LPS profiles from parent (Lane 1) and resistant to ERY (Lane 2), BKC (Lane 3), CHX (Lane 4) and TLN (Lane 5) *E. coli* O157 strains. M is a low range pre-stained SDS-PAGE standard 21.4 – 110 kDa.

4.3.5 Efflux Pumps

4.3.5.1 Inhibition by Reserpine

From the data presented in Table 4.6 it can be suggested that BKC and CHX resistance may be mediated by the presence of an active efflux pump in all strains investigated. For *Salm.* Typhimurium no conclusions can be drawn since the adaptation profile of the mutant is not sufficiently different to the original MIC of the parent strain.

Table 4.6: Results of efflux testing determined by reserpine inhibition.

Drug	Strain	Parent MIC in absence of reserpine	Resistant MIC in absence of reserpine	Parent MIC in presence of reserpine	Resistant MIC in presence of reserpine.
ERY	*E. coli* O157	256	1024	MIC >512	MIC >2048
	Salm. Virchow	32	512	64	4096
	Salm. Typhimurium	256	512	256	256
	Salm. Enteritidis	256	2048	256	MIC>1024
BKC	*E. coli* O157	16	128	32	32
	Salm. Virchow	4	256	16	16
	Salm. Typhimurium	32	128	32	32
	Salm. Enteritidis	32	256	32	32
TLN	*E. coli* O157	0.25	2048	16	MIC > 2048
	E. coli O55:H7	0.25	1024	1	512
	Salm. Virchow	16	1024	16	1024
	Salm. Typhimurium	8	256	4	64
	Salm. Enteritidis	16	512	4	256
CHX	*E. coli* O157	4	512	8	16
	Salm. Virchow	8	128	16	64

4.3.5.2 Inhibition by CCCP

According to Table 4.7 it can be concluded that all antibacterial resistances tested could be mediated by a presumptive efflux system. Again, it is noteworthy that for *Salm.* Typhimurium resistant to ERY any conclusions are difficult to be drawn since there is not much difference in the MIC of parent and resistant strains. In addition, two extra *E. coli* serotypes were tested with CCCP; *E. coli* O55:H7 resistant to CHX and *E. coli* K-12 (W3110) resistant to CHX. Of note, *E. coli* O157 has been re-passaged to CHX resistance for CCCP studies.

Table 4.7: Results of efflux testing determined by CCCP inhibition.

Drug	Strain	Parent MIC in absence of CCCP	Resistant MIC in absence of CCCP	Parent MIC in presence of CCCP	Resistant MIC in presence of CCCP
ERY	*E. coli* O157	256	1024	256	1024
	Salm. Virchow	32	512	32	64
	Salm. Typhimurium	256	512	256	256
	Salm. Enteritidis	256	2048	256	128
BKC	*E. coli* O157	16	128	16	16
	Salm. Virchow	4	256	16	16
	Salm. Typhimurium	32	128	32	32
	Salm. Enteritidis	32	256	32	32
TLN	*E. coli* O157	0.25	2048	2	2
	E. coli O55	0.25	1024	0.5	0.25
	Salm. Virchow	16	1024	16	16
	Salm. Typhimurium	8	256	4	4
	Salm. Enteritidis	16	512	16	32
CHX	*E. coli* O157	16	128	4	4
	Salm. Virchow	8	128	8	8
	E. coli O55:H7	16	128	16	16
	E. coli K-12 (W3110)	16	128	16	16

In summary, from Tables 4.6 and 4.7it can be concluded that resistance to ERY, BKC, CHX and TLN may be mediated by an efflux pump in all strains investigated, however those of BKC and CHX resistance could be mediated by an efflux pump belonging either to the MFS, ABC or RND superfamilies, since these resistances were reversed by both efflux pump inhibitors tested.

4.4. Discussion

There is a general consensus that antimicrobial resistance has emerged and the pace at which new antibacterials are being produced is slowing (Russell, 2002b). The cause of this trend is a direct consequence of the overuse and abuse of antibacterials (Gould, 1999; Levy, 2002a). Repeated exposure of bacteria to sub-lethal concentrations of antibiotics and/or biocides leads to the acquisition of bacterial drug resistance, since bacteria are capable to comprehend ways to fight and survive them. They are then free to multiply and cause infection resulting in an ill community with resistant strains. Understanding the mechanisms by which bacteria defend themselves has become essential as it will eventually lead to the preclusion of antimicrobial resistance (Maillard, 2002).

This study was undertaken to investigate bacterial defence towards a spectrum of biocides and erythromycin. Following exposure of *Salmonella enterica* and *E. coli* O157:H7 (12900) strains to benzalkonium chloride, chlorhexidine, triclosan and erythromycin, the investigation was centred on the elucidation of the mechanisms underlying resistance. The main resistance candidates examined in this study involved the possible presence of an active efflux pump, the cellular outer membrane and LPS composition and the cell surface hydrophobicity and charge.

4.4.1 Cell surface Hydrophobicity

Cell surface hydrophobicity is a vital factor in cellular adhesion mechanisms (van der Mei *et al.*, 1998). Alterations in cell surface hydrophobicity have been linked to changes in transmembrane penetration of some antimicrobial agents and therefore the CSH may play an important role in resistance (Kobayashi *et al.*, 1991). The physical

properties of antimicrobials including their hydrophobicity and charge play an important role for the penetration into the Gram-negative bacterial cell. According to Maillard (2002) one of the perceptible effects of the biocidal interaction with the bacterial cell is change in cell surface hydrophobicity, however, Loughlin *et al.* (2002) suggested that before lending excessive significance to any alterations obtained, it is very important to take under consideration the fact that cell surface hydrophobicity is extremely vulnerable to environmental changes. The origin of CSH can be determined by a MATH assay, which involves a comparison of the organisms' preference for the aqueous and hydrocarbon phases (van der Mei *et al.*, 1998). Thus, in this study a MATH assay was employed to determine CSH between the parent strain and the resistant strain to the highest concentration of antimicrobial agent.

Increased cell surface hydrophobicity was shown to be associated with erythromycin resistance in *Salm.* Enteritidis and *Salm.* Typhimurium, which was unexpected as erythromycin is a hydrophobic compound. This data suggested that ERY resistance and hydrophobicity are strain specific, as this was not the case in *Salm.* Virchow. To our knowledge, no other reports are available in the literature on CSH in *Salmonella enterica*. Data generated in this study also proposed that CSH was associated with BKC resistance as in all strains BKC did seem to make cells significantly more hydrophobic. Gram-negative bacteria tend to alter CSH when challenged by BKC (El Falaha *et al.*, 1985), which might be due to the cationic nature of BKC, which makes it more difficult to penetrate a hydrophobic bacterial cell wall. Tattawasart *et al.* (1999) examined *Pseudomonas stutzeri* strains resistant to chlorhexidine diacetate and cetylpyridinium chloride also proposed that some of the resistant strains were more hydrophobic than the parent strains. When the cell surface hydrophobicity was

examined only *E. coli* O157 strains resistant to CHX and TLN showed a significant increase in hydrophobicity suggesting that this may be a contributory factor to resistance. Increase in CSH has been shown to correlate with the presence of additional surface proteins (Parker and Munn, 1984). This is of interest especially when considered alongside the presence of any efflux activity, which has already been associated with ERY, BKC, CHX and TLN resistance. In this study, CSH was not associated with any change in the OMP profiles of resistant cells. Kobayashi *et al.* (1991) suggested that as cell surface hydrophobicity increases so does susceptibility to antimicrobial agents. The results of this investigation are in contrast to this however our study is in agreement with the findings of Tattawasart *et al.* (1999) and Loughlin *et al.* (2002).

4.4.2 Cell surface charge

The cell surface charge influences the entire cell polarity and is therefore vital in maintaining the optimal level of cell surface hydrophobicity necessary for cell function (Wilson *et al.,* 2001). Statistical significance was achieved for some strains but this was not consistent within one serotype or for any particular antimicrobial agent. It is unusual that a weak correlation was found between cell surface charge and hydrophobicity, as previous research suggests that the more negative the cell surface charge the greater the cell surface hydrophobicity (Wilson *et al.*, 2001). A number of studies have investigated whether there is a link between cell surface hydrophobicity and cell surface charge, however according to Smith *et al.* (1998) this depends to some extent on the type of hydrophobicity assay employed.

Cell surface charge was found to be significantly more negative in the ERY resistant *Salm.* Virchow when compared to the parent strain. In all BKC and TLN resistant strains there was a notable change in the charge of the cell although not reaching significance. This was a pattern followed in all biocides and strains tested, however at a lesser extent in *Salm.* Enteritidis. Loughlin *et al.* (2002) observed a change in zeta potential as *Pseudomonas aeruginosa* cells increased resistance to BKC however no reports are available regarding *Salmonella enterica.* It should be mentioned though that in 1977, Magnusson *et al.* suggested that a negative charge characterises the surface of rough *Salm.* Typhimurium strains that are sensitive to phagocytosis, whereas the absence of charge characterises the surface of smooth strains that are resistant to phagocytosis. This could be related to modifications in the LPS content; however this was not observed in this study.

All *E. coli* O157 resistant strains carried a negative charge; however there was no correlation between changes in the surface charge with the gradual acquisition of resistance. Cell surface charge was found to be significantly more negative, specifically in BKC resistant *E. coli* O157, however no overall general pattern emerged linking CSC and resistance

4.4.3 Outer membrane proteins & LPS

It is suggested that Gram-negative bacteria are protected from antimicrobial agents due to permeability barrier of the outer membrane (Nikaido, 2000). Lipopolysaccharides are the main components of the outer membrane of Gram-negative bacteria and are responsible for the cell impermeability characteristics (Denyer and Maillard, 2002). Thus, alterations in the outer membrane and LPS may contribute to the development of resistance, as they may prohibit influx of certain

antimicrobials. In this study LPS analysis was determined using SDS-PAGE in which the lipopolysaccharides were separated into polysaccharide chains of different lengths to produce a ladder pattern. Outer membrane and LPS profiles did not reveal any significant changes in all parent and resistant strains of *Salmonella enterica* and *E. coli* O157 strains investigated. A number of reports support that resistance in Gram-negative bacteria might be associated with changes in outer membrane, including LPS. Among them that of Loughlin *et al.* (2002) who reported that progression of resistance in *Pseudomonas aeruginosa* might be due to an alteration in some outer membrane and LPS structure. In addition, it has been proposed that resistance in *Pseudomonas stutzeri* is associated with alterations in the LPS composition (Tattawasart *et al.*, 2000). However, this study suggests that this is not a general Gram-negative phenomenon and in support, Elpec *et al.* (2001) concluded that no direct association between antibiotic resistance and OMPs including LPS in *Salm.* Typhimurium was obtained.

4.4.4 Efflux Pumps

Efflux mediated resistance is a frequent phenomenon in Gram-negative bacteria, although increased efflux alone might be insufficient to create a clinically relevant level of resistance. Such effects can enhance survival until further changes occur that result in resistance (Hughes, 2003). In this study the role of an active efflux system was assessed as a candidate resistance mechanism to biocides and erythromycin. To establish what possible efflux pump activity, if any, operate in *Salmonella enterica* and *E. coli* O157, experiments were performed using two known efflux pump inhibitors which are considered to inhibit different types of protein efflux pump systems. These included the plant alkaloid reserpine, which has been shown to inhibit

members of the major facilitator superfamily (MFS), the ATP binding cassette (ABC) and the resistance nodulation division (RND) family and the proton ionophore CCCP, which dissipates the proton motive force (pmf) across the cytoplasmic membrane that serves as the energy source for efflux. Carbonyl cyanide 3-chlorophenylhydrazone acts on efflux systems that use the proton motive force (pmf) such as the RND pumps AcrAB found in *E. coli* (Sanchez *et al.*, 1997; Ricci & Piddock, 2003).

The presence of reserpine, which by itself did not suppress the growth of *E. coli* O157, resulted in up to three folds decrease in the MIC of BKC and up to a four doubling dilution decrease in the CHX MICs. This data strongly suggest the involvement of an efflux mechanism in BKC and CHX resistance in *E. coli* O157 strains. By contrast, ERY- and TLN-resistant *Salmonella enterica* and *E. coli* O157 strains did not have their resistance reversed by reserpine, however, sensitivity was restored by CCCP.

The data obtained suggest that BKC and CHX resistance were mediated by an efflux pump belonging either to the MFS, ABC or RND superfamilies, since these resistances were reversed by both reserpine and CCCP. According to Bellido *et al.* (2002), the MFS and ABC families have been described only in Gram-positives, however, recently a macrolide ABC transporter, MacAB, has been reported in *E. coli* (Kobayashi *et al.*, 2001). In the case of ERY and TLN, our data suggests that the active efflux pump belongs to the RND superfamily, as sensitivity was restored in the presence of CCCP. Reserpine has been reported to block RND efflux pumps in Gram-negative bacteria however this was not observed in *Salmonella enterica* or *E. coli* O157 strains. It is possible that reserpine was unable to inhibit efflux as it is likely to

have difficulty traversing the Gram-negative cell wall (Keith Poole, personal communication).

Triclosan resistant *E. coli* O157 had acquired enhanced cross-resistance to ERY, BKC and CHX, indicating that these share at least one of the potential active efflux pumps involved in the acquisition of resistance. Probable links of cross-resistance between antibiotics and triclosan, or other biocides due to the presence of a presumptive efflux pump have been suggested previously (McMurry *et al.*, 1998; Chuanchuen *et al.*, 2001). The work of Sánchez *et al.* (1997) also suggests that RND transporters appear to have the widest substrate specificity and AcrAB efflux both positively charged (erythromycin) and negatively charged ampiphillic compounds.

In addition, reserpine studies suggest that an active efflux system was associated with BKC and CHX resistance in all *Salmonella enterica* strains investigated. More specifically, it was found that resistant strains returned to their parent MIC in the presence of reserpine, which consequently suggests the up-regulation of efflux in resistance. This was not the case however in ERY or TLN resistant strains according to studies with reserpine. Investigation with CCCP however proposed that an active efflux is associated with ERY, BKC, CHX, as well as with TLN resistance in all *Salmonella enterica* serotypes and *E. coli* O157 strains investigated. This leads to the conclusion that BKC resistance is mediated either by RND, MFS or ABC types of pumps as both efflux inhibitors restored sensitivity in all strains investigated, whereas ERY and TLN is mediated by an RND efflux system as only in the presence of CCCP resistance was sensitivity restored.

Resistance to BKC mediated by efflux pumps has been previously documented; Aase *et al.* (2000) found that BKC resistance was mediated by a proton motive force efflux pump in *Listeria monocytogenes*. Other pumps for BKC in Gram-positive organisms are also documented (Nikaido *et al.*, 1998). Specific Gram-negative pumps for BKC have not been as well studied, but it is thought that the AcrAB and the MexAB pumps of *E. coli* can efflux compounds with a positive charge (Nikaido, 1996). The mechanism of resistance to chlorhexidine in Gram-negative bacteria is still unclear; however evidence suggested that it could be similar to that of BKC (chapter 3). Fang *et al.* (2002) studied chlorhexidine resistance in *Klebsiella pneumoniae* strains and found that resistance was attributable to *cepA* encoding a cationic efflux pump.

Efflux activity was also inferred in *Salm.* Virchow strains resistant to chlorhexidine. In the presence of reserpine, the MIC for resistant strains returned to MICs similar to that seen in the parent strains. In the absence of reserpine the resistant strains maintained their increased level of resistance. In *Salm.* Virchow strains the development of BKC resistance conferred reduced susceptibility to chlorhexidine, which in turn suggests that the same efflux pump may be involved. Multidrug efflux can be responsible for cross-resistance between several groups of antimicrobial compounds (Levy, 2002b). In *Salm.* Typhimurium it was difficult for any firm conclusions to be drawn as the MICs for parent and resistant strains were close, however it is still highly likely that resistance is associated with the activity of an efflux pump, as resistant strains returned to their parent MIC in the presence of reserpine.

Erythromycin-resistant strains examined did not have their resistance reversed by reserpine, however CCCP was effective in restoring sensitivity. These results suggest that ERY resistance is mediated by an efflux pump belonging to the RND family. It might have been expected, therefore, that reserpine would have also restored sensitivity however reserpine is known to have difficulty traversing the cell wall which might explain this result. Previous investigations have also suggested the involvement of efflux in ERY resistance. Nikaido *et al.* (1998) showed that *Salm.* Typhimurium were resistant to a wide variety of antibiotics including erythromycin due to the presence of an AcrAB efflux pump. Erythromycin efflux due to AcrAB and MexAB has also been recorded by Nikaido (1996).

No efflux activity was observed in TLN-resistant strains as revealed by the inactivity of reserpine. In all cases the MIC of TLN towards resistant strains stayed much higher than that observed for the parent following reserpine inhibition. In the presence of CCCP, resistant strains were restored to sensitivity suggesting that RND type efflux was involved with TLN resistance in all strains investigated. Triclosan resistance has been observed previously. In TLN-resistant *E. coli* strains resistance was associated with a mutation in the *fabI* gene. The changes in this gene conferred changes to the triclosan target site, enoyl reductase (McMurry *et al.*, 1998b). In addition, mutations in the *marA* gene complex caused the over expression of the AcrAB pump, which is known to efflux triclosan (Levy, 2002b).

4.5 Conclusion

Although a great deal of work has been carried out on many aspects of antimicrobial resistance, questions still remain. There is a spectrum of possible mechanisms by which bacterial pathogens may become resistant to antimicrobial agents and this study aimed to investigate a number of candidate resistance mechanisms employed by *Salmonella enterica* and *E. coli* O157 strains.

Cell surface hydrophobicity played a significant role in some instances of *Salmonella enterica* and *E. coli* O157 resistance; parent strains were not hydrophobic, whereas resistant strains were hydrophobic. Modification in cell surface charge did not reveal any strong correlation with resistance. Resistance to ERY, BKC, CHX and TLN may be mediated by efflux activity in all isolates investigated. More specifically, results suggested that BKC and CHX resistance is mediated by an efflux pump belonging either to the MFS, ABC or RND superfamilies, since these resistances were reversed by both reserpine and CCCP. Of particular note in this study was the difficulty in predicting the likely response of an isolate following sub-lethal exposure to an antimicrobial even when data on a closely related strain existed. The variety and extent to which an individual isolate responded as it became less susceptible to sub-lethal exposure to an antimicrobial was largely characteristic of the specific isolate/antimicrobial interaction.

The emergence of biocide/antibiotic resistance is considered to have arisen by the widespread and indiscriminate use of biocides/antibiotics - both prophylactically and therapeutically. It is essential that responsibility is exercised in the use of

antimicrobial agents and a more detailed understanding of bacterial resistance mechanisms is necessary to prevent further development of antibacterial resistance.

Chapter 5: The molecular biology of *fabI* in *E. coli* O157

5.1 Introduction

It is currently considered that the mode of action of triclosan is via inhibition of enoyl-acyl carrier protein (ACP) reductase (FabI). *Escherichia coli* relies upon this enzyme to perform the final step in the elongation cycle of bacterial fatty acid biosynthesis. Thus, this study was undertaken in order to confirm mutations in the *fabI* is associated with the increased resistance to TLN and investigate subsequent alterations in the production of lipids or fatty acids.

5.1.1 FabI mutations

The NADH-dependent trans-2-enoyl-ACP reductase I, FabI (Figure 5.1), of *E. coli* composes an important regulator of the fatty acid biosynthetic pathway (Heath and Rock, 1995; Heath *et al.*, 2001); it catalyzes the last step in each cycle of fatty acid elongation in the type II fatty acid synthase systems (Marrakchi *et al.*, 2002b). Previous studies suggest that it can be inhibited by the action of the antibacterial agent, triclosan (McMurry *et al.*, 1998b). However, mutations, or overexpression of *fabI* can prevent this blockage (Heath *et al.*, 1998; McMurry *et al.*, 1998). Thus, in this study possible mutations in *fabI* were investigated in the TLN-resistant *E. coli* O157 mutant, in order to understand the mechanisms underlying the rapid adaptation of *E. coli* O157 to TLN and the cross-resistance to other antimicrobial agents obtained. In addition, the possibility of CHX resistance being mediated by alteration in *fabI* in *E. coli* O157 strains was also investigated, as in *E. coli* O157 co-resistance between TLN and CHX was observed.

Figure 5.1: Diagrammatic representation of the genes in the region surrounding FabI (position 2225824-2245823). The red arrow indicates FabI. The other annotated genes in the region are shown as blue arrows. FabI (*E. coli* O157:H7 EDL 933) enoyl-[acyl-carrier-protein] reductase [NADH] positions 2235429-2236217.

5.1.2 Polymerase chain reaction (PCR)

With PCR, genomic or cloned target sequences are specifically enzymatically amplified as directed by a pair of oligonucleotide primers (Williams, 1989). Segments of single-copy genomic DNA can be amplified >10 million-fold with very high specificity and fidelity. The PCR product can either be subcloned into a vector suitable for sequence analysis, or alternatively purified PCR products can be sequenced (Innis *et al.*, 1988). There are three basic steps in PCR. First, the target genetic material must be denatured that is, the strands of its helix must be unwound and separated by heating to 90-96°C. The second step is hybridization or annealing in which the primers bind to their complementary bases on the now single-stranded DNA. The third is DNA synthesis by a polymerase. The basic principles are shown in Figure 5.2.

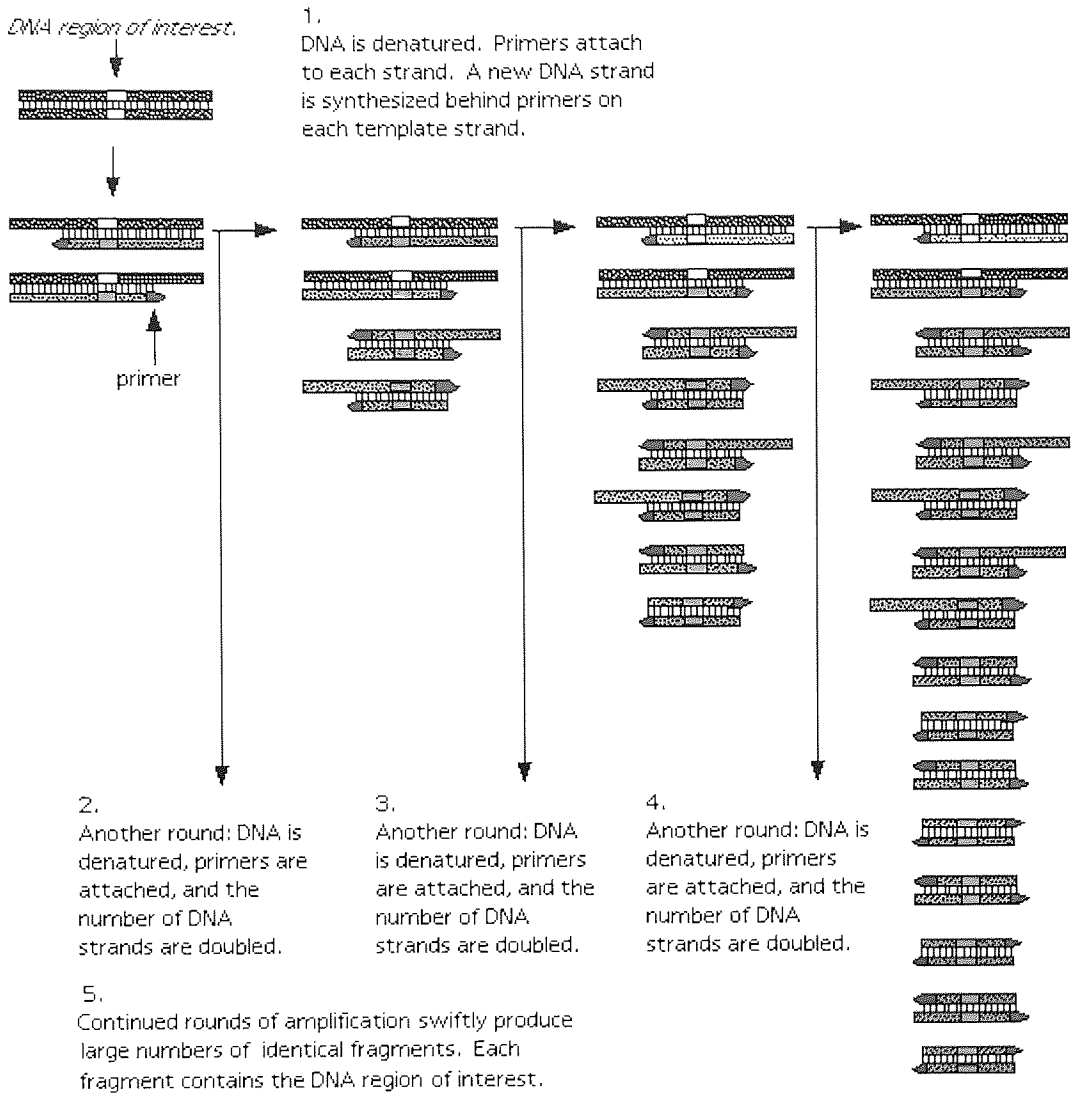


Figure 5.2: PCR amplifies a single DNA molecule into many billions of molecules; adapted from

URL: http://www.accessexcellence.org/AB/GG/polymerase.html (2004).

5.1.2.1 Practical Considerations of PCR

To perform a high fidelity PCR reaction it is vital that several parameters are taken into consideration. These include the composition of PCR reagents, dNTPs, buffers, the choice of thermal cycling primers and the concentration of *Taq* DNA polymerase.

Magnesium ion concentration plays an important role in PCR as too much $MgCl_2$ will result in high levels of non-specific amplification, whereas too little will inhibit the reaction. Annealing temperatures are critical; low temperature annealing increases non-specific amplification, whereas high temperatures can inhibit annealing but may also increase specificity.

5.1.3 Fatty acids

Differences between membrane fatty acids present in the mutant strains compared with the parent type have been reported previously (Persino and Lynchm 1982; Heath *et al.*, 2001). In this investigation the fatty acid profile was investigated in order to understand if there is any link between potential mutations arisen from *fabI* with the lipid or fatty acid content. In order to examine any alterations in the fatty acids, parent and TLN-resistant mutants were examined by gas chromatography. A summary of the system employed is shown in Figure 5.3

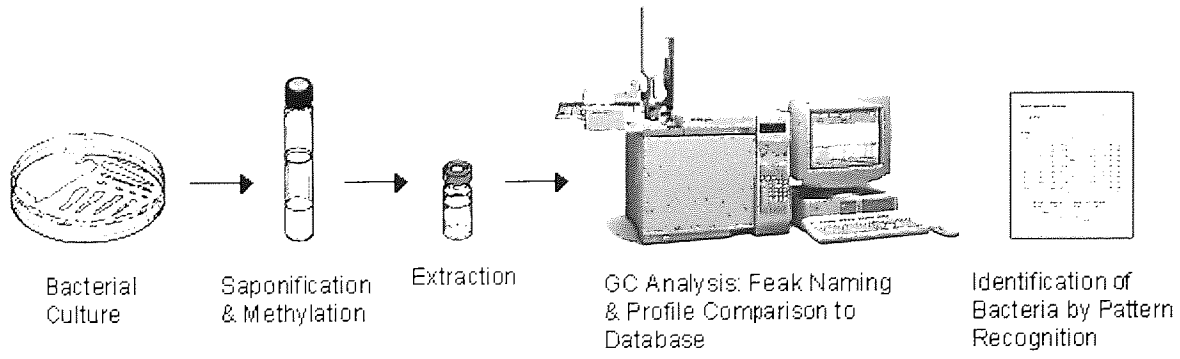


Figure 5.3: A Diagram of the system employed for the examination of fatty acids; adapted from MIDI, 2001.

5.1.4. Lipids

The introduction of chromatographic methods has facilitated the analysis of lipids. In this study thin layer chromatography was employed where lipids were removed by a

combination of chloroform, methanol and glacial acetic acid in preference to a mixture of chloroform/methanol and water as a second solvent system. According to Ratledge and Wilkinson, (1988) lipids are sparingly soluble in water but readily soluble in organic solvents such as chloroform, hydrocarbons, alcohols, ethers and esters. Visualisation of phospholipids was achieved by spraying with molybdenum blue reagent, while spraying with nihydrin and heating for a short period of time revealed those containing free amino groups such as the phosphatidylethanolamine (PE).

5.2 Materials & Methods

5.2.1 DNA Isolation

The methodology that was followed is based on that described to section 2.2.4.

5.2.2 PCR primers

The DNA of *E. coli* O157 was amplified by PCR using primers 1, 2, 3 and 4 initially (Table 5.1) (MWG Biotech AG, Ebensburg, Germany). Additional primers 5 and 6 were later employed in order to confirm the reproducibility of the data generated (Table 5.1.1).

Table 5.1: Design of primers 1, 2, 3 and 4

	Sequence	Length	Melting Temperature (T_m)	%G+C
Upstream-Primer 1	5'TAT TTT TAT CTT ATT CAT GGT GC3'	23bp	50.0°C	27.2%
Downstream-Primer 2	5'ACT TTC CCC AGT TCA GCG3'	18bp	50.2°C	55.4%
Upstream-Primer 3	5'CCG AAG ATG CCA GCA TCG3'	18bp	52.1°C	50%
Downstream-Primer 4	5'GAA AGG TAT AAC AGA GAT AAC G3'	22bp	52.5°C	38.4%

Table 5.1.1: Design of primers 5 and 6

	Sequence	Length	Melting Temperature (T_m)	%G+C
Upstream-Primer 5	$^{5'}$GAAAGGCCGCGTAGAAGAA$^{3'}$	19bp	52.0°C	40.9%
Downstream-Primer 6	$^{5'}$GCAATGCTGAAACCGCCG$^{3'}$	18bp	52.1°C	50.3%

5.2.3 PCR assay

The PCR amplification of *fabI* was carried out following the methodology described in section 2.4.5. The reaction mixtures were amplified in a PTC-100 thermocycler (MJ Research, INC. Waltham, MA, U.S.A) under the following conditions: (i): one cycle of five min at 94°C; (ii) five cycles consisting of 30sec at 94°C; one min at 50°C; one min at 72°C; and (iii) 29 cycles of one min at 94°C. The cycling was concluded with five min at 72°C and the reaction products stored at 4°C until required. The primers described in section 4.2.10 were used in this reaction. The expected size of the *fabI* amplicon was 1245bp.

5.2.4 DNA Analysis

The experiment was performed following the method described in section 2.2.6, only in this case an 85µl portion of the PCR reaction product was loaded onto a 1% polyacrylamide gel. The 1kbp ladder (Bioline, London, UK) served as the molecular weight (M) standard for determining the size of the PCR products. The DNA fragment generated in each case was excised from the agarose gel using a sterile scalpel blade.

5.2.5 DNA purification

For direct sequencing, the PCR products were purified using the QIAEX II gel extraction kit (150) (QIAGEN, Countaboeuf, France) following the manufacturer's recommendations.

5.2.5.1 Quantification and purity of the DNA preparation - Ethidium bromide fluorescence method

A 1µl volume of the DNA sample was mixed on a strip of Parafilm (Appleton Woods, Birmingham, UK) with an equal volume of TE (pH 7.6) containing 2µg/ml ethidium bromide. The fluorescence of the sample under UV excitation was visually compared to that of identically prepared standards of bacteriophage λ DNA (50µg/ml-3µg/ml) and the amount of DNA in the sample was estimated.

5.2.6 DNA sequencing and sequence analysis

The same set of primers used in the PCR analysis was used for sequencing purposes. Between 5-20ng of PCR product DNA was used as a template. Sequencing data was obtained from the Birmingham University Functional Genomics Lab, Birmingham, UK. The DNA and amino acid sequence following translation were compared with those previously described for *fabI* (coliBASE database: http://colibase.bham.ac.uk) using Biology Workbench database version 3.2 (http://workbench.sdsc.edu/) and Expasy-Translate tool database (http://us.expasy.org/tools/dna.html) for direct comparison of sequences and translation to amino acids, respectively.

5.2.7 Molecular Modelling of *fabI* enoyl reductase mutation

The FabI sequence was obtained from the National Center for Biotechnology Information Website (NCBI) employing PubMed; (2004) URL:http//www.ncbi.nim.nih.gov/PubMed. Atomic coordinates for the crystal structure of enoyl reductase (*fabI)* for *E. coli* O157:H7 ternary complex with TLN and NADH); accession number 1C14, were downloaded as a Protein Data Bank (pdb) file from Research Collaboration for Structural Bioinformatics site, 2004 (RCSB; URL:http//www.rcsb.org/pdb). Modelling of the G93→ V93 mutation was conducted using version 3.7 of the DeepView/Swiss PdbViewer (URL:http//www.ca.expasy.org/spdbv).

5.2.8 Preparation of Fatty acid Extracts

A 4mm loop was used to harvest approximately 20mg of bacterial cells from an overnight nutrient agar streaked plate. Cells were selected from the third quadrant of the plate and placed in a clean tube. A volume of 1ml of reagent 1 (3g sodium hydroxide, 10ml methanol and 10ml distilled H_2O) was added to each of the tubes containing the cells. The tubes were securely sealed with Teflon lined caps, vortexed briefly and boiled at 100°C for approximately 5 minutes. The tubes were then vigorously vortexed for 5-10 seconds and returned to boil to complete the 30 minute boiling process. Following cooling, the tubes were uncapped and 2ml of reagent 2 (10.83ml certified 6N hydrochloric acid and 9.16ml methanol) was added. The tubes were then capped and briefly vortexed. After vortexing, the tubes were heated for 10 minutes at 80°C; this step was critical with respect to both time and temperature. Addition of 1.25ml of reagent 3 (10ml hexane and 10ml diethyl ether) to the cooled tubes was followed by recapping and gentle mixing using a rotator (Spiramix 5, Denley, England) for approximately 10 minutes. The tubes were then uncapped and

the aqueous (lower) phase was discarded. Approximately 3ml of reagent 4 (0.24g sodium hydroxide dissolved in 20ml H_2O) was added to the organic phase remaining in the tubes and mixed for five minutes. Approximately, two-thirds of the organic phase was pipetted into a glass vial, was evaporated under nitrogen and was stored at -20°C until analysis.

5.2.8.1 Fatty acid analysis by Gas Chromatography

A volume of 1µl of each sample was loaded onto a Hewlett Packard HP-5890 Series II capillary column on a Hewlett Packard Chemstation series Gas Chromatograph (GC). The conditions used are summarised in Table 5.2. The peaks detected were integrated and the data was analysed by the HP Chemstation software package. Fatty acids were identified by comparing the retention times of the peaks with those of a mixed standard bacterial fatty acid methyl esters (FAME) containing 26 FAMEs, commonly found in bacteria (CP™ Mix), prepared based on the manufacturer's recommendations.

Table 5.2:Conditions used in Gas Chromatography of fatty acid samples

Conditions	Column HP 5890 Series II
Sample split	1:50
Mobile Gas phase	Helium
Column length (m) x column diameter (mm)	30 x 0.32
Film thickness	0.25µm
Linear velocity (hexane) cm/sec	20.8
Initial temperature	150°C/4 min
Program rate	4°C/min up to 250°C
Means of peak detection	Flame ionisation detector
Standard used	Bacterial acid methyl esters CP™ Mix

5.2.9 Whole Cell Lipids

5.2.9.1 Quantification of Lipids

A volume of 10ml of an overnight culture of parent and TLN-resistant mutants of *E. coli* O157 was freeze dried. The freeze-dried cells were weighed before being placed in glass tubes and a solvent of chloroform-methanol (3:1) added. The tubes were sealed with Teflon lined caps and mixed overnight on a rotary shaker. The following day, the cells were eliminated by filtration (Whatman 70mm, England, UK) through a glass funnel and the filtered solution was placed in a glass universal. An additional volume of 10ml of the same solvent was added. The lipids were extracted using a rotary evaporator (Rotavaporator R110, ORME Scientific Ltd, Middleton, UK) and the pre-weighed flasks containing the extracted lipids were weighed again, in order to calculate the proportion of lipids contained in the known dry weight of cells.

5.2.9.2 Preparation of whole cell lipids

A volume of 1 litre of an overnight bacterial suspension from parent and TLN-resistant mutants of *E. coli* O157 was centrifuged at 10,000x g for 10 minutes. The pellet was resuspended in 30ml of sterile distilled H_2O. Chloroform and methanol were mixed with the bacterial suspension in a ratio of one part bacterial suspension, one part chloroform and two parts methanol. The solution was mixed, covered with aluminium foil and left to stand overnight. The following day chloroform and dH_2O were added to the solution in the proportions; one part dH_2O, one part chloroform and one part solution. The new solution was mixed and allowed to separate into organic and aqueous phases. The lower phase was removed carefully and dried using a rotary

evaporator. The lipid residue was dissolved in 2ml solution of one part chloroform, two parts methanol and stored at -20°C in a glass vial covered with foil.

5.2.9.3 Separation and quality of whole cell lipids

Thin layer chromatography was employed for the separation of lipids. Approximately 2.5µl of the parent and TLN-resistant *E. coli* O157 mutants' lipids were spotted onto the bottom of a silica coated aluminium chromatography plate (20 x 20cm, layer thickness 200µm, particle size 2-25µm, pore size 60Å, Sigma, Poole, UK) using a syringe needle. Samples of standard phospholipids were spotted at equal concentrations. The plate was dried at room temperature and then placed in a glass chromatography tank (Sigma, Poole, UK). The plate was developed with the following solvents; either methanol-acetic acid-chloroform (25:8:65), or methanol-H_2O-chloroform (25:8:65). The tank was sealed with a glass lid and left to three quarters of the plate height; until the atmosphere within the tank began to saturate with the mobile phase vapour.

5.2.9.4 Identification of whole cell lipids

For the visualisation of those lipids containing amino groups, plates were sprayed with 0.2% ($^{v}/_{v}$) nihydrin (BDH Chemicals, Poole, UK) in ethanol and dry heated at 100°C for up to 2 minutes. Appearance of purple or brown spots revealed amino compounds. In addition, this method also allowed confirmation of the presence of phosphatidylethanolamine and to putatively identify its lyso form. Phospholipids were also visualised by spraying with molybdenum blue spray, a solution of 1.3% ($^{w}/_{v}$) molybdenum oxide in 4.2M H_2SO_4 (Sigma, Poole, UK), onto the dry plates.

5.2.10 Cell growth in M9 Broth

For studies on utilisation of exogenous fatty acids parent and resistant to CHX and TLN *E. coli* O157 mutants were grown in nutrient broth. Bacterial cells were harvested in log phase by centrifugation at 13,000x g for 4 min. The cells were then washed three times in sterile dH_2O and 0.25ml was added to 25ml M9 minimal medium (appendix 2). Once bacteria reached the log phase they were passaged again to fresh M9 minimal media in order to ensure that there was no excess of fatty acids.

5.2.11 Cell growth in M9 Agar

Growth on M9 agar was tested by the Stoke's method. Bacterial suspensions of parent and resistant to CHX and TLN *E. coli* O157 mutants were inoculated over the surface of an M9 minimal medium agar plate using a rotary plater. Volumes of 1µl, 2µl, 5µl and 10µl of fatty acid - containing disks were placed in the plates, which were incubated overnight at 37°C and examined for growth in the presence and absence of fatty acids supplemented to the M9 agar.

5.2.12 Statistical Analysis

The data for lipids analysis were checked for normality using the Kolmogorov-Smirnov test. All analysis was carried out using the Statistica Program (StatSoft, 2001, version 6, www.statsoft.com). SPSS was used to calculate a two-sample t-test. T-test was used to examine whether or not there were significant differences between the fatty acids present in parent and TLN-resistant *E. coli* O157 mutants.

5.3 Results

5.3.1 PCR –Buffer Optimisation

A PCR buffer titration was performed in order to select the optimal buffer for the amplification of the expected amplicon. In Figures 5.4 and 5.4.1 DNA from *E. coli* O157 (43888) was amplified in 11 buffers; primers 3 & 4 and 5 & 6 were used respectively. Data suggests that the most specific amplification was obtained using buffer 11; Lane 11 for primers 3 & 4 and buffer 9; Lane 9 for primers 5 & 6. The composition of buffers is shown in Chapter 2, Table 2.2.

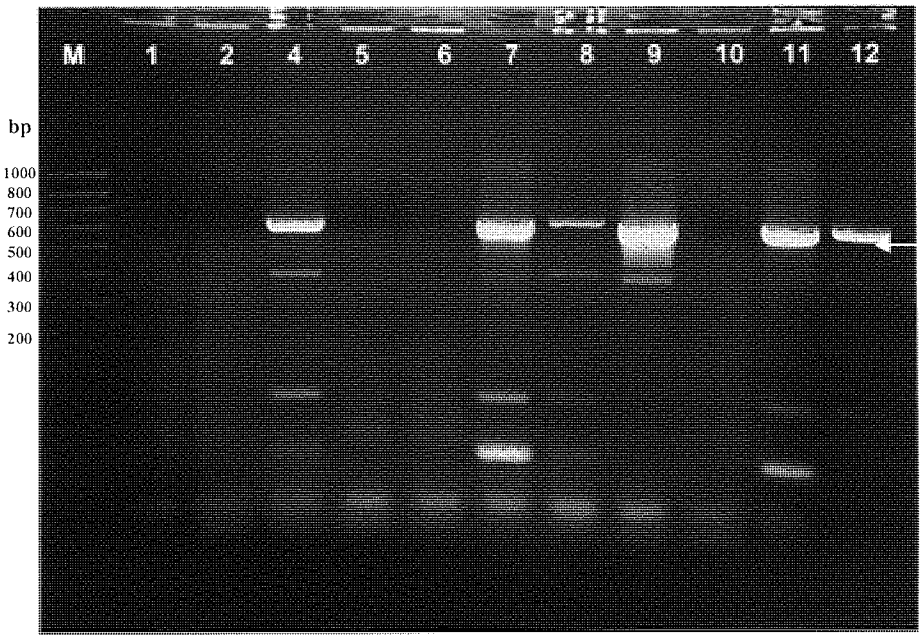


Figure 5.4: Visualization of PCR products using agarose gel electrophoresis analysis with primers 3 & 4 of *E. coli* O157 (43888) in a series of buffers. Buffer 11 (Lane 11) was used for further DNA analysis; precipitation and sequencing.

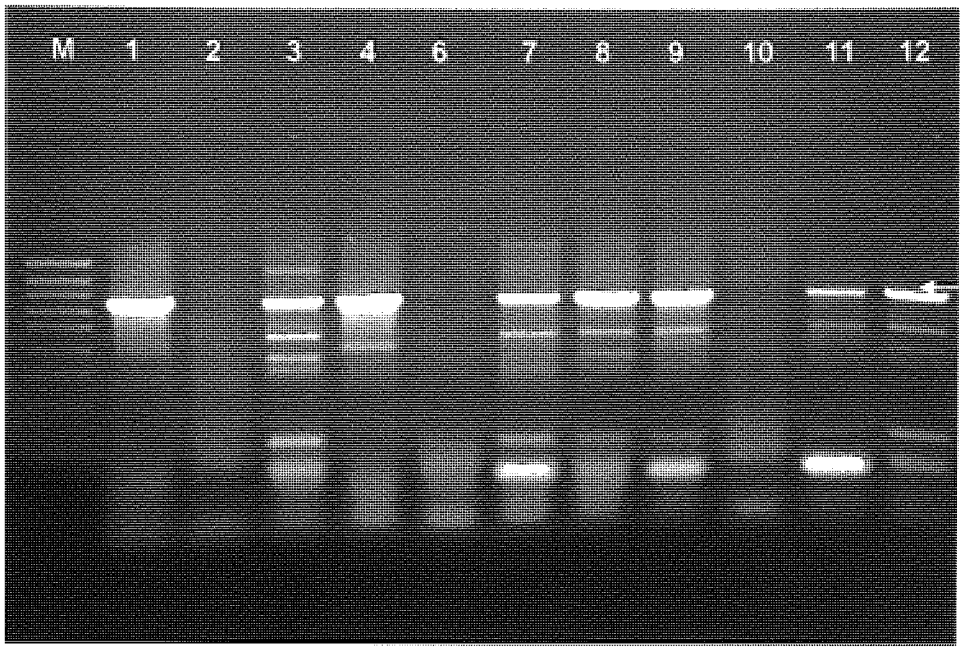


Figure 5.4.1: Visualization of the PCR products generated with primers 5 & 6 of *E. coli* O157 (12900) in a series of buffers. Buffer 9 (Lane 9) was used for further DNA analysis, precipitation and sequencing.

5.3.2 PCR Products

A PCR assay was employed to screen strains for the presence of *fabI*. *FabI* was amplified using two primer sets; primers 1 & 2 and primers 3 & 4. A third primer set, primers 5 & 6, was employed to confirm data from the second primer set. Agarose gel electrophoresis analysis of the products generated from *E. coli* O157 (12900) and *E. coli* O157 (43888) revealed the product bands; 638bp when primers 1 and 2 were used, 653bp when primers 3 & 4 were used and 630bp when primers 5 & 6 were used. An example is shown in Figure 5.5.

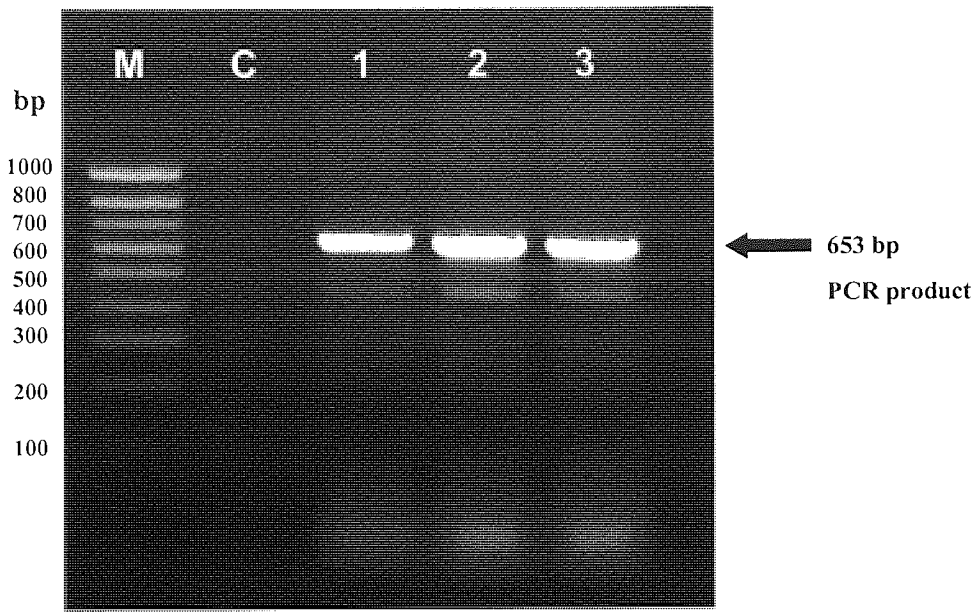


Figure 5.5: Visualisation of the PCR products generated using primers 3 and 4 in buffer 11 by agarose gel electrophoresis and ethidium bromide staining. Lane M, sizing ladder; Lane C, control; Lane 1, Parent *E. coli* O157 (12900); Lane 2, 1st passage to TLN, Lane 3, Resistant *E. coli* O157 (12900) to TLN.

5.3.3. Extraction of DNA product

Following PCR amplification of *fabI* from *E. coli* O157 (12900), the DNA band was excised from the agarose gel using a sterile scalpel blade for further DNA purification and preparation for sequencing. The product from *E. coli* O157 resistant to TLN is shown in Figure 5.5.1 and the product of *E. coli* O157 resistant to CHX is shown in Figure 5.5.2.

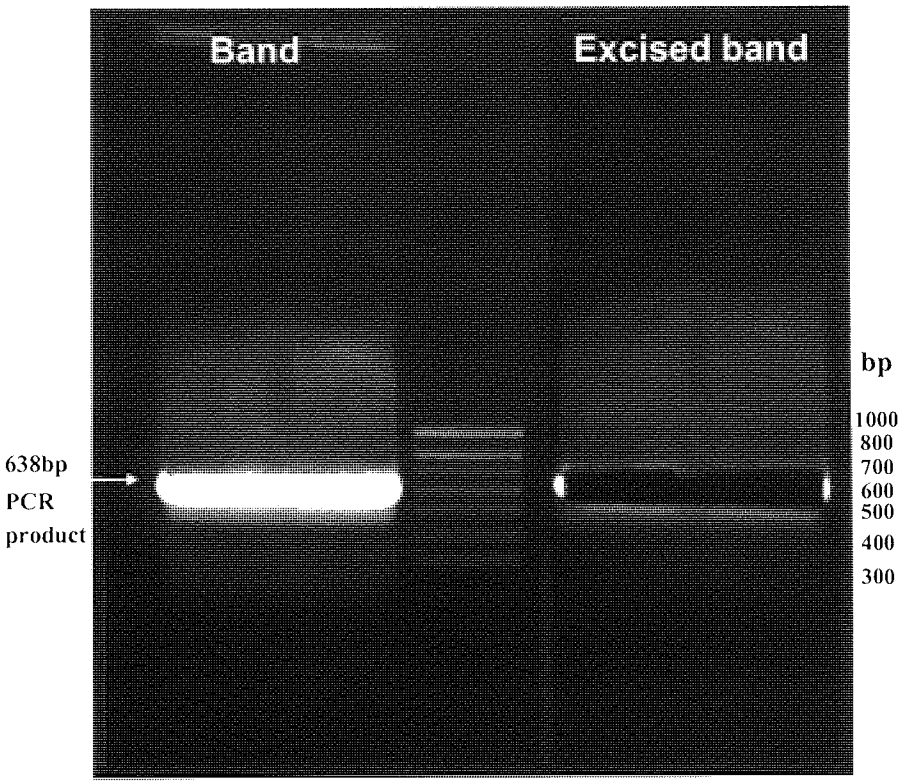


Figure 5.5.1: Visualisation of the PCR product generated using primers 1 and 2 with buffer 11 by agarose gel electrophoresis and ethidium bromide staining. *Escherichia coli* O157 (12900) resistant to TLN is loaded onto this gel.

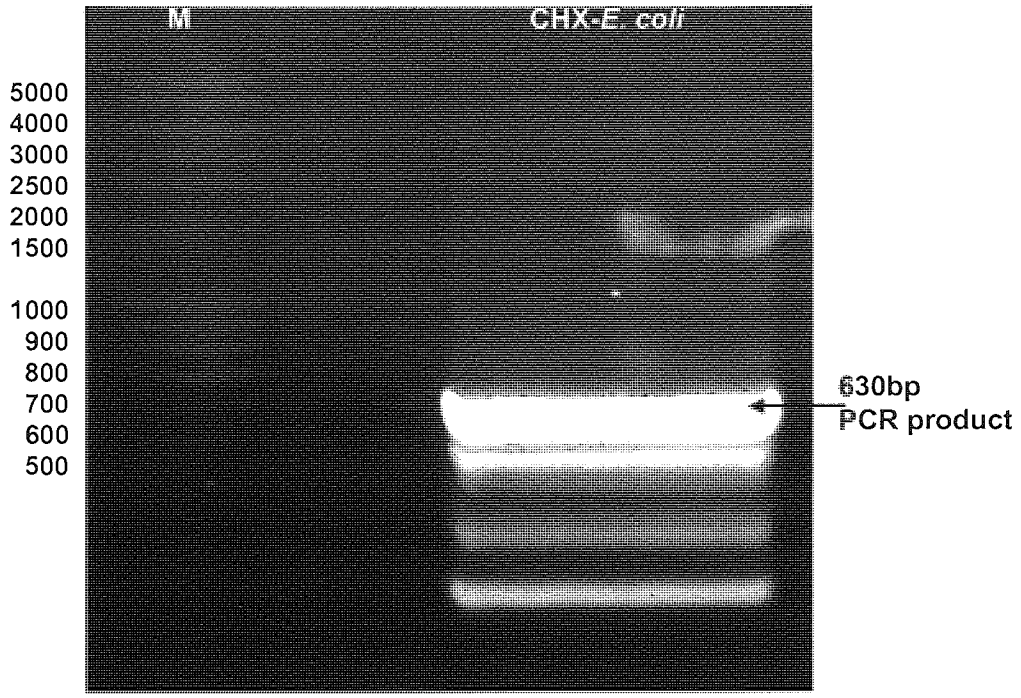


Figure 5.5.2: Visualisation of the PCR product generated using primers 5 and 6 with buffer 9 by agarose gel electrophoresis and ethidium bromide staining. *Escherichia coli* O157 (12900) resistant to CHX is loaded onto this gel.

5.3.4 Quantification and purity of the DNA preparation - ethidium bromide fluorescence method

In Figure 5.6 a strip of Parafilm containing the DNA sample in TE buffer containing 20μg/ml ethidium bromide is shown. It is apparent that the concentration of DNA of *E. coli* O157 resistant to TLN is between 25 and 12.5μg/ml.

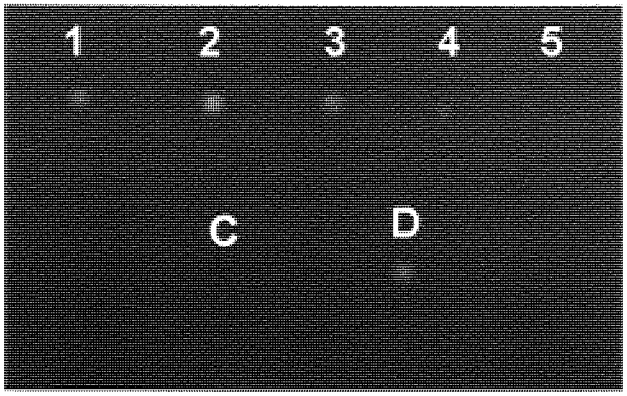


Figure 5.6: Ethidium bromide fluorescence method of DNA quantification. Here 1 refers to 50 μg/ml bacteriophage λ DNA; 2, 25μg/ml bacteriophage λ DNA; 3, 12.5μg/ml bacteriophage λ DNA; 4, 6μg/ml bacteriophage λ DNA; 5, 3.5μg/ml bacteriophage λ DNA; C, Negative control and D, DNA of *E. coli* O157 (12900) resistant to TLN and amplified by PCR using 3 & 4 primers and buffer 11.

5.3.5 DNA sequencing and sequence analysis of TLN-resistant *E. coli* O157

The *fabI* PCR products prepared from *E. coli* O157 (12900) and *E. coli* O157 (43888) resistant to TLN were sequenced. This experiment was repeated at least twice in each case. Sequencing of *fabI* revealed a single mutation; GGT to GTT at codon 93 of *fabI*. This mutation resulted in the replacement of glycine (hydrophobic amino acid) 93 by valine (hydrophobic amino acid) in FabI. The quality of the sequence data obtained in this study is illustrated in Figure 5.7; an example chromatogram of the sequence data obtained from 1st passage of *E. coli* O157 (12900) in TLN.

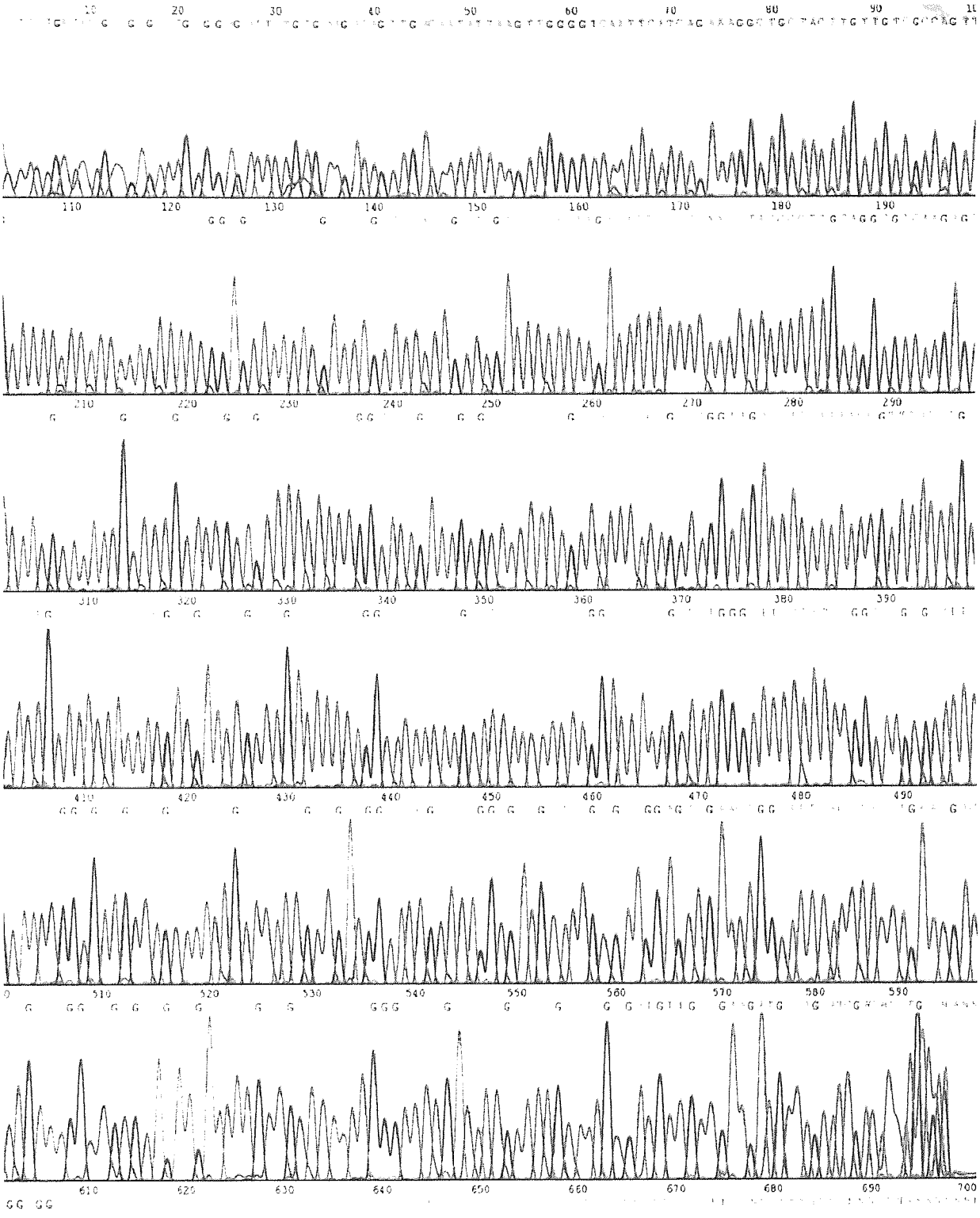
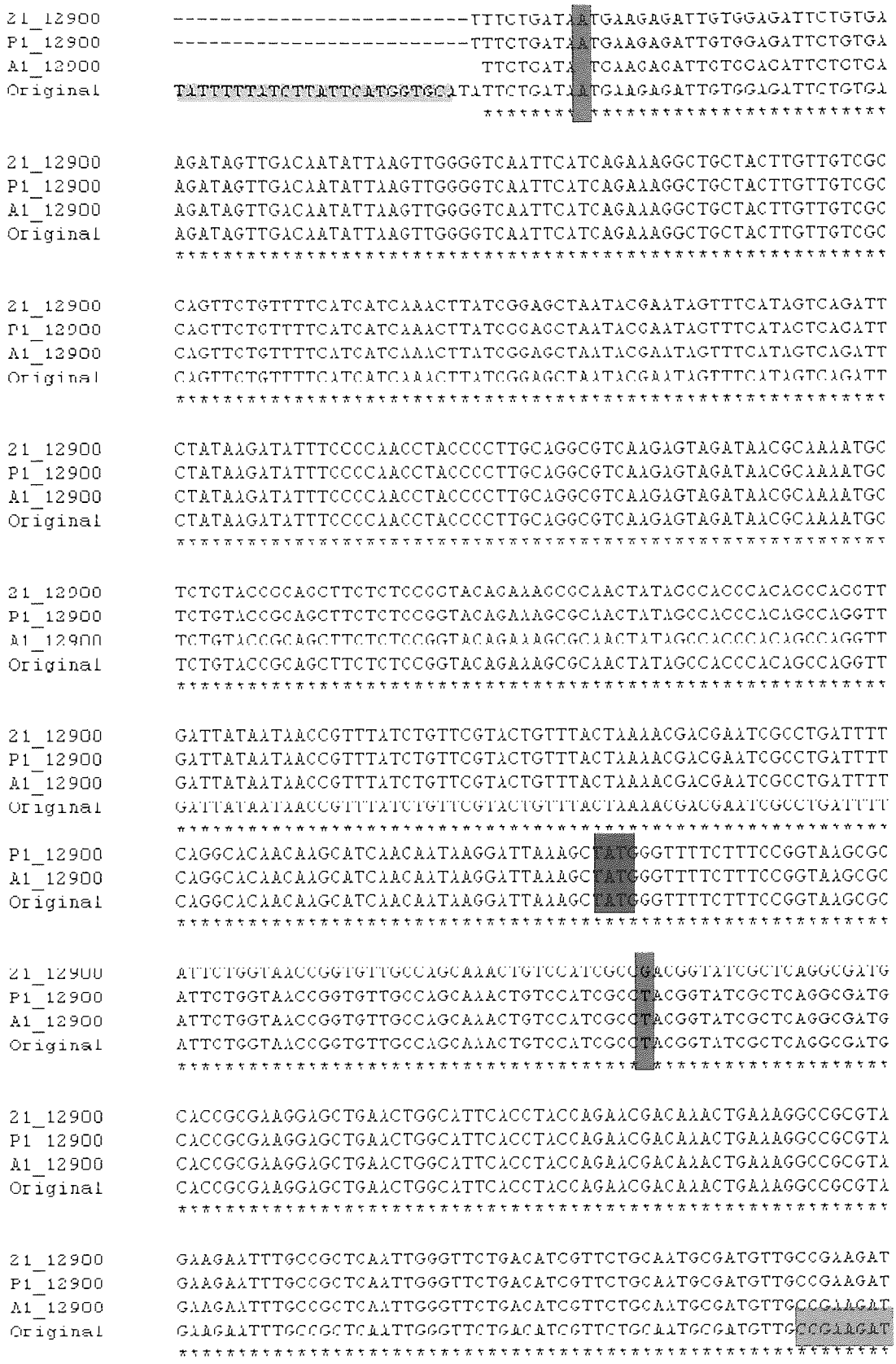


Figure 5.7: Data output from an automated sequencer. The sequence is represented by a series of peaks, one for each nucleotide position; green peak is an adenine 'A', blue is a cytosine 'C', black is a guanine 'G' and red is a thymine 'T'.

In Figure 5.8 the sequence alignments between each fragment of *fabI* sequence obtained from parent, 1st passage and resistant strains as well as the published sequence of *E. coli* O157:H7, EDL 933 is shown.


21_12900
P1_12900
A1_12900
Original

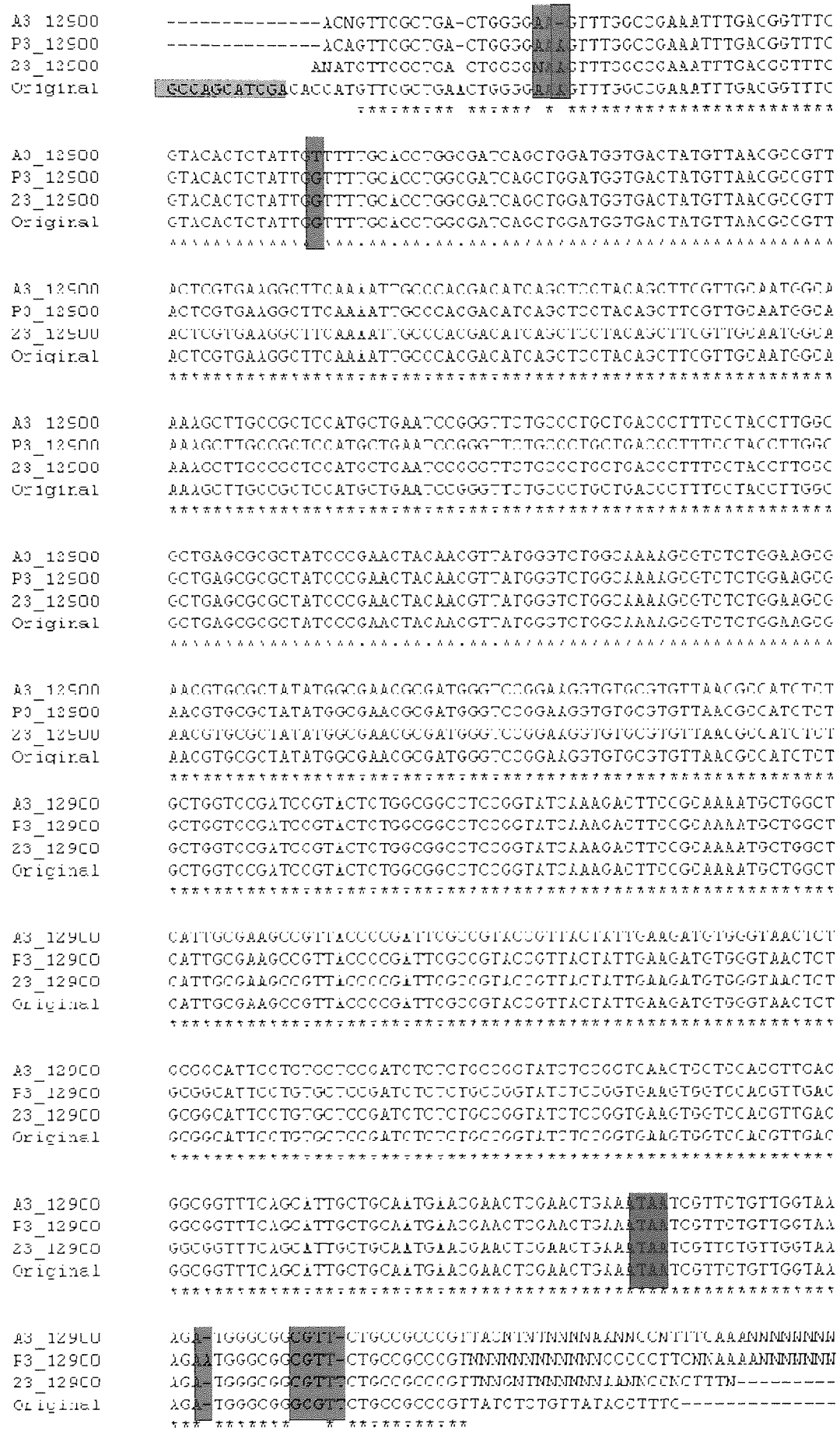


Figure 5.8: Sequence alignment between parent, 1st passage and resistant to TLN *E. coli* O157 (12900) mutants compared with the published sequence of *E. coli* O157:H7, EDL 933. Here Original: published *E. coli* O157 sequence, P3: Parent *E. coli* O157, 23: 2nd passage, A3: TLN-resistant *E. coli* O157, No. 3: results as with primer 3, nnnn: forward primer 1, nnnn: forward primer 3, nnn: open reading frame (ORF), nnnn: mutation, nnnn: artefact, nnnn: mutation? (Further investigation).

In Figure 5.9 a comparison of the sequence alignments between each fragment of *fabI* sequence obtained from parent, 1st passage and resistant *E. coli* O157 (43888) mutants to TLN as well as the published sequence of *E. coli* O157:H7, EDL 933 are shown.

```
Original    TATTTTTATCTTATTCATGGTGCATATTCTGATAATGAAGAGATTGTGGAGATTCTGTGA
P1_43888    -------------------------TNTTCTGATAATGAAGAGATTGTGGAGATTCTGTGA
A1_43888    --------------------------TTCTGATA-TGAAGAGATTGTGGAGATTCTGTGA
21_43888    -------------------------TTTCTGATAATGAAGAGATTGTGGAGATTCTGTGA
                                      ******* ************************

Original    AGATAGTTGACAATATTAAGTTGGGGTCAATTCATCAGAAAGGCTGCTACTTGTTGTCGC
P1_43888    AGATAGTTGACAATATTAAGTTGGGGTCAATTCATCAGAAAGGCTGCTACTTGTTGTCGC
A1_43888    AGATAGTTGACAATATTAAGTTGGGGTCAATTCATCAGAAAGGCTGCTACTTGTTGTCGC
21_43888    AGATAGTTGACAATATTAAGTTGGGGTCAATTCATCAGAAAGGCTGCTACTTGTTGTCGC
            ************************************************************

Original    CAGTTCTGTTTTCATCATCAAACTTATCGGAGCTAATACGAATAGTTTCATAGTCAGATT
P1_43888    CAGTTCTGTTTTCATCATCAAACTTATCGGAGCTAATACGAATAGTTTCATAGTCAGATT
A1_43888    CAGTTCTGTTTTCATCATCAAACTTATCGGAGCTAATACGAATAGTTTCATAGTCAGATT
21_43888    CAGTTCTGTTTTCATCATCAAACTTATCGGAGCTAATACGAATAGTTTCATAGTCAGATT
            ************************************************************

Original    CTATAAGATATTTCCCCAACCTACCCCTTGCAGGCGTCAAGAGTAGATAACGCAAAATGC
P1_43888    CTATAAGATATTTCCCCAACCTACCCCTTGCAGGCGTCAAGAGTAGATAACGCAAAATGC
A1_43888    CTATAAGATATTTCCCCAACCTACCCCTTGCAGGCGTCAAGAGTAGATAACGCAAAATGC
21_43888    CTATAAGATATTTCCCCAACCTACCCCTTGCAGGCGTCAAGAGTAGATAACGCAAAATGC
            ************************************************************

Original    TCTGTACCGCAGCTTCTCTCCGGTACAGAAAGCGCAACTATAGCCACCCACAGCCAGGTT
P1_43888    TCTGTACCGCAGCTTCTCTCCGGTACAGAAAGCGCAACTATAGCCACCCACAGCCAGGTT
A1_43888    TCTGTACCGCAGCTTCTCTCCGGTACAGAAAGCGCAACTATAGCCACCCACAGCCAGGTT
21_43888    TCTGTACCGCAGCTTCTCTCCGGTACAGAAAGCGCAACTATAGCCACCCACAGCCAGGTT
            ************************************************************

Original    GATTATAATAACCGTTTATCTGTTCGTACTGTTTACTAAAACGACGAATCGCCTGATTTT
P1_43888    GATTATAATAACCGTTTATCTGTTCGTACTGTTTACTAAAACGACGAATCGCCTGATTTT
A1_43888    GATTATAATAACCGTTTATCTGTTCGTACTGTTTACTAAAACGACGAATCGCCTGATTTT
21_43888    GATTATAATAACCGTTTATCTGTTCGTACTGTTTACTAAAACGACGAATCGCCTGATTTT
            ************************************************************

Original    CAGGCACAACAAGCATCAACAATAAGGATTAAAGCTATGGTTTTCTTTCCGGTAAGCGC
P1_43888    CAGGCACAACAAGCATCAACAATAAGGATTAAAGCTATGGTTTTCTTTCCGGTAAGCGC
A1_43888    CAGGCACAACAAGCATCAACAATAAGGATTAAAGCTATGGTTTTCTTTCCGGTAAGCGC
21_43888    CAGGCACAACAAGCATCAACAATAAGGATTAAAGCTATGGTTTTCTTTCCGGTAAGCGC
            ************************************************************

Original    ATTCTGGTAACCGGTGTTGCCAGCAAACTGTCCATCGCCTACGGTATCGCTCAGGCGATG
P1_43888    ATTCTGGTAACCGGTGTTGCCAGCAAACTGTCCATCGCCTACGGTATCGCTCAGGCGATG
A1_43888    ATTCTGGTAACCGGTGTTGCCAGCAAACTGTCCATCGCCTACGGTATCGCTCAGGCGATG
21_43888    ATTCTGGTAACCGGTGTTGCCAGCAAACTGTCCATCGCCTACGGTATCGCTCAGGCGATG
            ************************************************************

Original    CACCGCGAAGGAGCTGAACTGGCATTCACCTACCAGAACGACAAACTGAAAGGC-GCGT
P1_43888    CACCGCGAAGGAGCTGAACTGGCATTCACCTACCAGAACGACAAACTGAAAGGC-GCGT
A1_43888    CACCGCGAAGGAGCTGAACTGGCATTCACCTACCAGAACGACAAACTGAAAGGC-GCGT
21_43888    CACCGCGAAGGAGCTGAACTGGCATTCACCTACCAGAACGACAAACTGAAAGGCCGCGT
            ************************************************* ****

Original    AGAAGAATTTGCCGCTCAATTGGGTTCTGACATCGTTCTGCAATGCGATGTTGCCGAAGA
P1_43888    AGAAGAATTTGCCGCTCAATTGGGTTCTGACATCGTTCTGCAATGCGATGTTGCCGAAGA
A1_43888    AGAAGAATTTGCCGCTCAATTGGGTTCTGACATCGTTCTGCAATGCGATGTTGCCGAAGA
21_43888    AGAAGAATTTGCCGCTCAATTGGGTTCTGACATCGTTCTGCAATGCGATGTTGCCGAAGA
            ************************************************************
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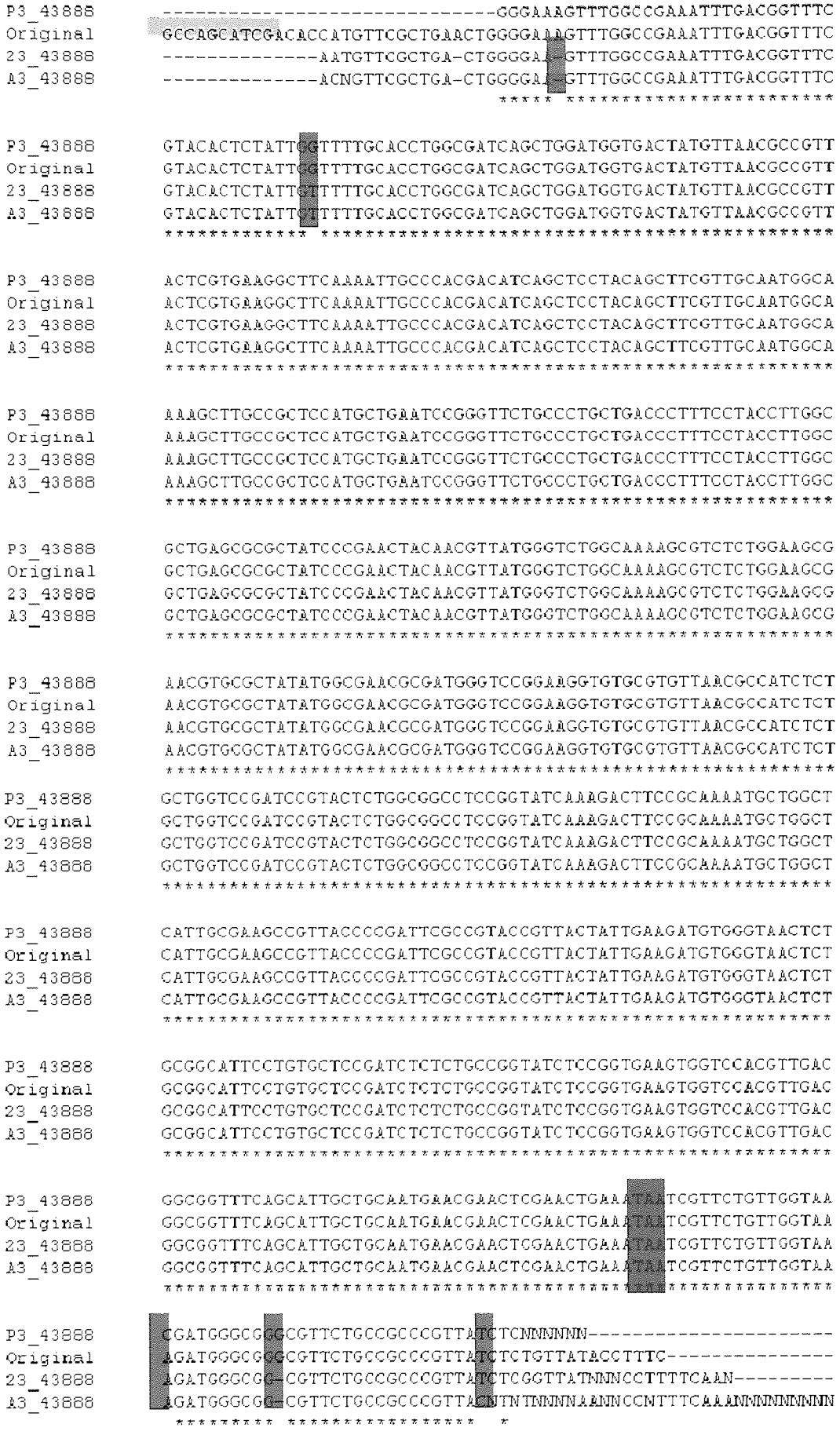

P3_43888 ----------GGGAAAGTTTGGCCGAAATTTGACGGTTTC
Original GCCAGCATCGACACCATGTTCGCTGAACTGGGGAAAGTTTGGCCGAAATTTGACGGTTTC
23_43888 ----------AATGTTCGCTGA-CTGGGGAA-GTTTGGCCGAAATTTGACGGTTTC
A3_43888 ----------ACNGTTCGCTGA-CTGGGGAA-GTTTGGCCGAAATTTGACGGTTTC
P3_43888 GTACACTCTATTCGTTTTGCACCTGGCGATCAGCTGGATGGTGACTATGTTAACGCCGTT
Original GTACACTCTATTCGTTTTGCACCTGGCGATCAGCTGGATGGTGACTATGTTAACGCCGTT
23_43888 GTACACTCTATTCTTTTTGCACCTGGCGATCAGCTGGATGGTGACTATGTTAACGCCGTT
A3_43888 GTACACTCTATTCTTTTTGCACCTGGCGATCAGCTGGATGGTGACTATGTTAACGCCGTT
P3_43888 ACTCGTGAAGGCTTCAAAATTGCCCACGACATCAGCTCCTACAGCTTCGTTGCAATGGCA
Original ACTCGTGAAGGCTTCAAAATTGCCCACGACATCAGCTCCTACAGCTTCGTTGCAATGGCA
23_43888 ACTCGTGAAGGCTTCAAAATTGCCCACGACATCAGCTCCTACAGCTTCGTTGCAATGGCA
A3_43888 ACTCGTGAAGGCTTCAAAATTGCCCACGACATCAGCTCCTACAGCTTCGTTGCAATGGCA
P3_43888 AAAGCTTGCCGCTCCATGCTGAATCCGGGTTCTGCCCTGCTGACCCTTTCCTACCTTGGC
Original AAAGCTTGCCGCTCCATGCTGAATCCGGGTTCTGCCCTGCTGACCCTTTCCTACCTTGGC
23_43888 AAAGCTTGCCGCTCCATGCTGAATCCGGGTTCTGCCCTGCTGACCCTTTCCTACCTTGGC
A3_43888 AAAGCTTGCCGCTCCATGCTGAATCCGGGTTCTGCCCTGCTGACCCTTTCCTACCTTGGC
P3_43888 GCTGAGCGCGCTATCCCGAACTACAACGTTATGGGTCTGGCAAAAGCGTCTCTGGAAGCG
Original GCTGAGCGCGCTATCCCGAACTACAACGTTATGGGTCTGGCAAAAGCGTCTCTGGAAGCG
23_43888 GCTGAGCGCGCTATCCCGAACTACAACGTTATGGGTCTGGCAAAAGCGTCTCTGGAAGCG
A3_43888 GCTGAGCGCGCTATCCCGAACTACAACGTTATGGGTCTGGCAAAAGCGTCTCTGGAAGCG
P3_43888 AACGTGCGCTATATGGCGAACGCGATGGGTCCGGAAGGTGTGCGTGTTAACGCCATCTCT
Original AACGTGCGCTATATGGCGAACGCGATGGGTCCGGAAGGTGTGCGTGTTAACGCCATCTCT
23_43888 AACGTGCGCTATATGGCGAACGCGATGGGTCCGGAAGGTGTGCGTGTTAACGCCATCTCT
A3_43888 AACGTGCGCTATATGGCGAACGCGATGGGTCCGGAAGGTGTGCGTGTTAACGCCATCTCT
P3_43888 GCTGGTCCGATCCGTACTCTGGCGGCCTCCGGTATCAAAGACTTCCGCAAAATGCTGGCT
Original GCTGGTCCGATCCGTACTCTGGCGGCCTCCGGTATCAAAGACTTCCGCAAAATGCTGGCT
23_43888 GCTGGTCCGATCCGTACTCTGGCGGCCTCCGGTATCAAAGACTTCCGCAAAATGCTGGCT
A3_43888 GCTGGTCCGATCCGTACTCTGGCGGCCTCCGGTATCAAAGACTTCCGCAAAATGCTGGCT
P3_43888 CATTGCGAAGCCGTTACCCCGATTCGCCGTACCGTTACTATTGAAGATGTGGGTAACTCT
Original CATTGCGAAGCCGTTACCCCGATTCGCCGTACCGTTACTATTGAAGATGTGGGTAACTCT
23_43888 CATTGCGAAGCCGTTACCCCGATTCGCCGTACCGTTACTATTGAAGATGTGGGTAACTCT
A3_43888 CATTGCGAAGCCGTTACCCCGATTCGCCGTACCGTTACTATTGAAGATGTGGGTAACTCT
P3_43888 GCGGCATTCCTGTGCTCCGATCTCTCTGCCGGTATCTCCGGTGAAGTGGTCCACGTTGAC
Original GCGGCATTCCTGTGCTCCGATCTCTCTGCCGGTATCTCCGGTGAAGTGGTCCACGTTGAC
23_43888 GCGGCATTCCTGTGCTCCGATCTCTCTGCCGGTATCTCCGGTGAAGTGGTCCACGTTGAC
A3_43888 GCGGCATTCCTGTGCTCCGATCTCTCTGCCGGTATCTCCGGTGAAGTGGTCCACGTTGAC
P3_43888 GGCGGTTTCAGCATTGCTGCAATGAACGAACTCGAACTGAAATAATCGTTCTGTTGGTAA
Original GGCGGTTTCAGCATTGCTGCAATGAACGAACTCGAACTGAAATAATCGTTCTGTTGGTAA
23_43888 GGCGGTTTCAGCATTGCTGCAATGAACGAACTCGAACTGAAATAATCGTTCTGTTGGTAA
A3_43888 GGCGGTTTCAGCATTGCTGCAATGAACGAACTCGAACTGAAATAATCGTTCTGTTGGTAA
P3_43888 CGATGGGCGCGCGTTCTGCCGCCCGTTATCTCNNNNNN----------
Original AGATGGGCGCGCGTTCTGCCGCCCGTTATCTCTGTTATACCTTTC----------
23_43888 AGATGGGCGC-CGTTCTGCCGCCCGTTATCTCGGTTATNNNCCTTTTCAAN----------
A3_43888 AGATGGGCGC-CGTTCTGCCGCCCGTTATCTNTNTNNNNAANNCCNTTTCAAANNNNNNNNNN

Figure 5.9: Sequence alignment between parent, 1st passage and resistant to TLN *E. coli* O157:H7 (43888) mutants compared with the published sequence of *E. coli* O157:H7, EDL 933. Where P1: parent *E. coli* O157, 21: second passage, A1: TLN-resistant *E. coli* O157, No. 1: results as with primer 1, nnnn: forward primer 1, nnnn: forward primer 3, nnn: ORF, nnnn: mutation, nnnn: artefact, nnnn: mutation? (Further investigation).

In Figure 5.10 a protein sequence alignment between the longest open reading frame of parent and TLN resistant mutants is shown. It is suggested that the frameshift mutation found results in the inhibition of FabI synthesis in the resistant mutant as a premature stop codon prevents the expression of the protein.

```
P1 Translated - Frame...  .MGFLSGKRILVTGVASKLSIAYGIAQAMHREGAELAFTYQNDKLKGRVEEFAAQLGSDIVLQCDVAEDASI
                consensus .MGFLSGKRILVTGVASKLSIAYGIAQAMHREGAELAFTYQNDKLKGRVEEFAAQLGSDIVLQCDVAEDASI
               confidence .*********************************************************************

A1 Translated - Frame...  DTMFAELGKFGRNLTVSYTLLFLHLAISWMVTMLTPLLVKASKLPTTSAPTASLQWQKLAAPC*TRVLPC*P
P1 Translated - Frame...  DTMFAELGKVWPKFDGFVHSIGFAPGDQLDGDYVVNAVTREGFKIAHDISSYSFVAMAKACRSMLNPGSALLT
                consensus DTMFAELGKFWPNFDGFYHLIGFHPGDQWDGDYINPITREGFKIPHDIAPYAFIQWQKACRPCLNPGLPCLP
               confidence **************988888888888888888888888888865555444555555554444433443333333

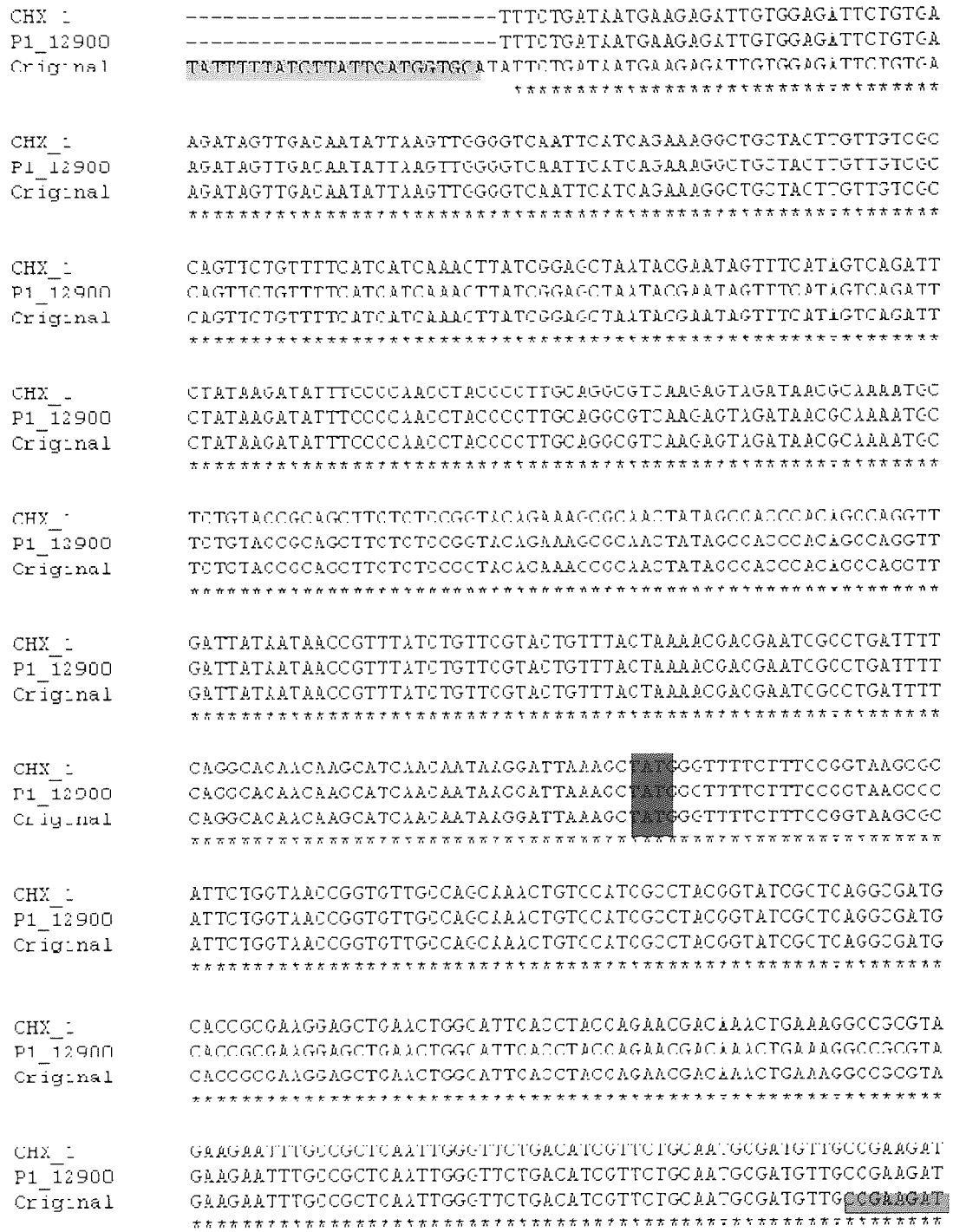
A1 Translated - Frame...  FPTl-alsaLSRTTTLWVWQKRLWKRTCAIWRTRWVRKVCVLTPSLLVRSVLWRPPVSKTSAKCWLIAKPLP
P1 Translated - Frame...  LSYlgaeraIPNYNVMGLAKASLEANVRYMANAMGPEGVRVNAISAGPIRTLAASGIKDFRKMLAHCEAVTP
                consensus FPYLGAERAIPNYNTMWIWQKRLWKNTCYMWNTRWPRGVCVNTPSAGPRRTLWRPPIKDFRKKCWHCEKPTP
               confidence 33220000123333333333333333333333333333344555555555555555555555555555555555

A1 Translated - Frame...  RFAVPLLLKMWVTLRHSCAPISLPVSPVKWSTLTAVSallq*tnsn*N.
P1 Translated - Frame...  IRRTVTIEDVGNSAAFLCSDLSAGISGEVVHVDGGFSiaammelelk*.
                consensus RFRTPTIEDMWNTARHLCAPISAPISPEKWHTDGGFSAAAQNENENKN
               confidence 555555555555555566555544443222222222211111111112
```

Figure 5.10: Amino acid sequence alignments between the longest translated frames of parent and resistant to TLN mutants. The premature stop codon is shown (plum).

5.3.6 DNA sequencing and sequence analysis in CHX-resistant *E. coli* O157

The *fabI* PCR products prepared from *E. coli* O157 (12900) and *E. coli* O157 (43888) CHX resistant mutants were sequenced. This experiment was repeated three times in each case. Sequencing of the *fabI* gene uncovered a frameshift mutation (deletion) identical to that obtained in the TLN-resistant strain. In addition, a second frameshift (insertion) within the same ORF was obtained. Further investigation is required to identify whether or not these are PCR artefacts.


CHX_1
P1_12900
Original

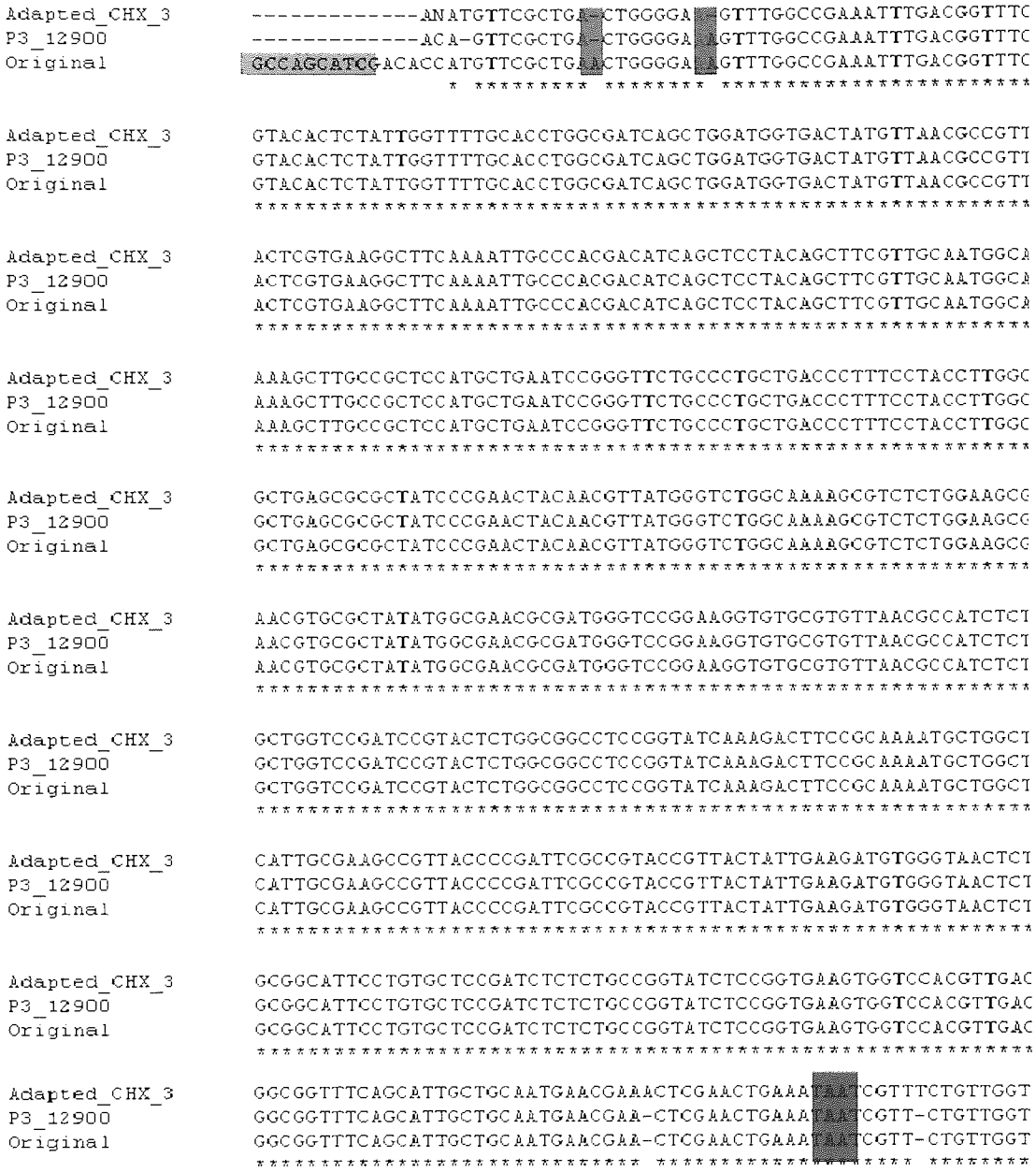


Figure 5.11: Sequence alignment between parent and resistant to CHX *E. coli* O157:H7 (12900) mutants compared with the published sequence of *E. coli* O157:H7, EDL 933. Here P3: Parenty E. coli O157, Adapted CHX 3: CHX-resistant strain, No. 3: results as with primer 3, nnnn: forward primer 1, nnnn: forward primer 3, nnn: ORF, nnnn: mutation, nnnn: artefact, nnnn: mutation? (Further investigation).

In Figure 5.12 a protein sequence alignment between the longest translated frame of the parent and resistant to CHX strains is shown. It is suggested that the frameshift mutation results in the inhibition of FabI synthesis in the resistant strain as a premature stop codon prevents the expression of the protein.

```
Merged_CHX_-Adapted_1...  .MGFLSGKRILVTGVASKLSIAYGIAQAMHREGAELAFTYQNDKLKGRVEEFAAQLGSDIVLQCDVAEDASI
P1 Translated - Frame...  .MGFLSGKRILVTGVASKLSIAYGIAQAMHREGAELAFTYQNDKLKGRVEEFAAQLGSDIVLQCDVAEDASI
                consensus  .MGFLSGKRILVTGVASKLSIAYGIAQAMHREGAELAFTYQNDKLKGRVEEFAAQLGSDIVLQCDVAEDASI
                confidence  .**********************************************************************

Merged_CHX_-Adapted_1...  DTMFADWGs-lacYRFRTLYWFCTWRSAGW*LC*RRYS*RLQNCPRHQLLQLRCNGKSLPLHAESGFCPAD
P1 Translated - Frame...  DTMFAELGkvwpkfdGFVHSIGFAPGDQLDGDYVNAVTREGFKIAHDISSYSFVAMAKACRSMLNPGSALLT
                consensus  DTMFADWGKVWPEFDGFRHLYWFCPWDQADWDYCNRRYREGFQNCHDHQLYQFRCNGKACPLHANPGFCPAD
                confidence  ****9744100012224444456***99999999999999999999999999999999999999999999999

Merged_CHX_-Adapted_1...  PFLPWR*ARYPELQRYGSGKSVSGSERALYGERDGSGRCAC*RHLCWSDPYSGGLRYQRLPQNAGSLRSRYP
P1 Translated - Frame...  LSYLGAERAIPNYNVMGLAKASLEANVRYMANAMGPEGVRVNAISAGPIRTLAASGIKDFRKMLAHCEAVTP
                consensus  PFYPWRERRYPNYNRYGLGKASLGANRRYYGNRDGPGGCRCNRHLCWPDPYLGGLGYQDFPQNAGHCRARYP
                confidence  888898899*********99776677767676666666666666666666666677777777777777666

Merged_CHX_-Adapted_1...  DSPYRYY*RCG*LCGIpvlrslcrylr*sgpr*rrfqhccnernsn*n.
P1 Translated - Frame...  IRRTVTIEDVGNSAAFlcsdlsagisgevvhvdggfsiaamnelelk*.
                consensus  DRPYRYYEDCGNLCGFPCLDLLCGYLGESGHRDGGFQHCCNNRNENKN
                confidence  6666666666632322222222222222222222111111111112
```

Figure 5.12: Amino acid sequence alignments between the longest translated frame of parent and resistant to CHX *E. coli* O157 strains. The premature stop codon is shown (plum).

5.3.7 Growth studies in minimal media

Growth of parent and *E. coli* O157 mutants resistant to CHX and TLN was determined by growing them in M9 minimal media broth and on M9 minimal media agar plates in the presence and absence of fatty acids (FA). All strains tested grew in minimal media, indicating that the resistant strains still produce their own fatty acids. Thus, the presence of the frameshift mutations (deletions) present in both CHX and TLN resistant mutants could be artefacts. In Figures 5.13 and 5.14 the growth of parent and *E. coli* O157 isolates resistant to TLN in the absence and presence of 2µl fatty acids are shown, respectively.

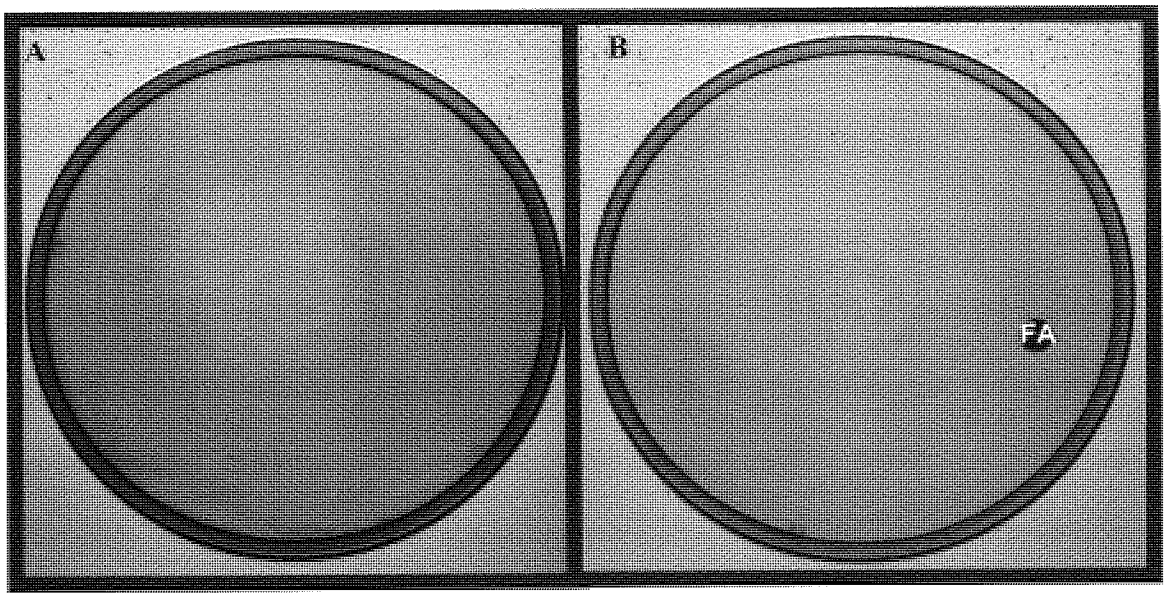


Figure 5.13: Growth of Parent *E. coli* O157 in the absence of fatty acids (A) and in the presence (B) of 2µl fatty acids (FA).

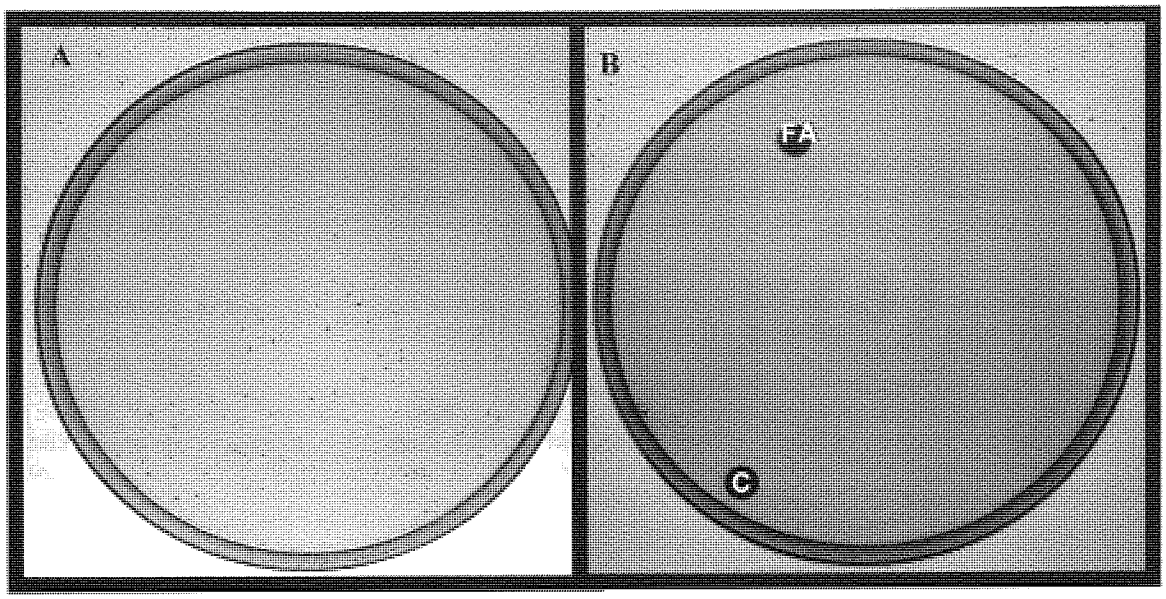


Figure 5.14: Growth of Resistant to TLN *E. coli* O157 in the absence (A) and presence (B) of 2µl fatty acids. FA; Fatty acids, C; Fatty acid free control.

5.3.8 Further DNA sequencing & sequence alignment in CHX- and TLN-resistant strains

The *fabI* PCR products prepared from *E. coli* O157 (12900) and *E. coli* O157 (43888) resistant to CHX and TLN, as well as the 1st passage in TLN were sequenced again by employing a different set of primers. This experiment was repeated once in each case. Sequencing of *fabI* revealed that the frameshift mutations (deletions) obtained previously were in fact artefacts since they were absent in all strains investigated. In the case of the CHX resistant mutant, the second frameshift obtained previously (by primers 3 and 4) was again absent in the new data generated. Thus, CHX resistant is not associated with accumulation of mutation in *fabI* in *E. coli* O157.

```
CHX_adapted_primer_5    ----------------------------------------------------
Original                GGCATTCACCTACCAGAACGACAAACTGAAAGGCCGCGTAGAAGAATTTG
Adapted_CHX_3           ----------------------------------------------------

CHX_adapted_primer_5    -TGCGCTATTGGGTTCTGACATCGTTCTGCAATGCGATGTTGCCGAAGAT
Original                CCGCTCAATTGGGTTCTGACATCGTTCTGCAATGCGATGTTGCCGAAGAT
Adapted_CHX_3           ----------------------------------------------------

CHX_adapted_primer_5    GCCAGCATCGACACCATGTTCGCTGAACTGGGGAAAGTTTGGCCGAAATT
Original                GCCAGCATCGACACCATGTTCGCTGAACTGGGGAAAGTTTGGCCGAAATT
Adapted_CHX_3           ----------ANATGTTCGCTGA-CTGGGGAA-GTTTGGCCGAAATT
                                  ********** ******* ***************

CHX_adapted_primer_5    TGACGGTTTCGTACACTCTATTGGTTTTGCACCTGGCGATCAGCTGGATG
Original                TGACGGTTTCGTACACTCTATTGGTTTTGCACCTGGCGATCAGCTGGATG
Adapted_CHX_3           TGACGGTTTCGTACACTCTATTGGTTTTGCACCTGGCGATCAGCTGGATG
                        ************************************************

CHX_adapted_primer_5    GTGACTATGTTAACGCCGTTACTCGTGAAGGCTTCAAAATTGCCCACGAC
Original                GTGACTATGTTAACGCCGTTACTCGTGAAGGCTTCAAAATTGCCCACGAC
Adapted_CHX_3           GTGACTATGTTAACGCCGTTACTCGTGAAGGCTTCAAAATTGCCCACGAC
                        ************************************************

CHX_adapted_primer_5    ATCAGCTCCTACAGCTTCGTTGCAATGGCAAAAGCTTGCCGCTCCATGCT
Original                ATCAGCTCCTACAGCTTCGTTGCAATGGCAAAAGCTTGCCGCTCCATGCT
Adapted_CHX_3           ATCAGCTCCTACAGCTTCGTTGCAATGGCAAAAGCTTGCCGCTCCATGCT
                        ************************************************

CHX_adapted_primer_5    GAATCCGGGTTCTGCCCTGCTGACCCTTTCCTACCTTGGCGCTGAGCGCG
Original                GAATCCGGGTTCTGCCCTGCTGACCCTTTCCTACCTTGGCGCTGAGCGCG
Adapted_CHX_3           GAATCCGGGTTCTGCCCTGCTGACCCTTTCCTACCTTGGCGCTGAGCGCG
                        ************************************************

CHX_adapted_primer_5    CTATCCCGAACTACAACGTTATGGGTCTGGCAAAAGCGTCTCTGGAAGCG
Original                CTATCCCGAACTACAACGTTATGGGTCTGGCAAAAGCGTCTCTGGAAGCG
Adapted_CHX_3           CTATCCCGAACTACAACGTTATGGGTCTGGCAAAAGCGTCTCTGGAAGCG
                        ************************************************

CHX_adapted_primer_5    AACGTGCGCTATATGGCGAACGCGATGGGTCCGGAAGGTGTGCGTGTTAA
Original                AACGTGCGCTATATGGCGAACGCGATGGGTCCGGAAGGTGTGCGTGTTAA
Adapted_CHX_3           AACGTGCGCTATATGGCGAACGCGATGGGTCCGGAAGGTGTGCGTGTTAA
                        ************************************************

CHX_adapted_primer_5    CGCCATCTCTGCTGGTCCGATCCGTACTCTGGCGGCCTCCGGTATCAAAG
Original                CGCCATCTCTGCTGGTCCGATCCGTACTCTGGCGGCCTCCGGTATCAAAG
Adapted_CHX_3           CGCCATCTCTGCTGGTCCGATCCGTACTCTGGCGGCCTCCGGTATCAAAG
                        ************************************************

CHX_adapted_primer_5    ACTTCCGCAAAATGCTGGGCTCATTGCGAAGCCGTTACCCCGATTCGCCG
Original                ACTTCCGCAAAATGCTGG-CTCATTGCGAAGCCGTTACCCCGATTCGCCG
Adapted_CHX_3           ACTTCCGCAAAATGCTGG-CTCATTGCGAAGCCGTTACCCCGATTCGCCG
                        ****************** ********************************

CHX_adapted_primer_5    TACCGTTACTATTGAAGATGTGGGTAACTCTGCGGCATTCCTGTGCTCCG
Original                TACCGTTACTATTGAAGATGTGGGTAACTCTGCGGCATTCCTGTGCTCCG
Adapted_CHX_3           TACCGTTACTATTGAAGATGTGGGTAACTCTGCGGCATTCCTGTGCTCCG
                        ************************************************

CHX_adapted_primer_5    ATCTCTCTGCCGGTTATCTCCGGTGAAGTGGTCCACGTTGA----GNTTT
Original                ATCTCTCTGCCGGT-ATCTCCGGTGAAGTGGTCCACGTTGACGGCGGTTT
Adapted_CHX_3           ATCTCTCTGCCGGT-ATCTCCGGTGAAGTGGTCCACGTTGACGGCGGTTT
                        ************ ************************    * ****
```

Figure 5.15: Sequence alignment between CHX-resistant *E. coli* O157:H7 (12900) strains generated by primers 3 & 4 and primers 5 & 6 compared with the published sequence of *E. coli* O157:H7, EDL 933. Where nnnn: forward primer 5, nnnn: artefact.

In Figure 5.16 a representation of *fabI* structure is shown. In Figures 5.17a and 5.17b the active sites of *fabI* showing the mutation, which causes the conversion of Gly93 to Val93 are illustrated. Note the steric clash between the side chains of Val93 and TCL (TLN) preventing effective inhibition. The bond crystal structure of TCL (TLN), NADH and *E. coli* FabI is also shown, which has been previously found by Stewart *et al,* (1999). From the Figures 5.17a and 5.17b it could be suggested that as valine is a structurally larger amino acid, when present, it prevents the binding of TLN to NADH, thus rendering TLN less effective. The *fabI* ribbon structure was omitted for simplification.

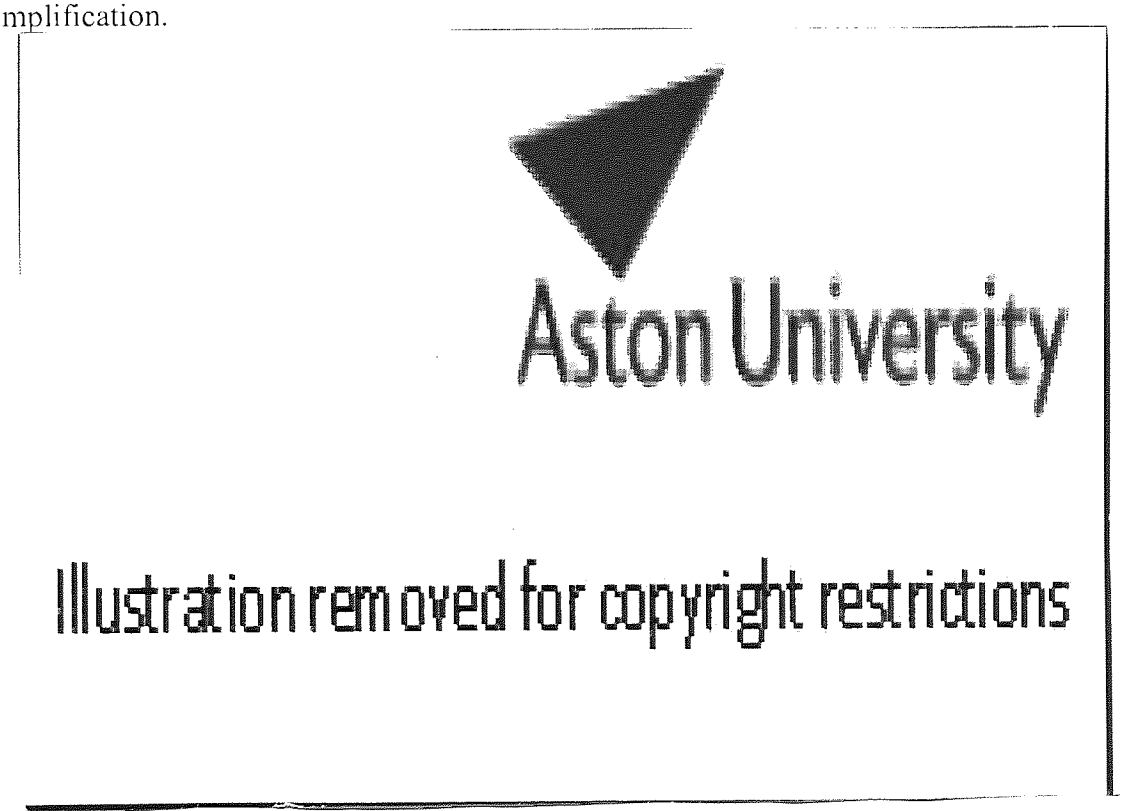
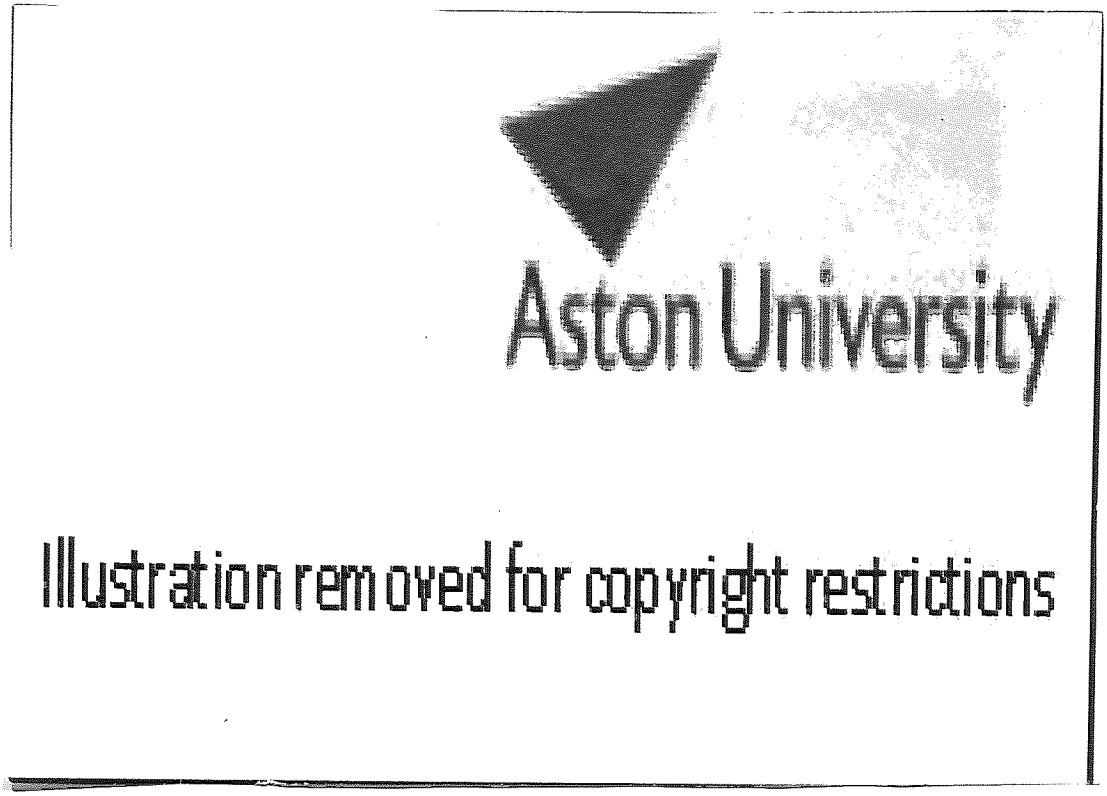


Figure 5.16: FabI ribbon structure (RCSB, 2004; URL:http//www.rcsb.org/pdb).



Figures 5.17a & b: Active site of *fabI* showing mutation; Gly93 to Val93 DeepView/Swiss PdbViewer, 2004 (URL:http//www.ca.expasy.org/spdbv).

5.3.9 Fatty acid analysis in TLN-resistant *E. coli* O157

In Figures 5.18 and 5.19 overlaid traces of standard bacterial fatty acid methyl esters present in parent and TLN resistant *E. coli* O157 strains between eight to fifteen and fifteen to twenty minutes are shown, respectively. By comparing the traces obtained for pre-and post- resistant strains it is suggested that significant differences are apparent. Table 4.10 lists the fatty acid methyl esters used in the standard, Table 4.11 lists those identified for parent strain and Table 4.12 lists those identified for the resistant *E. coli* O157 strains.

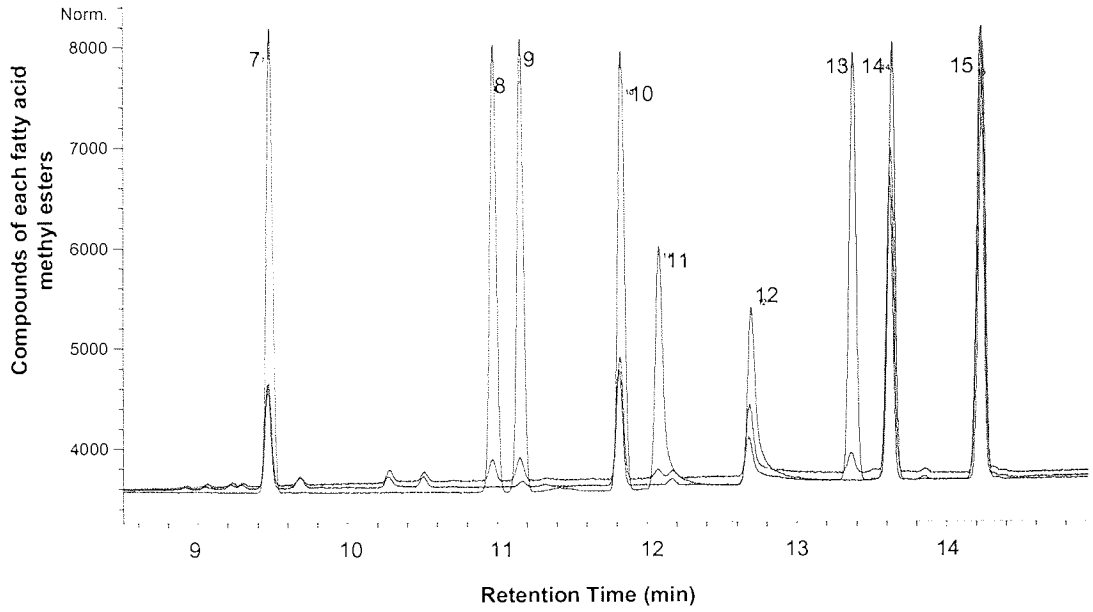


Figure 5.18: Gas Chromatography overlaid traces showing methyl esters of fatty acids present in *E. coli* O157 parent (green) and resistant to TLN (red) strains. In blue colour the standard bacterial fatty acid methyl esters CP^{TM}Mix are shown. Traces were obtained from eight to 15 minutes. Refer to Table 5.3 for fatty acid identitites.

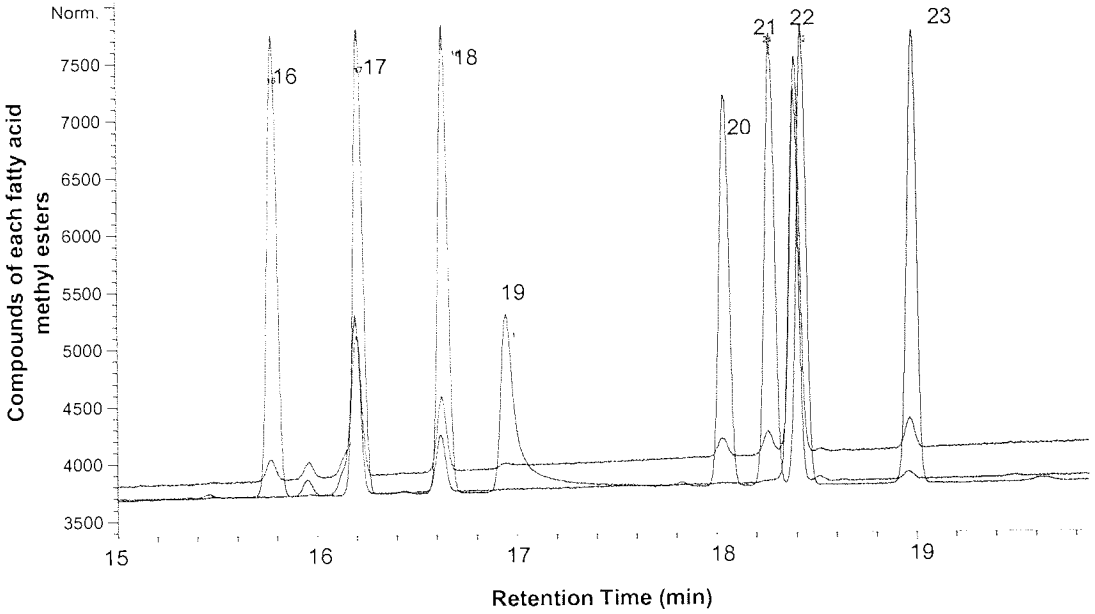


Figure 5.19: Gas Chromatography overlaid traces showing methyl esters of fatty acids present in *E. coli* O157 parent (green) and resistant to TLN (red) strains. In blue colour the standard bacterial fatty acid methyl esters CP^{TM}Mix are shown. Traces were obtained from 15 to 20 minutes. Refer to Table 5.3 for fatty acid identitites.

Table 5.3

List of FAMEs used in the standard			
Component	FAME	Component	FAME
1	11:0. undecanoate	14	16:1. Cis-9-hexadecenoate
2	2-OH 10:0. 2-hydroxydecanoate	15	16:0. hexadecanoate
3	12:0. dodecanoate	16	i-17:0 15-methylhexadecanoate
4	13:0. tridecanoate	17	17:0 9,10-methylenehexadecanoate
5	2-OH 12:0. 2-hydroxydodecanoate	18	17:0. heptadecanoate
6	3-OH 12:0. 3- hydroxydodecanoate	19	2-OH 16:0 2-hydroxyhexadecanoate
7	14:0.tetradecanoate	20	18:2. cis 9,10-octadecadienoate
8	i-15:0. 13-methyltetradecanoate	21	18:1 cis-9-octadecenoate
9	a-15:0 12-methyltetradecanoate	22	18:1 trans-9-octadecenoate
10	15:0.pentadecanoate	23	18:0 Octadecanoate
11	2-OH 14:0 2-hydroxytetradecanoate	24	19:0 cis-9,10-methyleneoctadecanoate
12	3-OH. 14:0 3-hydroxytetradecanoate	25	19:0 nonadecanoate
13	i-16:0 14-methylpentadecanoate	26	20:0-eicosanoate

From the data presented in Table 5.4 and 5.5, it is suggested that in the parent strain some fatty acids are not present, whereas in the resistant one the majority of them can be found. Thus, fatty acids synthesis is an active candidate for the resistance acquired by the resistant strain. In addition, four unknown peaks were common in both strains.

Table 5.4

List of FAMEs found in parent *E. coli* O157			
Component	**FAME**	**Component**	**FAME**
1	-[1]	13	-
2	-	14	**16:1. Cis-9-hexadecenoate**
3	**12:0. dodecanoate**	15	**16:0. hexadecanoate**
4	**13:0. tridecanoate**	16	-
5	-	X4	**Unknown**
6	-	17	**17:0 9,10-methylenehexadecanoate**
7	**14:0.tetradecanoate**	18	**17:0. heptadecanoate**
X1[2]	**Unknown**	19	-
X2	**Unknown**	20	-
X3	**Unknown**	21	-
8	-	22	**18:1 trans-9-octadecenoate**
9	**a-15:0 12-methyltetradecanoate**	23	**18:0 Octadecanoate**
10	**15:0.pentadecanoate**	24	**19:0 cis-9,10-methyleneoctadecanoate**
11	**2-OH 14:0 2-hydroxytetradecanoate**	25	-
12	**3-OH. 14:0 3-hydroxytetradecanoate**	26	**20:0-eicosanoate**

Table 5.5

List of FAMEs found in resistant *E. coli* O157			
Component	FAME	Component	FAME
1	-	13	-
2	-	14	**16:1. Cis-9-hexadecenoate**
3	**12:0. dodecanoate**	15	**16:0. hexadecanoate**
4	**13:0. tridecanoate**	16	i-17:0 15-methylhexadecanoate
5	2-OH 12:0. 2-hydroxydodecanoate[3]	X4	**Unknown**
6	3-OH 12:0. 3- hydroxydodecanoate	17	**17:0 9,10-methylenehexadecanoate**
7	**14:0.tetradecanoate**	18	**17:0. heptadecanoate**
X1	**Unknown**	19	-
X2	**Unknown**	20	18:2 cis 9,10-octadecadienoate
X3	**Unknown**	21	18:1 cis-9-octadecenoate
8	i-15:0 13-methyltetradecanoate	22	**18:1 trans-9-octadecenoate**
9	**a-15:0 12-methyltetradecanoate**	23	**18:0 Octadecanoate**
10	**15:0.pentadecanoate**	24	**19:0 cis-9,10-methyleneoctadecanoate**
11	**2-OH 14:0 2-hydroxytetradecanoate**	25	19:0 nonadecanoate
12	**3-OH. 14:0 3-hydroxytetradecanoate**	26	**20:0-eicosanoate**

[1]**The dash (-) indicates that the fatty acids were not found**

[2]**X indicates the presence of unknown peaks**

[3]**Violet-faced data indicates those fatty acids absent from the parent strain.**

5.3.9.1 Quantification of fatty acids

According to the results 100% of fatty acids were present in the parent strain, whereas in the TLN-resistant strain approximately 96.29% of fatty acids were found. In the

CHX-resistant mutant approximately 97.56% of fatty acids were present. This did not reach statistical significance (p=<0.05) in any case.

5.3.10 Quantification & Qualitation of lipids

In Figures 5.20 and 5.21 photographs of TLC plates visualised with nihydrin and molybdenum spray respectively are shown. The lipids obtained from parent and resistant *E. coli* O157 has not separated completely however, it is still evident that no significant differences between parent and resistant strains were obtained. In addition, lipids were extracted from CHX-resistant *E. coli* O157 and data generated suggests that no difference between parent and resistant strains exists. All experiments were carried out three times and a variety of solvents were used in each experiment.

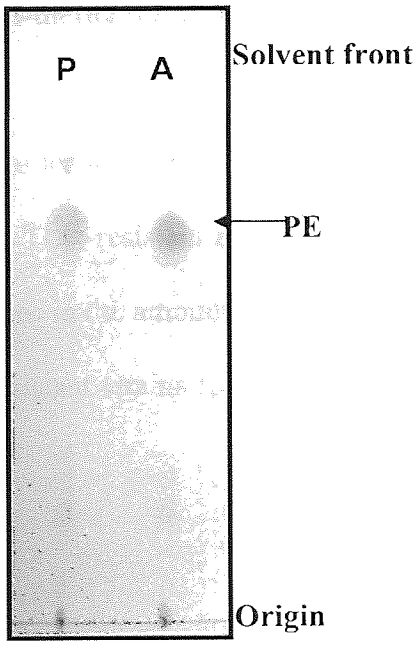


Figure 5.20: Lipids containing amino compounds visualised with nihydrin. 'P' refers to parent *E. coli* O157, 'A' refers to resistant *E. coli* O157. The solvent system employed was chloroform-methanol-acetic acid.

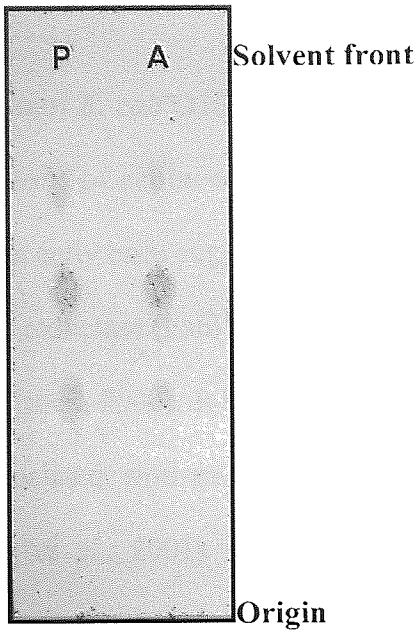


Figure 5.21: Whole cell phospholipids visualised with molybdenum blue spray. 'P 'refers to parent *E. coli* O157, 'A' refers to resistant *E. coli* O157. The solvent system employed was chloroform-methanol-acetic acid.

Experiments were carried out in order to quantify and compare the amount of lipid present in parent and in CHX- and TLN-resistant *E. coli* O157 strains. Data generated suggests that there are no differences in the amount of lipid present in the parent strain when compared to that of both resistant strains ($p=<0.05$). Experiments were carried out on three individual occasions.

5.4 Discussion

5.4.1 *fabI* mutations in TLN-resistant *E. coli* O157

It was initially believed that the mode of action of TLN was primarily on bacterial membranes, however it has more recently been suggested that it acts via the inhibition of enoyl-acyl carrier protein (ACP) reductase (FabI) in *E. coli* (Heath and Rock, 1995; McMurry *et al.*, 1998a; Heath and Rock, 2000). It has also been previously suggested that resistance to triclosan is associated with mutations in *fabI* (McMurry *et al.*, 1998a; Heath *et al.*, 1998; Heath *et al.*, 2001). Thus, this study investigated the presence of mutations in the resistant strain and more importantly whether a mutation could facilitate the acquisition of the rapid resistance observed in the resistant strain. This was examined by comparing parent, low-resistant (1^{st} passage) and fully-resistant strains.

From the data generated it is apparent that a mutation, GGT to GTT at codon 93 of *fabI* has taken place. This results in the substitution of glycine 93 to valine 93 in *fabI,* an observation which has been previously reported by McMurry *et al.* (1998a). Molecular studies of the *fabI* mutation suggested that because valine is large in structure amino acid, this mutation prevents TLN binding to the cofactor, NADH. Thus, the efficacy of TLN is eliminated and bacteria can resist even at high concentrations. It is also noteworthy that the same mutation was present in the low TLN-resistant mutant, which in turn suggests that this is not the mechanism accountable for the rapid resistance observed. In the low-TLN resistant strain it may be that only the mutation is responsible for the acquisition of resistance, whereas in the TLN (highly) resistant strain a combination of mechanisms may work; possibly the overexpression of an active efflux pump; marA, SoxS or acrAB which have

previously been associated with TLN-resistance in *E. coli* (McMurry *et al.*, 1998a; Walsh *et al.*, 2003). According to Gilbert & McBain (2002) work on FabI mutants and multidrug efflux pumps has led to the speculation that the continuous misuse of TLN might generate multiply antibiotic-resistant populations of bacteria.

The presence of a frameshift mutation in all strains of CHX- and TLN-resistant *E. coli* O157 was also observed. A deletion was present on every occasion and further *in silico* studies suggest that this may lead to the prevention of FabI expression, since a premature stop codon is introduced. Thus, further investigation is required in order to confirm whether or not this frameshift is genuine. Previous research has demonstrated that *E. coli* strains are capable of synthesizing phospholipids almost entirely from exogenous fatty acids supplied by the growth medium (Sinesky, 1971). Therefore even in the event that FabI was not expressed, *E. coli* cells may not lyse because they are utilising the lipids obtained from the media. One way this was further investigated was by growing parent and resistant strains in defined minimal media, where only the absolute essential sources for bacterial growth were present. If the frameshift was a true genetic event, the resistant strain should lyse since it would not be possible to produce phospholipids and there are no other alternative fatty acid biosynthetic pathway known to be present in *E. coli*. At the same time additional primers were designed in order to verify whether or not the frameshift was present at the same position of the gene on a different amplicon.

Growth curves were performed in minimal broth and it was concluded that the resistant strain grew with a significantly lower growth rate when compared with the parent strain. Growth studies on minimal agar media were undertaken whereby both

strains grew, suggesting that *fabI* was still expressed. This was confirmed when the sequencing data was re-analysed; the frameshift mutation was absent from both CHX- and TLN-resistant strains. Thus, it can be concluded that CHX resistance is not involved in *fabI* and that TLN resistant strains arose from a substitution mutation converting glycine 93 to valine 93 as previously discussed.

5.4.2 Quantification and qualitation of fatty acids & lipids in *E. coli* O157

Lipids of TLN resistant *E. coli* O157 were extracted by two different solvent systems; chloroform/methanol and glacial acetic acid, or chloroform/methanol and water and characterised by thin layer chromatography. Lipid standards examined included PE, PG and DPG. Those classes of lipids characterised as PE gave a positive reaction with both molybdenum blue and nihydrin, whilst those characterised as PG and DPG gave a positive reaction with molybdenum blue and a negative one with nihydrin.

Although the solutes were not successfully separated, it was still apparent that no quantitative or qualitative differences existed in the phospholipids or aminophospholipids between parent and resistant strains. More specifically, the composition of nihydrin- or molybdenum blue-positive phospholipids hardly differed between susceptible and resistant strains of *E. coli* O157. The fact that a complete separation of lipids was not achievable could be a result of different sources of error in TLC, such as overloading, tailing or changes in the temperatures (Wharton and McCarty, 1972). Another error occasionally encountered in TLC rests in the use of solvent systems; however this could not be case in the current investigation since the solvents employed had worked reproducibly on *E. coli* in previous investigations (Subrahmanyam and Cronan Jr., 1998).

In addition, quantitative examination revealed that there were no significant differences in the relative amount of chloroform/methanol extractable lipids collected from parent and *E. coli* O157 resistant to TLN strains ($p=<0.05$).

The precise fatty acid compositions of parent and resistant to TLN *E. coli* strains were determined by gas chromatography. Gas chromatography has two advantages over other forms of chromatography; separations are feasible in a shorter period of time and the technique can be made quantitatively precise (Wharton and McCarty, 1972).

In Gram-negative bacteria, the major saturated fatty acid is usually 16:0 and 14:0 or to a lesser extent 18:0. The major unsaturated fatty acids are 16:1 and 18:1 and hydroxylated fatty acids have also been reported (Ratledge and Wilkinson, 1988). This is in agreement with the suggestions made by Subrahmanyam and Cronan Jr. (1998), according to whom *E. coli* synthesizes four major fatty acids including C14:0 (myristate), 16:0 (palmitate, 16:1 (palmitoleate) and 18:1 (cis-vacceate). Laurate 12:0 and 3-hydroxymyristate are found in lipid A, whereas palmitate, palmitoleate and cis-vaccenate are phospholipids components.

In the current investigation potential qualitative and quantitative changes in the production of fatty acids in TLN- resistant cells of *E. coli* were examined. Fatty acid profiles presented in section 4.3.9 proved the presence of the major fatty acids usually found in *E. coli*, as discussed above, in both parent and resistant strains. However, consistent qualitative differences were also obtained between the fatty acid compositions of parent and resistant to TLN *E. coli* O157 strains. Most notable was

the fact that in resistant strains seven fatty acids were found that were absent in the parent strain. It could be possible that the presence of triclosan could trigger the synthesis of these fatty acids, which could facilitate the acquisition of the rapid resistance observed in the resistant strain. In 1982, Persino and Lynchm also suggested that alterations in the fatty acids biosynthesis were found to accompany triclosan resistance observed in *E. coli* (Persino and Lynchm, 1982). No quantitative differences in fatty acid production between parent and resistant mutants were observed.

This study has demonstrated that sub-lethal levels of TLN selects for mutants in FabI. Triclosan shares this target with some current therapeutic agents such as the antituberculosis drug, isoniazid suggesting that sub-inhibitory triclosan exposure could select for resistance to such antimicrobial agents. Of note, in *Mycobacterium smegmatis* resistance to isoniazid has been associated with mutations in InhA, which is a homologue of FabI (McMurry *et al.*, 1999). However, similar studies have suggested that this is not the case in *M. tuberculosis* suggesting that although they share the same target their interactions remain distinct (McBain *et al.*, 2002). If this is the case in TLN-resistant *E. coli* O157 strains, the increased degree of cross-resistance exhibited, might be explained as a result of alterations in the fatty acid production observed. In the resistant strains approximately seven additional fatty acids were present, indicating that these fatty acids might reduce the ability of those antibiotics to which *E. coli* O157 resistant to TLN showed reduced susceptibility, to permeate cells, leading to the acquisition of resistance and potential cross-resistance.

5.5 Conclusions

In this study the phenomenon by which *E. coli* O157 responded to sub-lethal exposure to TLN was investigated further, particularly to try to explain the rapid jump in resistance following first exposure. Mutation in *fabI* of *E. coli* K-12 is accepted as an important event in TLN resistance and this gene was investigated further in *E. coli* O157 for evidence to support the resistance characteristics observed. Using a primer set designed for amplifying *fabI* and sequencing of the amplicons, the previously reported Glycine to Valine substitution was observed as expected but an additional novel insertion resulting in a premature stop codon was also discovered. Further analysis using a second primer set suggested that the insertion was possibly a PCR artefact, which was supported by growth of the isolate on M9 minimal media. This result however is intriguing as the first primer set repeatedly and reproducibly revealed the single nucleotide insertion. Confirmation of the validity of the data generated by each primer set could come from analysis of gene expression by Reverse Transcription PCR (RT-PCR). However, time constraints prevented taking this further.

A similar observation was obtained from amplification of *fabI* of *E. coli* O157 isolates resistant to CHX; an insertion in an identical position leading to a premature stop codon but in this resistance the Glycine to Valine substitution was absent.

Thin layer chromatography revealed identical lipid profiles between parent and resistant strains. This was confirmed quantitatively using solvent extraction. Gas chromatography revealed seven fatty acids present in the resistant strain but absent

from the parent. This may be as a consequence of an auxotrophic phenotype in the resistant isolate, which requires further investigation.

Chapter 6: Final Discussion

In this investigation the potential for resistance to erythromycin, benzalkonium chloride, chlorhexidine and triclosan was investigated in *Salmonella enterica* serovars Enteritidis, Typhimurium and Virchow and in *Escherichia coli* O157. Following sub-lethal exposure, potential mechanisms of resistance and cross-resistance, if any were examined further.

Through serial passage in increasing sub-lethal concentrations of antimicrobial agent resistance was acquired in all isolates investigated. Of particular interest was the observation that *E. coli* O157 while initially sensitive to extremely low concentrations (0.25μg/ml) of TLN, rapidly decreased susceptibility; growth at extremely high concentrations (1024μg/ml) was observed. This is of particular concern given the widespread incorporation of TLN into many household products where their utility is questionable. Coupled with the lack of control of usage it is not impossible to envisage a situation where microoorganisms are exposed to sub-lethal concentrations of an antimicrobial. While it is accepted that laboratory investigations cannot entirely model microbe/biocide interactions in the environment. It is certainly an indication that such events leading to a reduction in susceptibility are possible.

The resistance was stable for up to 30 days when strains were passaged in antibiotic/biocide free media in all cases investigated. This is an interesting observation in the fact that it suggests that in the event that such products were to be reduced in their application, the resistance mechanisms would be maintained. The

actual duration of retention is difficult to predict but on the basis of this investigation it appears to be for a considerable time.

Although a great deal of work has been carried out on many aspects of antimicrobial resistance, questions still remain. There is a spectrum of possible mechanisms by which bacterial pathogens may become resistant to antimicrobial agents and this study aimed to investigate a number of resistance mechanisms possibly employed by *Salmonella enterica* and *E. coli* O157 strains.

Previous research suggested that resistance in Gram-negative bacteria might be associated with changes in outer membrane, including LPS (Loughlin *et al.*, 2002). However, outer membrane and LPS profiles did not reveal any significant changes between all parent and resistant strains of *Salmonella enterica* and *E. coli* O157 strains investigated.

Cell surface hydrophobicity played a significant role in some instances of *Salmonella enterica* and *E. coli* O157 resistance; parent strains were not hydrophobic, whereas resistant strains were hydrophobic. This might imply the possibility of the presence of an extra protein in the cell surface. However this was not revealed in OMP profiles. Modification in cell surface charge did not reveal any strong correlation with resistance although this is probably dependent upon the change of the antimicrobial being treated.

Resistance to ERY, BKC, CHX and TLN was strongly associated with up regulation of efflux activity in all strains investigated. More specifically, results suggested that BKC and CHX resistance was mediated by an efflux pump belonging either to the

MFS, ABC or RND superfamilies, since sensitivity was restored by both reserpine and CCCP, whereas ERY and TLN resistance could be mediated by pumps belonging to the RND family as only CCCP inhibited the pump involved in this resistance. The observation that exposure to the relatively narrow range of antimicrobial agents investigated in this study stimulates the up-regulation of generic efflux mechanisms has rather broader implications than a mere reduction in the sensitivity of isolates. One might suspect that a much wider range of substrates could be more efficiently removed from the cell. For instance, might there be an associated pH/osmotic resistance (Au).

It is highly likely that the reduction in sensitivity observed in the isolates investigated is not attributable to a single resistance mechanism; indeed the two phase development of resistance to TLN in *E. coli* O157 supports this. In order to investigate this further *fabI* was examined as it is accepted as a key modification in resistant isolates. A series of genetic studies suggested that *E. coli* O157 resistant to TLN strain arose via substitution mutations in the *fabI* converting glycine 93 to valine 93. The same mutation occurred in the 'low-resistant' TLN-resistant strain, which indicated that was not the result of the rapid resistance observed. Molecular modelling suggested that due to this mutation TLN was not able to bind to NADH. Genetic evidence also suggested that *fabI* of *E. coli* O157 is not a target for CHX.

In TLN resistant *E. coli* O157, the presence of an active efflux in association with the mutations in the FabI could explain the acquisition of the rapid high levels of resistance to TLN. In order to confirm that, one obvious experiment would be to investigate whether an active efflux system was present in the 'low-resistant' TLN-

resistant strain, since the mutation was common in both of them. However, with the assays employed it would be difficult to draw any conclusions regarding the presence of an active efflux in the 'low-resistant strain' since the MIC of the parent did not differ greatly from that of the first passage. A recent collaboration between our laboratory and Dr Sima Yaron of the Institute of Technology, Israel has provided a library of GFP reporter plasmids carrying acrAB, marRAB, soxR, soxS, robA and micF promoters. It is anticipated that these will provide a more accurate determination of efflux activity especially at low levels of expression. Unfortunately, time was not available within the duration of this study to use the reporter plasmids, but this will be undertaken in the future.

Studies on the production of fatty acids were carried out by comparing parent and *E. coli* O157 resistant to CHX and TLN strains. Data suggested that in TLN resistant strains seven fatty acids were found that were absent from the parent isolates, however no quantitative differences in the fatty acid production between parent and *E. coli* O157 resistant to TLN were observed. In addition, fatty acid examination in the CHX-resistant strain did not reveal any significant changes.

Also, possible differences in the production of lipids were investigated in parent and in both CHX- and TLN- resistant *E. coli* O157 strains. Data generated suggested that the presence of FabI mutation did not result in any quantitative or qualitative differences in the lipid production between parent and CHX- or TLN- resistant strains.

This study demonstrated links between biocide and antibiotic resistance. More specifically it was shown that some types of biocide resistance could provide cross-resistance to other antimicrobial agents. However, of note is that this cross-resistance was serotype specific and not in any case antibacterial or strain specific. For instance, *Salm.* Enteritidis did not exhibit any reduced susceptibility to any antimicrobial agent when exposed to BKC, whereas the rest of *Salmonella enterica* serotypes investigated in this study.

Cross-resistance of TLN-resistant *E. coli* to a panel of antimicrobial agents was also tested in *E. coli* O157, *E. coli* O55 and *E. coli* K-12. Data obtained suggested that there was a degree of cross-resistance in *E. coli* O55 and *E. coli* K-12, however to a lesser extent when compared with *E. coli* O157. Triclosan-resistant *E. coli* O157 showed decreased susceptibility to CHL, ERY, IPM, TET, TMP, CHX and BKC, whereas *E. coli* O55 demonstrated cross-resistance only to TMP and *E. coli* K-12 just to CHL. It is particularly difficult to predict the likely response of an isolate following exposure at sub-inhibitory concentrations of an antimicrobial even when data on a closely related isolate is available. It is highly dependent on the specific microbe/antimicrobial interaction.

In addition, previous studies indicate that TLN resistant *E. coli* O157 had acquired enhanced cross-resistance to ERY, BKC and CHX, indicating that these share at least one of the potential active efflux pumps involved in the acquisition of resistance. The same study showed that in *Salm.* Virchow strains the development of BKC resistance conferred reduced susceptibility to chlorhexidine, which in turn might suggest that the same efflux pump is up-regulated. Multidrug efflux can be responsible for cross-

resistance between several groups of antimicrobial compounds. In *Salmonella* Typhimurium it was difficult for any firm conclusions to be drawn as the MICs for parent and resistant strains were close, however it is still highly likely that resistance is associated with the activity of an efflux pump, as resistant strains returned to their parent MIC in the presence of reserpine. Again, GFP reporter plasmids will help clarify the involvement of efflux.

The high degree of cross-resistance in TLN-resistant in *E. coli* O157 could be attributed to the seven additional fatty acids present in the cell wall. Probable links of cross-resistance between antibiotics and triclosan, or other biocides due to the presence of a presumptive efflux pump have been suggested previously.

Exposure to sub-inhibitory concentrations of biocides is feasible especially when they become dissipated to the environment. Thus, it is possible biocide resistance to emerge, which is related to a number of distinct mechanisms. This study investigated those mechanisms that promote biocide resistance and subsequent effects on antibiotic susceptibility in *Salmonella enterica* and *E. coli* O157. From the evidence generated it appears possible that stable resistance to biocides can derive from the up-regulation of an efflux system and changes in the hydrophobicity in certain isolates. A particular concern was that triclosan can select for mutants in the *fabI* of *E. coli* at sub-lethal concentrations, which probably coupled with the presence of an active efflux system, could confer cross-resistance to various therapeutic antibiotics. A summary of the results obtained throughout this study for *Salmonella enterica* and *Escherichia coli* is shown in Tables 6.1 and 6.2, respectively.

Although these observations have not been supported by retrospective analysis of isolates taken from hospitals or the domestic environment they are still of major concern and discourage the uncontrolled use of biocides in the every day life.

Table 6.1

Bacterial Isolates And antimicrobial agent	Possible Mechanisms of Resistance in *Salmonella enterica*					
	Modified Growth Rate	Change in Cell Surface Hydrophobicity	Modified Cell Surface Charge	Upregulated Efflux	Modification of Outer Membrane Proteins	Modification of LPS
Salm. Enteritidis ERY	√	√	-	√	-	-
Salm. Enteritidis BKC	-	√	-	√	-	-
Salm. Enteritidis TLN	√	-	-	√	-	-
Salm. Typhimurium ERY	√	√	-	√	-	-
Salm. Typhimurium BKC	√	√	√	√	-	-
Salm. Typhimurium TLN	√	-	-	√	-	-
Salm. Virchow ERY	√	-	√	√	-	-
Salm. Virchow BKC	-	√	-	√	-	-
Salm. Virchow CHX	-	N/A	N/A	√	-	-
Salm. Virchow TLN	-	-	_	√	-	-

Where √=A mechanism for which data supports its involvement in resistance
-= A mechanisms which appears to not be involved in resistance
N/A= A mechanism not investigated with this isolate/antimicrobial combination

Table 6.2

Bacterial Isolates & antimicrobials	Possible Mechanisms of Resistance in *Escherichia coli*								
	Modified Growth Rate	Change in Cell Surface Hydrophobicity	Modified Cell Surface Charge	Upregulated Efflux	Modification of Outer Membrane Proteins	Modification of LPS	*fabI* mutations	Changes in Lipids	Changes in Fatty acids
E. coli O157 ERY	√	-	-	√	-	-	N/A	N/A	N/A
E. coli O157 BKC	√	-	√	√	-	-	N/A	N/A	N/A
E. coli O157 CHX	√	√	-	√	-	-	N/A	N/A	N/A
E. coli O157 TLN	√	√	-	√	-	-	√	-	√
E. coli O55 TLN	-	-	√	√	N/A	N/A	N/A	N/A	N/A
E. coli K-12 TLN	N/A	N/A	N/A	√	N/A	N/A	N/A	N/A	N/A

Where √=A mechanism for which data supports its involvement in resistance
-= A mechanisms which appears to not be involved in resistance
N/A= A mechanism not investigated with this isolate/antimicrobial combination

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APPENDICES

Appendix 1: An example of microelectrophoresis raw data from control, KCl, parent & resistant strains.

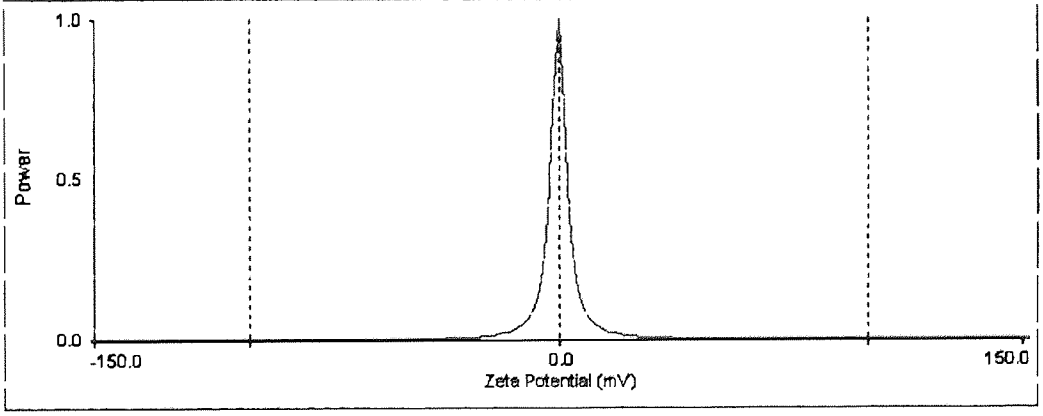
Brookhaven Instruments Corp.
Zeta Potential Analyzer Ver. 3.23

Date: May 15, 2002
Time: 09:29:51

Sample ID **Control (Run 10)**
Operator ID **Maria**
Notes

Measurement Parameters:			
Avg. Zeta Potential	= 0.00 mv	Liquid	= Aqueous
Avg. Mobility	= 0.00 (μs) / (V/cm)	Temperature	= 25.0 deg. C
pH	= 5:50	Viscosity	= 0.890 cP
Conductance	= 308 μS	Refractive Index	= 1.330
Concentration	= 1.00 mg/mL	Dielectric Constant	= 78.54
		Particle Size	= 0.0 nm

Instrument Parameters:			
Sample Count Rate	= 145 kcps	Current	= 1.78 mA
Ref. Count Rate	= 3216 kcps	Electric Field	= 15.95 V/cm
Sampling Time	= 256 μs	User1	= 0.00
Wavelength	= 659.0 nm	User2	= 0.00



Run	Zeta Potential (mV)	Half Width (mV)
1	0.00	2.71
2	0.00	2.77
3	0.00	2.64
4	0.00	2.75
5	0.00	2.59
6	0.00	2.59
7	0.00	2.56
8	0.00	2.59
9	0.00	2.61
10	0.00	2.60
Mean	0.00	2.64
Std. Error	0.00	0.02

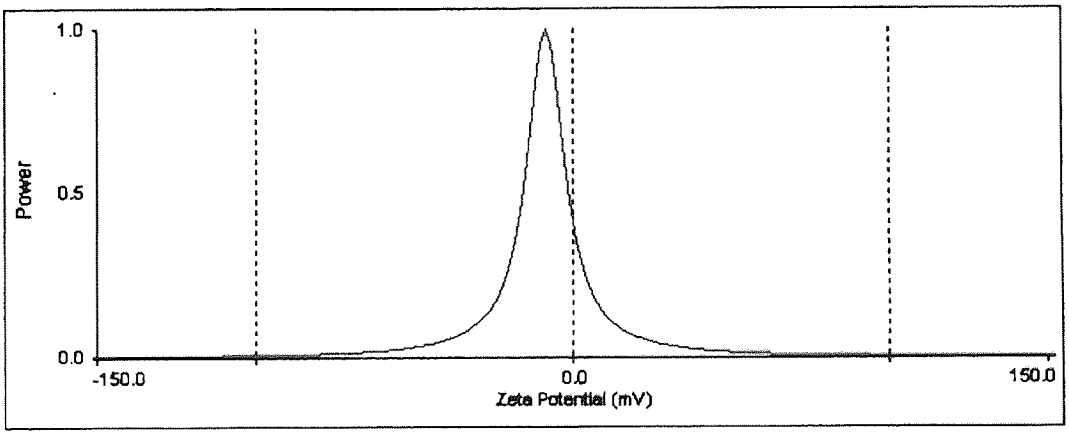
Brookhaven Instruments Corp.
Zeta Potential Analyzer Ver. 3.23

Date: May 15, 2002
Time: 09:44:01

Sample ID **KCL 1mM (Run 10)**
Operator ID **MB**
Notes

Measurement Parameters:			
Avg. Zeta Potential	= -1.12 mv	Liquid	= Aqueous
Avg. Mobility	= -0.09 (μ/s) / (V/cm)	Temperature	= 25.0 deg. C
pH	= 5.50	Viscosity	= 0.890 cP
Conductance	= 282 μS	Refractive Index	= 1.330
Concentration	= 1.00 mg/mL	Dielectric Constant	= 78.54
		Particle Size	= 0.0 nm

Instrument Parameters:			
Sample Count Rate	= 434 kcps	Current	= 1.64 mA
Ref. Count Rate	= 3532 kcps	Electric Field	= 14.13 V/cm
Sampling Time	= 256 μs	User1	= 0.00
Wavelength	= 659.0 nm	User2	= 0.00



Run	Zeta Potential (mV)	Half Width (mV)
1	0.00	3.00
2	0.00	3.92
3	0.00	3.00
4	0.00	2.86
5	0.00	2.95
6	0.00	3.11
7	0.00	3.39
8	-2.76	4.19
9	0.00	3.44
10	-8.40	7.30
Mean	-1.12	3.73
Std. Error	0.85	0.42

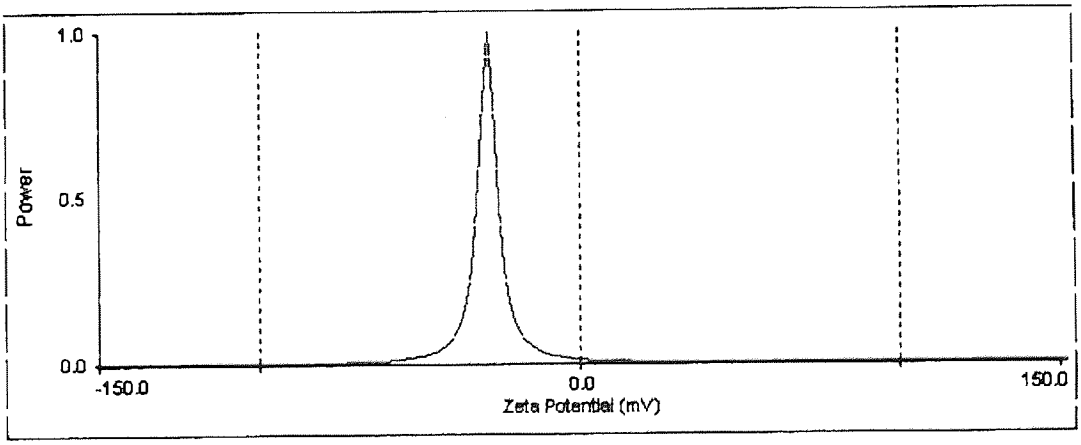
Brookhaven Instruments Corp.
Zeta Potential Analyzer Ver. 3.23

Date: May 15, 2002
Time: 10:00:47

Sample ID Parent
Operator ID Maria
Notes

Measurement Parameters:			
Avg. Zeta Potential	= -27.13 mv	Liquid	= Aqueous
Avg. Mobility	= -2.12 (μs) / (V/cm)	Temperature	= 25.0 deg. C
pH	= 5:50	Viscosity	= 0.890 cP
Conductance	= 385 μS	Refractive Index	= 1.330
Concentration	= 1.00 mg/mL	Dielectric Constant	= 78.54
		Particle Size	= 0.0 nm

Instrument Parameters:			
Sample Count Rate	= 904 kcps	Current	= 2.19 mA
Ref. Count Rate	= 2610 kcps	Electric Field	= 16.01 V/cm
Sampling Time	= 256 μs	User1	= 0.00
Wavelength	= 659.0 nm	User2	= 0.00



Run	Zeta Potential (mV)	Peak Width (mV)
1	-32.52	2.50
2	-1.67	2.90
3	-28.30	2.77
4	-24.64	3.46
5	-29.02	2.68
6	-36.83	3.12
7	-31.51	2.91
8	-29.02	5.02
9	-29.29	4.13
10	-28.46	3.21
Mean	-27.13	3.27
Std. Error	3.00	0.24

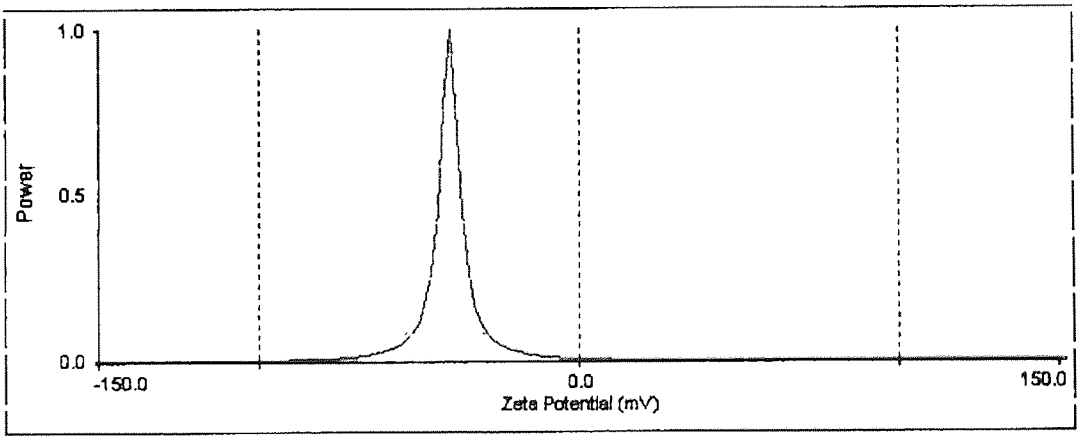
Brookhaven Instruments Corp.
Zeta Potential Analyzer Ver. 3.23

Date: May 15, 2002
Time: 10:43:28

Sample ID Resistant
Operator ID Maria
Notes

Measurement Parameters:			
Avg. Zeta Potential	= -36.57 mv	Liquid	= Aqueous
Avg. Mobility	= -2.86 (μ/s) / (V/cm)	Temperature	= 25.0 deg. C
pH	= 5.50	Viscosity	= 0.890 cP
Conductance	= 805 μS	Refractive Index	= 1.330
Concentration	= 1.00 mg/mL	Dielectric Constant	= 78.54
		Particle Size	= 0.0 nm

Instrument Parameters:			
Sample Count Rate	= 671 kcps	Current	= 4.46 mA
Ref. Count Rate	= 3315 kcps	Electric Field	= 14.23 V/cm
Sampling Time	= 256 μs	User1	= 0.00
Wavelength	= 659.0 nm	User2	= 0.00



Run	Zeta Potential (mV)	Half Width (mV)
1	-43.17	4.11
2	-36.70	3.89
3	-35.37	3.69
4	-35.37	7.30
5	-29.28	4.09
6	-36.89	8.27
7	-36.33	5.77
8	-42.28	10.34
9	-30.07	3.83
10	-40.23	3.54
Mean	-36.57	5.46
Std. Error	1.45	0.76

Appendix 2: M9 Media

Per liter:

To 750 ml of sterile deionised H_2O add the following:

5x M9 salts	200ml
Sterile distilled H_2O to 1 liter	
1M $MgSO_4$	2 ml
20% solution of the appropriate carbon source (20% glucose)	20ml
1M $CaCl_2$	0.1 ml

5x M9 Salts:

Dissolve the following in distilled H_2O to a final volume of 1 liter:

$Na_2HPO_4 \cdot 7\ H_2O$	64g
KH_2PO_4	15g
NaCl	2.5g
NH_4Cl	5.0g

The salt solution is divided into 200 ml aliquots and sterilised by autoclaving for 15 min at 15 lb/sq. in. on liquid cycle.

The $MgSO_4$ and $CaCl_2$ solutions should be prepared separately, sterilised by autoclaving, and added after diluting the 5x M9 salts to 1 liter with sterile distilled water. Glucose should be sterilised by filtration before it is added to the diluted M9 salts.

Adapted from: Sambrook, Fritsch and Maniatis. (2) Molecular Cloning: Laboratory Manual, 2nd Ed.

CONFERENCES & WORKSHOPS ATTENDED

July 2004	*SfAM* Summer Meeting – Diary and Food microbiology: Challenges and Opportunities, Cork, Ireland.
June 2004	3rd ESCMID School of Clinical Microbiology and Infectious Diseases, Athens, Greece.
May 2004	14th European Congress of Clinical Microbiology and Infectious Diseases, Prague, Czech Republic.
October 2003	5th International Symposium on the Epidemiology and Control of Foodborne Pathogens in Pork, Crete, Greece.
October 2003	2003 Workshop on the Mathematical Modelling on Safety and Spoilage of Meat, Crete, Greece.
September 2003	Society for General Microbiology 153rd Meeting, Manchester, UK.
September 2003	ASM Conference on *Salmonella*, Alghero, (Sardinia), Italy.
June 2003	1st FEMS Congress of European Microbiologists, Ljubljana, Slovenia.
June 2003	5th International Symposium on 'Shiga Toxin (Verocytoxin) –Producing Escherichia coli Infections' VTEC Conference, Edinburgh, UK.
April 2003	Society for General Microbiology 152nd Meeting, Edinburgh, UK.
January 2003	*SfAM* January Meeting – Lab on a Chip, Birmingham, UK.
September 2002	Society for General Microbiology 151st Meeting, Loughborough, UK.
April 2002	Society for General Microbiology 150th Meeting, Warwick, UK.

LIST OF PUBLICATIONS

Full papers

Braoudaki M. & A. C. Hilton (2004). Review on cross-resistance between antibiotics and biocides.
(Review in preparation for the Journal of Hospital Infection)

Braoudaki M. & A. C. Hilton (2004). Mechanisms of resistance in *Salmonella enterica* adapted to erythromycin, benzalkonium chloride and triclosan.
(Accepted for publication to International Journal of Antimicrobial Agents)

Braoudaki M. & A. C. Hilton (2004). Low level of cross-resistance between triclosan and antibiotics *in Escherichia coli* K-12 and *E. coli* O55 compared to *E. coli* O157.
(FEMS Microbiol. Letters, 235: 305-309).

Braoudaki M. & A.C. Hilton. (2004). Adaptive Resistance to Biocides in *Salmonella enterica* and *Escherichia coli* O157 and Cross-resistance to Antimicrobial Agents.
(J. Clin. Microbiol. 42: 73-78).

Extended Abstracts

Braoudaki M. & A. C. Hilton. (2003). Adaptive resistance to Biocides and Cross-Resistance to Antimicrobial Agents in *Salmonella enterica.*
(Presented as an oral presentation at the Safe Pork conference; the extended abstract is published at the proceedings).

Braoudaki M. & A. C. Hilton. (2003). Mechanisms of adaptive resistance in *Salmonella enterica.*
(Presented as a poster presentation at the Safe Pork conference; the extended abstract is published at the proceedings).

Abstracts & Poster presentations

Braoudaki M. & A. C. Hilton. (2004). Triclosan Resistance strategies in *Escherichia coli* O157:H7.
(To be presented as a poster presentation at the Food Microbiology meeting of the Society for Applied
Microbiology (S*f*am); the abstract is published at the proceedings).

Braoudaki M. & A. C. Hilton. (2004). Mechanisms of resistance in *Salmonella enterica.*
(Presented as a poster presentation at the 104th General Meeting of American Society for Microbiology; the abstract is published at the proceedings).

Braoudaki M. & A. C. Hilton. (2004). Antibiotic and Biocide resistance strategies in *Escherichia coli* O157.
(Poster presentation at ESCMID conference; the abstract is published at the proceedings).

Braoudaki M. & A. C. Hilton. (2003). Adaptive resistance to biocides in *E. coli* O157 and *Salmonella enterica* and cross-resistance to other antimicrobials.
(Presented as a poster at the 152nd meeting of Society for General Microbiology (SGM); the abstract is published at the proceedings).

Braoudaki M. & A. C. Hilton. (2003). *Salmonella enterica* and *Escherichia coli* adaptive resistance to erythromycin, benzalkonium chloride, chlorhexidine, and triclosan:Effect on the susceptibility to other antimicrobials.
(Accepted as a poster at the 103rd General Meeting of ASM; the abstract is published at the proceedings).

Braoudaki M. & A. C. Hilton. (2003). Adaptive resistance mechanisms to antibiotics and biocides and cross-resistance to other antimicrobials.
(Presented as a poster at 5th International Symposium on Shiga toxin (Verocytotoxin) – producing *Escherichia coli* infections; the abstract is published at the proceedings).

Braoudaki M. & A. C. Hilton. (2003). Adaptive resistance to biocides and cross-resistance to antimicrobial agents in *Salmonella enterica* and *Escherichia coli.*
(Presented as a poster at 1st European Congress of FEMS; the abstract is published at the proceedings).

Braoudaki M. & A. C. Hilton. (2003). Mechanisms of adaptive resistance in *Salmonella enterica.*
(Presented as a poster at the *Salmonella* conference of the ASM; the abstract is published at the proceedings).

Braoudaki M. & A. C. Hilton. (2003). Adaptive resistance to Biocides and Cross-Resistance to Antimicrobial Agents in *Salmonella enterica.*
(Presented as a poster at the *Salmonella* conference of the ASM; the abstract is published at the proceedings).

Oral presentations

Braoudaki M. Antibiotic and Biocide Resistance in foodborne pathogens. (2004).
(Aston University, Birmingham, UK).

Braoudaki M. Triclosan resistance strategies in *Escherichia coli* O157. (2004).
(ESCMID workshop, Athens, Greece).

Braoudaki M. & A. C. Hilton. (2003). Adaptive resistance to Biocides and Cross-Resistance to Antimicrobial Agents in *Salmonella enterica.*
(Safe Pork conference, Crete, Greece).

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0095-1137/04/$08.00+0 DOI: 10.1128/JCM.42.1.73–78.2004

Vol. 42, No. 1

Adaptive Resistance to Biocides in *Salmonella enterica* and *Escherichia coli* O157 and Cross-Resistance to Antimicrobial Agents

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Received 13 June 2003/Returned for modification 11 July 2003/Accepted 18 September 2003

44-121-3590578. E-mail: a.c.hilton@aston.ac.uk.

whether there is any potential link between adaptive resistance

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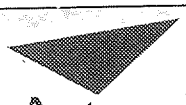

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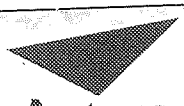

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FEMS Microbiology Letters 235 (2004) 305–309

FEMS MICROBIOLOGY Letters

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Low level of cross-resistance between triclosan and antibiotics in *Escherichia coli* K-12 and *E. coli* O55 compared to *E. coli* O157

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First published online 8 May 2004

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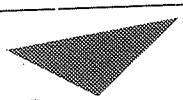

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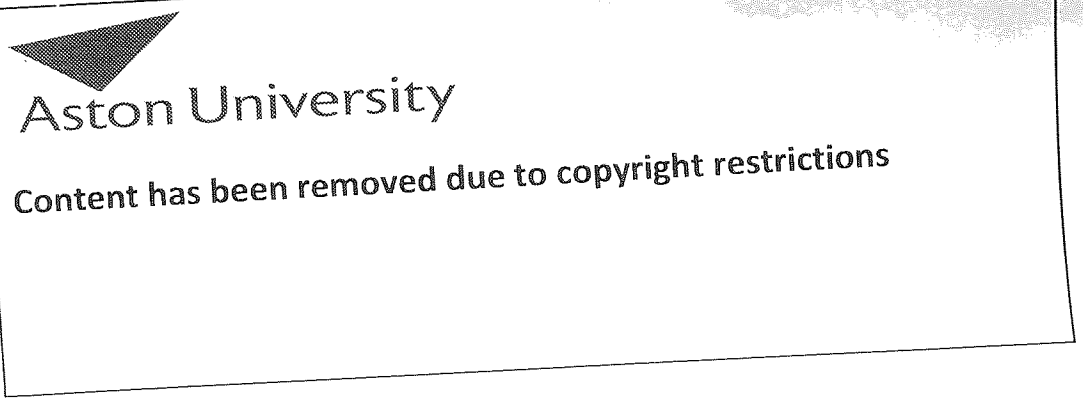
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Adaptive resistance to biocides in *Escherichia coli* O157 and *Salmonella enterica* and cross-resistance to other antimicrobials.

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Dept. of Microbiology, Life & Health Sciences, Aston University, Birmingham, U.K.

Introduction

This study investigates adaptive resistance to commonly used QAC's in *Salmonella* and *Escherichia coli* O157 to identify mechanisms underlying resistance and cross-resistance to antibiotics.

Methods

Bacterial strains. *S. enterica* serovar Typhimurium NCTC 12023, *S. enterica* serovar Typhimurium NCTC74 and *E. coli* O157, a Gram-negative strain, NCTC 12900.

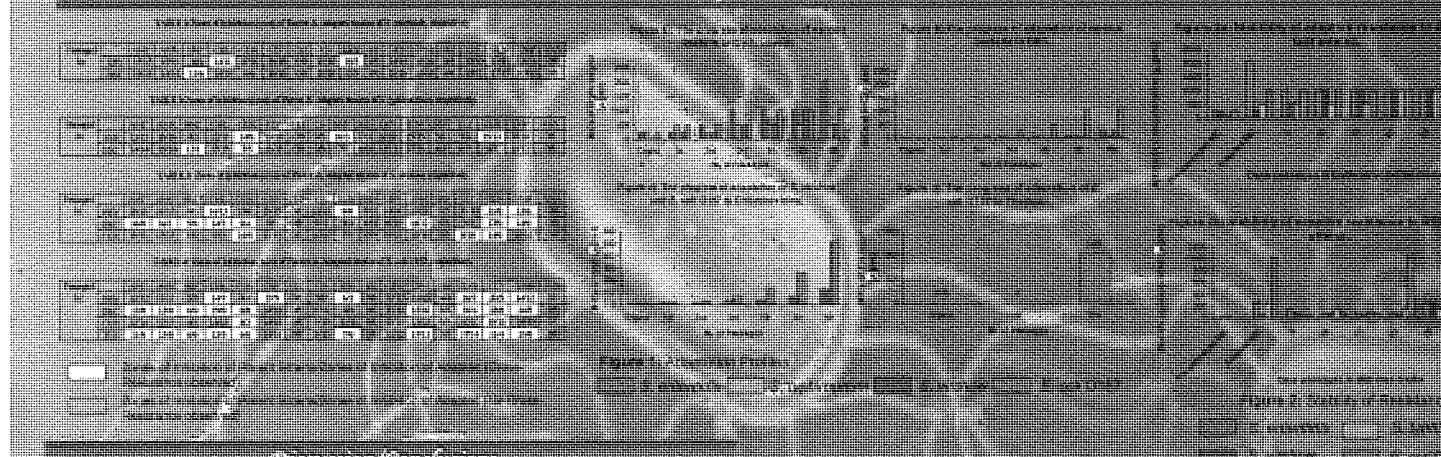
Determining the Minimum Inhibitory Concentration (MIC). The MIC was determined using the broth dilution method, which was carried out using a two-fold dilution of each antibacterial agent. The MIC was determined as the lowest concentration of the antibacterial/biocide inhibiting growth.

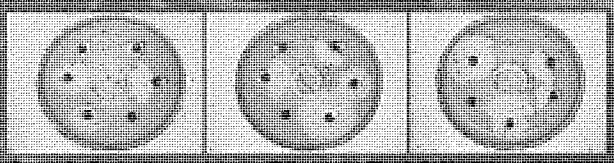
Adaptive Resistance. The first tube showing growth below the MIC was selected and used to inoculate increasing concentrations of an antibacterial. This procedure took place daily until a significant increase in the MIC (4 log) had occurred. (See Figure 1).

Cross-Resistance to Antimicrobial Agents & Biocides. Test Panel 1 (P1) Cross-resistance was examined using the Stokes' method. A suspension of the bacterial strain was inoculated on the surface of a Mueller-Hinton agar plate, and selected strains on the surface using a sterile swab. Antibiotic and biocide impregnated test discs were placed on the inoculated plates and incubated overnight at 37°C (Figure 3).

Stability of Adaptive Resistance. The stability of the adaptive resistance in the mutant was tested on blood agar and determined by passaging a selected colony and checking their MIC's. Passaging was repeated daily for 30 days. The MIC was determined every 5 days (see Figure 2).

Results





Discussion/Conclusions

Cross-resistance to different antimicrobial agents including quaternary ammonium compounds, triclosan, and some classes of antibiotics is a common phenomenon in Gram-negative bacteria (2,3). In the present study there was clear evidence that the acquired in some of the organisms under investigation. In *S. enterica* serovar Typhimurium NCTC 12023 and *E. coli* O157 cross-resistance between antibacterial agents and biocides was seen. Cross-resistance between antibacterial agents/biocides and TLV was common in *S. enterica* and *E. coli* O157 and a low incidence in *S. typhimurium*. It has been proposed that permeability changes in the outer membrane are involved in the development of such cross-resistance. This is a Gram-negative bacteria the cells membrane represents a significant barrier to the entrance of agents (4).

Cross-resistance between antibiotics and QAC's has been previously reported in other serotypes of *S. enterica*, however this is the first report of resistance observed in O157. This is of concern given the effectiveness in veterinary practice in treating serotypes of gram-negative bacteria. The widespread and inappropriate use of antimicrobial products containing biocides has been suggested as a possible cause of cross-resistance to certain antibacterial agents (1).

Salmonella and *E. coli* O157 were adapted to a range of biocides and its expression and promotion of resistance was readily achieved. The adapted mechanisms underlying resistance were active for up to 30 days when strains were passaged in antimicrobial free media. These results support the concern that residues of biocides not only promote adaptive resistance but also confer a decreased sensitivity to antibiotics.

This study is supported by

References

Adaptive Resistance Mechanisms to Antibiotics and Biocides and Cross-Resistance to other Antimicrobials in *E. coli* O157 and O55.

M. Braoudaki, A. C. Hilton.

Microbiology, Life & Health Sciences, Aston University, Birmingham, U.K.

Introduction

There has been an alarming increase in bacterial resistance to most of the existing antibacterials. Two strains investigated in this study, belonging to serotypes O55 and O157, are readily implicated in food poisoning outbreaks and sporadic cases. The aim of this study was to investigate the development of adaptive resistance to biocides and antibiotics in these strains and to determine the cross-resistance to other antimicrobials.

Methods

Determining the Minimum Inhibitory Concentration (MIC). The MIC was determined using the broth dilution method, which was carried out using a two-fold dilution of each antimicrobial agent. The MIC was determined as the lowest concentration of the antibiotic/biocide inhibiting growth.

Adaptive Resistance. The first slow growing broth below the MIC was selected and used to investigate increasing concentrations of antibiotic/biocide. This procedure took place daily until a significant increase above MIC (10 fold) occurred (see Figure 1).

Bacterial Identification. Random Amplification of Polymorphic DNA (RAPD) was performed on pre and post adapted strains to confirm strain continuity (see Figure 3).

Cross-Resistance to Antimicrobial Agents & Biocides. Table 1). Cross-resistance was determined using the Stokes' method. A suspension of the parent strain was inoculated onto the centre portion of a Mueller Hinton agar plate, and agarose strips with the perimeter using a sharp sterile scalpel. Antibiotic and biocide impregnated discs were placed at the interface between the two agents (Figure 2). Sensitivity to the antibiotic / biocide was determined by the zone of inhibition (measured in millimetres) around the impregnated disc.

Stability of Adaptive Resistance. The stability of the adaptive resistance of the most resistant bacterial cells was determined by passage in antibiotic-free and biocide-free broth. The procedure was repeated every 24 hours for 30 days. The MIC was determined every 5 days.

Results

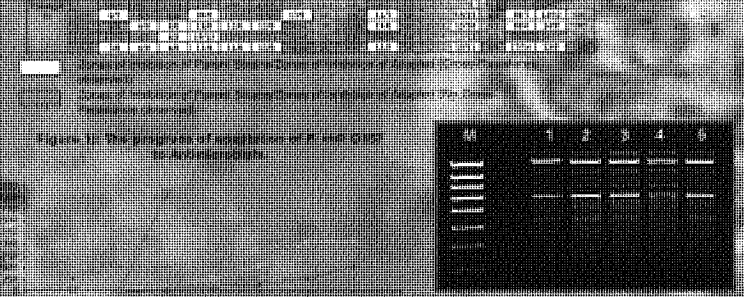
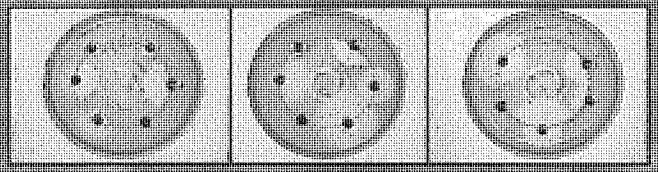


Figure 1a The progress of adaptation of *E. coli* O157 to Ciprofloxacin.

Figure 3 RAPD Profiles generated from *E. coli* O157 pre (lane 1) and post adaptation to Ciprofloxacin (lane 2), Triclosan (lane 3), Chlorhexidine (lane 4) and Benzalkonium chloride (lane 5). Lane M is the molecular weight marker.



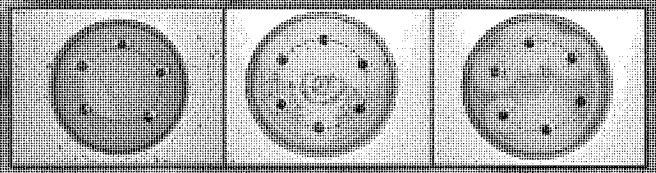


Figure 2a Cross-resistance between *E. coli* O157 strains and biocides/antibiotics. Zones of inhibition between parent and adapted strains.

Discussion/Conclusions

Cross-resistance to different antibacterial agents including antibiotics and biocides, and cross-resistance between biocides and antibiotics is a common phenomenon in Gram-negative bacteria (Russell, 1999). In this study, adaptive resistance in *E. coli* O157 was readily achieved by passage in sub-lethal concentrations of antibacterial agents. High cross-resistance to other antibiotics and biocides, particularly, adaptive resistance to CHX by *E. coli* O157, appeared to confer a marked increase in MIC to AMC, AMX and IPM and to a lesser degree to CIP, CS, CS-SDS. This phenomenon has been observed in *Pseudomonas aeruginosa* when adapted to CHX (9). Differences between the adaptive and cross-resistance profiles between O55 and O157 suggest that strain specific rather than serotype specific factors are underlying the resistances observed, some of which may be facilitated by the additional genes O157 harbours in prophage CP-933 and potentially O55 (1). Permeability changes in the outer membrane (10), including altered LPS (2), surface charge and hydrophobicity (5), and upregulated efflux (11) are the most likely candidates for investigation.

Cross-resistance between antibiotics and TLN has been reported previously in other serotypes of *E. coli* (7). However, this is the first report of resistance observed in O157. Of note is the study by Levy (1998) in which resistance to very high concentrations of TLN was acquired. The widespread and indiscriminate use of antimicrobial products containing TLN has been suggested as a possible cause of cross-resistance to other antibacterial agents (3), which is supported by the findings of this study. The observed resistant *E. coli* have been shown to be less readily taken up by TLN (8) and this might be a mechanism involved in the resistance of O157 to elevated concentrations of TLN (see figure 4).

E. coli O157 were subjected to a range of biocides and to erythromycin and the effects of resistance were readily achieved. The acquired resistance to erythromycin was assumed to be stable for over 30 passages in antibiotic-free media. These findings support the concern that repeated exposure of bacteria to biocides not only promotes adaptive resistance but also confers a decreased susceptibility to antibiotics.

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A-074

Mechanisms of Adaptive Resistance in *Salmonella enterica*.

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Introduction

Bacterial resistance to antibacterials is an emerging problem as a consequence of employing these in recent cell practices, farming practices and animal husbandry. This study investigated the potential for adaptive resistance to commonly used biocides in *Salmonella enterica*, to identify mechanisms underlying resistance.

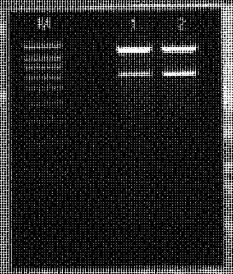
Methods

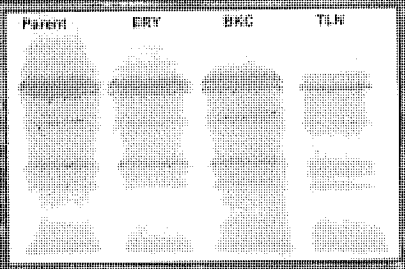
Antimicrobial Susceptibility Testing & The Reserpine Effect. Standard broth dilution method was carried out using a twofold dilution of each antibacterial agent and reserpine for the MIC of ~106 bacteria. The MICs of ciprofloxacin, chlorhexidine digluconate and benzalkonium chloride were also obtained in the presence and absence of reserpine 20 μg/ml together with the antibacterials (Fig. 2). After the overnight incubation period at 37°C bacterial growth was assessed by observing turbidity in the wells.

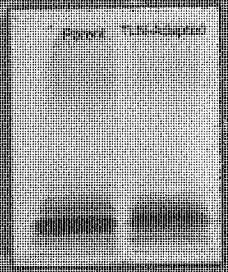
Adaptive Resistance. The first tube showing growth below the MIC was selected to inoculate increasing concentrations of antimicrobials. This procedure took place daily until a significant increase in the MIC (>2-fold) was observed (Fig. 1).

Strain Continuity. Random Amplification of Polymorphic DNA (RAPD) was performed on pre and post adaptive strains to confirm strain continuity (Fig. 3).

Results







Discussion/Conclusion

Bacterial resistance to antimicrobial drugs has created a growing public health problem. Bacteria continue their natural evolution and develop resistance mechanisms at an incredible speed. The aim of this study was to investigate these resistance mechanisms so that new control strategies will be soon developed. The following possible resistance strategies showed that:

- LPS examination did not reveal any significant changes between parent and adapted strains.
- Cell surface charge was significantly more negative in the BKC-adapted Salm. Typhimurium when compared to the parent.
- Increased CSH was associated with ERY resistance in Salm. Enteritidis and Salm. Typhimurium which seems unusual as ERY is a hydrophobic compound.
- An efflux system was the most likely mechanism of BKC and CHX resistance in all strains investigated.

It appears possible that the most effective strategy for reducing resistance in the community may be trying to understand the mechanisms underlying resistance.

References

Antibiotic & Biocide Resistance strategies in *Escherichia coli* O157.

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Introduction

This study investigated adaptive resistance to commonly used antibacterials erythromycin (ERY), benzalkonium chloride (BKC), chlorhexidine (CHX) and triclosan (TLN) in *Escherichia coli* O157 to identify mechanisms and challenge resistance.

Methods

Determining the Minimum Inhibitory Concentration (MIC). The MIC was determined using the broth dilution method, which was carried out using a two-fold dilution of each antibacterial agent. The MIC was determined as the lowest concentration of the antibiotic/biocide inhibiting growth.

Adaptive Resistance. The first tube showing growth below the MIC was selected and used to inoculate increasing concentrations of antibiotic/biocide. This procedure took place daily until a significant increase in the MIC (>1-log) had occurred (Fig. 1).

Stability of Adapted Cultures. The stability of adaptive resistance was determined for each adapted strain by repeated subculture of *E. coli* O157 in non-selective broth. This procedure was repeated every 24 hours for 30 days. The MIC was determined every 5 days (Fig. 2).

Bacterial Identification. Strains were characterised genotypically by Random Amplification of Polymorphic DNA assays (2) to confirm strain continuity during passage (Fig. 3).

Cell Surface Hydrophobicity. The cell surface hydrophobicity was determined by: i. Microbial adhesion to Hydrocarbon (MATH) assay (4) employing n-hexadecane as the hydrocarbon phase (Fig. 4).

Analysis of outer membrane proteins (OMP) and lipopolysaccharide (LPS). Outer membrane protein was prepared by a modified method based on Lambert *et al.*, (3). A 30µl sample of membrane was loaded in loading buffer and electrophoresed at 200 V for 45 minutes in an 11% polyacrylamide gel using Bio-Rad Mini Protean II apparatus (Bio-Rad Laboratories, Hercules, CA). The LPS bands were visualised by silver staining (1) (Fig. 5) and the OMPs by Coomassie blue (Fig. 6).

PCR amplification, DNA sequencing and analyses of sequence information. PCR was performed as described previously (5) to pre- and post-adapted to TLN *E. coli* O157. PCR products were visualised by electrophoresis on 1% agarose gels and staining with 1% ethidium bromide. For direct sequencing, the PCR products were purified with the QIAquick PCR purification kit (Qiagen). Sequencing data were collected from the Birmingham University Functional Genomics Lab UK (Fig. 7 & 8).

Results

Figure 1: The progress of adaptive of *E. coli* 0157 to antimicrobials.

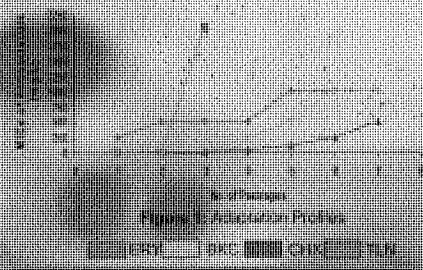
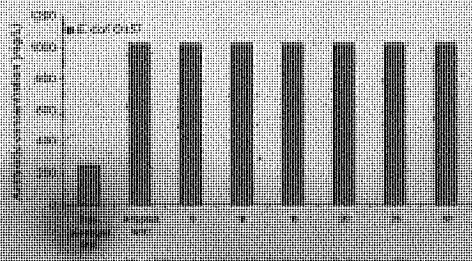


Figure 2: Stability of adapted *E. coli* O157 to TLN.



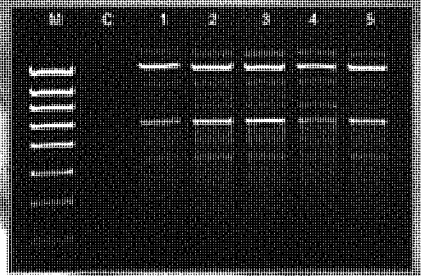
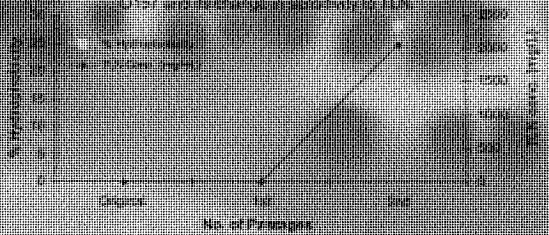


Fig. 3): RAPD Profiles derived from *E. coli* O157 pre-(Lane 1) and post- adapted to BKC (Lane 2), BKC (Lane 3), CHX (Lane 4), TLN (Lane 5). Lane M is a 1kb base pair weight marker and Lane C is a control.

Figure 4: The relationship between % hydrophobicity of *E. coli* O157 and adaptation to exposure to TLN.



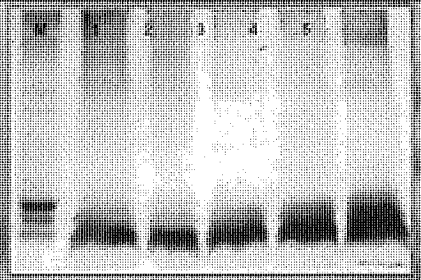


Figure 5: Silver stained SDS-PAGE (urban form) pre- (Lane 1) and post-adapted to ERY (Lane 2), BKC (Lane 3), CHX (Lane 4) and TLN (Lane 5) *E. coli* O157 strains. M is a low range pre-stained SDS-PAGE standard (14.4 – 200 kDa).

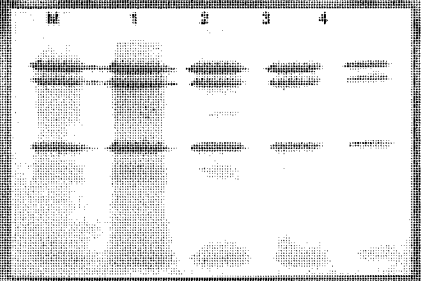


Fig. 6: Coomassie blue stained SDS-PAGE (urban form) pre- (Lane 1) and post-adapted to ERY (Lane 2), BKC (Lane 3), CHX (Lane 4) and TLN (Lane 5) *E. coli* O157 strains.

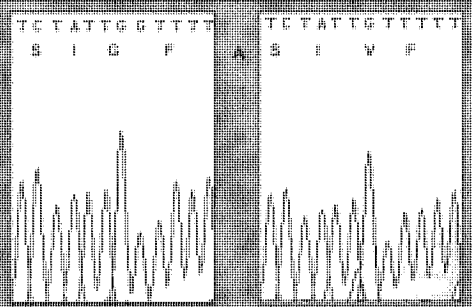


Figure 7: Chromatogram showing DNA sequencing results of *E. coli* O157 pre-(P) and post-adapted (A) to TLN. Mutation in the *fabI* gene is the base change from G to A, resulting in the amino acid change from Glycine (G) to Valine (V).

Figure 8: Location of residues conferring resistance against triclosan, adapted from Stewart *et al.*, 1999.

Conclusions

- Stable adaptive resistance to all antimicrobials tested were easily achieved.
- Outer membrane & LPS examination did not reveal any significant changes between parent and adapted strains.
- Cell surface charge did not reveal any distinct pattern.
- Parent strains were not hydrophobic, whereas adapted strains were.
- In all cases antimicrobial resistance was mediated by an active efflux pump, those of BKC & CHX in common could belong to MFS, ABC or RND superfamilies, since these resistances were reversed by both reserpine and CCCP.
- TLN resistance was found to be associated with mutation (Gly93Val) in the *fabI* gene, which encodes enoyl reductase, an enzyme that catalyses fatty acid synthesis. The residue is involved in the formation of the co-enzyme binding site.

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Triclosan resistance strategies in *Escherichia coli* O157:H7.

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Introduction

A series of genetic and biochemical tests were performed to identify mechanisms underlying resistance in *E. coli* O157 resistant to triclosan mutants.

Methods

Determining MICs & Stable Adaptive resistance. The MIC was determined using the broth dilution method, which was carried out using a two-fold dilution of each antibacterial agent. The first tube showing growth below the MIC was selected and used to inoculate increasing concentrations of antibiotic/biocide. This procedure took place daily until a significant increase in the MIC (> 1 log) had occurred (Fig. 1).

Bacterial Identification. Strains were characterised genotypically by Random Amplification of Polymorphic DNA assays (2) to confirm strain continuity during passage.

Cell Surface Hydrophobicity & Charge. The cell surface hydrophobicity was determined by microbial adhesion to hydrocarbon (MATH) assay (3) employing a hexadecane as the hydrocarbon phase, whereas cell surface charge was determined by microelectrophoresis employing a Zetamaster Particle Electrophoresis Analyser, (Brookhaven Instruments, New York, U.S.A) (Table 1).

The Use of Reserpine and CCCP in Efflux Assessment. Growth inhibition studies were conducted by standard broth dilution method using a two-fold dilution of the antibacterial and/or (20µg/ml) reserpine, or (5µM) Carbonyl cyanide m-chlorophenylhydrazone (CCCP) (Table 1).

Analysis of outer membrane proteins (OMP) and lipopolysaccharide (LPS). Outer membrane extracts were prepared by a modified method based on Lambert *et al.*, (3). A 30µl sample of membrane suspension in loading buffer was electrophoresed at 200 V for 42 minutes in an 11% polyacrylamide gel using Bio-Rad Mini Protean II apparatus (Bio-Rad, Hemel Hempstead, UK). The LPS bands were visualised by silver staining (4) and the OMP by Coomassie blue.

PCR amplification, DNA sequencing and analyses of sequence information. PCR was performed as described previously (2) to pre- and post-adapted *E. coli* O157. PCR products were visualized by electrophoresis on 1% agarose gels and staining with 1% ethidium bromide. For direct sequencing, the PCR products were purified with the QIAquick PCR purification kit (Qiagen). Sequencing data were obtained from the Birmingham University Functional Genomics Lab,UK (Fig. 2).

Identification of whole cell lipids. Thin layer chromatography (TLC) was employed for the separation of lipids. For the visualisation of those lipids containing amino groups, TLC plates were sprayed with 0.2 % w/v ninhydrin, whereas phospholipids were visualised by spraying with molybdenum blue spray, a solution of 1.3 % w/v molybdenum oxide in 4.2 M H_2SO_4 (Fig. 4).

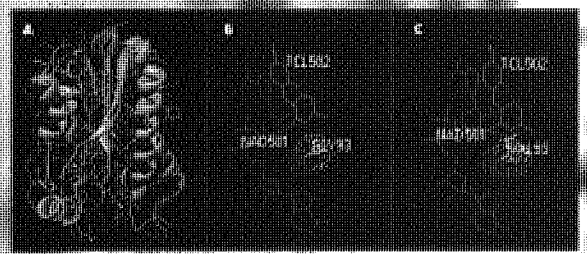
Fatty acid analysis by Gas Chromatography (GC). Samples were loaded onto a Hewlett Packard HP-5890 Series II capillary column on a Hewlett Packard Chemstation series CC and Fatty acids were identified by comparing the retention times of the peaks with those of a standard bacterial fatty acid methyl esters (FAME) (Fig. 5A & B).

Results

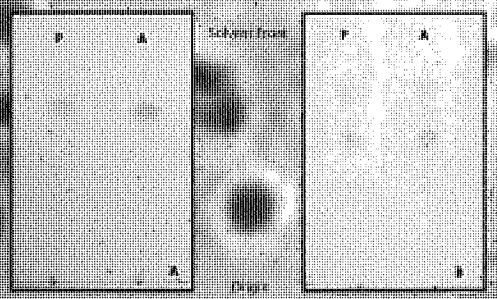
Mid Range of 30S, 3P and Phenicols is of 50S and resistance to it.

Organisation of the gene encoding the target enzyme			Mechanism(s) of resistance			Associated resistance to	
Name	No. of Copies	Location	Mutation	No. of Mutations	Location	Antibiotic	Biocide
fabI	1	Chromosome	Point mutation	1	Chromosome	None	Triclosan

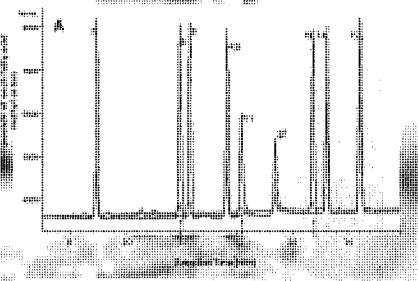
Figure 2: Sequence alignment of *fabI* gene. Sequences are shown from forward primer at the focus where the mutations (red pink arrow) occurred. Codon 93: GGC (Gly) → GTC (Val).



Figures 3A, B & C. A: FabI ribbon structure. B&C: Active site of FabI showing mutation that causes the conversion of Gly 93 to Val 93. Note steric clash between side chains of Val 93 and TCL preventing effective inhibition. Bond of crystal structure of H_2O, NADP and *E. coli* FabI (5). Brookhaven Protein Data Bank accession number 1G14. FabI ribbon structure omitted for simplification.



Figures 4A & B. A: Lipids containing amino compounds visualised with ninydrin and B: phospholipids visualised with molybdenum blue. *P* refers to parent *E. coli* O157 and *A* refers to adapted *E. coli* O157.



Figures 5A & B. GC overlay traces showing methyl esters of fatty acids in parent *E. coli* O157 pre- (green) and post-adapted to TLN (red) strains. To blue the standard bacterial fatty acid methyl esters are shown. Traces were obtained from 9 to 14 & 15 to 20 minutes, respectively.

Summary of Findings

- Stable adaptive resistance to all antimicrobials tested were easily achieved.
- Outer membrane & LPS examination did not reveal any significant changes between parent and adapted strains.
- Cell surface charge did not reveal any distinct pattern.
- Adapted strains were not hydrophobic, whereas adapted strains became.
- Triclosan resistance are mediated by the same efflux pump (reserpine sensitive), however active efflux systems were not implicated with ERY & TLN resistance (CCCP results).
- Triclosan resistance was found to occur through mutations in the *fabI* gene, which encodes enoyl reductase, an enzyme involved in fatty acid synthesis.
- No significant changes were observed in the quantification or quantitation of lipids, however changes in the fatty acids analysis were detected.

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Adaptive resistance to Biocides and Cross-Resistance to Antimicrobial Agents in *Salmonella enterica* and *Escherichia coli.*

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Introduction

This study investigated adaptive resistance to commonly used biocides in *Salmonella* and *Escherichia coli* O157, to identify mechanisms underlying resistance and any cross-resistance to antibiotics.

Methods

Antimicrobial Susceptibility Testing. The MIC was determined using the broth dilution method, which was carried out using a two-fold dilution of each antibacterial agent. The MIC was determined as the lowest concentration of the antibiotic/biocide inhibiting growth.

Adaptive Resistance. The first tube showing growth below the MIC was selected to inoculate increasing concentrations of antibiotic/biocide. This procedure took place daily until a significant increase in the MIC [illegible] had occurred (Fig. 1).

Bacterial Identification. Random Amplification of Polymorphic DNA (RAPD) was performed on pre- and post- adapted strains to confirm strain continuity (Fig. 2).

Cross-Resistance to Antimicrobial Agents & Biocides (Tables 1-4). Cross-resistance was determined using the Stokes' method. A suspension of the parent strain was inoculated in the centre portion of a Mueller-Hinton agar plate, and adapted strains on the periphery [illegible]. Antibiotic and biocide impregnated discs were placed at the interface between the two strains (Fig. 3a/b).

Stability of Adaptive Resistance. The stability of the adaptive resistance was determined by passage in antibiotic- and biocide-free broth. The procedure was repeated every 24 hours for 30 days. The MIC was determined every 5 days (Fig. 4).

Results

TABLE 1: [illegible]

[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

TABLE 2: Zones of inhibition (mm) of [illegible]

[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

TABLE 3: Zones of inhibition (mm) of [illegible]

[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

TABLE 4: Zones of inhibition (mm) of [illegible]

[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

Zones of Inhibition of Parent Strains/Zones of Inhibition of Adapted [illegible] (Cross-Resistance observed).

Zones of Inhibition of Parent Strains/Zones of Inhibition of Adapted [illegible] (Cross-Protection observed).

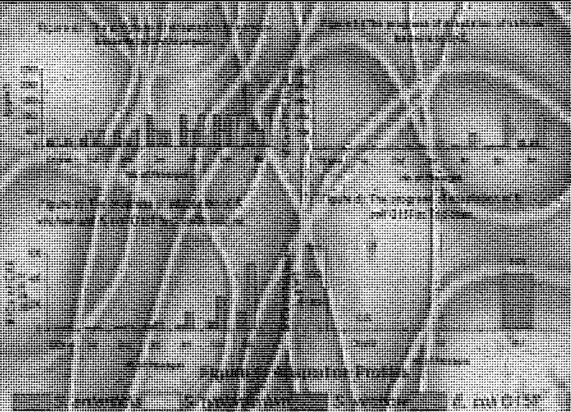


Figure 1: Adaptive Profiles

Figure 2: RAPD profiles obtained from E. coli O157 parent (Lane 1) and post-adapted to ERY (Lane 2), BKC (Lane 3), CHX (Lane 4), TLN (Lane 5) strains. Lane M is a 1kbp molecular weight marker.

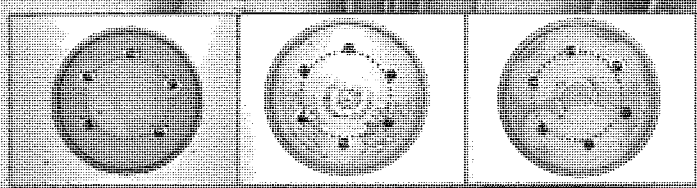


Figure 3a: Cross-resistance between TLN-resistant [illegible] to several agents.

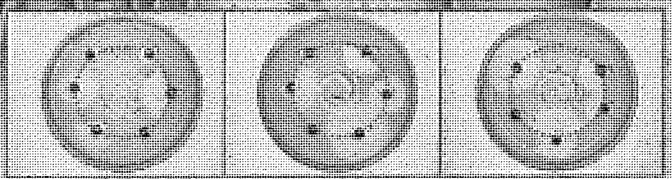
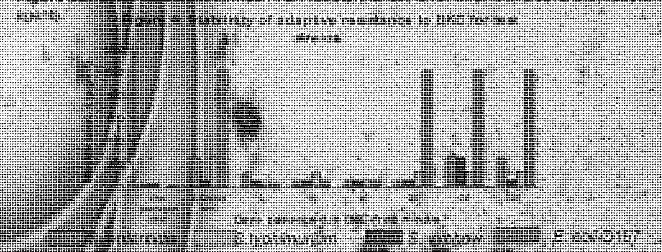


Figure 3b: Cross-resistance between TLN-resistant E. coli O157 strains and several antibacterial agents.

Figure 4: Stability of adaptive resistance to BKC for test strains.



Discussion/Conclusions

Cross-resistance is a common [illegible] Gram-negative bacteria (5). In the present study cross-[illegible] only in S. typhimurium, S. virchow and E. coli O157. Cross-resistance between antibiotics and TLN was [illegible] E. coli O157 and in one [illegible] in S. typhimurium. [illegible] changes in the outer membrane are involved in the development of [illegible] cross-resistance, since in Gram-negative bacteria the outer membrane represents a significant barrier to entry of antibacterial agents (4).

Cross-resistance between antibiotics and TLN has been previously reported in other serotypes of E. coli (3), however this is the first report of resistance observed in O157. This is important given the different mechanisms [illegible] observed in different serotypes of other Gram-negative bacteria. The widespread and indiscriminate use of antimicrobial products containing triclosan has been suggested as a possible cause of cross-resistance to other antibacterial agents (1).

Abbreviations: [illegible] chloramphenicol [illegible] (OS 25µg/ml), [illegible] (GEN 10µg/ml) [illegible] rifampicin (RIF [illegible]) [illegible] (CMP [illegible]) [illegible] 5µg/ml), erythromycin (ERY [illegible]), benzalkonium chloride (BKC 1024 µg/ml), chlorhexidine (CHX 512 µg/ml), triclosan (TLN 1024 µg/ml).

References

[illegible]

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Adaptive resistance to Biocides and Cross-Resistance to Antimicrobial Agents in *Salmonella enterica*.

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Introduction

This study investigated adaptive resistance (AR) induced by road biocides in *Salmonella enterica* serovar Enteritidis, Typhimurium and Virchow, in seeking mechanisms underlying resistance and any cross-resistance to antibiotics.

Methods

Minimum Inhibitory Concentrations. The MIC was determined using exponential phase, broth cultures, which was carried out using a two-fold dilution of each antibiotic or agent. The MIC was recorded as the lowest concentration of the antimicrobial inhibiting growth.

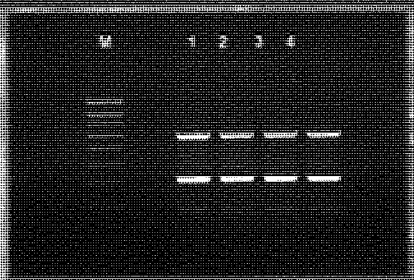
Serial Passage. The first tube showing growth (i.e. the MIC) was selected to provide increasing concentrations of antimicrobials. The procedure took place daily until a significant increase in the MIC (> 2-fold) had occurred. (Table 1)

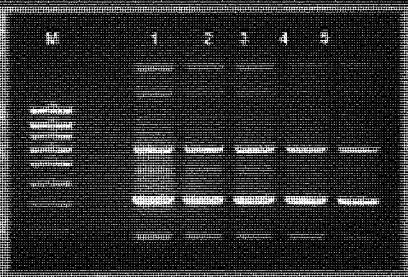
Bacterial Identification. Pre- and post- adapted strains were differentiated biochemically and genotypically using API 20E and Random Amplification of Polymorphic DNA (RAPD) assays to confirm strain continuity. (Fig. 1 A&B)

Cross-Resistance to Antimicrobial Agents & Biocides. (Tables 1-3). Cross-resistance was determined using the Stokes method. A suspension of the parent strain was inoculated on the entire surface of a Mueller-Hinton agar plate, and sterile discs on the perimeter using a rotary platter. Antibiotic and biocide discs and their concentrations were selected on the basis of clinical relevance.

Stability of Adaptive Resistance. The stability of the adaptive resistance was determined on passage in antibiotic and biocide-free broth. The procedure was repeated every 24 hours for 20 days. The MIC was determined every 5 days (Fig. 2&3).

Results





Discussion/Conclusions

References

Acknowledgements