# CLONING AND CHARACTERISATION OF THE *lpxA* GENE IN *Campylobacter jejuni.*

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A Thesis

submitted in partial fulfilment

of the requirements for the Degree

of

Master of Science in Microbiology

in the

University of Canterbury

by

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University of Canterbury

1997

# ACKNOWLEDGEMENTS.

I would like to thank the following people for the time, help and support they have given me over the last eighteen months.

Firstly and primarily I would like to thank my supervisor John Klena for his encouragement, ongoing support and enthusiasm, which were most appreciated. Also many thanks to Jack Heinemann for his input and helpful suggestions.

I am most indebted to Megan Mahan for her technical expertise, advice and friendship. I am also most grateful to Craig Galilee for his ongoing technical support.

I am most grateful to Dougal Holmes for his time and photographic expertise in producing the quality of photos shown in this thesis.

Thanks to everyone in John's laboratory for their help and friendship over the last year and a half, and to members of other laboratories for their additional advice and support.

A special thanks goes out to my parents, Lucille and Richard, and my sister Alison for all their support and understanding, without which I would never have made it this far.

I would also like to acknowledge those that helped in the production of this thesis. This includes Martin Jarvis and Trevor Lyttle for their fine proof-reading skills; Mark Silby for his advice and critical skills, especially over the last couple of months; Hamish Reid for his never-ending supply of jokes; and Andrew Calder for his unparalleled computer knowledge and expertise.

For their friendship and moral support I would like to thank Simon Adkins, Angela Price, and Mark Schreiber.

And last, but certainly not least, I would like to thank Jenny Armstrong, Michelle Adamson, and Rebekah Owen for their continued friendship, encouragement and support.

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# LIST OF ABBREVIATIONS.

| | |
|---|---|
| [α-$^{35}$S] dATP | 2'-deoxy adenosine triphosphate, with $^{35}$S at the α phosphate position |
| aa | amino acid |
| APS | ammonium persulphate |
| bp | base pairs |
| CCDA | *Campylobacter* charcoal differential agar |
| $dH_2O$ | distilled water |
| FA | fatty acid |
| GlcNAc | *N*-acetylglucosamine |
| kb | kilobase pairs |
| kDa | kilodalton |
| kPa | kilopascal(s) |
| LPS | lipopolysaccharide |
| min | minutes |
| NTP | nucleotide triphosphate |
| dNTP | 2'-deoxy nucleotide triphosphate |
| $OD_{600}$ | optical density at 600 nm |
| rpm | revolutions per minute |
| SDS-PAGE | sodium dodecyl sulphate-polyacrylamide gel electrophoresis |
| sec | second(s) |
| TEMED | N, N, N', N'-tetramethylethylenediamine |
| ts | temperature sensitive |
| UDP | uridine diphosphate |
| UMP | uridine monophosphate |
| UV | ultraviolet |
| v/v | volume per volume |
| w/v | weight per volume |
| // | transformed with plasmid X |

# ABSTRACT.

Lipopolysaccharides (LPS) are important structural components of the outer membrane of Gram-negative bacteria. Lipid A anchors LPS molecules in the outer membrane and is also the sole LPS component necessary for endotoxic shock induction. UDP-*N*-acetylglucosamine acyltransferase is encoded by the *lpxA* gene. In *Escherichia coli* K12 and *Salmonella typhimurium*, this enzyme completes the first step in lipid A synthesis. The *lpxA* gene product is essential for the production of LPS in these organisms as null mutations in *lpxA* are lethal. The aim of the study was to characterise lipid A biosynthesis in *Campylobacter jejuni*. *C. jejuni* appears to contain three lipid A types, one of which is similar to the lipid A species in *E. coli*. In this study it was proposed that *C. jejuni* contains an enzyme that is functionally analogous to LpxA from *E. coli*. The initial aim was to clone the gene necessary for the production of LpxA in *C. jejuni*. An *E. coli* temperature-sensitive mutant defective in LpxA activity provided a model with which to study the effect of acyltransferase deficiency on cell viability. A *C. jejuni* plasmid expression library was electroporated into *E. coli lpxA* mutant SM101 cells and transformants were screened for their ability to survive at the previous non-permissive temperature (42°C) on minimal media containing ampicillin. Plasmid DNA from one transformant able to restore the full length LPS in *lpxA* defect in SM101 was analysed. Nucleotide sequence has shown the gene order to be *fabZ-lpxA* as in *E. coli* and the predicted amino acid sequence from the '*lpxA*' gene from *C. jejuni* displayed strong amino acid similarity to the 3' end of the UDP-*N*-acetylglucosamine acyltransferase protein from *E. coli*, *S. typhimurium*, *Rickettsia rickettsii*, *Yersinia enterocolitica* and *Haemophilus influenzae*. While this gene from *C. jejuni* F38011 was capable of rescuing the temperature-sensitive defect of KLC 4177 (SM101), growth curves and viable plate counts reveal that rescue is not complete. A second allele of *lpxA*, cloned from *C. jejuni* strain NZRM 1958 has shown a similar arrangement of genes (*fabZ-lpxA*) although differences in primary amino acid sequence between the alleles were revealed.

# CHAPTER I.

# INTRODUCTION.

## 1.1 General Introduction.

*Campylobacter jejuni* and related species have been known to be causative agents of human enterocolitis since the 1970's (Skirrow 1977), being first selectively isolated from the diarrhoeal stools of humans in 1972 (Butzler *et al.* 1973). In Britain and Wales, *Campylobacter* spp. are the most common enteric bacterial pathogen reported and have now overtaken *Salmonella* spp. as the principal cause of bacterial gastroenteritis (Jones and Telford 1991b; Southern *et al.* 1990; Humphrey 1995). Moreover, as disease caused by *C. jejuni* is not statutorily notifiable in Britain, the problem has the potential to be up to 100 times greater than indicated by the number of reported cases (Jones and Telford 1991b). Studies in other developed countries such as Australia, Belgium, Canada, the Netherlands, Sweden and the United States have shown *C. jejuni* in stools of 4%-14% of patients with diarrhoea and of <1% of asymptomatic persons (Blaser and Reller 1981). Studies in developing countries, such as Bangladesh, Peru, Rwanda, and The Gambia suggest that *C. jejuni* may be even more routinely isolated during diarrhoeal illness than in developed countries. However the rate of asymptomatic infection in these developing countries is often also very high (Blaser and Reller 1981; Blaser *et al.* 1983). This is suggestive of differences in infection rates in these two environments. Children in third world countries are constantly exposed to *C. jejuni* and thus may be living in an equilibrium with *C. jejuni* more so than people in the first world (Blaser and Reller 1981). From the first studies in the developing world, it was recognised that *Campylobacter* species could be isolated from children who were well as often as from children who had diarrhoea. In one study performed in Bangui, Central African Republic, 127 infants were monitored from birth to 6 months of age. Eighty-two *Campylobacter* infections were observed in

42% of the children, but there was no difference in isolation rates between children with and without diarrhoea (D. N. Taylor 1992).

## 1.2 *Campylobacter* Biology.

In New Zealand campylobacteriosis was made notifiable in 1981. Campylobacteriosis incidents are assumed to be the result of *C. jejuni* infection (Brieseman 1990) as *C. jejuni* is responsible for greater than 90% and *Campylobacter coli* for less than 10% of human infections. These two organisms are clinically indistinguishable and most laboratories do not routinely attempt to distinguish them (Nachamkin 1992). *Campylobacter* is the most commonly notified bacterial disease agent (Brieseman 1990). Rates have been rising steadily from a national average of 14 cases per 100 000 in 1981 to 150 cases per 100 000 in 1992, and 240 cases per 100 000 in 1993 (Brieseman 1994). Infection rates in Canterbury are consistently higher than the rest of New Zealand with 291 cases per 100 000 in 1992 and 382 cases per 100 000 in 1993 (Brieseman 1994).

## 1.3 Description of the Genus.

### 1.3.1 MORPHOLOGICAL OVERVIEW.

The generic name *Campylobacter* comes from the Greek and means 'curved rod'. Members of the genus *Campylobacter* are seen as curved or helical rods 0.2-0.9 μm wide by 0.5-5 μm long. *Campylobacter* contain a single polar flagellum present at either one or both poles of the cell which provides motility to the organism. They are members of the Spirillum group, family *Spirillaceae*. Members of this genus are animal and human parasites. They are microaerophilic, unable to replicate in the level of oxygen present in air (21%) and are generally cultured in a gaseous atmosphere containing 6% oxygen, 10% carbon dioxide, and 84% nitrogen. Skirrow *et al.* (1991; cited from Corry *et al.* 1995) reported that the presence of hydrogen at not less than 7% improved the primary isolation of *C. jejuni* from faeces.

*C. jejuni* is able to survive in the aquatic environment in a viable but nonculturable coccoid form (Rollins and Colwell 1986; Moran and Upton 1986). It is thought that this morphological transition is a survival adaptation for unfavourable environments and a great deal of interest has been generated in this area of physiology and the role of coccoid cells in disease transmission (Moran and Upton 1986). There is some evidence that *C. jejuni* is infectious to neonatal mice in this form, but this property may differ between strains (Jones *et al.* 1991a).

### 1.3.2 EPIDEMIOLOGY.

The sources of *Campylobacter* infection have been studied extensively in the United States as well as in New Zealand (Tauxe 1992; Hasell 1994; Eberhart-Phillips 1995). Four sources account for nearly all epidemic cases: consumption of chicken and chicken products, unpasteurized milk, contact with pets and other animals, and impure water (Rollins and Colwell 1986; Jones *et al.* 1991a; Park *et al.* 1981; Smith and Muldoon 1974; Brieseman 1994; Doyle and Roman 1982; Lovett *et al.* 1983; Brieseman 1984; Altekruse *et al.* 1994). In addition, other food sources have also been implicated in *Campylobacter* infections including mushrooms, watermelon, papaya, and shellfish (Doyle and Schoeni 1986; Castillo and Escartin 1994; Wilson and Moore 1996).

*C. jejuni* is carried as part of the intestinal flora of many cattle, sheep, pigs, dogs, cats, poultry and other animals with animal faeces representing a major source of contamination (Rosef *et al.* 1983; Weijtens *et al.* 1993; Brennhovd *et al.* 1992). *C. jejuni* has been isolated from birds such as crows, gulls, domestic pigeons, migratory ducks, sandhill cranes and Canada geese (Kapperud and Rosef 1983; Pacha *et al.* 1988). Houseflies can also act as vectors for *C. jejuni* (Rosef and Kapperud 1983). There is some evidence to link contact with puppies and kittens to campylobacteriosis, especially to children (Hasell 1994). In another case study, the rise in the rate of *C. jejuni* infection was attributed to the consumption or handling of milk that had been attacked by birds, such as magpies and jackdaws (Southern *et al.* 1990).

### 1.3.3 TEMPERATURE AND STRESS.

Most *Campylobacter* species have an optimum replication temperature of 30 to 37°C. *C. jejuni, C. coli, C. lari* and *C. upsaliensis* are exceptions in that optimum replication rates occur at 42°C and thus they are considered to be thermophilic. Paradoxically, *Campylobacter* spp. survive storage at refrigerated temperatures better than at room temperature (National Advisory Committee on Microbiological Criteria for Foods 1995). Cells are sensitive to freezing, drying and to salt concentrations above 1% sodium chloride (National Advisory Committee on Microbiological Criteria for Foods 1995). *Campylobacter* spp. are sensitive to standard concentrations of common disinfectants, and *C. jejuni* is also sensitive to ultraviolet and gamma irradiation (National Advisory Committee on Microbiological Criteria for Foods 1995). *Campylobacter* spp. do not form spores, however as previously mentioned, may undergo a morphological transition to coccoid cells over time or under stress (Jones *et al.* 1991a; Griffins 1993; Boucher *et al.* 1994).

The most frequently isolated *Campylobacter* spp. with regard to human enteric pathogens are *C. jejuni, C. fetus, C. coli,* and *C. lari* (Walker *et al.* 1986; Skirrow 1977; Faoagali 1984; Stanier *et al.* 1984; Nachamkin 1992; National Advisory Committee on Microbiological Criteria for Foods 1995). *C. jejuni* is so called because it was first isolated from the jejunum of calves with diarrhoea in 1931 (Rees *et al.* 1993).

### 1.3.4 THE GENETICS OF *Campylobacter* SPECIES.

*Campylobacter* spp. have a small genome of about 1600-1800 kb, an exception being *C. upsaliensis* with a genome of approximately 2000 kb, which is the largest *Campylobacter* genome recognised to date (Bourke *et al.* 1995). *C. jejuni* and *C. coli* genomes are only 36% of the size of the *Escherichia coli* chromosome (4600 kb) (Smith *et al.* 1987). The chromosome of *C. jejuni* is circular and approximately 1700 kb in circumference (Nuijten *et al.* 1990; Chang and Taylor 1990; Kim *et al.* 1992). The DNA is extremely AT-rich with a mol % GC of approximately 30% compared with the genome of *E. coli,* which has a mol % GC of 50% (Chang and Taylor 1990; Kim *et al.* 1992; Kim *et al.* 1993). The small genome size of *Campylobacter* spp. is

consistent with their small and fastidious nature; these organisms require numerous supplements for growth, cannot ferment most ordinary carbohydrates or degrade complex substances, and appear biologically inert (D. E. Taylor 1992). Conjugative plasmids and bacteriophages have been reported in campylobacters and these extrachromosomal elements may contain essential genes required by campylobacters due to their small genome (D. E. Taylor 1992).

## 1.4 *Campylobacter* Diseases.

### 1.4.1 PATHOLOGY AND CLINICAL SYMPTOMS OF CAMPYLOBACTERIOSIS.

Once established *C. jejuni* infections in humans can manifest in several different forms. Symptomatic infection due to *C. jejuni* is shown by a gastrointestinal illness and is usually self-limited. Mild *C. jejuni* infections may produce symptoms similar to those during viral gastroenteritis and may only last for 24 hours (Blaser *et al.* 1983). However the illness can last up to three weeks, with an incubation period of 2-11 days (Skirrow 1977). Symptoms of *Campylobacter* gastroenteritis include diarrhoea (often bloody), nausea, fever, occasional vomiting, headache, malaise, muscular aches and pains, and acute abdominal pain (Stehr-Green *et al.* 1991; Eberhart-Phillips *et al.* 1995) (Table 1). Many patients have at least one day with eight or more bowel movements. Acute abdominal pain in some circumstances may mimic acute appendicitis and may result in unnecessary surgery (Blaser and Reller 1981; Blaser *et al.* 1983). In one reported case, a patient with a suspected bowel perforation was operated upon, only to find inflammation of the ileum and colon. An unknown *Campylobacter* spp. was subsequently isolated from the diarrhoeal fluid (Skirrow 1977).

Most afflicted people recover in less than a week; however, the illness may follow a biphasic course, with up to 20% of cases undergoing a relapse or suffering from a prolonged or severe illness (Skirrow 1977; Blaser and Reller 1981; Blaser *et al.* 1983). Infrequently, death may occur in elderly or debilitated patients (Tauxe 1992; Blaser *et al.* 1983). Untreated patients may continue to excrete the organism in faeces for two

to three weeks but transmissibility is low. Chronic carriage is unknown in healthy people in industrialised countries (Skirrow 1990).

**Table 1:** Frequency of symptoms reported by cases in the New Zealand multi-centre analysis of gastroenteritis induced by *Campylobacter* (MAGIC) study (Adapted from Eberhart-Phillips *et al.* 1995).

| Symptoms | Number (n=621) | Percent (%) |
|---|---|---|
| Diarrhoea | 612 | 98.6 |
| Stomach pains/cramps | 558 | 89.9 |
| Fever | 454 | 73.1 |
| Nausea | 394 | 63.4 |
| Blood in stool | 219 | 35.3 |
| Vomiting | 194 | 31.3 |
| Headache | 152 | 24.5 |
| Lethargic/tired | 94 | 15.1 |
| Body aches and pains | 68 | 11.0 |

Generally hundreds to thousands of bacteria are usually required for infection by *C. jejuni*. However, studies indicate that even low doses of *C. jejuni* can produce infection and illness in humans. In one milk-borne outbreak it was estimated that only about 500 organisms had caused infection (Jones and Telford 1991b), and in a separate study, the minimum number of *C. jejuni* ingested by volunteers was 800 organisms, and this resulted in diarrhoeal illness (Black *et al.* 1988). *Campylobacter* spp. that have been ingested can be killed by gastric acid. Conditions which reduce acidity in the stomach, such as gastric surgery or peptic ulcer treatment, increases a person's susceptibility to *Campylobacter* infection. Milk buffers gastric acid and thus fewer organisms are needed for infection.

The incidence of infection follows a bimodal age distribution, with the highest incidence recorded in infants and young children with a second peak in young adults 20 to 40 years of age (Nachamkin 1992). A seasonal pattern is also shown with increased notifications occurring during spring and summer for both the northern and southern hemispheres.

*Campylobacter* infections can be effectively treated with antibiotics if provided early enough during an infection. Erythromycin is the antibiotic of choice for several reasons including organism susceptibility, ease of administration, high host uptake, and the lack of serious toxicity to host. It can shorten the duration of illness in some cases by rapidly killing organisms, as seen by dramatic decreases in culturable counts of *C. jejuni* excreted in the faeces within 48 hours. However, erythromycin does not seem to prevent relapse or cure persistent illness. There is evidence that ciprofloxacin also kills campylobacters and works rapidly (Blaser and Reller 1981; Jones and Telford 1991b; Wallis 1994).

### 1.4.2 *Campylobacter* EXTRAINTESTINAL DISEASES.

Extraintestinal infections caused by *C. jejuni* have been reported. *C. jejuni* may penetrate the intestinal wall and invade a patient's bloodstream. Ladrón de Guevara *et al.* have reported 7 cases of bacteraemia caused by *C. jejuni* or *C. coli* (Ladrón de Guevara *et al.* 1994). *C. jejuni* bacteraemia has also occurred after a bone marrow transplant (Hagensee *et al.* 1994). Other extraintestinal infections that may occur due to *C. jejuni* include septicaemia, urinary tract infection, cholecystitis, reactive arthritis, Reiter's syndrome (Walker *et al.* 1986), empyema, encephalopathy (Yuki *et al.* 1994), bursitis, endocarditis, peritonitis, erythema nodosom, pancreatitis, abortion and neonatal sepsis (Nachamkin 1992). *C. jejuni* has also recently been isolated from bone marrow (Ruiz-Arenas and Arroyo-Gomez 1994). Neurologic complications due to *C. jejuni* infections are infrequent, but some which do occur are meningitis, stroke, Guillain-Barré syndrome (GBS) and Miller-Fisher Syndrome (MFS), the latter two are forms of involuntary paralysis.

Guillain-Barré Syndrome (GBS) has replaced polio as the most common cause of acute neuromuscular paralytic disease in the developed world (Ropper 1992; Rees *et al.* 1993; Yuan *et al.* 1993; Yuki *et al.* 1994). Up to two-thirds of GBS cases are preceded by symptoms of an infectious illness, most frequently of the respiratory or gastrointestinal tract (Rees *et al.* 1993; Rees *et al.* 1995b). Campylobacteriosis is the most common antecedent event associated with GBS (Rees *et al.* 1993; Mishu *et al.* 1993; Kuroki *et al.* 1993; Rees *et al.* 1995a). In the United States GBS has an annual incidence of 1.7 per 100 000 people (Mishu *et al.* 1993) and this reflects the annual

incidence in most other parts of the world as well, with 1 to 2 per 100 000 members of a population suffering from GBS (Mishu and Blaser 1994). GBS usually occurs 1-3 weeks after bacterial or viral infections (Ropper 1992). About a quarter of all patients require mechanical ventilation. GBS has a mortality rate of 1-18% and 20% suffer prolonged residual disability (Rees *et al.* 1993). *C. jejuni* has been recovered from Japanese patients with GBS, and most of these isolates belong to a single serotype, heat stable antigen (HS) 19 (88%). HS19 only accounts for 1-3% of all campylobacteriosis which suggests that certain *Campylobacter* serotypes are associated with this syndrome (Kuroki *et al.* 1993). It has been observed that LPS from some HS strains of *C. jejuni* mimic the structure of human gangliosides and this ganglioside mimicry is thought to be of significance in the aetiology of GBS (Aspinall *et al.* 1992; Aspinall *et al.* 1994a). Ganglioside-like structures have been identified at the terminal regions of the core oligosaccharide (OS) of the lipopolysaccharide (LPS) molecule (Aspinall *et al.* 1994b). OS structures which have human ganglioside $G_{M1}$ and $G_{D1a}$ structures have been found in serostrains for serotypes HS4 and HS19 (Aspinall *et al.* 1992; Aspinall *et al.* 1994b; Yuki *et al.* 1994) and $G_{M2}$-like OS structures occur in the LPS from serostrains for serotypes HS1, HS23 and HS36 (Aspinall *et al.* 1992). A *C. jejuni* strain of serotype HS10 isolated from a patient suffering from MFS had an OS structure reflecting the terminal region of human ganglioside $GD_3$ (Salloway *et al.* 1996). Chemical analysis of the LPS revealed that the core oligosaccharide had a terminal trisaccharide epitope consisting of two molecules of sialic acid linked to galactose, and this trisaccharide is also present in LPS cores of serotype HS14 strains from patients with GBS, but not in cores of nonneuropathic *C. jejuni*. This suggests a possible role for the trisaccharide in the etiology of neuropathies (Salloway *et al.* 1996).

In the United States between 1982 and 1989, extraintestinal infections accounted for only 0.3% of all the isolates of *Campylobacter*. In another example, the frequency of extraintestinal infections due to *C. jejuni* was also very low at 0.62% (Ruiz-Arenas and Arroyo-Gomez 1994).

*C. jejuni* is the most common enteric bacterial pathogen in the United States and New Zealand and notifications of campylobacteriosis have increased dramatically in many

countries including New Zealand since becoming mandatory (Hasell 1994). The increase in campylobacteriosis is usually attributed to improved laboratory isolation techniques for culturing *Campylobacter* and increased awareness of the disease within the community at large (Hasell 1994). However, campylobacteriosis incidence is still on the increase. If more patients are seeking medical attention, could it be due to the severity of the symptoms of campylobacteriosis compared to salmonellosis? Is *C. jejuni,* in comparison with other organisms, more capable of producing infection in immunocompromised hosts? These questions still remain to be answered. A possibility for the increased number of cases may be a change in the virulence or host adaptation of at least some strains of *Campylobacter* due to *Campylobacter* introduction into the human population through increased poultry consumption (Hasell 1994). Human adapted strains could perhaps spread more readily to other humans than would poultry-adapted strains. In developing countries *Campylobacter* strains have been found that produce toxins which are not associated with strains from industrialised countries. This could indicate that strains may acquire some type of virulence factor. If this was associated with adaptation to a human host, then infections with increased severity could result in more people seeking medical help than before (Hasell 1994).

## 1.5 Virulence and Virulence Factors.

### 1.5.1 FLAGELLA AND OTHER PROTEINS.

For a pathogenic bacterium to be successful, it must be able to invade a susceptible host; adhere to host tissue; avoid destruction by the host's immune system; and replicate and transmit progeny to a new, susceptible host. In most cases specific structures on the pathogen's surface are critical in adherence and avoidance.

The mechanisms by which *C. jejuni* causes disease are poorly understood. Cell surface structures such as flagella, fimbriae and LPS have been implicated in the virulence of enteric pathogens, such as *E. coli, Salmonella* spp. and *C. jejuni* (Nikaido and Vaara 1985; Walker *et al.* 1986; Preston *et al.* 1996; Somerville *et al.* 1996).

In this section I will discuss factors produced by *Campylobacter* spp. that are implicated or proven to be important for the pathogenicity of the organism. These factors (flagella, LPS, toxins, fimbriae and others) are briefly examined below.

**1.5.1.1** Flagella.

As a result of pathological changes due to campylobacters colonising the intestine clinical symptoms are observed in patients, most notably in the form of diarrhoea. *Campylobacter* spp. are usually associated with food or water, and therefore must enter the host intestine by way of the stomach. *C. jejuni* must be able to survive the acid barrier of the stomach before they are able to proceed to the intestine and colonise the distal ileum and colon (Ketley 1995). This colonisation step requires the production of flagella, which are the best characterised virulence determinants of *Campylobacter* spp. Mutants without flagella and flagellated nonmotile strains have been found to be unable to colonise animal models (D. E. Taylor 1992). Unsheathed flagella are phase and antigenically variable and are highly immunogenic, with patients producing antibodies to the flagella shortly after infection (D.E. Taylor 1992). Microfilaments, coated pit formation and *de novo* bacterial protein synthesis for invasion are all suggested mechanisms and pathways by which campylobacters invade host cells, but none have been consistently involved in the process of invasion apart from the flagellum (Ketley 1995).

The intestinal mucus layer is a major barrier to penetration by enteric organisms, but locomotion aided by the spiral shape of *Campylobacter* spp. is thought to facilitate penetration through this viscous layer (Walker *et al.* 1986). *C. jejuni* has the unusual ability to remain highly motile in mucus and it is thought that adhesion to host cells is not always necessary as the bacterium is able to remain in the intestine by colonising the mucus layer overlying the epithelium. Some studies have shown that the flagellum may contain adhesins for epithelial cells (McSweegan and Walker 1986; Walker *et al.* 1986; Wallis 1994), but other experiments have shown that the flagellum does not seem to have a role in the adhesion in *C. jejuni* and *C. coli* (Ketley 1995). Production of fimbriae has not been observed (Walker *et al.* 1986), but various outer-membrane proteins and LPS (McSweegan and Walker 1986) have been described that bind to eucaryotic cells. These molecules have not been characterised yet as to their role in virulence (Ketley 1995).

**1.5.1.2** Cytotoxins.

After colonisation of the mucus, campylobacters disrupt the normal absorptive capacity of the intestine by damaging epithelial cells either by cell invasion, the production of toxin(s), or both (Wallis 1994). The occasional bacteraemia case (Ladrón de Guevara *et al.* 1994) and inflammation of the intestine strongly suggest that cell invasion is an important pathogenic mechanism. Toxins may contribute to the disease process. Campylobacters have been reported to produce various toxic activities, including a cholera-like toxin (CJT) and several cytotoxins (Walker *et al.* 1986; Suzuki *et al.* 1994), including a cytolethal distending toxin (CLDT), a shiga-like toxin (SLT) and a haemolysin (Wallis 1994; Ketley 1995). However, other studies have refuted the significance of these findings (Konkel *et al.* 1992). Many reports have also suggested that actin polymerisation is required for *Campylobacter* invasion because drugs that inhibit actin polymerisation also inhibit invasion (Wooldridge and Ketley 1997). However other studies report an absence of polymerised actin in association with sites of bacterial attachment (cited in Wooldridge and Ketley 1997).

**1.5.1.3** LPS.

LPS is a virulence determinant in many species of Gram-negative bacteria. It has been shown to contribute to several areas of the pathogenic process including serum resistance, resistance to phagocytic killing, and cell toxicity (Ketley 1995; Rietschel and Brade 1992; Nikaido and Vaara 1985). Much progress has been made on the biochemical characterisation of *C. jejuni* LPS and most of the attention has been directed towards the utilisation of LPS in serotyping (Moran *et al.* 1991; Mills *et al.* 1992a). *C. jejuni* LPS consists of a low molecular weight fraction and in some cases it may also contain a high molecular weight fraction (Preston and Penner 1989; Mills *et al.* 1992b; Aspinall *et al.* 1992). McSweegan and Walker detailed the role of LPS as an important adhesin with results that indicate that the short O side chains of *C. jejuni* LPS molecules are involved in adhesion to INT 407 epithelial cells and mucus (McSweegan and Walker 1986). LPS was extracted from radiolabelled *C. jejuni* HC and used in a INT 407 cell adhesion assay. Tritiated LPS specifically bound to cells, verifying the role of LPS in attachment to epithelial cells. Periodate oxidation of the LPS reduced the binding of LPS to cells which suggests that binding of the LPS is through the carbohydrate portion of the LPS molecule (McSweegan and Walker

1986). LPS extracted from *C. jejuni* HC was also effective in blocking the attachment of whole *C. jejuni* HC cells to epithelial cells.

*C. jejuni* is unusual in that many strains, eg., HS1, HS2, HS4, HS19, HS23, and HS36, do not display LPS with an extensive O side chain (Aspinall *et al.* 1994a; Yuki *et al.* 1994). The presence of a rough LPS phenotype in *C. jejuni* may be a significant factor in its ability to colonise the epithelium, unlike strains with a smooth LPS phenotype (McSweegan and Walker 1986). The epithelial cell surface of the intestine appears to be the preferred binding site of both the flagellum and LPS, suggesting that the epithelial cell surface contains better *Campylobacter* receptors than does the intestinal mucus (McSweegan and Walker 1986).

LPS is involved in the virulence of other Gram-negative pathogens and gives an intellectual basis for the search of such a role in *C. jejuni.* The role of the flagella in colonisation and invasion has been confirmed with defined genetic mutants (D. E. Taylor 1992), but similar studies have yet to be performed for the LPS from *C. jejuni.*

## 1.6 Overview of Lipopolysaccharides.

### 1.6.1 GENERAL INTRODUCTION.

With the major exception of mycoplasmas, bacteria produce cell walls. In both Gram-positive and Gram-negative bacteria the distinctive heteropolymer known as peptidoglycan is the component which contributes to the mechanical rigidity of the cell wall. All Gram-negative bacteria contain an additional layer in the cell wall structure, termed the outer membrane, which is located on the environmental side of the peptidoglycan layer (Nikaido and Vaara 1985; Stanier *et al.* 1984). The outer membrane is important in protecting Gram-negative bacteria from certain environmental and host-parasite interactions. Components of the outer membrane of the Gram-negative bacterial cell wall include phospholipids, LPS, proteins including the porins, the OmpA protein and a few enzymes including phospholipase $A_1$ and proteases(s) (Stanier *et al.* 1984; Nikaido and Vaara 1985). The outer membrane is very important in the physiology of Gram-negative bacteria as it provides resistance to host defence factors such as lysozyme, B-lysin, and various leukocyte proteins which are highly toxic to Gram-positive bacteria. Resistance to these elements correlates well with the presence of unusual structural components, LPS and porins (Nikaido and Vaara 1985). The outer membrane is a very effective barrier, providing protection to cells from the detergent action of bile salts and degradation by digestive enzymes found in intestinal tracts of animals (Nikaido and Vaara 1985). The outer membrane of some Gram-negative bacteria is able to act as a strong permeability barrier to many antibiotics that are effective against other bacteria (Tamaki *et al.* 1971; Rietschel and Brade 1992). Another important function of the outer membrane is to endow the bacterial surface with strong hydrophilicity which is important in evading phagocytosis, complement resistance, and the capacity to avoid a specific immune attack by altering the surface antigen constitution (Nikaido and Vaara 1985; Schnaitman and Klena 1993). Most work on the LPS has centred on the enteric bacteria *E. coli* and *Salmonella typhimurium* due to the well-established genetic systems available in these organisms. The remainder of this chapter is a basic overview of LPS, mainly derived from *E. coli* and *S. typhimurium,* as these two organisms are the models which most other studies on LPS have been based.

### 1.6.2 PATHOLOGY AND VIRULENCE.

LPS is the major component of the outer membrane of Gram-negative bacteria (Wang and Reeves 1994; MacLachlan *et al.* 1991) accounting for 3-8% of the dry weight of the cell (Hancock *et al.* 1994). LPS is an immunodominant antigen for most Gram-negative bacteria and is the essential component of bacterial endotoxins (Schnaitman and Klena 1993). Since their discovery in the late 1800's, bacterial endotoxins have been among the most widely studied of all bacterial components, due, in large part to the diversity of biological responses which this molecule elicits both *in vitro* and *in vivo* (Hitchcock *et al.* 1986). Endotoxins can elicit disease symptoms ranging from chills and fever to irreversible shock (circulatory failure leading to malfunctioning of organs throughout the body), and death. Paradoxically, these same endotoxins can enhance the body's overall immune resistance to bacterial and viral infections and cancer (Rietschel and Brade 1992). The realisation that endotoxins have a unique structure to which immunological and biological properties appeared to be related led to considerable research being carried out in the 1950's and 1960's on LPS structure. Most of the studies focused on enteric bacteria including various members of the *Salmonella* and *Escherichia* genera, especially *E. coli.* As a result of this research there is now a near thorough characterisation of the biosynthesis, immunochemistry, chemical composition, genetic regulation and structure of LPS species produced by various isolates of *Salmonella* and *E. coli.*

### 1.6.3 HETEROGENEITY OF LPS.

Microorganisms exposed to the selective pressure of an immune system need to generate and maintain cell surface diversity for their survival, often within extremely diverse and inhospitable habitats. LPS chemical heterogeneity may be important at the species or subspecies level to generate diversity for survival. Through genetic variation it is possible that LPS may vary within a single isolate. Mills *et al.* (1992b) have reported a shift in the antigenic spectrum of *C. jejuni* within one host, and in *Neisseria gonorrhoeae* it has been shown that not only can lipooligosaccharide (LOS) structure vary between strains, but that each strain is also able to produce an assortment of LOS, depending on the environment that the organism is exposed to (Schneider *et al.* 1984; Kerwood *et al.* 1992).

The LPS molecule is generally conserved in its overall structure. It consists of three main regions: a lipid A region, a non-repeating core oligosaccharide region and a polymeric O antigen (Stanier *et al.* 1984; Wang and Reeves 1994; Crowell *et al.* 1986) (Figure 1). The molecular weight of individual LPS molecules can vary from about 3,000-54,000 Da according to the lack of or presence of variable numbers of the repeating saccharide units that comprise the O antigenic polysaccharide. However, certain bacterial species, including *Neisseria* spp., *Haemophilus influenzae* and *Bordetella pertussis*, do not produce O antigen containing polysaccharides. Instead the carbohydrate portion attached to lipid A consists of approximately 10 monosaccharides and these molecules are termed lipooligosaccharides (also called R-type or rough LPS) (Hancock *et al.* 1994).

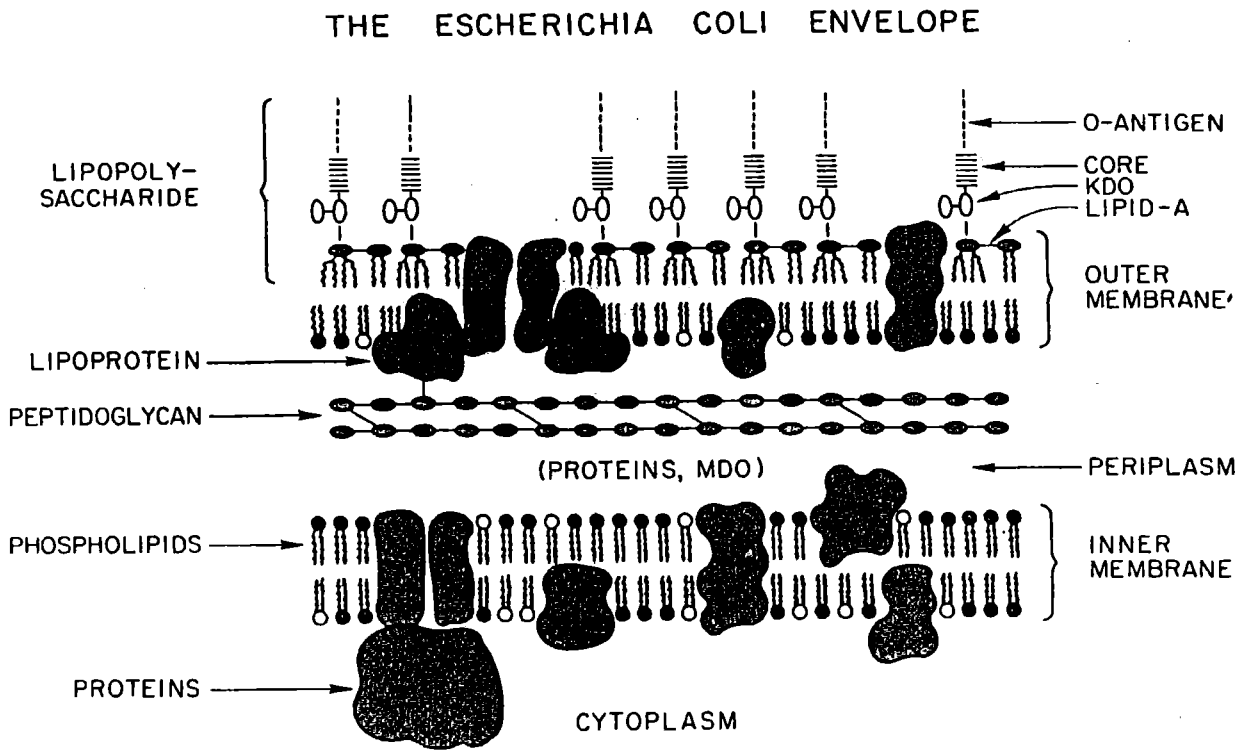



**Figure 1.** Schematic representation of the membrane lipids and proteins in the *Escherichia coli* envelope. Glycerophospholipids are depicted showing fatty acyl chains (as zig zags) with a polar head group sphere attached; sugar residues are drawn as ovals or short lines. The proteins of the inner and outer membranes are unique, and there are 50-100 distinct proteins in each membrane. The membrane-derived oligosaccharides (MDOs) are found in the periplasm. The minimal lipopolysaccharide required for growth of *E. coli* consists of the lipid A domain and two phosphorylated 2-keto-3-deoxyoctulosonic acid (KDO) residues (Adapted from Raetz 1986).

## 1.7 LPS and Cellular Viability.

In enteric and non-enteric bacteria the innermost region of the LPS appears to be essential to bacterial survival. This region consists of lipid A, and at least two residues of 2-keto-3-deoxy-D-*manno*-octulosonic acid (KDO) from the core (Raetz 1986). This next section presents an overview of the different regions of LPS.

### 1.7.1 LIPID A.

Lipid A is a non-conventional hydrophobic phospholipid. Eubacterial phospholipids are glycerol esters, however lipid A is composed of two molecules of *N*-acetylglucosamine (GlcNAc) containing 3'-hydroxy linked fatty acids (FAs) which helps to anchor the LPS in the outer membrane. In *E. coli* and *Salmonella* spp. this structure is relatively conserved with regard to both FAs and GlcNAc composition (Raetz 1986).

Variation may exist in the length of FA branches and/or non-stoichiometric substituents located on the GlcNAc residues. Non-enteric bacteria that grow at lower temperatures (for example, *N. gonorrhoeae* and *Pseudomonas aeruginosa*), possess lipid A molecules with somewhat shorter fatty acyl chains. This alteration might function to optimise the physical properties of lipid A for growth at lower temperatures (Takayama *et al.* 1986; Raetz 1990). Gram-negative bacteria such as *P. aeruginosa* may differ in the FA composition of their lipid A, sometimes containing *S*-2-hydroxy FAs in acyl oxyacyl linkage as opposed to *R*-3-hydroxy FAs that are attached to the glucosamine backbone (Raetz 1990).

### 1.7.2 NON-REPEATING LPS CORE.

The core is a hydrophilic non-repeating oligosaccharide consisting of an inner and outer region. The core oligosaccharide is composed of a variety of sugars, including certain unique species that are characteristic of LPS, such as KDO and L-*glycero*-D-*manno*-heptose (Hep) (Reeves 1994). The eight-carbon sugar KDO, is linked α2,6' to the lipid A disaccharide. This attachment is catalysed by the enzyme KDO transferase, which is encoded by the *kdtA* gene in *E. coli* (Belunis *et al.* 1995). In *E. coli*, strains that are defective in lipid A or KDO biosynthesis have only been isolated as conditional

lethal mutants. KDO has not been found in mammalian cells. Mutants unable to produce KDO are non-viable and thus this sugar is a promising target in the design of novel antibacterial agents (Hammond *et al.* 1987). Two synthetic antimicrobial agents have been designed which specifically target CMP-KDO synthetase, encoded by the gene *kdsB*. These agents inhibit LPS synthesis and cause accumulation of lipid A precursors, resulting in growth stasis and eventually, bacterial death (Goldman *et al.* 1987; Hammond *et al.* 1987). These agents directed against KDO metabolism ought to be endowed with a high degree of selective toxicity (Hammond *et al.* 1987).

Other core sugars include glucose, galactose and GlcNAc (Raetz 1990). The core region is relatively conserved among related Gram-negative bacteria (Raetz 1986), especially the inner core region (Rietschel and Brade 1992). Because this innermost region is more conserved than the O antigen in Gram-negative organisms, it is a major target for generation of antibodies and therapeutic agents as a means to antagonise Gram-negative sepsis (Dasgupta *et al.* 1994). Various monoclonal antibodies directed against the LPS core have been reported, and some researchers have demonstrated that core-lipid A-specific monoclonal antibodies can confer protection against experimental Gram-negative sepsis (Dasgupta *et al.* 1994).

The exact structures of lipid A and the inner-core region of completed LPS molecules are still not known in *E. coli* K-12 or *S. typhimurium*. Non-stoichiometric modifications which are best characterised include a 4-aminoarabinose attached to the 1-phosphate of lipid A and a phosphoryl-ethanolamine (PEA) substituent attached to the 7 position of KDO (Raetz 1990; Schnaitman and Klena 1993).

### 1.7.3 O ANTIGEN.

The O antigen is a hydrophilic polymer composed of from one to five carbohydrates, commonly including hexoses, amino-hexoses, dideoxyhexoses and methylpentoses (Reeves 1994). These sugars are often found in repeating tetra- or pentasaccharide units, although monosaccharides exist as well. The O antigen occurs as a side chain attached to the non-reducing end of the LPS core (Schnaitman and Klena 1993); ie. it is present at the cell surface. Genetic studies using bacteriophages have demonstrated that bacterial cells unable to produce an O antigen are viable and only have a slightly

reduced growth rate when compared to wild-type strains (Raetz 1986). However, rough variants of smooth strains are often avirulent (LPS of bacteria containing O antigens are denoted as smooth (S), and those which lack O antigens are denoted as rough (R) (Hitchcock *et al.* 1986). The O specific chain is the most variable segment of LPS when compared among genera, species and subspecies of bacteria (Rietschel and Brade 1992). Alterations of the O antigen (for example, phage conversion in *Salmonella*) change the properties of the LPS, in some cases by altering bacterial surface charge or by masking or unmasking surface exposed proteins. The O antigen is generally the heat-stable molecule used in serotyping - again exploiting this variability (Raetz 1990; Schnaitman and Klena 1993).

## 1.8 Lipid A.

In the next section, I will describe key features of the biochemistry and genetics of lipid A biosynthesis in *E. coli*. I will describe features of lipid A biosynthesis in other organisms when it has been described.

### 1.8.1 SIGNIFICANCE OF LIPID A AND ITS PRECURSORS:

The lipid A moiety is responsible for most of the biological activities and toxic properties associated with LPS. Mature lipid A (ie. lipid A with KDO disaccharide attached) is a potent toxin capable of inducing a severe shock syndrome in humans and other large mammals. Lipid A and many of its precursors stimulate proliferation of β-lymphocytes and these molecules activate macrophages, an event that is accompanied by prostaglandin release (Hampton *et al.* 1991). Monosaccharide precursors of lipid A (for example lipid A precursors that contain only a single GlcNAc residue) are nontoxic, but retain some of the "beneficial" immunostimulatory effects. The precursor lipid X (see section 1.8.2.1) has been shown to desensitize animals, such as mice and sheep, against the lethal toxicity of purified mature lipid A alone or LPS (Raetz 1986). This finding may have important clinical applications (Raetz 1986). In another study it was found by enzyme-linked immunosorbent assay (ELISA) that lactoferrin binds directly to the isolated lipid A portion of intact LPS of clinically

relevant bacteria. This event may result in bacterial growth reduction and/or killing, as well as in decreased endotoxicity of LPS occurring both in the bloodstream and at mucosal surfaces. Further studies of LPS as an anti-endotoxin agent are therefore warranted (Appelmelk *et al.* 1994).

### 1.8.2 LIPID A SYNTHESIS IN *E. coli* AND *S. typhimurium.*

The predominant species of lipid A in *E. coli* and *S. typhimurium* (Figure 2) is a β(1-6)-linked disaccharide composed of two *N*-acetylglucosamine (GlcNAc) residues, with *R*-3-hydroxymyristoyl substitutions at positions 2, 3, 2' and 3', and phosphate substitutions at positions 1 and 4' (Galloway and Raetz 1990; Anderson and Raetz 1987). Attached at position 6' of the non-repeating GlcNAc through an α glycosidic linkage are two KDO molecules. All other sugars (if present) are attached to the LPS at these KDO moieties (Anderson and Raetz 1987) (Figure 3).

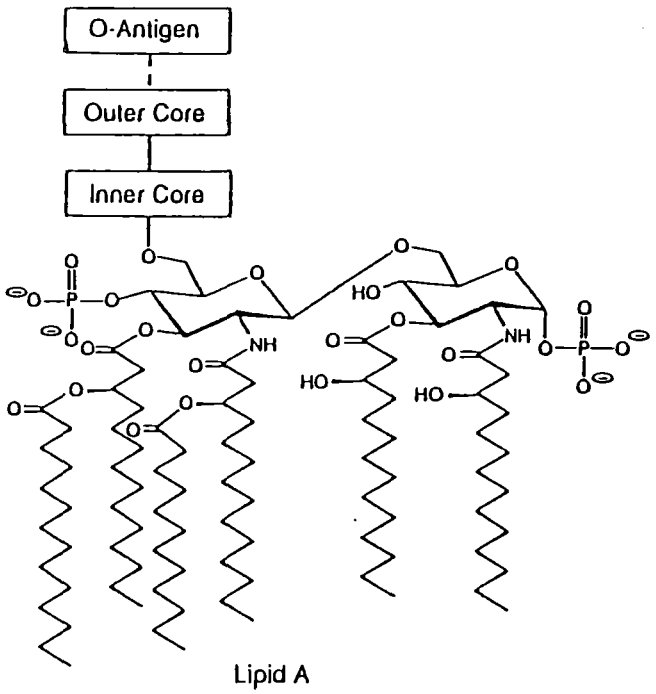



**Figure 2.** Structure of *E. coli* and *S. typhimurium* lipid A. Positions 1 and 4' may be substituted by either phosphate, phosphatidylglycerol or 4-aminoarabinose at non-stoichiometric levels (Adapted from Raetz 1990).

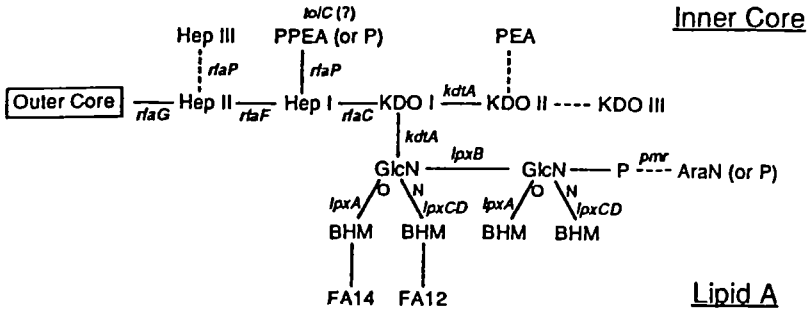



**Figure 3.** Carbohydrates that compose lipid A and inner core regions of enteric bacteria and the genes involved in their synthesis. The lipid A -inner core region is a composite of *E. coli* K-12 and *S. typhimurium* structures. Partial substituents are indicated by dashed lines. The abbreviations BHM, FA12, and FA14 represent β-hydroxymyristate, laurate, and myristate substituents respectively; P represents phosphate. GlcN represents glucosamine; PEA a phosphorylethanolamine, and PPEA a pyrophosphorylethanolamine; AraN 4-aminoarabinose (Adapted from Schnaitman and Klena 1993).

### 1.8.2.1 Overview of Synthesis.

UDP-GlcNAc is an important intermediate in cell surface biosynthesis and is situated at a biosynthetic branchpoint in *E. coli* leading either to peptidoglycan or lipid A synthesis (Figure 4). *R*-3-hydroxymyristoyl-acyl carrier protein (ACP), the other unique early precursor of lipid A, is also a common intermediate since it is able to be incorporated into both lipid A and glycerol-based phospholipids (Schnaitman and Klena 1993). It comes as no surprise that the elucidation of the biosynthetic pathway of lipid A was discovered during a search for genes involved in early steps of glycerophospholipid synthesis (Raetz 1986). Raetz and co-workers found that certain phosphatidylglycerol-deficient mutants of *E. coli* accumulate monosaccharide precursors of lipid A. This led to the discovery of a lipid with the structure 2,3-diacylglucosamine-1-phosphate. This lipid was given the name lipid X and the structure suggested that it might be a biosynthetic precursor of the reducing end unit of mature lipid A. Raetz and co-workers were led to postulate a biosynthetic role for lipid A (Anderson *et al.* 1985) (Figure 5).

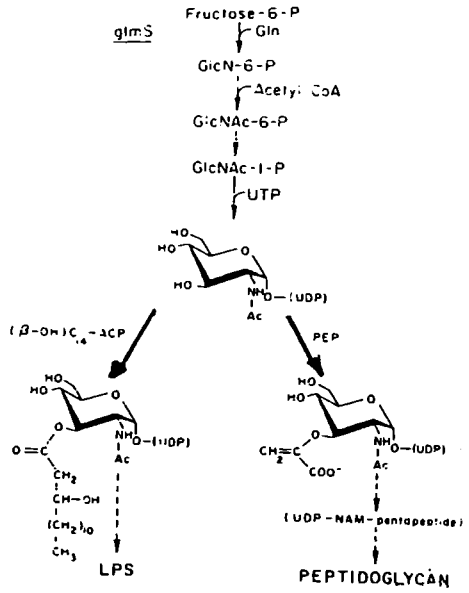



**Figure 4.** Biosynthesis and partitioning of UDP-GlcNAc in *E. coli*. UDP-GlcNAc is situated at a branchpoint in *E. coli* leading to either lipid A or peptidoglycan (for details see text). Abbreviations: GlcN, glucosamine; acetyl CoA, acetyl coenzyme A; ACP, acyl carrier protein; PEP, phosphoenolpyruvate; NAM, *N*-acetylmuramic acid; LPS, lipopolysaccharide (Adapted from Raetz 1987).

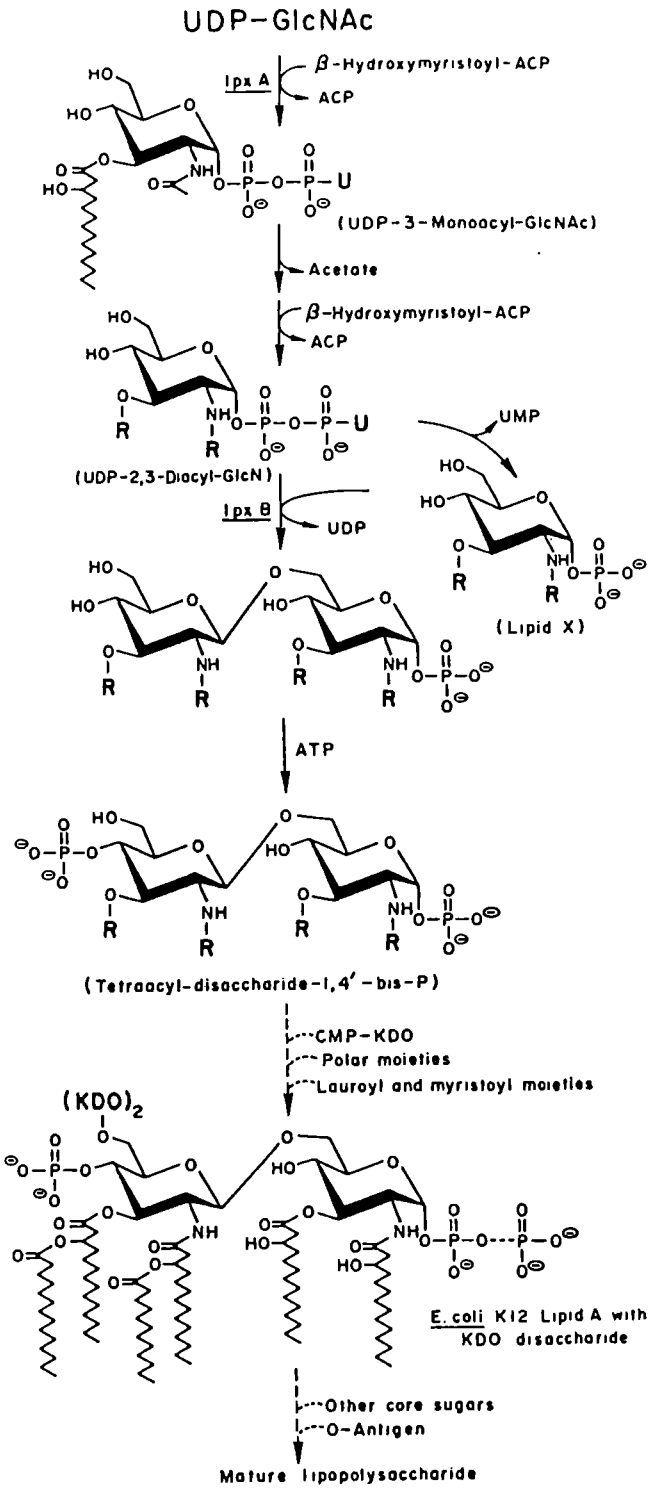



**Figure 5.** Postulated scheme for the biosynthesis of lipid A in *E. coli.* In the figure, R designates a β-hydroxymyristoyl moiety and U designates uridine (Adapted from Ray and Raetz 1987).

**1.8.2.2** Role of *lpxA*.

The first step of lipid A biosynthesis in *E. coli* and *S. typhimurium* involves the transfer of a FA, *R*-3-hydroxymyristic acid, from an acyl carrier protein (ACP) to the 3-OH group of the glucosamine ring of UDP-GlcNAc (Coleman and Raetz 1988). UDP-*N*-acetylglucosamine (UDP-GlcNAc) acyltransferase is encoded by the *lpxA* gene, which has been cloned and sequenced in *E. coli* (Coleman and Raetz 1988) and maps to the minute 4 region of the *E. coli* K-12 map (Galloway and Raetz 1990). The acyltransferase gene was discovered by the isolation of a tight (ie. not easily revertable) conditional *lpxA* mutant in which UDP-*N*-acetylglucosamine acyltransferase activity was absent in cell extracts. This strain is temperature-sensitive (ts) for growth. Characterisation of this mutation provided the evidence that UDP-GlcNAc acyltransferase catalyses the initial step for lipid A biosynthesis in *E. coli* and that lipid A is an essential molecule (Galloway and Raetz 1990). The (ts) phenotype can be corrected by *lpxA*$^+$-bearing plasmids which overproduce the UDP-GlcNAc acyltransferase enzyme (Raetz 1987).

The *lpxA* gene is part of a cluster of 11 genes organised into an operon called the macromolecular synthesis II operon (see below) (Schnaitman and Klena 1993). The amino acid sequence from *lpxA* reveals similarity to LpxD, the enzyme catalysing the third step of LPS biosynthesis, and also to several unrelated bacterial acetyl transferases. The regions of similarity in common to all of these proteins include a repeating sequence motif which has been termed a "hexapeptide repeat" or an "isoleucine patch" (Vuorio *et al.* 1991; Vuorio *et al.* 1994) (see section 1.8.2.3).

**1.8.2.3** Lipid X Formation.

Step two in the lipid A pathway involves *N*-deacetylation of the O-acylated UDP-GlcNAc, catalysed by the enzyme UDP-3-O-(*R*-3-hydroxymyristoyl)-acyl-acetylglucosamine deacetylase (*envA*/*lpxC* gene product). Step three in the pathway involves the transfer of a second acyl group from *R*-3-hydroxymyristoyl-ACP to the amino group to form UDP-2,3-diacylglucosamine (as a result of LpxD) which is the precursor of the nonreducing end of lipid A (Dicker and Seetharam 1992). In step four, two hydrolases (one a CDP-diglyceride hydrolase and one as yet uncharacterised) cleave UDP-2,3-diacylglucosamine to generate lipid X and uridine-monophosphate

(UMP) (Bulawa and Raetz 1984a). Anderson *et al.* carried out short-term labelling of *E. coli* with $^{32}P_i$ to reveal that the precursor of lipid X was UDP-2,3-diacylglucosamine (Anderson *et al.* 1985).

The LpxD protein from *E. coli* and *S. typhimurium* shares an unusual region of homology termed an 'isoleucine patch' or 'the acetyltransferase motif' with LpxA, LacA, CysE, DapD of *E. coli*, NodL from *Rhizobium leguminosarum*, the three hypothetical proteins Yglm of *E. coli*, its analogue Tms of *Bacillus subtilis* as well as Yerm of *B. sphaericus*, and FirA and LpxA from *Rickettsia rickettsii*. LpxA, LacA, CysE, NodL and LpxD are all acyltransferases (Vuorio *et al.* 1991; Vaara 1992; Shaw and Wood 1994; Vuorio *et al.* 1994; Vuorio and Vaara 1995). Periodicity repeat themes are very rare in enzymes. It is plausible to expect that the hexad regions of LpxA and LpxD form a structural (non-catalytic) part of the protein. The crystal structure for LpxA in *E. coli* has been determined and from the structure the roles of the conserved residues of the hexapeptide repeat sequence motif have been identified (Raetz and Roderick 1995). The secondary structure is composed of an $NH_2$-terminal domain of predominantly parallel β-sheet structure and a COOH-terminal domain containing four α helices. The predominance of β-structure is found in the conserved hexapeptide region. Thus, the repeating hexapeptide pattern observed in *lpxA* is important is secondary structure folding allowing formation of a well-packed and nearly symmetric hydrophobic core (Raetz and Roderick 1995).

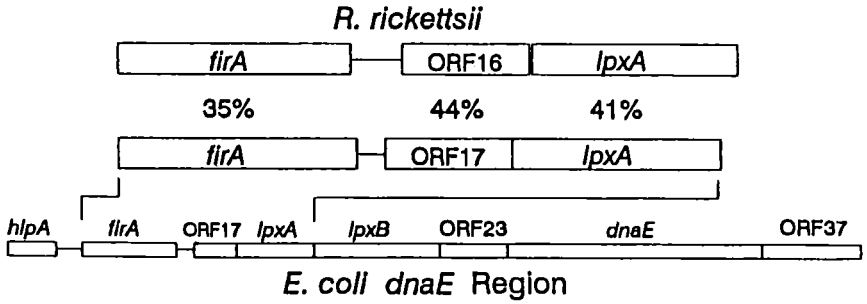



**Figure 6.** Spacing of the *Rickettsia rickettsii* and *Escherichia coli firA-lpxA* region of genes. This schematic demonstrates the relative size and spacing of the *R. rickettsii* genes with respect to the analogous *E. coli dnaE* region. Boxes represent the coding sequences and lines represent the nucleotides separating them with sizes drawn to scale. The percentages indicate the percent of identical amino acids between *E. coli* and *R. rickettsii* proteins. *orf17* is now known to be *fabZ* (Adapted from Shaw and Wood 1994).

**1.8.2.4** Lipid $IV_A$ Assembly.

The fifth step in the biosynthesis of lipid A (figure 5) is catalysed by the protein product of the *lpxB* gene, lipid A disaccharide synthase (Raetz 1986). Disaccharide synthase (*lpxB* gene product) condenses a molecule of 2,3-diacyl-GlcN-1-P (lipid X) with a molecule of UDP-2,3-diacylglucosamine to give tetraacyldisaccharide-1-phosphate. The disaccharide synthase cannot condense two molecules of UDP-2,3-diacylglucosamine directly. Tetraacyldisaccharide-1-phosphate is phosphorylated in *E. coli* at position 4' yielding the tetraacyldisaccharide-1,4'-bisphosphate (lipid $IV_A$).

Mutations in *lpxB* are interesting and non-lethal and provide further evidence for the lipid A biosynthetic scheme. *lpxB1* causes a 100-fold reduction in the specific activity of the lipid A disaccharide synthase (Ray *et al.* 1984). High levels of 2,3-diacylglucosamine-1-phosphate (lipid X) and UDP-2,3-diacylglucosamine accumulate in cells containing the *lpxB1* mutation (Bulawa and Raetz 1984b). Mutants defective in the *lpxB* gene are still capable of generating mature lipid A. It is thought that the accumulation of the precursor molecules compensates for the reduction in the level of disaccharide synthase (Raetz 1986).

*lpxB* (*pgsB*) has been sequenced in *E. coli* and is adjacent to *lpxA* within the operon (Coleman and Raetz 1988). *lpxA* and *lpxB* have been shown to be transcribed in a clockwise direction and it is thought that these two genes are cotranscribed (Crowell *et al.* 1986). The biological significance of the *lpx* operon is not yet certain, but it may be of importance in the control of *E. coli* envelope assembly (Raetz 1987). The *lpxA* and *lpxB* genes map in close proximity to *dnaE*, the gene encoding the α subunit of DNA polymerase III (Tomasiewicz and McHenry 1987). Other genes located in this region include *lpxD* (*firA*), and *orf*17 (*fabZ*) (Mohan *et al.* 1994). The product of the *lpxD* gene is involved in step 3 of lipid A biosynthesis (figure 5). At least two other genes are part of this complex operon. One, located downstream of *lpxB*, is an open reading frame coding for a 23-kilodalton (kDa) polypeptide, and the other is another open reading frame, downstream of *dnaE*, able to code for a 37-kDa protein (Figure 7).

To date, work on lipid A biosynthesis has primarily been carried out only with *E. coli*, but the structure of lipid A from *Salmonella typhimurium* and *S. minnesota* is virtually the same as that of *E. coli* (Ray *et al.* 1984; Bulawa and Raetz 1984a; Anderson *et al.*

1985). Although several notable structural differences exist between the lipid A of *E. coli* and *Rhizobium leguminosarum*, a study by Price *et al.* (1994) concluded that the early steps in lipid A biosynthesis are conserved and that divergence leading to rhizobial lipid A may occur at a later stage in the pathway, presumably after attachment of the KDO residues (Price *et al.* 1994).

All of the enzymes in the lipid A biosynthetic scheme through to the disaccharide synthase are recovered in the cytoplasmic cellular fraction (Anderson *et al.* 1985). However it is thought that these early enzymes of lipid A biosynthesis function as peripheral membrane proteins on the inner surface of the inner membrane, due to the fact that lipid X is membrane bound (Raetz 1987). In *E. coli* the first integral membrane protein in the lipid A pathway is the 4' kinase in the sixth step of the pathway. This was determined by fast atom bombardment (FAB) mass spectrometry and by $^{1}H$ and $^{31}P$-NMR spectroscopy (Ray and Raetz 1987). The first five substrates in lipid A biosynthesis have been shown to occur in cell extracts, and the structures of the metabolites have been verified by nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry, and chemical analysis (Raetz 1986).

**1.8.2.5** "Lipid $IV_A$-KDO: Minimal Unit for Viability".

Lipid $IV_A$ is the minimal substrate to which KDO residues are attached. The tetraacyldisaccharide-1,4'-bisphosphate intermediate is of considerable interest, not only as a precursor in the pathway to mature lipid A, but also because it possesses many of the biological activities of lipid A and LPS, especially the immunostimulatory properties (Raetz *et al.* 1985; Raetz 1987).

Attachment of the KDO residues and the "piggy-backed" fatty acids (3-hydroxy-acyl fatty acids) to the nonreducing end of lipid A is less well characterised. The gene that codes for the bifunctional KDO transferase has been cloned (*kdtA*). It maps near minute 81 between *rfa* and *pyrE* (Clementz and Raetz 1988, cited from Raetz 1990). This transferase transfers two KDO residues from two CMP-KDO molecules to lipid $IV_A$, termed $(KDO)_2$-$IV_A$. Brozek and Raetz have demonstrated the presence of enzymes in *E. coli* extracts that transfer laurate and/or myristate residues from lauroyl or myristoyl-acyl carrier protein (ACP) to $(KDO)_2$-$IV_A$. These acyltransferases are

unable to utilise lipid $IV_A$ alone as a substrate, showing that they possess novel KDO recognition domains (Brozek and Raetz 1990).

Temperature-sensitive mutants of *S. typhimurium* that are deficient in KDO biosynthesis accumulate disaccharide precursor(s) of lipid A under non-permissive conditions (Raetz *et al.* 1985). The conditional lethality of KDO-deficient mutants may be due to the absence of mature lipid A or due to the accumulation of the disaccharide precursors in the inner membrane (Raetz 1986).

## 1.9 *C. jejuni* LPS.

### 1.9.1 OVERVIEW.

The LPS of *C. jejuni* is biologically active in humans, results in endotoxic shock and has been identified in mediating attachment of the bacterium to epithelial cells, acting as an adhesin (McSweegan and Walker 1986).

Biochemical characterisation of the *C. jejuni* LPS has revealed the presence of typical components such as D-glucose, D-galactose, phosphate, KDO, Hep, D-glucuronic acid, glucosamine (GlcN), D-galactosamine, ethanolamine, ethanolamine-phosphate, and fatty acids (Conrad and Galanos 1990; Moran *et al.* 1991a; Moran *et al.* 1991b). However these fatty acids are shorter than those seen in *E. coli*. Fatty acids reported in four *C. jejuni* strains were tetradecanoic acid, hexadecanoic acid, and 3-hydroxytetradecanoic acid (Moran 1995). In addition, more variety in the core region has been noted amongst *C. jejuni* isolates than with other bacterial species (Moran 1995).

D-mannose is reported to be present in the LPS of *C. jejuni* biotype 2 but absent in that of the more common *C. jejuni* biotype 1 (Moran *et al.* 1991a). *N*-acetyl neuraminic acid (Neu5Ac) as well as KDO was found in the LPS of three strains of *C. jejuni*. Presence of Neu5Ac was unexpected; Neu5Ac is found only rarely in procaryotes but, when it is present, it usually occurs as a component of capsular polysaccharide (cited from Moran *et al.* 1991a). Neu5Ac is chemically related to KDO but uncommon in

LPS. Neu5A is a chainlinked polymer when present in capsular polysaccharide and confers on the capsular polysaccharide low or no immunogenicity because of structural mimicry to mammalian host components (Moran *et al.* 1991a). It is unknown whether the presence of Neu5Ac in *C. jejuni* LPS contributes to serum resistance as it does in *Rhodobacter* spp.

Electrophoretic analyses indicate that wild-type strains of *C. jejuni* produce S (smooth)-form LPS, structurally distinct low molecular weight LPS containing sialylated oligosaccharides, or both (Moran 1995). The low molecular weight LOS type is similar to that found in pathogens such as *Neisseria, Haemophilus* and *Bordetella* spp. (Walker *et al.* 1986) showing that there is a variety of core/LPS structures in different bacteria.

### 1.9.2 LIPID A OF *C. jejuni.*

The lipid A component of *C. jejuni* LPS also has unusual chemical properties in comparison with that of *E. coli* and *Salmonella* spp. As mentioned before, *E. coli* and *Salmonella* spp. have a rather conserved lipid A structure characterised by a β(1-6)-linked D-glucosamine (GlcN) disaccharide backbone with attachments of phosphate groups and fatty acids. LPS analysed from bacteria having diverse origins, using techniques like high voltage paper electrophoresis (HVPE), serological studies, chemical studies and structural studies, has shown the occurrence of structurally deviating lipid A molecules with monosaccharide or disaccharide backbones composed of other amino acids such as 2,3-diamino-2,3-dideoxy-D-glucose (GlcN3N) or glucosaminuronic acid (Weckesser and Mayer 1988). In *C. jejuni* lipid A may contain both GlcN and GlcN3N (Moran *et al.* 1991a). Previous work by Moran and coworkers has shown that *C. jejuni* CCUG 10936 (serotype HS2) LPS contains three lipid A backbones composed of GlcN-GlcN, GlcN3N-GlcN3N, and a hybrid disaccharide GlcN3N-GlcN in the ratio 1:1.2:6 respectively (Moran *et al.* 1991b). Techniques showing the breakdown of the LPS into the separate lipid A portions included chromatographic techniques such as HVPE, thin-layer chromatography (TLC), gas-liquid chromatography/mass spectrometry (GLC-MS), laser desorption mass spectrometry (LDMS), and $^{31}$P-NMR spectroscopy, as well as chemical degradation procedures (Moran *et al.* 1991b). This study also showed that *C. jejuni*

LPS purified using the hot phenol-water technique, possesses 50% lower lethal toxicity, 30- to 50-fold lower pyrogenicity and a 100-fold lower ability to induce tumour necrosis factor (TNF) secretion than *Salmonella* spp. LPS (Moran 1995).

As *C. jejuni* lipid A differs in its backbone composition, and possesses acyl chains of different lengths (Moran *et al.* 1991a; Moran *et al.* 1991b), it is thought that these structural differences might account for the lower biological activities of *C. jejuni* LPS compared with *Salmonella* LPS (Moran 1995). The presence of both D-glucosamine and 2,3-diamino-2,3-dideoxy-D-glucose in *C. jejuni* lipid A is a novel finding as it was thought that only D-glucosamine was the major amino sugar component in the lipid A of *C. jejuni* (Moran *et al.* 1991b).

## 1.10 Objectives of this study.

Most Gram-negative bacterial LPS contains a lipid A-like substance. Even diverse phototrophic bacteria, assumed to have emerged relatively early in evolution, contain lipid A (Weckesser and Mayer 1988). Lipid A biosynthesis in *C. jejuni* appears to involve novel pathways with unique intermediates. Given the conservation of the lipid A pathway in *E. coli* and other related Gram-negative bacteria it is relevant to investigate the biosynthesis of lipid A in *C. jejuni*. Work on the organisation and regulation of LPS biosynthetic genes in this organism may help in deducing the role LPS plays in virulence relationships. There is also interest in identifying *C. jejuni* genes and proteins in the LPS which may be useful in the development of diagnostic assays or vaccine formulations. A nontoxic, effective vaccine would be desirable in both developed and developing countries as *C. jejuni* infection is widespread and economically expensive, in terms of lost production time, sick-day pay and many other reasons (Konkel *et al.* 1996).

UDP-*N*-acetylglucosamine acyltransferase is encoded by the *lpxA* gene. The *lpxA* gene product is essential for the production of LPS in *E. coli* as null mutations in *lpxA* are lethal. The initial aim of this study was to characterise the *lpxA* gene hypothesised to

be necessary for synthesis of at least one type of lipid A in *C. jejuni*. It was proposed that *C. jejuni* contains an enzyme that is functionally analogous to LpxA from *E. coli*.

Genetic manipulations were originally performed in *E. coli* as the genetics of this system is well characterised. *C. jejuni* is not a well characterised genetic system and identification of putative LpxA analogs was facilitated by interspecies complementation analysis in *E. coli*. This was achieved by tracnsformation of a genomic plasmid expression library containing *C. jejuni* DNA into the strain *E. coli* KLC 4177 (*lpxA*$^-$).

### 1.10.1 AIMS:

- To determine whether *C. jejuni* has an *lpxA*-like gene;
- To sequence the *lpxA* gene in *C. jejuni*;
- To characterise the uniqueness of the *lpxA* gene in the *C. jejuni* chromosome;
- To identify the *lpxA* gene product, UDP-*N*-acetylglucosamine acyltransferase, from *C. jejuni* and assess its ability to restore growth in interspecies complementation analyses; and as time permitted,
- To attempt to create a 'null mutation' in the *lpxA* gene of *C. jejuni* using a Km$^r$ cassette by homologous recombination and assess function of LpxA protein.

These aims were carried out to determine if *C. jejuni* has a conserved lipid A biosynthetic pathway as shown in many other Gram-negative bacteria, not just the enterics. An *E. coli* temperature-sensitive mutant defective in LpxA activity provides a system with which to study the effect of acyltransferase deficiency on cell viability. Temperature-complementation of the *lpxA* defect within this mutant *E. coli* strain enables identification of an *lpxA*-like gene from *C. jejuni*. By sequencing the *lpxA* gene from *C. jejuni* it will be shown whether the sequence differs from that in *E. coli* as suspected, and whether the predicted LpxA protein from *C. jejuni* shows the conserved isoleucine patches as in other bacteria. Sequencing will also play a large role in constructing a 'knockout' mutation within the *C. jejuni* chromosome if time permits.

# CHAPTER II.

# MATERIALS AND METHODS.

## 2.1 BACTERIAL STRAINS AND PLASMIDS.

The bacterial strains and plasmids that have been used in this study are listed in Table 2.

**Table 2.** Bacterial strains and plasmids used.

| Strain | Description of Relevant Genotype | Source |
|---|---|---|
| *Escherichia coli* | | |
| KLC 4000 | DH5α; *rec*A1 | Laboratory collection |
| KLC 4176 | SM105; F⁻ *thr*-1 *ara*-14 *tsx*-78 Δ(*gal*K-*att*λ)99 *his*G4(Oc) *rfb*D1 *rps*L136(str$^R$) *xyl*A5 *mtl*-1 *thi*-1 | *E. coli* Genetic Stock Centre (EGSC) |
| KLC 4177 | SM101; F⁻ *thr*-1 *ara*-14 *tsx*-78 Δ(*gal*K-*att*λ)99 *his*G4(Oc) *rfb*D1 *rps*L136(str$^R$) *xyl*A5 *mtl*-1 *thi*-1 *lpx*A2(ts) | EGSC |
| KLC 4178 | KLC 4177//pCI1 Ap$^r$ | This study |
| KLC 4179 | KLC 4177//pCI2 Ap$^r$ | This study |
| KLC 4180 | KLC 4177//pCI3 Ap$^r$ | This study |
| KLC 4181 | KLC 4177//pCI4 Ap$^r$ | This study |
| KLC 4182 | KLC 4177//pCI5 Ap$^r$ | This study |
| KLC 4183 | P678-54T; F- *thr leu thi sup*E *lac*Y *ton*A *gal mal xyl ara mtl min* | H. K. Mahanty |
| KLC 4184 | KLC 4183//pCI1 Ap$^r$ | This study |
| KLC 4185 | KLC 4183//pCI2 Ap$^r$ | This study |
| KLC 4186 | KLC 4183//pCI3 Ap$^r$ | This study |

**Table 2.** Continued.

| Strain | Description of Relevant Genotype | Source |
|---|---|---|
| KLC 4187 | KLC 4183//pCI4 $Ap^r$ | This study |
| KLC 4188 | KLC 4183//pCI5 $Ap^r$ | This study |
| KLC 4189 | KLC 4183//pBIISK(+) $Ap^r$ | Laboratory collection |
| KLC 4190 | KLC 4000//pCI6 $Ap^r$ | This study |
| KLC 4199 | KLC 4000//pCI102 $Ap^r$ | This study |
| KLC 4200 | KLC 4000//pCI103 $Ap^r$ | This study |
| KLC 4201 | KLC 4000//pCI104 $Ap^r$ | This study |
| KLC 4203 | KLC 4000//pCI112 $Ap^r$ | This study |
| KLC 4204 | KLC 4000//pCI113 $Ap^r$ | This study |
| KLC 4205 | KLC 4000//pCI114 $Ap^r$ | This study |
| KLC 4026 | CSR 603 Maxicell parent strain. | Laboratory collection |
| KLC 4027 | KLC 4026//pBIISK(+) $Ap^r$ | Laboratory collection |
| JM109 | *rec*A1 *end*A1 *gyr*A96 *thi hsd*R17 ($r_{K-}$, $m_{K+}$) *rel*A1 *sup*E44 Δ(*lac-pro*AB) [F', *tra*D36, *pro*AB, *lac*$I^qZ\Delta M15$] | Promega |
| *Campylobacter jejuni* | | |
| KLC 4132 | NZRM 1958 (NCTC 11168) (Faeces, human enteritis) | CDC, Wellington |
| F38011 | human gastroenteritis | M. E. Konkel (Washington State University) |
| Plasmids | | |
| pBluescriptSKII+ | $Ap^r$, *col*E1ori | Stratagene |
| pGEM®-T | $Ap^r$, *col*E1ori, PCR cloning vector | Promega |

**Table 2.** Continued.

| Plasmid | Description of Relevant Genotype | Source |
|---|---|---|
| pCI1 | pBIISK(+)::1.5 kb '*fabZ-lpxA*' fragment (*C. jejuni* F38011) $Ap^r$ | This study |
| pCI2 | pBIISK(+)::300 bp '*fabZ-lpxA*' fragment (*C. jejuni* F38011) $Ap^r$ | This study |
| pCI3 | pBIISK(+)::450 bp '*fabZ-lpxA*' fragment (*C. jejuni* F38011) $Ap^r$ | This study |
| pCI4 | pBIISK(+)::2.7 kb '*fabZ-lpxA*' fragment (*C. jejuni* F38011) $Ap^r$ | This study |
| pCI5 | pBIISK(+)::2.5 kb '*fabZ-lpxA*' fragment (*C. jejuni* F38011) $Ap^r$ | This study |
| pCI6 | pGEM-T::'*fabZ-lpxA*' (*C. jejuni* NZRM 1958) $Ap^r$ | This study |
| pCI102 | pBIISK(+)::0.6 kb *Eco*RI/*Eco*RI fragment from pCI1 $Ap^r$ | This study |
| pCI103 | pBIISK(+)::0.7 kb *Eco*RI/*Eco*RI fragment from pCI1 $Ap^r$ | This study |
| pCI104 | pBIISK(+)::1 kb *Eco*RI/*Eco*RI fragment from pCI1 $Ap^r$ | This study |
| pCI112 | pBIISK(+)::1.1 kb *Hind*III/*Hind*III fragment from pCI1 $Ap^r$ | This study |
| pCI113 | pBIISK(+)::0.9 kb *Hind*III/*Hind*III fragment from pCI1 $Ap^r$ | This study |
| pCI114 | pBIISK(+)::0.3 kb *Hind*III/*Hind*III fragment from pCI1 $Ap^r$ | This study |

## 2.2 BUFFERS AND MEDIA.

Solutions and media used in this study were prepared as described in Appendices 1 and 2.

### 2.2.1 ANTIBIOTICS AND SUPPLEMENTS.

Antibiotics and colourimetric reagents were added to liquid or solid media where necessary, to maintain selection pressure or to identify strains and constructs, in the concentrations shown in Table 3.

**Table 3.** Antibiotics and supplements.

| Antibiotic | Abbreviation | Concentration |
|---|---|---|
| ampicillin | Ap | 50 mg/ml |
| kanamycin | Km | 30 mg/ml |
| cycloserine | Cs | 50 mg/ml |
| 5-bromo-4-chloro-3-indoyl-ß-D-galactopyranoside | X-gal | 40 mg/ml |

## 2.3 BACTERIOLOGICAL METHODS.

### 2.3.1 CULTURE CONDITIONS.

*Escherichia coli* strains KLC 4177 and KLC 4176 were incubated aerobically at 30°C on M9 minimal salts media (MM) including histidine (His) and threonine (Thr) + 1.5% agar unless otherwise stated. All other *E. coli* strains were grown aerobically at 37°C in Luria Broth (LB) or LB broth + 1.5% agar (LBA). All *Campylobacter jejuni* strains were grown in a reduced oxygen environment (10% $CO_2$) at 42°C on Oxoid Campylobacter blood-free selective agar base (Modified CCDA-Preston) containing the antibiotic cefoperazone (3.2g/L) or on Mueller-Hinton Media. *C. jejuni* was confirmed by Gram stain.

### 2.3.2 OVERNIGHT CULTURES.

Single *E. coli* colonies were resuspended in 5 ml LB broth containing antibiotics as required (see Table 2), with aeration (200 rpm) and incubated at the appropriate temperature for 16 hours before harvesting.

### 2.3.3 STORAGE OF STRAINS.

For day to day use strains were maintained on agar plates supplemented with antibiotics as required and stored at 4°C for three weeks. Long term storage of strains was achieved by mixing 0.5 ml of an overnight culture with 0.5 ml glycerol (20% final concentration) and storage at -80°C.

### 2.3.4 BACTERIAL GROWTH CURVES.

Overnight cultures of KLC 4176, KLC 4177 and KLC 4178 were grown at 30°C in LB broth with aeration. Cultures of KLC 4178 were also supplemented with antibiotics as necessary. Following a 1:100 dilution into fresh LB broth, cells from each strain were incubated at 30°C until $A_{600}$ was ~0.2. Aliquots of these cultures were then incubated at 30 or 42°C (as per Galloway and Raetz 1990). Absorbances were measured every hour for ten hours and dilutions were spread plated onto LB or LB + Ap agar plates to determine numbers of viable bacterial cells at each absorbance measurement. Spread plates were counted after overnight incubation at 30°C.

## 2.4 TECHNIQUES FOR STRAIN CONSTRUCTION.

### 2.4.1 CALCIUM CHLORIDE TRANSFORMATIONS.

#### 2.4.1.1 Preparation of competent cells.

*E. coli* strains were transformed using either the calcium chloride or electroporation method. Competent cells were prepared using a variation of the method of Sambrook *et al.* Briefly, overnight cultures of *E. coli* were diluted 1:100 into 10 ml of LB broth and incubated aerobically at the appropriate temperature to mid-log phase. Cultures were centrifuged at 10,000 rpm, 4°C, for 10 minutes, resuspended in one-half volume

of ice-cold 100 mM $CaCl_2$ and incubated on ice for 60 minutes. Cell suspensions were centrifuged as above, resuspended in 1/20 of the original volume with ice-cold 100 mM $CaCl_2$ and left to sit on ice for approximately 60 minutes before exposure to plasmid DNA. At this stage, aliquots of competent cells were used immediately, stored at 4°C overnight for next day use, or mixed with glycerol (final concentration 20%) and frozen at -80°C for long term (6 months) storage.

**2.4.1.2** Transformation.

0.5 µg of pre-chilled plasmid DNA was added to 100 µl of ice-cold $CaCl_2$-competent cells, gently mixed and incubated on ice for 15 minutes. Cell mixtures were heat shocked at 42°C for 2 minutes without shaking before incubation at room temperature for a further 12-15 minutes. Transformants were diluted in 200 µl LB broth and incubated at the appropriate temperature without shaking for 60 minutes to allow for protein expression (for example, antibiotic resistance proteins). Transformants were spread using a sterile glass rod onto LBA containing selective antibiotics and incubated at the appropriate temperature overnight. Well-isolated transformants were purified by streaking onto fresh LBA selective plates to obtain single colonies. Well-isolated single colonies were restreaked onto selective plates to obtain highly purified isolates. Plasmid DNA from these colonies was isolated as described below (section 2.6.2) and analysed using restriction endonucleases to verify the presence of the initial construct.

**2.4.2** ELECTROPORATION TRANSFORMATION.

*E. coli* strains KLC 4176 and KLC 4177 were incubated at 30°C and not at 37°C as usual for the transformation procedures described below.

**2.4.2.1** Preparation of competent cells.

Electroporation was found to be a more efficient method of plasmid DNA transfer into *E. coli* strains KLC 4176 and KLC 4177 than the calcium chloride method. Competent cells were prepared using a variation of the protocol supplied with the Bio-Rad Gene Pulser (Bio-Rad, Richmond, CA). Cells from an overnight culture were initially diluted 1:100 with LB broth, incubated aerobically at the appropriate temperature to early-mid log phase and chilled on ice for 20 minutes. Ice-cold cells were collected by centrifugation at 11,000 rpm, 4°C, for 10 minutes. The cell pellet was resuspended in one volume of ice-cold sterile distilled water ($dH_2O$) and collected

by centrifugation as before. The cell pellet was resuspended in 1/2 volume cold $dH_2O$ before centrifugation as above. The pellet was resuspended in 1/50 volume sterile, ice-cold 10% glycerol, transferred to a sterile 1.5 ml Eppendorf tube and centrifuged as before. At this stage the supernatant was carefully removed and the pellet suspended in 1/500 of the original volume using sterile, cold 10% glycerol. 40 µl aliquots of electrocompetent cells were used immediately or stored at -80°C in glycerol (20% final concentration) until required. When required, frozen cells were allowed to thaw on ice. All solutions were sterile and ice-cold to maintain competence.

2.4.2.2 Electroporation.

Sterile electroporation cuvettes (0.2 cm gap width) and the chamber slide were chilled on ice prior to electroporation. Thawed competent cells were mixed with approximately 100 ng of DNA and the mixture left on ice for 60 seconds. The mixture was transferred to an ice-cold cuvette, placed in the chilled chamber slide and pulsed in a Biorad Gene Pulser set to 25 µF, 2.5 kV and with a resistance of 250 ohms. These settings usually generated a time constant between 3.9 - 4.6 msec. An 1 ml aliquot of LB broth + glucose (20 mM final concentration) was added immediately to the electrically disrupted sample and the suspension was transferred to a sterile Eppendorf tube. Transformants were grown aerobically at the appropriate temperature for 60 minutes with shaking and aliquots were spread using a glass spreader onto LBA + antibiotic plates. Plates were incubated overnight at the appropriate temperature.

## 2.5 PROTEIN COMPLEMENTATION.

### 2.5.1 TEMPERATURE-SENSITIVE COMPLEMENTATION.

Successful complementation of the temperature-sensitive (ts) *lpxA* defect was determined by the ability of transformants to survive and replicate at the non-permissive temperature of 42°C. *E. coli* strain KLC 4177 was transformed with a *C. jejuni* (F38011) plasmid expression library by electroporation and spread plated onto MM supplemented with His, Thr and antibiotics as necessary. Transformants were incubated at the permissive temperature (30°C) overnight. Transformants were replica plated onto MM supplemented with His and Thr containing antibiotics as required at

the non-permissive and permissive temperatures for 24-48 hours. The ability of a transformant to grow at 42°C on selective media indicated that the transforming plasmid expressed a gene encoding a protein which restored wild-type lipopolysaccharide expression.

## 2.6 DNA Manipulation and Cloning Techniques.

### 2.6.1 PREPARATION OF DNA.

#### 2.6.1.1 Preparation of total genomic DNA.

The method of Berg *et al.* (1994) resulted in the greatest yield of chromosomal DNA from *E. coli*. A 48 hour streak plate of *C. jejuni* (approximately 1 x $10^9$ cells) was overlaid with 3 ml of solution 1 (see Appendix II) and the cells scraped into a sterile 30 ml centrifuge tube using a sterile glass spreader. Cells were centrifuged for 10 minutes, 4°C, at 10,000 rpm. The pellet was suspended in 200 µl lysozyme solution (10 mg/ml) and incubated for 5 minutes at room temperature. 3 ml of solution II was added, mixed by pipette and incubated for a minimum of 2 hours at 55°C. RNAase was added to a final concentration of 20 µg/ml and the mixture was incubated for 10 minutes at 55°C to degrade RNA. The solution was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and centrifuged for 10 minutes, 4°C, at 10,000 rpm. The top layer was removed to a fresh Eppendorf tube. A 1/10 volume of 3 M sodium acetate was added and the DNA was precipitated with two volumes 95% ethanol, centrifuged at 10,000 rpm, 4°C, for 10 minutes. The DNA pellet was washed in 70% ethanol, and air dried before dissolving the pellet in 100 µl of $dH_2O$.

#### 2.6.1.2 Method for Extraction of Chromosomal DNA from *Campylobacter jejuni.*

(adapted from Giesendorf *et al.* (1994)).

The surface of a Mueller-Hinton agar plate (supplemented with 5-10% defribrinated sheep blood) containing a 48 hour streak culture of *C. jejuni* cells was scraped using a sterile glass rod and 10 ml $dH_2O$ into a sterile 30 ml centrifuge tube. Cells were centrifuged for 10 minutes at 1500 x g at ambient temperature. Supernatant was removed and the bacterial pellet washed with 10 ml sterile, cold $dH_2O$, and centrifuged as before. Supernatant was removed and cells resuspended in 10 ml sterile, cold

$dH_2O$. Cells were then aliquoted into 2 x 0.5 ml, 2 x 1.0 ml and 2 x 1.5 ml aliquots in 1.5 ml Eppendorf centrifuge tubes to determine the volume of cells resulting in the highest yield of chromosomal DNA. Cells were centrifuged for 2 minutes at 12,000 x g, supernatants removed and the bacterial pellet resuspended in 150 µl solution I (50 mM glucose, 10 mM EDTA, 25 mM Tris, pH 8.0). 10 µl of lysozyme (125 mg/ml) was added to the resulting solution and the cell lysates incubated at ambient temperature for 5 minutes. After addition of 12.5 µl proteinase K (1 mg/ml) and 12.5 µl SDS (10% wt/vol), cell lysates were incubated at 37°C for 30 minutes (volume at this stage approximately 185 µl). Nucleic acids were extracted with an equal volume of Tris-saturated phenol (pH 8.0), vortexed, and centrifuged at 37°C for 5 minutes before transfer of the aqueous phase to a clean Eppendorf tube. Nucleic acids were extracted two additional times with an equal volume of Tris-saturated phenol (pH 8.0), vortexed, and centrifuged for 5 minutes at 4°C. The resulting supernatant containing the nucleic acids was precipitated with 0.1 volume 3 M sodium acetate (pH 5.2) and 2.5 volumes 100% ethanol.

Nucleic acids were collected by centrifugation for 10 minutes at 12,000 x g (4°C) and the pellet washed with 70% ethanol before resuspension in 100-200 µl $dH_2O$ without RNAase. Samples were analysed by electrophoresis through an agarose gel (0.8%), stained with ethidium bromide and detected with UV light. Comparison of unknown concentration with known concentrations of 1 kb marker DNA were used to quantify samples.

**2.6.1.3** Alkaline Extraction of Plasmid DNA. (Sambrook *et al.* 1989).

Plasmid DNA was extracted from bacterial cells as described below.

An overnight culture was grown aerobically at the required temperature in 5 ml LB broth containing the appropriate antibiotics. 1.5 ml aliquots of the cells were centrifuged at 13,000 rpm (room temperature) for 1-2 minutes, supernatants removed, leaving the bacterial pellet as dry as possible. Bacterial pellets were resuspended in 100 µl of ice-cold solution I (Appendix I) by vigorous pipetting and left to sit at room temperature for 5 minutes. 200 µl of solution II (Appendix I) was added and the contents mixed by inverting the Eppendorf tube rapidly five times, followed by 5 minutes incubation on ice. The addition of 150 µl ice-cold solution III was followed

by inversion of the tube rapidly ten times and the tube stored on ice for 5 minutes. Cellular debris was collected by centrifugation at 12,000 rpm (4°C) for 10 minutes, and the supernatant transferred to a fresh Eppendorf tube. To extract protein contaminants from the plasmid DNA an equal volume of phenol/chloroform/isoamyl alcohol (IAA) was added, emulsified, and centrifuged at 12,000 rpm for 4 minutes at room temperature. The aqueous phase was transferred to a fresh tube where the plasmid DNA was precipitated by the addition of two volumes of 100% ethanol, mixed, and incubated at room temperature for 5 minutes. Plasmid DNA was collected by centrifugation at 12,000 rpm for 10 minutes at 4°C, washed with 1 ml of 70% ethanol, and air dried before resuspending plasmid DNA in $dH_2O$ containing RNAase A (20 µg/µl).

For plasmid DNA that was to be sequenced, further purification was accomplished using the Geneclean III kit (Bio 101, La Jolla, CA) according to the manufacturer's protocol.

### 2.6.2 RESTRICTION ENDONUCLEASE DIGESTION OF DNA.

#### 2.6.2.1 Restriction endonuclease digestions.

Restriction endonuclease digestions were carried out according to the manufacturer's recommended protocol using supplied buffers. Generally, reactions were carried out in 15 µl final volumes and incubated for 2-3 hours at 37°C unless otherwise specified. For digestions that employed more than one enzyme the most suitable buffer for the enzymes was used according to the manufacturer's protocol.

#### 2.6.2.2 Partial digests.

Partial restriction enzyme digestions were used to clone segments of DNA where the restriction endonuclease sites occurred more than once within the DNA fragment. Reactions were carried out in 15 µl final volumes and incubated at 37°C for 15, 30 or 45 minutes. The degree of digest achieved was determined by visualisation of reaction products after electrophoresis and ethidium bromide staining.

### 2.6.3 DEPHOSPHORYLATION OF LINEARISED PLASMID DNA USING CIAP.

To prevent the self-ligation of vector or insert DNA, calf intestinal alkaline phosphatase (CIAP) was added to restriction endonuclease digested DNA to remove 5' phosphate overhangs. The final volume of the reaction mixture was 25 µl. The mixture contained 20 µl of genecleaned DNA (concentration about 2 mg/ml of dsDNA), 2.5 µl of 10x dephosphorylation buffer (Boehringer Mannheim), 1 µl of CIAP (Boehringer Mannheim) and $dH_2O$ making up the remaining volume. The reaction mixture was mixed thoroughly and incubated at 37°C for 1 hour before heat inactivation (incubation at 65°C for 10-20 minutes) of CIAP.

### 2.6.4 DNA LIGATION.

Ligation was performed in a total volume of 20 µl. 1 µl of T4 DNA ligase (Gibco-BRL or Promega) and 4 µl of 5x DNA ligase buffer (Gibco-BRL or Promega) were used to ligate vector DNA to a desired DNA fragment. Ligation mixtures were incubated overnight (12-24 hours) at 12°C. Ligation efficiency was usually checked by UV visualisation of ligation products after agarose gel electrophoresis and ethidium bromide staining, before ligated DNA was transformed into electrocompetent cells. A vector DNA to insert DNA ratio of 1:3 was favoured for high intermolecular ligation and low concatamer formation.

### 2.6.5 AGAROSE GEL ELECTROPHORESIS OF DNA.

#### 2.6.5.1 Gel Electrophoresis.

Agarose dissolved in 1x TAE buffer (appendix 2) was used for gel electrophoresis of DNA. The agarose content varied between 0.8% for restriction endonuclease digested DNA fragments, to 2% for small (less than 800 bp) PCR products. DNA was mixed with 2-3 µl of 6x bromophenol blue gel dye before loading into the agarose gel. DNA fragments were separated by exposure to an electrical current of 65 volts/$cm^2$ for 1.5-2.5 hours in a Bio-rad mini sub or DNA sub cell apparatus. Ethidium bromide (0.5 µg/ml) in 1x TAE was used to stain gels for 10-20 minutes. DNA bands were visualised using a Sigma T2210 UV Transilluminator (302 nm). Known

concentrations of 1 kb marker DNA were used as a standard reference of DNA size and concentration.

2.6.5.2 Elution of DNA Fragments from Agarose Gels.

After visualisation of DNA on an agarose gel by ethidium bromide staining and UV transillumination, desired fragments were excised from agarose using a sterile scalpel blade and placed into sterile 1.5 ml Eppendorf tubes. DNA was extracted from the agarose matrix using the Geneclean III kit (Bio 101, La Jolla, CA) according to the manufacturer's instructions. DNA was eluted from the silica (Glassmilk) matrix using $dH_2O$. A modification to the manufacturer's protocol was to add a third elution step to ensure complete absence of the silica matrix from the eluted DNA.

## 2.7 PLASMID CONSTRUCTS.

Plasmids containing *col*E1 replication origins commonly used for molecular biology (such as pBIISK(+), pGEM-T) were used as vectors for the insertion of foreign DNA segments. These constructs were used for temperature-sensitive mutational complementation, DNA sequencing analysis and Southern hybridisation, as well as for use in protein expression analyses. This section describes the important plasmids constructed in this study.

**pCI1-pCI5**

*C. jejuni* F38011 chromosomal DNA was partially restriction endonuclease digested with *Sau*3A and analysed by agarose gel electrophoresis followed by ethidium bromide staining and UV detection. DNA fragments ranging from 2-3 kb were excised from the agarose, the agarose removed and the resultant DNA ligated into the unique *Bam*HI site of pBIISK(+). Each construct complements the *lpxA* mutation in KLC 4177. $Ap^r$.

**pCI6**

A 0.8 kb PCR amplicon of *C. jejuni* strain NZRM 1958 was amplified by PCR using primers 96-24 and 96-25 (see table 4) and ligated into vector pGEM-T. Used for sequencing. $Ap^r$.

Templates were needed for sequencing and these were generated by partial restriction endonuclease digests of pCI1 using *Eco*RI and *Hind*III in separate digests.

**pCI101-104**

pCI1 was restriction endonuclease digested with *Eco*RI for 15 minutes and the resultant fragments were allowed to religate in the original vector. Used for sequencing. $Ap^r$.

**pCI111-114**

pCI1 was restriction endonuclease digested with *Hind*III for 15 minutes and the subsequent fragments were allowed to religate in the original vector. Used for sequencing. $Ap^r$.

## 2.8 SEQUENCING.

DNA sequencing of the *lpxA* gene of *C. jejuni* F38011 was achieved by the Sanger dideoxy sequencing method (Sanger *et al.* 1977). Part of the nucleotide sequence of the *fabZ-lpxA* gene segment was obtained using an Applied Biosystems Model 733A version 1.2.0 automated sequencing apparatus at the Centre for Gene Research, Biochemistry Department, University of Otago. Plasmids used for sequencing containing inserts of the *lpxA* gene of *C. jejuni* of varying lengths, were prepared from either *E. coli* strains KLC 4000 or KLC 4177. Both of these strains gave a good yield of plasmid DNA. Primers used for sequencing are listed in table 4. DNA templates sequenced by the Sanger method were prepared for electrophoresis using reagents and enzymes supplied with the Sequenase Version 2.0 DNA sequencing kit (Amersham). [$\alpha$-$^{35}$S]dATP was purchased from NEN Life Science Products or Amersham.

### 2.8.1 TEMPLATE PREPARATION.

Plasmids used as templates for Sanger sequencing were prepared to a high level of purity. This was accomplished by first obtaining the plasmid from strains via alkaline lysis and then removal of all trace amounts of phenol via use of the Geneclean III kit (Bio 101, La Jolla, CA) according to the manufacturer's protocol. Double stranded

plasmid DNA template was denatured into single stranded DNA by addition of 1 N NaOH and mixed gently before incubation at room temperature for 5 minutes.

### 2.8.2 PRIMER ANNEALING.

To the above solution the following reagents were added sequentially: single stranded DNA sequencing primer (30 pmol), 3 M potassium acetate (pH 4.5) (final concentration 0.45 M), and $dH_2O$ to a total volume of 20 µl. Each reagent was mixed thoroughly by gentle pipetting upon addition to the solution. The resulting solution was precipitated immediately with 1 volume isopropanol, mixed, and incubated, for 5 minutes at 4°C. Reactions were centrifuged for approximately 10-12 minutes at 12,000 rpm, 4°C, and resultant pellets washed with 70% ethanol. The resulting pellet was air dried at 37°C and resuspended in 12 µl of 1x sequenase buffer. At this stage 2 µl of the template mix was removed and analysed by electrophoresis through a 0.8% agarose gel, ethidium bromide stained and UV visualised to determine the presence and concentration of plasmid DNA. This was done to ensure that there was no loss of DNA due to the process. Annealed templates were usually labelled immediately.

**Table 4.** Primers used for PCR and sequencing.

| Primer name | Sequence | Target gene/organism |
|---|---|---|
| pUC Forward | 5'-GTAAAACGACGGCCAGT | plasmid vector pBIISK(+) |
| pUC Reverse | 5'-GGAAACAGCTATGACCATG | plasmid vector pBIISK(+) |
| 96-24 | 5'-GCTCTACTAAGTCTATCT | *lpxA* in *C. jejuni* F38011 |
| 96-25 | 5'-GTCCGTCCTGGAGATAGGC | *fabZ* in *C. jejuni* F38011 |

### 2.8.3 LABELLING REACTIONS.

To the annealed DNA template-primer mix, the following reagents were added: 1 µl of DTT (0.1 M), 2 µl of labelling nucleotide mix (dGTP), and 0.5 µl [$\alpha$-$^{35}$S]dATP. Each reagent was mixed thoroughly before the addition of 2 µl Sequenase Enzyme 2.0 (diluted 1:8 in sequenase dilution buffer, Amersham). Reactions were pulsed in a microfuge and incubated for 5 minutes at room temperature.

2.5 µl of the individual dideoxy termination nucleotides (ddG, ddA, ddT, ddC) were added to tubes labelled 'G', 'A', 'T', or 'C', and pre-warmed to 37°C. 3.5 µl of the completed labelling reactions were added to each of the four tubes 'G', 'A', 'T', and 'C' and incubated at 37°C for 5 minutes. Reactions were stopped by the addition of 4 µl of Stop Solution. Completed reactions were stored on ice if they were to be used immediately or at -20°C until use. All sequencing samples were denatured at 95°C for 3 minutes before loading onto 5% polyacrylamide gels containing urea.

### 2.8.4 ELECTROPHORESIS AND AUTORADIOGRAPHY.

Electrophoresis of dye sample through 5% denaturing polyacrylamide gels containing urea was performed for 20 minutes at 1750 volts. DNA samples were heated to 95°C for 3 minutes and 3 µl of each sample was loaded into the appropriately designated well in the gel (21 cm x 40 cm) and samples were electrophoresed at 1700-2000 volts for approximately 3 hours. 1x TBE was used as the electrophoresis buffer. Once electrophoresis was completed, plates were separated and the PAGE gel was fixed (10% acetic acid and 10% isopropanol) for 15 minutes before being dried on to Whatman 3 mm paper at 80°C for 2-5 hours using a Dry Gel Sr slab gel drier SE1160 (Hoeffer Scientific Instruments, San Francisco). Labelled DNA was then visualised in one of two ways. Method one involved exposing the dried sequencing gel to Kodak Autoradiography film for 2-3 days in an autoradiography cassette before development of the film. The film was developed in Agfa G-150 developer for 5 minutes, washed in water for 1 minute, and the image fixed in Agfa G-334 fixer for five minutes. Fixed autoradiographs were rinsed in water and air dried. In the second method dried gels were used to expose a STORM (Molecular Dynamics) phosphoimager screen (covered in gladwrap) for 12-24 hours. This screen was scanned using red beam fluorescence at 635 nm and the image analysed on an Apple Macintosh using ImageQuant software.

### 2.8.5 COMPUTER SOFTWARE FOR DNA SEQUENCING ANALYSIS.

Computer software used for analysing DNA sequence obtained from sequencing gels consisted of three programs: DNASIS, CLUSTALV and BOXSHADE. DNASIS was used for nucleotide comparison of the various sequences. CLUSTALV and BOXSHADE performed multiple sequence alignment of the various translated

sequences and identified similar and conserved proteins. The BLAST database was used for comparison of *C. jejuni* nucleotide sequence/protein sequence to that of other organisms.

## 2.9 POLYMERASE CHAIN REACTION (PCR).

PCR of both plasmid and chromosomal DNA was carried out using a Corbett Research FTS-320 Thermal Sequencer. Buffers, dNTPs and enzymes were purchased from Gibco-BRL, and primers were purchased from Amrad-Pharmacia (Table 4).

### 2.9.1 PCR AMPLIFICATION OF DNA TARGETS.

#### 2.9.1.1 PCR of plasmid DNA.

0.5 µl thin-walled PCR tubes were used for each reaction. For each PCR reaction approximately 100 ng/ml of plasmid DNA was used. Primers were diluted 1:10 in $dH_2O$ to give a final concentration of 30 pmol. dNTPs were diluted to give a final concentration of 0.08 mM each (ddG, ddA, ddT, ddT) per reaction. For the PCR mix, 5-10 µl of template DNA was added to 12 µl of the dNTP mix and 5 µl of each primer. To this was added 15 µl of 10x PCR buffer ($Mg^{2+}$ free), 12 µl of 25 mM $MgCl_2$ (final concentration 2 mM) and $ddH_2O$ up to a final volume of 149.5 µl. 0.5 µl of *Taq* DNA Polymerase was added to each tube and the mixture gently mixed, pulsed briefly in a microfuge and overlayed with 100 µl of paraffin oil. The DNA was amplified for 32 cycles in the thermal cycler. Each amplification cycle consisted of three steps: denaturation for 1 min at 94°C, annealing for 1 min at 37°C (55°C if homologous DNA was used depending on T*m* of primer - T*m* of primers was often less than 55°C), and extension of PCR product for 1.5 min at 72°C. An initial cycle of 94°C for 3 min was performed so that the target DNA was completely denatured. The program concluded with a soak step that incubated the tubes at 4°C before removal for storage at 4°C or -20°C, minus paraffin oil. 5-10 µl from each PCR reaction was loaded on a 2% agarose gel, subjected to an electrical field (50-100 $V/cm^2$), and stained with ethidium bromide before visualisation by UV to observe the sizes of PCR products.

2.9.1.2 PCR of Chromosomal DNA.

PCR reactions either utilised as a template extracted chromosomal DNA or whole bacterial cells (colony-PCR). For extracted chromosomal DNA the PCR method is as for plasmid DNA. For whole cell PCR reactions, a colony of bacteria (about $10^6$ cells) was selected from a spread plate and dispersed in the reaction mix described for PCR of plasmid DNA minus the 10x buffer ($Mg^{2+}$ free) and *Taq*. This mix was boiled for 10 minutes, briefly cooled to room temperature and mixed with 10x buffer containing *Taq* DNA Polymerase. Each tube was gently mixed, pulsed briefly in a microfuge and overlaid with 100 µl paraffin oil. Amplification conditions were as described for plasmid DNA.

## 2.10 MAXICELL ANALYSIS OF PLASMID ENCODED PROTEINS. (Silhavy *et al.* 1984)

Bacterial proteins were analysed using the method of Silhavy *et al.* (1984). Briefly, cells of strain KLC 4026 were made calcium chloride competent and transformed with the plasmid of interest. Transformants were incubated in the dark at 37°C on LBA containing antibiotic supplements. Transformants were purified before proceeding with the maxicell protocol. Culture tubes containing 5 ml M63 medium, 1% casamino acids, 0.4% glucose and the appropriate antibiotic, in this case ampicillin, were inoculated with a single colony of the transformant and incubated overnight at 30°C with aeration. KLC 4026 and KLC 4027 were used as controls. On day 2, 0.2 ml of the overnight culture was inoculated into 11 ml of the M63 medium, supplemented as above and shaken at 37°C until the culture reached an $OD_{600}$ of 0.7. The cells were irradiated in the dark with a UV light dose of 300 erg/mm$^2$, with occasional mixing. 10 ml of irradiated cells were inoculated into a foil-wrapped flask and shaken for 30 minutes at 37°C before addition of 40 µl of a 50 mg/ml stock of cycloserine, and incubation continued overnight. On day 3, 0.1 ml of cells were spread onto an LBA plate supplemented appropriately and incubated overnight at 37°C to check for survivors; the rest of the cells were collected by centrifugation at 1500 g for 10 minutes before resuspension in 10 ml of M63 medium. Cells were recentrifuged at

1500 g for 10 minutes, resuspended in 2 ml of M63 medium containing 0.4% glucose, and incubated at 37°C for 1 hour with aeration. After the addition of 10 µl of [$^{35}$S] methionine (25-100 µCi/ml) to the cells incubation was continued at 37°C for 30 minutes, before centrifuging at 1500 g for 10 minutes and the unincorporated radioactive supernatant discarded. The cell pellet was resuspended in 0.5 ml of M63 medium and transferred to a sterile 1.5 ml Eppendorf tube and centrifuged for 5 minutes in a benchtop microfuge at 13 000 rpm. Supernatant was discarded, the cells resuspended in 0.5 ml M63 medium and centrifuged for 5 minutes at 13 000 rpm in a benchtop microfuge. The supernatant was discarded and the cell pellet resuspended in 0.1 ml of 2 x loading buffer. Tubes containing the cells were boiled for 5 minutes in a waterbath before storage at -20°C, in preparation for electrophoresis on an SDS-polyacrylamide gel (SDS-PAGE).

**2.10.1** SDS-PAGE (Silhavy *et al.* 1984).

Proteins were separated by electrophoresis through a 12% polyacrylamide gel (16 x 20 x 0.75 cm) comprised of a 12% acrylamide separating gel and a 3% stacking gel in a Hoeffer SE600 gel apparatus (Hoeffer) using a sodium dodecyl sulphate (SDS)†-†glycine running buffer system for both top and bottom buffers. Continuous voltage at 100 volts/cm$^2$ was carried out through the stacking gel until the dye front reached the separating gel. At this point voltage was increased to 200 volts/cm$^2$ and the protein samples electrophoresed until the dye front (bromophenol blue) migrated to the bottom of the gel. The acrylamide gel was removed from the glass plates and placed in coomassie brilliant blue stain for 30-60 min before destaining. The gel was destained initially for 15 minutes in a 5% methanol and 7% acetic acid solution with gentle shaking. Destaining solution was discarded, fresh solution added and gels were gently shaken for 1 hour. Used destaining solution was discarded, fresh solution was added and gels were left in the destain solution overnight. After destaining steps, the protein gel was placed on an acetate and photographed. In a second method, dried gels were used to expose a STORM (Molecular Dynamics) phosphoimager screen (covered in gladwrap) for 12-24 hours. This screen was scanned using red beam fluorescence at 635 nm and the image analysed on an Apple Macintosh using ImageQuant software.

## 2.11 SOUTHERN HYBRIDISATION.

### 2.11.1 SOUTHERN TRANSFER.

DNA was digested to completion (usually a minimum of five hours) and separated by electrophoresis through a 1% agarose gel in a DNA sub cell or Biorad mini sub cell apparatus. Ethidium bromide (0.5 μg/ml) in 1 x TAE was used to stain gels for 20-30 minutes and gels were photographed using a Sigma T2210 UV Transilluminator (302 nm) using 1 kb marker as the standard. DNA was transferred to a 10 x 15 cm nylon membrane (Boehringer Mannheim) using a Hoefer Trans-Vac TE80 vacuum blotter attached to a Cole-Parmer Air Cadet vacuum/pressure station at a negative pressure of 125 mm Hg (-15 kPa). The next three steps were also performed under vacuum. Nylon membranes containing DNA were soaked in depurination solution (appendix II) for 10 minutes prior to removal and the addition of denaturation solution (appendix II) to the membrane which was left to soak for a further 10 minutes. Denaturation solution was removed and the membrane exposed to neutralisation solution (appendix II) for 10 minutes before removal of the neutralisation solution. DNA was transferred in 20 x SSC for 60 minutes. The agarose gel was restained in ethidium bromide and visualised on a UV transilluminator to determine the efficiency of transfer. The membrane was gently blotted dry using Whatman 3 mm paper. Single stranded DNA was cross-linked to the membrane by exposure to UV for 30 seconds at 1200 kJ in a UVC-515 Ultraviolet Multilinker (Ultra-lum). Membranes were either probed immediately or wrapped in foil and stored at room temperature until required.

### 2.11.2 PROBE LABELLING AND HYBRIDISATION.

The non-radioactive digoxigenin-dUTP (DIG) DNA labelling and detection kit (Boehringer Mannheim) was used for all Southern hybridisations. The labelling reaction was carried out as follows: to 300 ng of phenol/chloroform-purified DNA in a 1.5 ml microcentrifuge tube $ddH_2O$ was added to make a final volume of 16 μl. DNA was denatured by placing the tube in a boiling waterbath for 10 minutes. The tube was immediately placed on ice for 5 minutes before the addition of 4 μl DIG High-Prime to the denatured DNA. Contents of the tube were mixed by gentle tapping or pipetting and briefly centrifuged in a microcentrifuge before incubation at 37°C overnight to

ensure the greatest yield of DIG-labelled DNA. A colourimetric test was used to check efficiency of incorporation of DIG into the probe DNA. Dilutions of probe DNA were visually compared to known amounts of DIG-labelled DNA contained on a test-strip, and the amount of DIG-labelled probe was calculated based upon the known concentrations of DNA on the test-strip.

### 2.11.3 PRE-HYBRIDISATION.

Hybridisations were performed as described in the DIG users manual (Boehringer Mannheim). Membranes were placed in a roller hybridisation bottle containing 10 ml of DIG Easy Hyb and incubated at 42°C in a Hybaid LTD Micro-4 HB-MCR4 for 2 hours. The amount of probe required for 10 ml hybridisation buffer to give a final concentration of 25 ng/ml was calculated. The probe volume was made up to 50 µl with $ddH_2O$ and the probe denatured in a boiling waterbath for 10 minutes and placed immediately on ice.

### 2.11.4 HYBRIDISATION.

The 1.5 ml microfuge tube containing the probe was briefly centrifuged in a microcentrifuge before addition to 15 ml of fresh DIG Easy Hyb that had been prewarmed to 42°C. The hybridisation solution containing the probe was filtered using a 0.45 µm nitrocellulose disposable filter into a sterile universal bottle. Membranes were left in hybridisation solution overnight at 42°C with constant rotation. Following hybridisation, membranes were removed from the hybridisation solution and washed for 15 minutes in low stringency wash buffer (2 x SSC, 0.1% SDS) at room temperature with occasional shaking. Care was taken to not allow membranes to dry out. This wash was repeated with fresh solution. This step is performed to remove unbound probe. Membranes were washed further (twice for 15 minutes) in high stringency buffer (0.5 x SSC, 0.1% SDS) at 68°C with constant shaking. After performing stringency washes, membranes were rinsed briefly (1-3 minutes) in 1 x washing buffer (50-100 ml to equilibrate) before incubation for 30 minutes at room temperature in 1 x blocking solution (see Appendix 2). Anti-DIG-AP conjugate (antibody) was centrifuged for 5 minutes at 10 000 rpm before being diluted 1:10000 in 1 x blocking solution. Membranes were incubated in diluted antibody solution for

30 minutes in a separate, clean container, washed twice (for 15 minutes) in 1 x washing buffer, and equilibrated for 2-5 minutes in 1 x detection buffer. The membrane was placed on an acetate (DNA side up) and 20 drops (approx. 1 ml) of CSPD, ready-to-use solution, was applied, dropper-form, along the side of the membrane and the membrane was immediately covered with a second acetate and CSPD spread evenly over the membrane with a damp cloth. After incubation at room temperature for 5 minutes any excess substrate was squeezed out at the edges of the acetate sandwich and sealed, using a heat-sealer. The damp membrane was incubated at 37°C for 15 minutes to enhance the luminescent reaction, before the acetate was wiped down with 70% ethanol and exposed to Lumifilm (in the dark room) for 30 minutes at room temperature and developed.

# CHAPTER III.

# RESULTS.

## 3.1. Cloning of a functional analog of the *E. coli* gene *lpxA* from *Campylobacter jejuni.*

### 3.1.1. SCREENING OF A *C. jejuni* PLASMID LIBRARY FOR *lpxA* FUNCTIONAL ANALOGS.

A plasmid expression library made from *C. jejuni* F38011 DNA cloned into the vector pBIISK(+) was obtained from Dr. M. E. Konkel (Washington State University). The expression library was constructed from a partial *Sau*3A digest of C. *jejuni* F38011 genomic DNA, with subsequent *Sau*3A/*Sau*3A fragments ligated into the *Bam*HI site of pBIISK(+). The library consisted of either F38011 DNA inserts of 2-3 kb (F2) or, in the case of F3, 3-4 kb inserts of F38011 DNA. This library has been successfully used to isolate several LPS and non-LPS genes from *C. jejuni* (Konkel *et al.* 1996; J. Klena, pers. comm.).

### 3.1.2 TEMPERATURE-SENSITIVE MUTANT: SELECTION FOR GROWTH AT 42°C.

KLC 4177 is a temperature-sensitive strain of *E. coli* K12 that lacks the ability to produce the enzyme UDP-*N*-acetylglucosamine acyltransferase, the protein product of the *lpxA* gene, at 42°C but not at 30°C. Due to this defect in lipid A synthesis, KLC 4177 is unable to grow on MM supplemented with His and Thr at the non-permissive temperature of 42°C (figure 7). In order to identify a gene whose product is capable of restoring viability of KLC 4177 temperature-sensitive mutants at 42°C, the F38011 plasmid library was transformed into KLC 4177. The plasmid expression library was introduced into electro-competent KLC 4177 cells and transformants were selected on MM supplemented with His, Thr and Ap at 30°C.

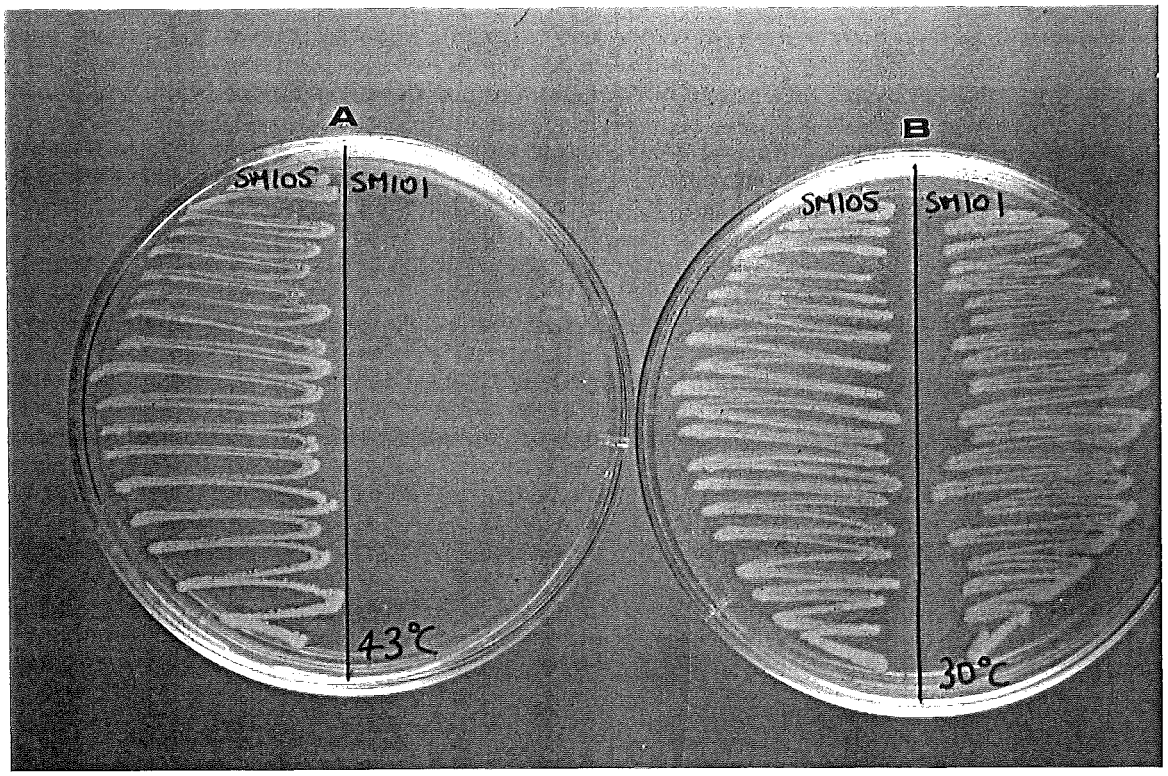



**Figure 7.** Growth phenotype of *E. coli* strains KLC 4176 and KLC 4177 at 30°C and 42°C. Strains were incubated on MM supplemented with His, Thr and Ap at 42°C (A) and 30°C (B). KLC 4176 (wild-type) is shown on the left and the temperature-sensitive mutant KLC 4177 on the right of each plate.

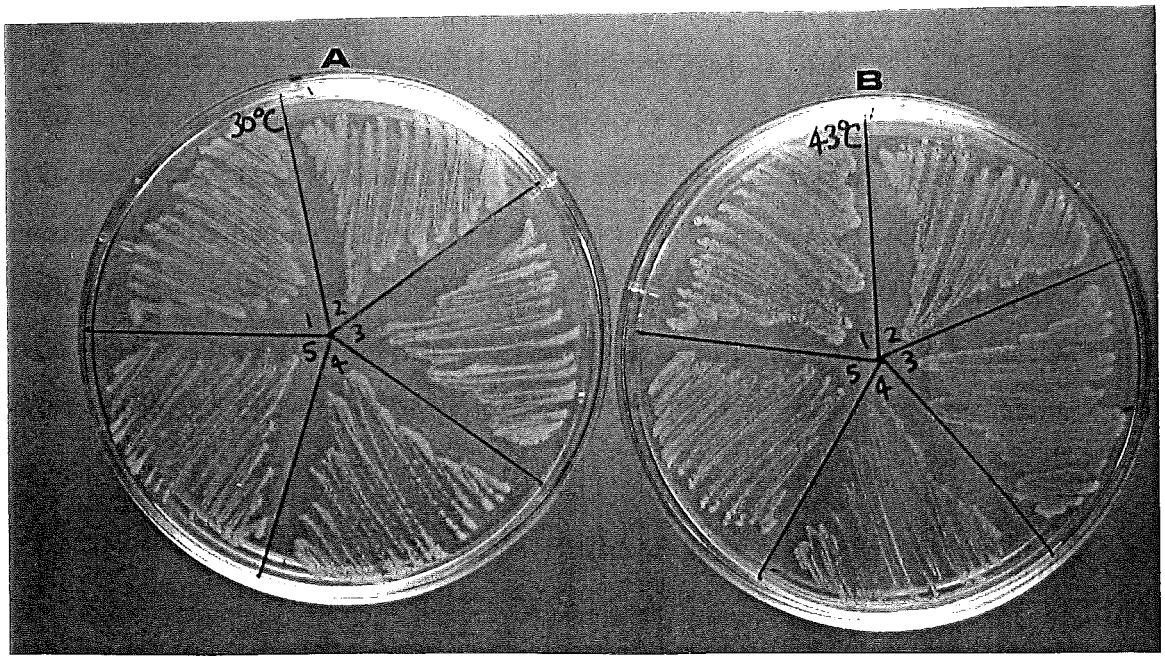



**Figure 8.** Growth phenotype of transformants KLC 4178-4182 showing viability at both 30°C and 42°C. Strains were incubated on MM supplemented with His, Thr and Ap at 30°C (A) and 42°C (B). KLC 4178 - KLC 4182 are labelled 1-5 respectively on each plate.

This was done to ensure that viable transformants were produced and contained a plasmid enabling them to survive ampicillin selection at the permissive temperature. In order to select transformants containing a *lpxA* analog, transformants were replica plated to two plates of MM + His, Thr and Ap; one plate was incubated at 30°C (permissive) while the other replica plate was incubated at 42°C (non-permissive). Restoration of the ability of transformants to replicate at 42°C should be indicative of a gene product from *C. jejuni* F38011 that overcomes the *lpxA* defect in *E. coli* K12. From the thousands of transformants screened at both 30°C and 42°C (data not shown) five $Ap^r$ transformants of KLC 4177 generated with the F2 expression library arose at 42°C (KLC 4178-KLC 4182) (figure 8). Plasmid DNA isolated from these transformants was designated pCI1, pCI2, pCI3, pCI4, and pCI5. The control plate containing KLC 4177 cells transformed with pBIISK(+) alone produced growth at 30°C but not at 42°C.

To ensure that the ability to grow at 42°C was due to genetic determinants expressed from the plasmids and not reversion of the *lpxA* mutation in KLC 4177, electro-competent KLC 4177 cells were transformed with plasmids pCI1-5 and selected on MM + His, Thr and Ap a second time. If growth at 42°C is due to reversion and this event is infrequent in a population (as the *lpxA* mutation is tight), then a difference should be seen in the number of transformants arising at 42°C compared to provision of complementing activity from a plasmid (*in trans*). In other words, all transformants receiving a complementing plasmid should grow at 42°C, while only a small population of cells in a population with a revertant (depending on when reversion occurred) should arise at 42°C. Strain KLC 4177 was taken from fresh stock each time it was needed and shown to be unable to form colonies at 42°C to ensure that parental stocks had not reverted. Colony formation was determined at both 30°C and 42°C and in all five cases each transformant arising originally at 30°C, containing either pCI1 through to pCI5, was able to replicate at 42°C. Growth of KLC 4177 transformants containing pBIISK(+) were observed at 30°C only indicating no reversion in the *lpxA* parental strain during cell competence preparation. In calcium chloride transformations of KLC 4177 with pCI1-pCI5, approximately 600 transformants arose containing pCI1 and pCI4 at 30°C and at 42°C. It was observed that fewer transformants arose at 30°C

and 42°C containing the plasmids pCI2 and pCI5, and that no transformants grew containing pCI3 at 30°C and 42°C. The fewer number of transformants for plasmids pCI2 and pCI5 and the lack of transformants from pCI3 may have been due to the efficiency of the transformation technique, or perhaps the quality of the plasmid preparation used. However when visualised by agarose gel electrophoresis and ethidium bromide staining, pCI3 appeared as concentrated as all other pCI plasmids used. From electroporation transformation data, KLC 4177 transformants containing plasmid pCI3 arose at a lower frequency relative to the other pCI plasmids as determined by visual observation of colony numbers both at 30°C and at 42°C. Several reasons might explain this: 1) the *C. jejuni* DNA fragment containing *lpxA* on these plasmids may also contain a gene whose product is toxic to *E. coli*; 2) a partial gene contained on the clone may produce an incomplete product and this product interferes with *E. coli* growth; or 3) possibly the most interesting theory is perhaps the presence of a gene whose product can modify the LPS of *E. coli* in such a fashion that growth may occur, but this is due to synthesis of a novel lipid A species or intermediate and not *lpxA* complementation. This last scenario would be most exciting as it might enable genes involved in assembly of other lipid A analogs in *C. jejuni* to be identified.

### 3.1.3 RESTRICTION ENZYME MAPPING OF pCI1-5.

Restriction endonuclease digestions were carried out on plasmids pCI1-5 using a number of different enzymes. The purpose of these digestions was to determine if a common fragment existed among the cloned *C. jejuni* inserts. A common fragment within the inserts may rule out one or two of the scenarios stated earlier, and perhaps indicate where the gene responsible for complementary activity is located.

A double digest with *Kpn*I and *Sac*I was performed to determine sizes of the *C. jejuni* F38011 chromosomal DNA inserts within pCI1-pCI5 as these two enzymes are known to digest DNA on either side of the insert (see figure 10 of pCI1). Insert sizes differed for all five plasmids. The insert in pCI1 was shown to be 1.4-1.6 kbp in size; pCI2 produced three fragments, two of approximately 2.9 kb and one of 300 bp; pCI3 generated three fragments from the digestion, two of 2.9 kb and one of 450 bp; the fragment of pCI4 was 2.7-2.8 kb in size; and the insert seen in pCI5 was 2.5 kb (figure

9). From the pattern observed for pCI2 and pCI3 the insert has probably been cut twice by either *Kpn*I or *Sac*I.

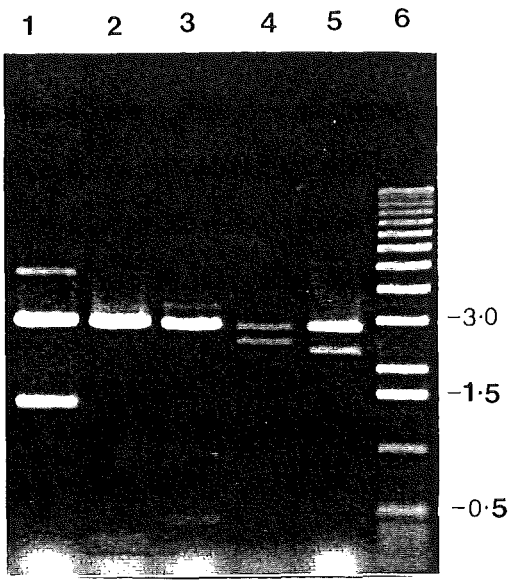



**Figure 9.** Plasmids pCI1-pCI5 restriction endonuclease digested with *Kpn*I and *Sac*I to visualise the *C. jejuni* F38011 DNA insert from the plasmid vector pBIISK(+). Lane 1, pCI1; Lane 2, pCI2; Lane 3, pCI3; Lane 4, pCI4; Lane 5, pCI5; Lane 6, 1 kb standard marker. Sizes (kb) of the bands in lanes 1-5 were estimated by comparing migration of digested DNA to that of the known 1 kb standard migration. DNA was electrophoresed on a 0.8% agarose gel, stained with ethidium bromide and visualised under UV light. Bands larger than 3 kb are due to uncut or partially digested plasmid DNA. Bright bands at the bottom of the gel are indicative of RNA that has not been completely removed by RNAase A. The uniform band at approximately 3.0 kb is the vector pBIISK(+).

Subsequent restriction endonuclease digestions of these plasmids with the enzymes *Eco*RI and *Hind*III in separate digests revealed that pCI1 was digested 4 times by *Eco*RI; once in the pBIISK(+) vector and three times within the insert, resulting in DNA fragments of approximately 3.2 kb, 700 bp, 500 bp and 50 bp (summarised in figure 10). From the *Hind*III digestion of pCI1, the enzyme cut five times, once in the vector and four times in the inserted fragment generating 3 kb, 900 bp, 400 bp, 200 bp, and 150 bp DNA fragments (figure 10). As the vector is 3.0 kb itself, the addition of the other bands suggested an insert of 1.4-1.6 kb in size for pCI1. Plasmids pCI2 and pCI3 restriction endonuclease digested with *Eco*RI and *Hind*III in separate reactions displayed a similar banding pattern to each other when analysed by agarose gel electrophoresis. A doublet of 2.9-3.0 kb in size was observed in both pCI2 and pCI3 suggesting that the inserts within these plasmids are approximately twice the size of the insert in pCI1. The digestion pattern for pCI2 and pCI3 also indicates that each insert must contain only a single *Eco*RI and *Hind*III site. When plasmids pCI4 and pCI5

were digested each revealed a unique restriction pattern. pCI4 digested with *Eco*RI generated at least a 3 kb, 800 bp and 600 bp fragment. pCI4 was cut five times with *Hind*III producing fragments 3 kb, 900 bp, 700 bp, 200 bp and 150 bp in size. pCI5 was cut twice by *Eco*RI, once in the vector and once in the insert, generating a 3 kb and a 600 bp doublet fragment, as well as a 4.5 kb fragment which may be the result of uncut or only partially digested DNA. The *Hind*III profile for pCI5 showed a doublet at 3 kb and two smaller fragments at 900 bp and 400 bp. When the sizes of the *Eco*RI and *Hind*III fragments were summed up and compared for pCI4 and pCI5 they did not agree (data not shown).

Restriction mapping revealed only one common fragment for all five plasmids; the vector, pBIISK(+). pCI1 appeared to contain the smallest, most consistently sized *C. jejuni* insert capable of restoring growth at the non-permissive temperature in KLC 4177. pCI1 also contained many restriction endonuclease sites not present in the other five plasmids. Although both pCI2 and pCI3 generated similar restriction enzyme fragments, it was shown by the digestion with *Kpn*I and *Sac*I that the two plasmids contained different sized inserts. Common fragments shared by plasmids were the 900 bp, 400 bp and 150 bp *Hind*III fragments observed for pCI1 and pCI4.

As a result of the data generated from the restriction endonuclease digests and the growth of transformants on MM, plasmids pCI1 and pCI4 were chosen for further analysis as they were the plasmids containing the least amount of *C. jejuni* DNA with the most restriction sites (important for ease of sequencing and manipulation), shared sizes of some restriction fragments and appeared to produce the best complementation (shown by the greatest number of transformants). Time was too limited to take a closer look at all the other plasmids to determine the differences between them.

## 3.2 Characterisation of pCI1 and pCI4 F38011 chromosomal DNA.

### 3.2.1 RESTRICTION MAPPING.

Plasmids pCI1 and pCI4 were further digested with the following restriction enzymes, *Bam*HI, *Bgl*II, *Bst*XI, *Dde*I, and *Eco*RV, to construct a physical map.

Results generated for pCI1 included internal restriction enzyme sites for *Bgl*II, *Bst*XI, *Dde*I, and *Eco*RV (see figure 10). When pCI1 was digested with *Bst*XI, two fragments, 3.5 kb and 1.0 kb, were generated, indicating that the enzyme has a restriction site within the insert as well as the site known within the vector. *Eco*RV appeared to cut once within the insert of pCI1 and once in the vector, producing bands of 1.5 kb and 3.0 kb. The restriction enzyme *Dde*I cut within pCI1 a number of times producing bands of 600 bp, 500 bp and 400 bp as well as many smaller fragments which were difficult to resolve due to contaminating RNA. As expected *Sau*3A cut a number of times within the insert generating many fragments of varying sizes.

Initially, pCI4 had an unusual restriction pattern; when digested with *Hind*III, *Sac*I, *Eco*RI, *Xho*I and *Eco*RV, doublets of fragments were observed ranging in size between 3-5 kbp. This was unexpected as the average size of the inserts in the F2 library are between 2-3 kb and the fragments observed for pCI4 were much larger than 2-3 kb. *Eco*RI and *Eco*RV digests of pCI4 DNA also produced smaller sized fragments of 0.5 kbp-1.5 kbp as well as the 3-5 kbp fragment. An explanation for these unexpected restriction patterns is that there may have been two plasmids present within the original colony and both were isolated initially, resulting in restriction profiles that were similar to each other but not identical. Further purification of the pCI4 plasmid followed by restriction endonuclease digests have resulted in the disappearance of the plasmid fragment sized at 3-5 kbp. This lends further support to the hypothesis of two plasmids being initially isolated and digested.

PCR was performed using pCI1 and pCI4 and the pUC forward and reverse primers as another method to help in determining of sizes of the insert within these plasmids. For pCI1 a PCR fragment was observed measuring 1.5 kbp in size. For pCI4, a PCR fragment of 1.9 kb was observed, close to the size of the fragment produced from the restriction digest mapping (data not shown). For pCI1, the restriction digestion patterns using *Eco*RI and *Hind*III and the results from PCR were encouraging as they were consistent with the mapping studies done above. The patterns consistently showed an insert of 1.4-1.6 kbp and identical placement of restriction sites (*Hind*III, *Eco*RI) in pCI1 (data not shown). Due to the initial difficulties encountered with pCI4, plasmid pCI1 was chosen for further analysis.

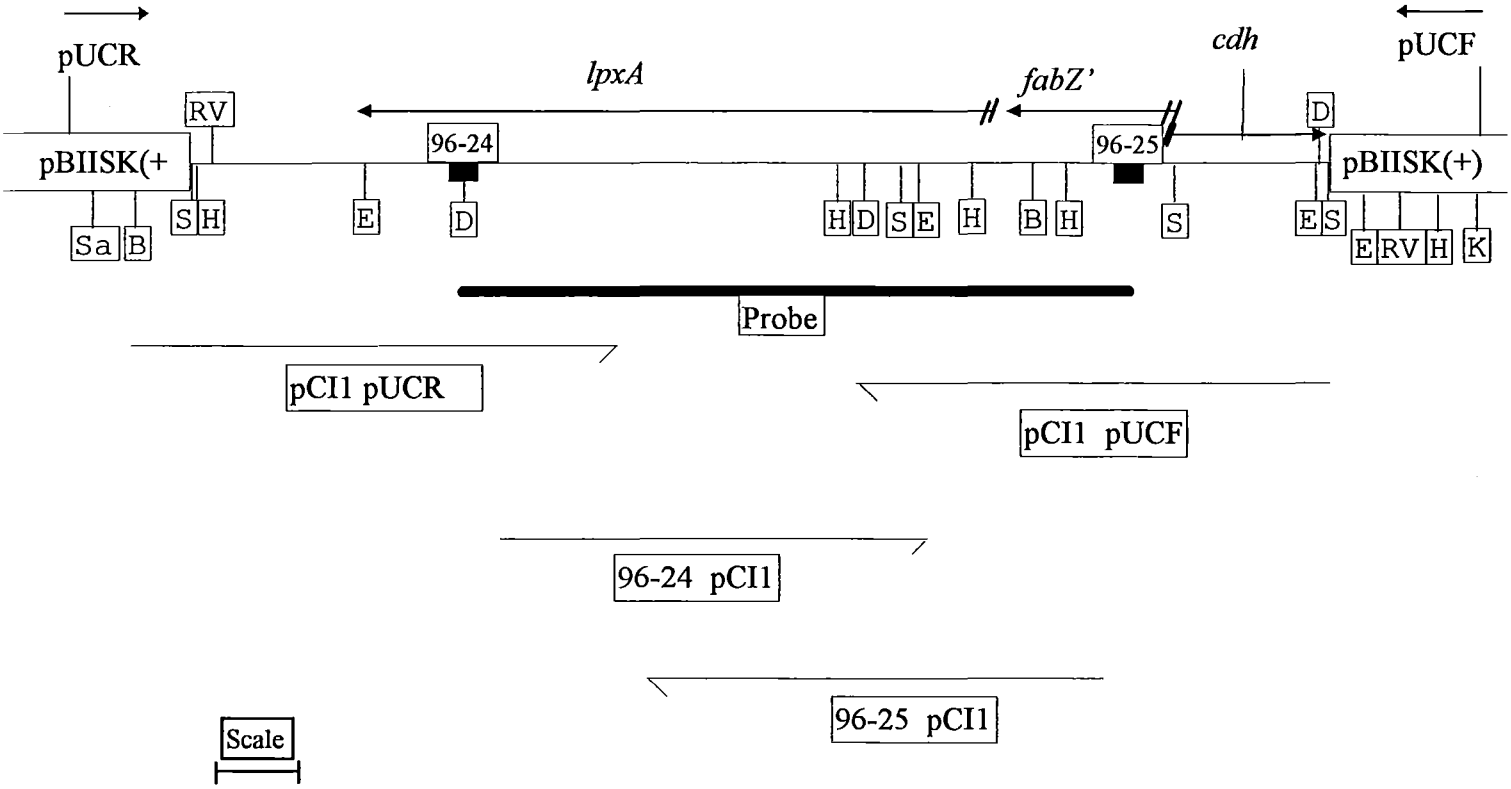



**Figure 10.** Linear map showing *C. jejuni* F38011 DNA insert in pCI1. One complete and two partial ORFs are contained on the fragment; a portion of the citrate synthase gene (cdh); the 3' end of *fabZ*; and the *lpxA* gene. Arrows show direction of transcription. Letters in boxes indicate restriction enzyme sites; H = *Hind*III; E = *Eco*RI; D = *Dde*I; B = *Bst*XI; S = *Sau*3A; RV = *Eco*RV; K = *Kpn*I; Sa = *Sac*I. The arrows beneath restriction enzyme map indicate nucleotide sequence of pCI1 generated from primers pUCF, pUCR, 96-24 and 96-25. The vector insert is cloned into pBIISK(+). Solid line represents size and position of probe obtained by PCR and used in Southern hybridisation. Solid boxes on line indicate hybridisation position of primers 96-24 and 96-25. Scale: 1cm = 100 base pairs.

### 3.2.2 DNA SEQUENCING OF THE *lpxA* REGION OF *C. jejuni.*

To determine whether the gene(s) responsible for restoration of the ability of KLC 4177 to grow at 42°C was in fact *lpxA*, the nucleotide sequence of the *C. jejuni* F38011 insert contained in pCI1 was determined.

DNA sequence from pCI1 was initially produced using universal primers pUC forward and pUC reverse (section 2.8) by Sanger dideoxy sequencing, as the insert was cloned into the pBIISK(+) vector. About 500-600 bp of sequence was obtained from both the forward and the reverse primer, however these nucleotide sequences were non-overlapping at their respective 3' ends. From this *C. jejuni* F38011 DNA sequence, oligonucleotide primers were constructed in order to obtain the DNA sequence from the middle region of the F38011 DNA insert in pCI1 (section 2.8). Lastly, subclones were constructed from pCI1, using *Eco*RI and *Hind*III partial digestions, solely for sequencing purposes, in order to tidy up the middle region of the insert (pCI103 and pCI112).

The nearly complete sequence of the F38011 chromosomal DNA insert from pCI1 is shown in Figure 11. It consists of 792 nucleotides and has a G+C content of about 30%, consistent with that reported for other *C. jejuni* genes.

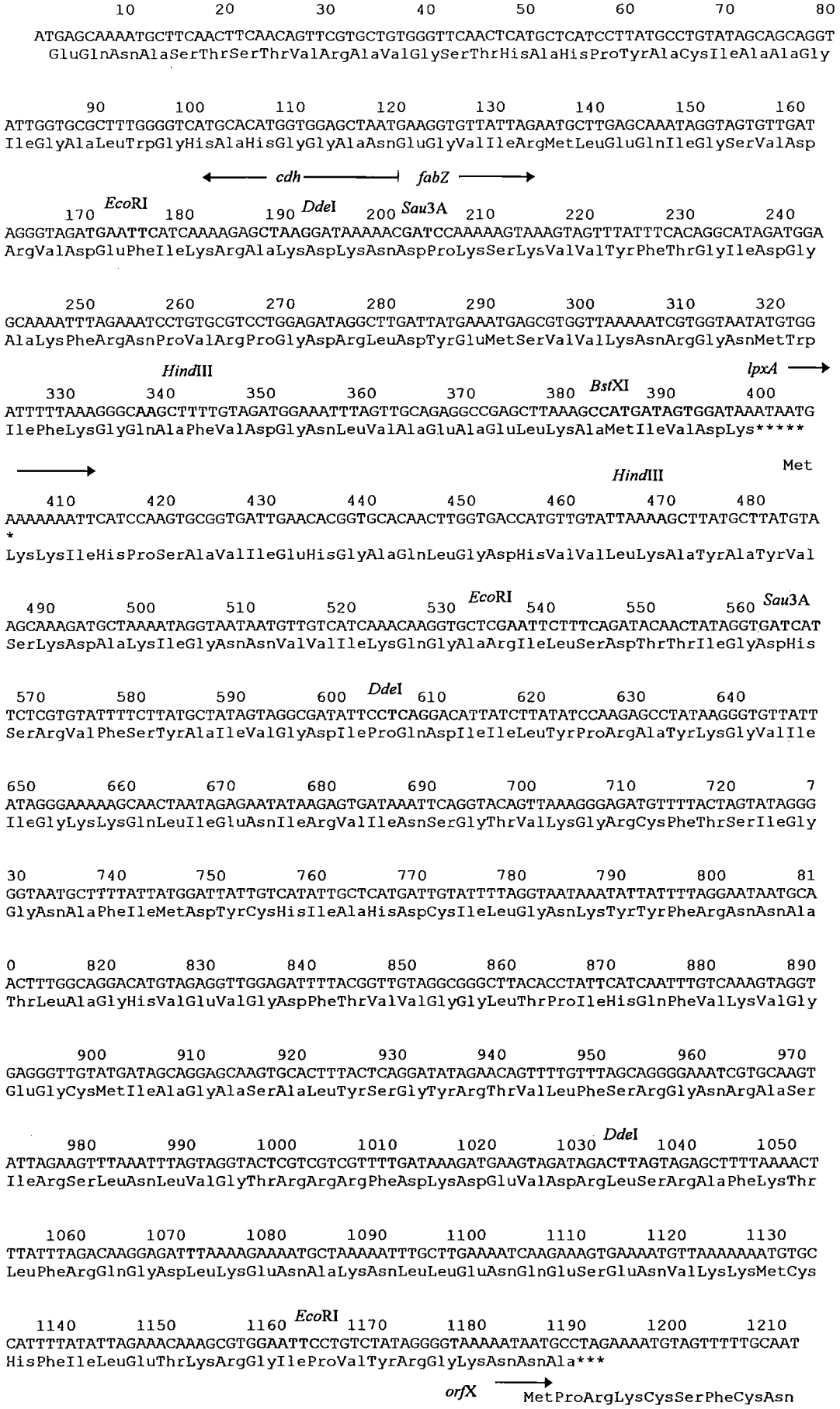
DNAsis analysis of the sequence generated from *C. jejuni* F38011 revealed one complete open reading frame (ORF), and two partial ORFs. The sequence of the *lpxA* gene from *E. coli* K12 and other Gram-negative bacteria has been shown previously to consist of from 786 to 792 bp, encoding a protein ranging from 258 to 264 amino acids. In pCI1, *lpxA* amino acid sequence appears to be transcribed and translated from the pUCF direction.

DNA sequence from pCI1 was sent to the BLAST server to search for possible nucleotide sequence and amino acid similarities with other known DNA and amino acid sequences. Translated amino acid sequence from the pUC forward end of the DNA sequence revealed protein similarity to two genes: citrate synthase (*cdh*); and *fabZ* from *E. coli, R. rickettsii, H. influenzae, Y. enterocolitica* and *S. typhimurium.* Mohan *et al.*(1994) found significant homology between *orf17* (*fabZ*) and *fabA*

(encodes a dehydrase necessary for introduction of *cis* unsaturation into *E. coli* fatty acids) of *E. coli* which suggested that *orf17* also encodes a dehydrase, presumably one involved in saturated fatty acid biosynthesis. *orf17* has therefore been designated *fabZ* in *E. coli*. It is this gene, *fabZ*, that appears to be located directly upstream of *lpxA* in *C. jejuni* F38011. These open reading frames are transcribed in the same direction and this correlates with the order of genes in *E. coli*. In *E. coli*, the termination codon of *fabZ*, UGA overlaps with the start codon GUG of *lpxA* and it is thought that these genes may be translationally coupled (Coleman and Raetz 1988). This situation appears similar in F38011 (figure 11).

BLAST analysis of F38011 DNA sequence revealed another incomplete ORF upstream of the putative *fabZ* sequence. This ORF displayed strong amino acid similarity to the citrate synthase gene from *Bartonella* spp., *Rickettsia* spp., *Streptococcus* spp. and *Rhizobium* spp. This gene appears to be a cloning artifact of the original *C. jejuni* F38011 plasmid expression library construction. This library was originally constructed by *Sau*3A partial digestions ligated into pBIISK(+). The citrate synthase gene in pCI1 is located solely on a 204 bp *Sau*3A/*Sau*3A fragment. There is no evidence for linkage of citrate synthase with *fabZ* or *lpxA* in other organisms. Additionally, the open reading frame of the citrate synthase gene is located within the coding region of *fabZ* and is apparently transcribed in the opposite orientation to *fabZ* and represents a central portion of the Cdh protein.

Translation of nucleotide sequence nearest to the pUC reverse primer revealed no DNA sequence similarity to *lpxA* sequence from any other organism. The closest match was similarity to part of the *Methanococcus jannaschii* genome with a probability match of 0.075 (the closer to 1 the probability is, the more improbable the match is). However predicted amino acid sequence from the complete insert sequence displayed strong amino acid identity (beginning about 400 nucleotides from pUCF and ending about 220 nucleotides in from the pUCR primer) to the UDP-N-acetylglucosamine acyltransferase protein (LpxA) of *E. coli, S. typhimurium, R. rickettsii, Y. enterocolitica* and *Haemophilus influenzae,* revealing the characteristic hexapeptide repeat motif observed in all other *lpxA* protein sequences to date (figure 12).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90 100 110 120 130 140 150 160
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IleGlyAlaLeuTrpGlyHisAlaHisGlyGlyAlaAsnGluGlyValIleArgMetLeuGluGlnIleGlySerValAsp
cdh
fabZ
EcoRI
170 180 190 DdeI 200 Sau3A 210 220 230 240
AGGGTAGATGAATTCATCAAAAGAGCTAAGGATAAAAACGATCCAAAAAGTAAAGTAGTTTATTTCACAGGCATAGATGGA
ArgValAspGluPheIleLysArgAlaLysAspLysAsnAspProLysSerLysValValTyrPheThrGlyIleAspGly
250 260 270 280 290 300 310 320
GCAAAATTTAGAAATCCTGTGCGTCCTGGAGATAGGCTTGATTATGAAATGAGCGTGGTTAAAAATCGTGGTAATATGTGG
AlaLysPheArgAsnProValArgProGlyAspArgLeuAspTyrGluMetSerValValLysAsnArgGlyAsnMetTrp
HindIII
lpxA
330 340 350 360 370 380 BstXI 390 400
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IlePheLysGlyGlnAlaPheValAspGlyAsnLeuValAlaGluAlaGluLeuLysAlaMetIleValAspLys*****
Met
HindIII
410 420 430 440 450 460 470 480
AAAAAAATTCATCCAAGTGCGGTGATTGAACACGGTGCACAACTTGGTGACCATGTTGTATTAAAAGCTTATGCTTATGTA
*
LysLysIleHisProSerAlaValIleGluHisGlyAlaGlnLeuGlyAspHisValValLeuLysAlaTyrAlaTyrVal
490 500 510 520 530 EcoRI 540 550 560 Sau3A
AGCAAAGATGCTAAAATAGGTAATAATGTTGTCATCAAACAAGGTGCTCGAATTCTTTCAGATACAACTATAGGTGATCAT
SerLysAspAlaLysIleGlyAsnAsnValValIleLysGlnGlyAlaArgIleLeuSerAspThrThrIleGlyAspHis
570 580 590 600 DdeI 610 620 630 640
TCTCGTGTATTTTCTTATGCTATAGTAGGCGATATTCCTCAGGACATTATCTTATATCCAAGAGCCTATAAGGGTGTTATT
SerArgValPheSerTyrAlaIleValGlyAspIleProGlnAspIleIleLeuTyrProArgAlaTyrLysGlyValIle
650 660 670 680 690 700 710 720 7
ATAGGGAAAAAGCAACTAATAGAGAATATAAGAGTGATAAATTCAGGTACAGTTAAAGGGAGATGTTTTACTAGTATAGGG
IleGlyLysLysGlnLeuIleGluAsnIleArgValIleAsnSerGlyThrValLysGlyArgCysPheThrSerIleGly
30 740 750 760 770 780 790 800 81
GGTAATGCTTTTATTATGGATTATTGTCATATTGCTCATGATTGTATTTTAGGTAATAAATATTATTTTAGGAATAATGCA
GlyAsnAlaPheIleMetAspTyrCysHisIleAlaHisAspCysIleLeuGlyAsnLysTyrTyrPheArgAsnAsnAla
0 820 830 840 850 860 870 880 890
ACTTTGGCAGGACATGTAGAGGTTGGAGATTTTACGGTTGTAGGCGGGCTTACACCTATTCATCAATTTGTCAAAGTAGGT
ThrLeuAlaGlyHisValGluValGlyAspPheThrValValGlyGlyLeuThrProIleHisGlnPheValLysValGly
900 910 920 930 940 950 960 970
GAGGGTTGTATGATAGCAGGAGCAAGTGCACTTTACTCAGGATATAGAACAGTTTTGTTTAGCAGGGGAAATCGTGCAAGT
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980 990 1000 1010 1020 1030 DdeI 1040 1050
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1140 1150 1160 EcoRI 1170 1180 1190 1200 1210
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HisPheIleLeuGluThrLysArgGlyIleProValTyrArgGlyLysAsnAsnAla***
orfX
MetProArgLysCysSerPheCysAsn

```
     1220       1230       1240       1250       1260       1270       1280       1290
GAAGTGGAGAATCCACAAAGAAGAATTTTAGCAAATGAAAATGATGATGCTTTTATTTGTGAATATTGCGTTGAAGGT
GluValGluAsnProGlnArgArgIleLeuAlaAsnGluAsnAspAspAlaPheIleCysGluTyrCysValGluGly
                                                                        EcoRV
       1300       1310       1320       1330       1340       1350       1360       1370
GCTTATAGTATTATTTATGGAGAAGAAAAAGAATTTAAAGAACCTAAACAAAGTTATAATACAGAATTTAAAGATATC
AlaTyrSerIleIleTyrGlyGluGluLysGluPheLysGluProLysGlnSerTyrAsnThrGluPheLysAspIle

            HindIII        Sau3A
          1380       1390     1398
ACTCCAAAAGAATTAAAAGCTTATTTAGATC 3'
ThrProLysGluLeuLysAlaTyrLeuAsp
```

**Figure 11.** *C. jejuni* F38011 DNA sequence of pCI1. The *C. jejuni* insert includes one complete open reading frame (ORF) and two partial ORFs with the possibility of a fourth putative ORF (ORFX). Restriction endonuclease sites within the insert are shown in bold and labelled accordingly. Arrows show the direction of transcription of the three ORFs; citrate synthase is transcribed in the opposite direction to *fabZ* and *lpxA*. A fourth ORF is thought to be present at the termination of the *lpxA* gene. There is speculation about whether ORFX is the *lpxB* gene and whether *lpxB* is coupled with the *lpxA* gene in *C. jejuni*, after the observation of this order of genes in *E. coli*.

The mol % GC content of the *lpxA* gene of *E. coli* is around 50%, while the mol % GC content of the *C. jejuni lpxA* gene is approximately 30%.

Sequence analysis did not reveal a putative promoter for *lpxA*; this may be because *lpxA* is transcribed together with *fabZ*, as suggested by the enteric bacteria. The initiation codon for the *C. jejuni* F38011 *lpxA* gene appears to be the standard AUG, and overlaps with the double termination codon of *fabZ*. In other organisms, the initiation codon of *lpxA* is not AUG but GUG (this would normally be the amino acid valine). The order of the genes *fabZ* and *lpxA* in *C. jejuni* F38011 appears to be the same as that published for *E. coli*. No *Sau*3A restriction site occurs between the 3' end of *fabZ* and the 5' end of *lpxA* in F38011, according to the sequence, indicating no cloning anomaly present.

```
BablpxA         MSKSMKETFiHPTALvEPGVElGQGVSvGPFCHvQSGAIiGNDCElMSHV
RrilpxA         MSN--SN--iHTTAViAEGAKlGKNVKiGPYCIiGPEVVlNDNVElKSHV
EcolpxA         MID--KSAFiHPTAIvEEGASiGANAHiGPFCIvGPHVEiGEGTVlKSHV
YenlpxA         MID--KTAViHPSSIvEEGAViGAGVHiGPFCFvGSQVEiGAGTElKSHV
StylpxA         MID--KSAFiHPTAIvEDGAViGANAHiGPFCIvGPQVEiGEGTVlKSHV
CjejlpxA        MKK------iHPSAViEHGAQlGDHVVlKAYAYvSKDAKiGNNVViKQGA
HinflpxA        MIH--PSAKiHPTALiEEGAViGEDVFiGPFCIiEGTVEiKARTVlKSHV
                *       .* .... *  .*    . ..  .     .     .

BablpxA         ViTGATTlGAGTKvYPHAIlGCDPQNNKHKGGPTRLNvGVNCIiREGVTm
RrilpxA         ViEGITEiGENTViYPGASiGQPPQILKYANERSSTIiGSNNTiREYVTv
EcolpxA         VvNGHTKiGRDNEiYQFASiGEVNQDLKYAGEPTRVEiGDRNRiRESVTi
YenlpxA         VvNGITKiGCDNQiYQFASiGEANQDLKYAGEPTRVEiGDRNRiRESVSi
StylpxA         VvNGQTKiGRDNEiYQFASiGEVNQDLKYAGEPTRVEiGDRNRiRESVTi
CjejlpxA        RiLSDTTiGDHSRvFSYAIvGDIPQDIILPRAYKGVIiGKKQLiENIRVi
HinflpxA        VvRGDTViGEDNEiYQFTSiGEVNQDLKYKGEATKTIiGNSNKiREHVTi
                 . . * .*    ..  . .*   *           .*    * .   .

BablpxA         HKGSDNARGYTSiGDNCSFLAYAHvAHDCDiGGHYVTFSNNVMiGGHTSi
RrilpxA         QAGSQGGGMMTRvGNNNLFMVGVHiGHDCKiGNNVV-FANYVSlAGHIGv
EcolpxA         HRGTVQGGGLTKvGSDNLlMINAHiAHDCTvGNRCI-lANNATlAGHVSv
YenlpxA         HRGTVQGGGLSKvGSDNLiMINAHiAHDCIiGDRCI-iANNATlGGHVEi
StylpxA         HRGTVQGGGLTKvGSDNLlMINAHvAHDCTvGNRCI-lANNATlAGHVSv
CjejlpxA        NSGTVKGRCFTSiGGNAFiMDYCHiAHDCIlGNKYY-FRNNATlAGHVEv
HinflpxA        HRGTIQGCGITAiGNNNLlMINVHvAHDCQiKNNCI-lANNATlAGHVEl
                . *.  .   . .* .  ..   *..*** .      . *   ..**  .

BablpxA         GHHAIlGGGAAvHQFVRvGHHAFiGGLAAvVSDLIPYGMAIGVHAHLGGL
RrilpxA         GDYAIiGGLSAvHQYARiGEYSMiGGLSPvGADVIPFGLVSSKRAVLEGL
EcolpxA         DDFAIiGGMTAvHQFCIiGAHVMvGGCSGvAQDVPPYVIAQGNHATPFGV
YenlpxA         DDFAIiGGMTAiHQFCViGAHVMvGGCSGvAQDVPPFVIAQGNHATPFGI
StylpxA         DDFAIiGGMTAvHQFCIiGAHVMvGGCSGvAQDVPPYVIAQGNHATPFGV
CjejlpxA        GDFTVvGGLTPiHQFVKvGEGCMiAGASAlYSGYRTVLFSRGNRASIRSL
HinflpxA        DDFVIvGGMSAiHQFVIvGAHVMlGGGSMvSQDVPPYVMAQGNHARPFGV
                ..  ..** ...**.  .*    ..* . .  .        . .*   ..

BablpxA         NIIGMKRSGMERKEIHNLRHAVRMLFDRTKPIRQRAQDVLAAIPDSPTVS
RrilpxA         NLIGMNRKGFDKVKSLSALKALEEIFSGEGNFAERIKQVAEKYNNNSIVI
EcolpxA         NIEGLKRRGFSREAITAIRNAYKLIYRSGKTLDEVKPEIAELAETYPEVK
YenlpxA         NIEGLKRRGFDKESLHAIRNAYKLLYRSGRTLDEVKPEIAELADQHPAVQ
StylpxA         NIEGLKRRGFSREGLVAIRNAYKLLYRSGKTLDEAKLEIAELAEKHPEVK
CjejlpxA        NLVGTRRR-FDKDEVDRLSRAFKTLFRQG-DLKENAKNLLENQESEN-VK
HinflpxA        NLEGLKRRGFDKPTMHVIRNIYKMLYRSGKTLEEVLPEIEQIAETDSAIS
                *. *  *    .           ..    . .  ..         .
```

```
BablpxA        DMISFINVDTKRAYCTPPLDAAHGGAGHDSDED
RrilpxA        QIIDFLNQDSSRAFCRF--------------EK
EcolpxA        AFTDFFAR-STRGLIR-----------------
YenlpxA        AFIDFFAR-STRGIIR-----------------
StylpxA        AFTEFFER-STRGPIR-----------------
CjejlpxA       KMCHFIL-ETKRGIPVY----------RGKNNA
HinflpxA       FFVEFFKR-STRGIIR-----------------
                   *.    . *.
```

**Figure 12.** Multiple amino acid sequence alignment of the UDP-*N*-acetyltransferases (*lpxA*) of *Brucella abortus* (Bab), *Rickettsia rickettsii* (Rri), *Escherichia coli* (Eco), *Yersinia enterocolitica* (Yen), *Salmonella typhimurium* (Sty), *Haemophilus influenzae* (Hinf), with the putative *Campylobacter jejuni* (Cjej) *lpxA* gene. Identical residues are indicated by an *, conserved residues by a ., and dashes indicate regions of unobtained amino acid sequence (or gaps). Lower case letters show the conserved hexapeptide repeat observed in enteric organisms for *lpxA* (and seen in other genes (section 1.8.2.3)).

## 3.3. PCR ANALYSIS.

Due to problems encountered with importing the *C. jejuni* F38011 strain from the US, work shifted to a well-characterised *C. jejuni* clinical strain from the NZ collection, strain NZRM 1958.

A PCR reaction was performed using the *fabZ-lpxA* primers 96-24 and 96-25, previously used for DNA sequencing of *C. jejuni* F38011 DNA (section 3.2.2). This experiment was designed to show that *lpxA* was found on the genome of *C. jejuni* NZRM 1958. For pCI1 and pCI4 plasmid DNA, and genomic DNA from lysed colonies of *C. jejuni* NZRM 1958, an approximately 800 bp PCR amplicon was observed using these two primers (Figure 13a). PCR amplicons generated from pCI1, pCI4 and *C. jejuni* NZRM 1958 were restriction endonuclease digested with *Eco*RI and *Hind*III in separate reactions. An identical restriction digestion pattern was observed for both strains of *C. jejuni* with either *Eco*RI and *Hind*III (Figures 13b and 13c). To rule out amplification of the *E. coli lpxA* gene by the primers 96-24/96-25 in the plasmid PCR samples, an *E. coli* control was performed. No PCR amplicon was generated in this experiment (figure 13a).

The 800 bp amplicon from *C. jejuni* NZRM 1958 was cloned into the PCR vector pGEM-T (pCI6) and the nucleotide sequence determined by Sanger dideoxy

sequencing using the pUC forward and reverse primers. Approximately 600 bp of sequence out of a possible 800 nucleotides was obtained. Nucleotide sequence was sent to the BLAST server, revealing amino acid similarity to the *lpxA* genes of *H. influenzae, R. rickettsii, S. typhimurium, Y. enterocolitica, E. coli* and *Brucella abortus.*

The putative amino acid sequence translated from *C. jejuni* 1958 DNA sequence revealed the characteristic hexapeptide motif observed in the other LpxA proteins (Figure 14). This region shows clear amino acid sequence identity to the same region of the LpxA protein of strain F38011, although noticeable differences are apparent as well. It should be noted that some of the NZRM 1958 sequence was most likely erroneous (note large gaps in Figure 14, part A). However, despite these gaps, sequence differences were still observed.

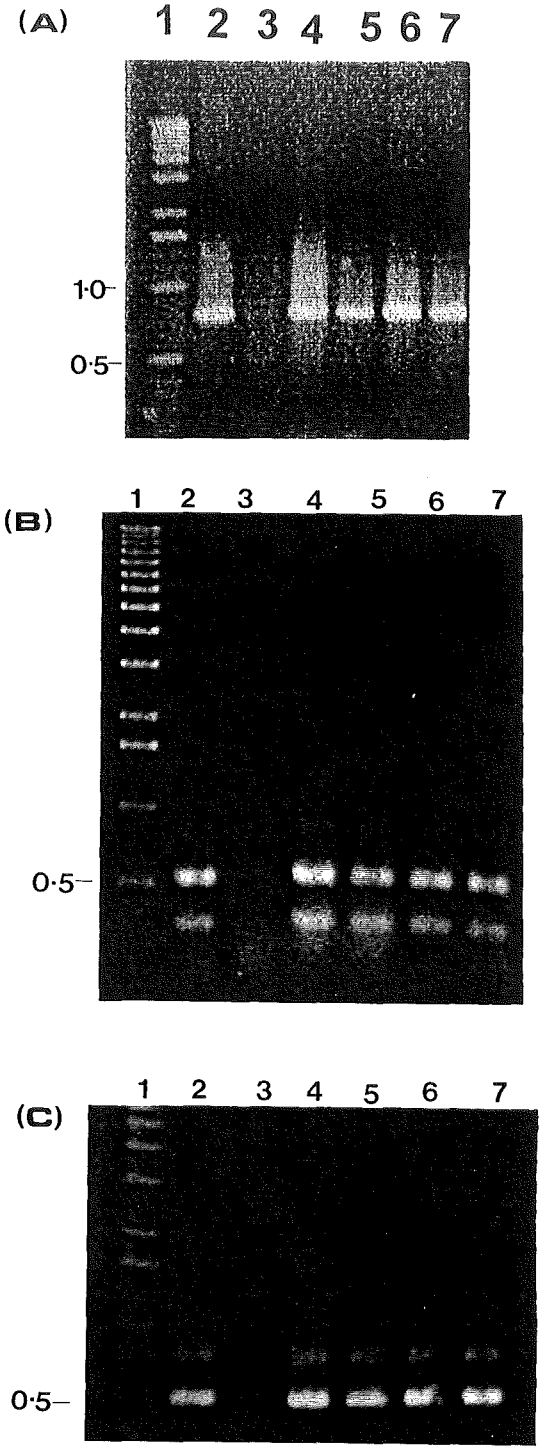



**Figure 13.** (A) Lane 1, 1 kb marker; Lanes 2-7 - PCR products resulting from primers 96-24 and 96-25. In lane 2, the DNA template was a lysed colony of *C. jejuni* NZRM 1958; Lane 3, DNA template was a KLC 4177 lysed colony; Lane 4, DNA template was a lysed colony of KLC 4178; Lane 5, DNA template was a lysed colony of KLC 4181; Lane 6, DNA template was pCI1 plasmid DNA; Lane 7, DNA template was pCI4 plasmid DNA. For PCR conditions see Materials and Methods. (B) PCR products from (A) digested with *Eco*RI. Lane 1, 1 kb standard marker; Lanes 2-7 are the same as in (A). (C) PCR products from (A) digested with *Hind*III. Lane 1, 1 kb standard marker; Lanes 2-7 are the same as in (A).

(A)

```
1958            GATCATTCTCCTCTATTTTCTTATTCTATACTACGTGATTTTCCTCAGGA
F38011          GATCATTCTCGTGTATTTTCTTATGCTATAGTAGGCGATATTCCTCAGGA
                ********** * *********** ***** ** * *** ******* **

1958            CATT-TCTTATACACAATATCAAATTAGCGGTGTTGTTATTTGGAAAAAT
F38011          CATTATCTTATAACCAAGAGCCTATAAGCGGTGTTATTATAGGGAAAAA-
                **** *******  *** * *  ** ********* ****  *******

1958            GCAACTTTTAAAAAATTTGC-ATCCATAAATTCAGGTTCACCTAAAGGAT
F38011          GCAACTAATAGAGAATATAAGAGTGATAAATTCAGGTACAGTTAAAGGGA
                ******  ** * *** *   *   ************ **  ******

1958            ATGGTTTTACTCCTATATGCCATAATGCCTTTATTATCTCTTAATGTCAT
F38011          GATGTTTTACTAGTATAGGGGGTAATGCTTTTATTATGGATTATTGTCAT
                   ********  **** *   ****** ********   *** ******

1958            ATTGCTCATGATTGTCTTTTATGTTATTA-TATTACTTTATCTCATAATG
F38011          ATTGCTCATGATTGTATTTTAGGTAATAAATATTATTTTAGGA-ATAATG
                *************** ***** ** ** * ***** ****    ******

1958            CTACTTTGGCATGAC-TCTTCACCTTTCATATTTTACGGTTCTATGCTGC
F38011          CAACTTTGGCAGGACATGTAGAGGTTGGAGATTTTACGGTTGTAGGCGGG
                * ********* *** * *  *  **  * ********** ** ** *

1958            CTCTCCCCTACTCATCAATTTGTCAAACTTTGTGATGGTTGTATCATACC
F38011          CTTACACCTATTCATCAATTTGTCAAAGTAGGTGAGGGTTGTATGATAGC
                **  * **** ***************** *  **** ******** *** *

1958            ACGAACAACTCCTCTTTCTCC------TCGGATAATCTTCCCTCTCTTTT
F38011          AGGAGCAAGTGCACTTTACTCAGGATATAGAACAGTTTTGTTTAGCAGGG
                * ** *** * * ****   *      * * * * * **   *  *

1958            T---TTCCCCAAACGAAAATCCCCCCACCTTTTTCTGAAAATTTCAATAT
F38011          GAAATCGTGCAAGTATTAGAAGTTTAAATTTAGTAGGTACTCGTCGTCGT
                    *    ***     *       *  **  *  * *    **    *

1958            TCCTAT-----TCTCGTTATTCTCCTTAGTCTTTTTTTTTCAATCTTCTT
F38011          TTTGATAAAGATGAAGTAGATAGACTTAGTAGAGCTTTTAAAACTTTATT
                *   **     *   **   *   **   *     ****  **  ** **

1958            C---CCATTCTATCTTTCT
F38011          TAGACAAGGAGATTTAAAA
                    * *    ** *
```

(B)

```
1958            DHSPLFSYSILRDFPHDISYTQYQISGVVIWKNATFKKFASINSGSPKGY
F38011          DHSRVFSYAIVGDIPQDIILPR-AYKGVIIGKKQLIENIRVINSGTVKGR
                *** .***.*. *.*.**   .    **.* *.  . ..  ****. **

1958            GFTPICHNAFIIS-CHIAHDCLLC--YYITLSHNATLA----LFTFHILR
F38011          CFTSIGGNAFIMDYCHIAHDCILGNKYY--FRNNATLAGHVEVGDFTVVG
                 **.*  ****.  *******.*   **  . .*****    .  * ..

1958            FYAASPLLINLSNFVMVVS-------YHEQLLFLLGSSLSFFPQTKIPPP
F38011          GLTPIHQFVKVGEGCMIAGASALYSGYRT-VLFSRGNRASIRSLNLVGT-
                  ..   ......  *. .       *.  .**  *.  *. .   .

1958            FSENFNIPILVILLPLFFSIFFPFYLS
F38011          -RRRFDKDEVDRLSRAFKTLFRQGDLK
                    *.   .  *   * ..*    *
```

**Figure 14.** Comparison of *C. jejuni* 1958 and *C. jejuni* F38011 nucleotide (A), and amino acid (B) sequence from nucleotide 561 (amino acid 187) to nucleotide 1077 (aa 359) of *C. jejuni* F38011. * indicates identical amino acids or nucleotides; a full stop indicates conserved identities; and dashes indicate regions of unobtained amino acids or nucleotides (or gaps). Bold letters indicate conserved hexapeptide repeat observed in LpxA in other enteric organisms.

## 3.4 Southern Hybridisation Analysis of Two *C. jejuni* strains.

Southern hybridisation analyses of plasmid DNA from *C. jejuni* strain F38011 and from genomic DNA obtained from *C. jejuni* strain NZRM 1958 were performed to confirm that the order of genes on the *C. jejuni* chromosome was *fabZ-lpxA*, and that the genes were contained as a single copy on the *C. jejuni* genome. A probe was constructed from the PCR amplicon generated from genomic *C. jejuni* NZRM 1958 DNA using the primers 96-24 and 96-25 (see section 3.3, figure 10). The probe was used to hybridise to *C. jejuni* NZRM 1958 genomic DNA that had been restriction endonuclease digested in separate reactions with *Eco*RI, *Hind*III and *Pst*I (data not shown).

The probe was found to hybridise to two fragments in pCI1 digested with *Eco*RI (figure 15). These two *Eco*RI fragments, 700 bp and 500 bp were predicted based on

the deduced nucleotide sequence from *lpxA* of F38011. The probe hybridised to three fragments in pCI1 digested with *Hind*III, a 900 bp fragment, a 400 bp fragment and a 200 bp fragment. These three DNA fragments were also anticipated to be detected as predicted from the nucleotide sequence. A fourth *Hind*III fragment, of 150 bp, was anticipated but during DNA transfer it was apparently lost from the end of the blot and not therefore detected.

Differences were observed in the Southern blot profile of pCI4 digested with *Eco*RI or *Hind*III when compared to pCI1; in pCI4 the probe hybridised to two *Eco*RI fragments of 700 bp and 3 kb, and three *Hind*III fragments of 200 bp, 900 bp and 3 kb. As in pCI1 a fourth *Hind*III fragment, of 150 bp, was anticipated for pCI4 but during DNA transfer it was apparently lost off the end of the blot and not detected. The differences observed between the number of fragments produced for each plasmid from digestions, and hybridised to in pCI1 and pCI4 suggest that the portion of the citrate synthase gene sequenced from pCI1 is not present in pCI4 and the orientation of the DNA insert in pCI4 is in the opposite direction to that of pCI1. This is deduced from the *Eco*RI and *Hind*III digests of pCI4 compared to pCI1. The only difference between pCI4 and pCI1 probe hybridisation from the *Hind*III digestions was a 450 bp fragment observed for pCI1. This would be observed for pCI4 if the insert in pCI4 was orientated in the same direction as for pCI1. If the insert in pCI4 was orientated in the opposite direction to pCI1 no 450 bp fragment would be observed and a slightly larger vector band would be observed and hybridised to by the 96-24/96-25 probe. This is what was actually observed for pCI4. In the *Eco*RI digestions a common fragment was observed for both pCI1 and pCI4 at 700 bp. This fragment was hybridised to by the probe for both plasmids. This was expected from restriction mapping data. If the orientation of the F38011 insert in pCI4 is indeed in the opposite direction a fragment would be expected to be observed at approximately 3.2 kb. This is what was observed for pCI4. A fragment of a slightly smaller size (3 kb) was hybridised to but not strongly in pCI1, indicating partially digested DNA. However for pCI4 a strong band is seen, suggesting more than just partially digested DNA. This also suggests that pCI4 contains more *C. jejuni* F38011 DNA downstream of *lpxA* than is apparent in pCI1. Due to the number of restriction fragments originally seen for pCI4 from digestions with *Eco*RI and the observation that only two of these

fragments were hybridised to, a suggestion is made that there are two additional *Eco*RI restriction enzyme sites present downstream of *lpxA* in *C. jejuni* F38011. This downstream region may include the start or contain the whole *lpxB* gene of *C. jejuni* if the order of genes continues as in *E. coli*. One *Eco*RI site observed in restriction endonuclease digestions in pCI1 is non-existent in pCI4. This would be expected if the citrate synthase gene was absent upstream of *fabZ*, as the *Eco*RI site is in *cdh*. Faint hybridisation to fragments from pCI1 of approximately 3 kbp in size is an indication of partially digested plasmid DNA. Ambiguities are found in terms of insert size when comparing restriction mapping data and Southern blot material for pCI4.

The hybridisation of the probe to a single *Pst*I restriction fragment (approx. 10 kb) from *C. jejuni* NZRM 1958 chromosomal DNA suggests that the *lpxA* gene is located on the genome of this strain as a single copy. *lpxA* was predicted to lie on a single *Pst*I fragment only as no *Pst*I fragment was present within the DNA sequence. A single large band was seen for an *Eco*RI fragment of digested chromosomal DNA (>14 kb). This was partially unexpected (expected two fragments - a 700 bp fragment and another fragment as probe overlaps 700 bp on one side) as *Eco*RI digestion was expected to generate a fragment of 700 bp. The *Eco*RI restriction site (GAATTC) is not a frequent restriction site in *C. jejuni*, and though digestions were incubated at 37°C for 5-7 hours, only a large *Eco*RI fragment was detected. This result was repeated two separate times. More conclusive evidence of the genomic DNA containing a single copy of the *lpxA* gene would be shown by a *Hind*III digest of *C. jejuni* NZRM 1958 genomic DNA. However difficulties were encountered in obtaining genomic DNA digested with *Hind*III (data not shown). A second Southern hybridisation was attempted with *Hind*III-digested genomic NZRM 1958 DNA and a band was observed at approximately 1.0 kb. It was expected that more fragments would have been observed ranging in size from 150 bp upwards. More *Hind*III-digested genomic DNA needs to be obtained and worked with. From this data the *Pst*I digest suggests one copy of the *lpxA* gene, but *Eco*RI/*Hind*III digests did not reveal additional information.

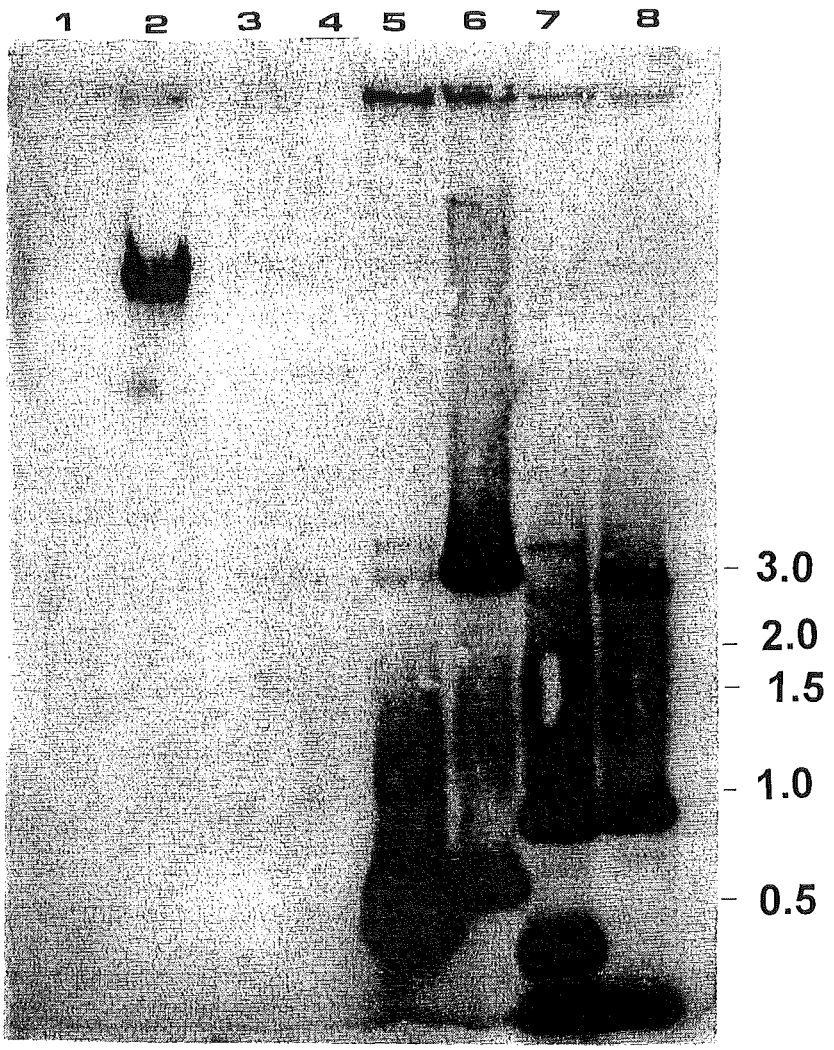



**Figure 15.** Southern hybridisation. Plasmid DNA from pCI1 and pCI4 was digested with *Eco*RI and *Hind*III in separate digests and probed with an 800 base pair PCR amplicon generated using the F38011-derived primers 96-24 and 96-25. Lane 1, 1 kb marker; lane 2, *C. jejuni* NZRM 1958 chromosomal DNA digested with *Eco*RI; lane 3, *C. jejuni* NZRM 1958 chromosomal DNA digested with *Hind*III; lane 4, , *C. jejuni* NZRM 1958 chromosomal DNA digested with *Pst*I; lane 5, pCI1 digested with *Eco*RI; lane 6, pCI4 digested with *Eco*RI; lane 7, pCI1 digested with *Hind*III; lane 8, pCI4 digested with *Hind*III. Sizes (kb) of the bands shown in lanes 1-4 were estimated by comparing migration of plasmid DNA to that of the known 1 kb standard migration.

## 3.5 Bacterial Growth Curve Data.

Measurements of bacterial growth ($OD_{600}$ absorbance changes with respect to time) were performed over a ten hour period to determine whether the plasmid pCI1 was capable of complete restoration of the growth defect in KLC 4177. It was hypothesised that if complete restoration of the temperature sensitive defect of KLC 4177 occurred, the replication of KLC 4178 would be restored to the level of KLC 4176 (wild-type) as reported by Galloway and Raetz (1990). Intermediate levels would suggest incomplete restoration of *E. coli* LPS.

Aliquots of KLC 4176, KLC 4177 and KLC 4178 replicating exponentially at 30°C in LB broth were shifted to 42°C as per Galloway and Raetz (1990). Absorbance at 600 nm was measured hourly. The KLC 4176 parent replicates to high cfus at both permissive and non-permissive temperatures (figure 16a). At 30°C both KLC 4177 and KLC 4178 appeared to replicate on a par with KLC 4176 and therefore had optical density measurements comparable to that of the wild-type KLC 4176 (see figure 16a). Strain KLC 4177 failed to replicate at the non-permissive temperature of 42°C as predicted. Analyses (at a confidence interval of 99% using Student's T-test) showed a significant difference in cell viability and optical density between KLC 4177 and the wild-type *E. coli* K12 strain KLC 4176 at 42°C. The presence of a plasmid encoding the *C. jejuni lpxA* gene (pCI1) restored high temperature growth and also presumably UDP-*N*-acetylglucosamine acyltransferase activity in KLC 4178 as seen by replication of KLC 4178 at 42°C. KLC 4178, containing F38011 *lpxA* did not replicate as well as KLC 4176 at 42°C (figure 16b), but it did replicate significantly better than KLC 4177 (confidence level of 99%), thus indicating that the *C. jejuni lpxA* is capable of restoring replication in the temperature-sensitive mutant. Cell viability of KLC 4178 at 42°C was also significantly better than KLC 4177 at 42°C. This was judged by plating efficiency (see section 2.3.1).

**Figure 16a.** Changes in optical density of *E. coli* K12 strains KLC 4176, KLC 4177 and KLC 4178 at 30°C and 42°C with respect to time. Results are an average of triplicate measurements. Arrow indicates time of temperature shift. Solid symbols indicate strains at 30°C; hollow symbols indicate strains at 42°C.

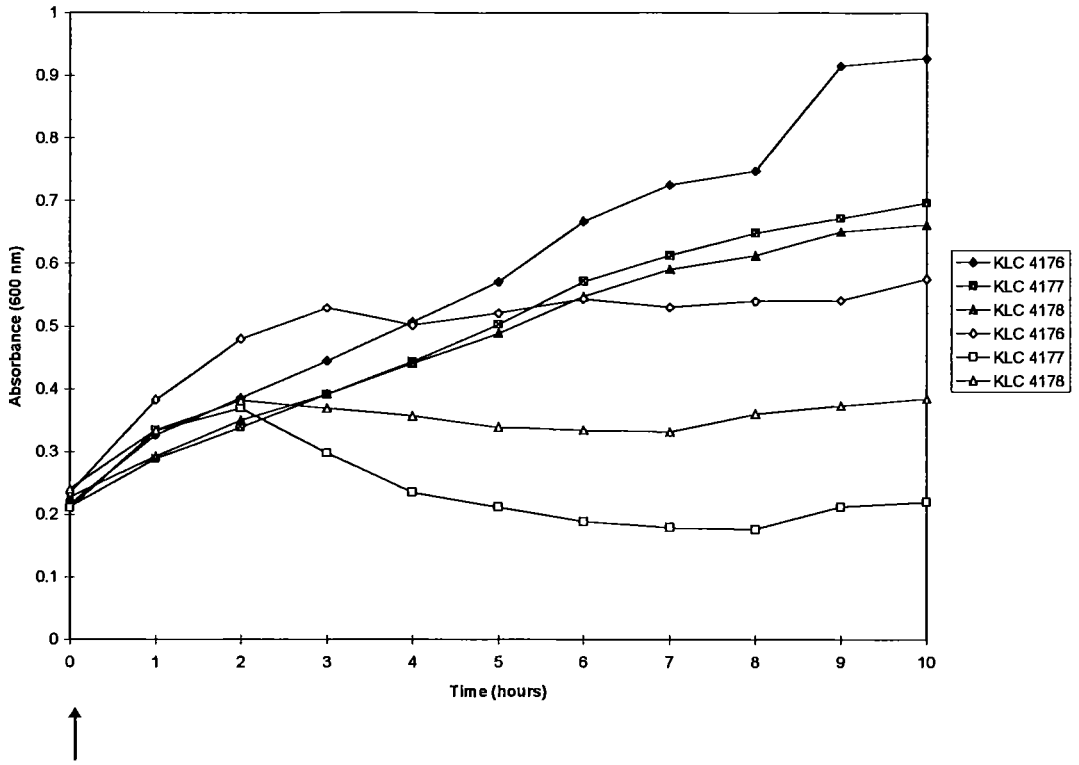
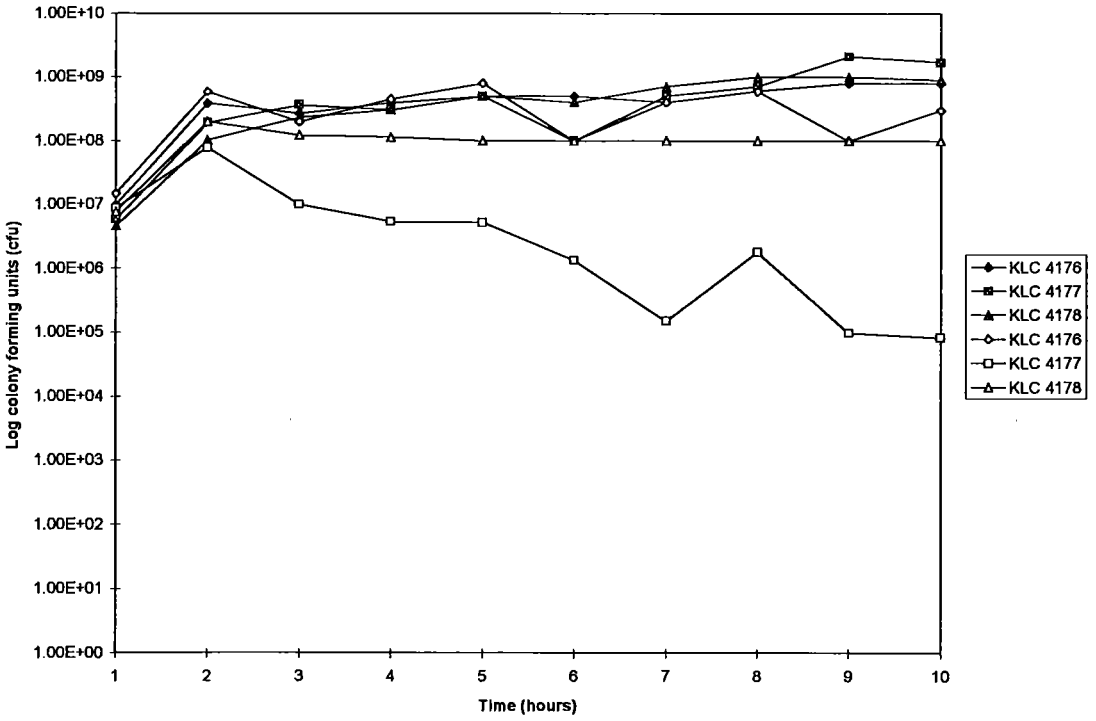



**Figure 16b.** Colony forming units of KLC 4176, KLC 4177 and KLC 4178 at 30°C and 42°C over a ten hour sampling period. Results are an average of triplicate samples. Solid symbols indicate strains at 30°C; hollow symbols indicate strains at 42°C.



## 3.6 SDS-PAGE Analysis of Proteins.

SDS-PAGE analysis was performed to investigate proteins expressed from the plasmid pCI1. Maxicell analysis (see materials and methods) using strains KLC 4026, KLC 4027 and KLC 4178, was performed in an attempt to observe the presence of unique polypeptide products produced from KLC 4178, a strain containing pCI1.

Analysis of KLC 4178 containing the plasmid construct pCI1, displayed a protein profile no different to those of KLC 4026 and KLC 4027 (controls) (see figure 17). Protein expression was inconclusive and time limits prevented further analysis of the protein product.

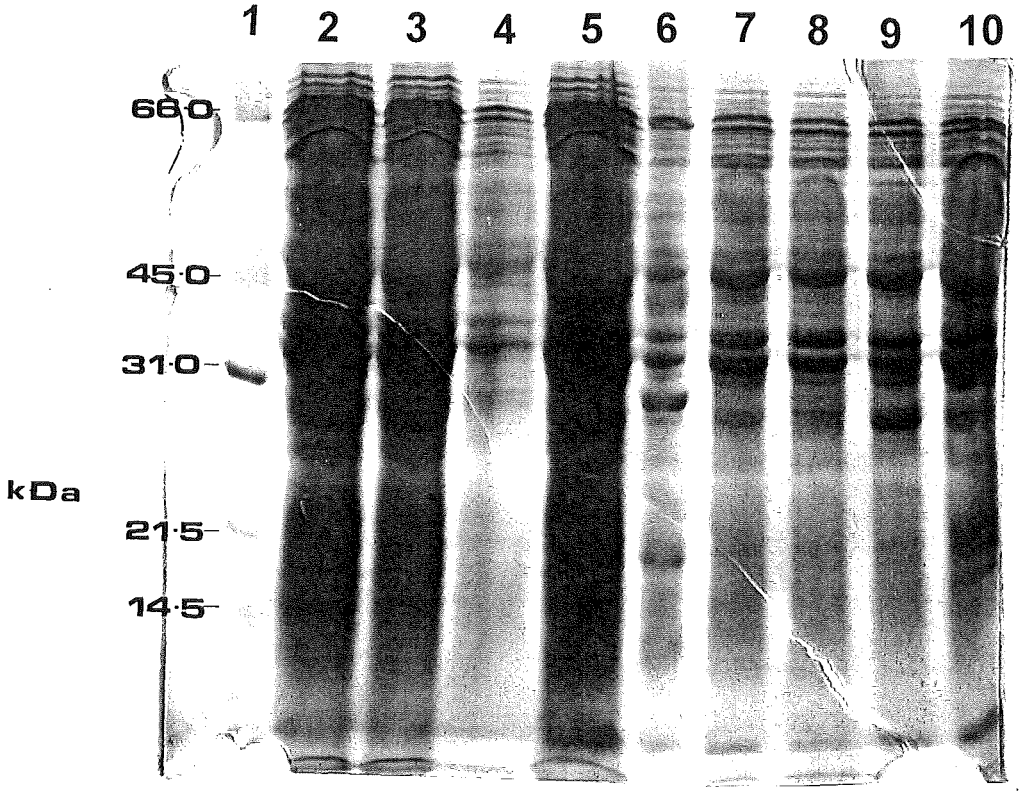



**Figure 17**. Coomassie-blue stained 12% SDS-PAGE gel showing proteins isolated from KLC 4026, KLC 4027, KLC 4178 and KLC 4181. Lane 1, protein standard marker; lanes 2-5 show 20 µl of sample loaded and lanes 7-10 show 10 µl of sample loaded. Lane 2, KLC 4026; lane 3, KLC 4027; lane 4, KLC 4178; lane 5, KLC 4181; lane 6, protein standard marker; lane 7, KLC 4026; lane 8, KLC 4027; lane 9, KLC 4178; lane 10, KLC 4181. Autorad picture not shown due to the fact that nothing was seen when the phosphoimager screen was scanned and read.

# CHAPTER IV.

# DISCUSSION.

LPS is an integral part of the Gram-negative outer membrane and has been an object of intensive research during the last decade. The most extensive studies involving the genetics of LPS biosynthesis have been carried out with *E. coli* K12, *S. typhimurium* LT2 and *Salmonella minnesota*. Lipid A is the most conserved portion of LPS with most of the variation either in the chain length of the glucosamine substituted fatty acids or the number and chain length of acyl oxyacyl substituted fatty acids. Various lipid A defective strains have been constructed and characterised (Raetz 1990), and such studies have demonstrated the importance of lipid A (required for cell viability and endotoxicity). In this study I have isolated and characterised a gene encoding an enzyme that is likely to be involved in the biosynthesis of the lipid A region of LPS in *Campylobacter jejuni* strains F38011 and NZRM 1958.

## 4.1 Temperature-sensitive complementation studies.

The *lpxA* gene product, UDP-*N*-acetylglucosamine acyltransferase is known in *E. coli*, *S. typhimurium* and a number of other Gram-negative organisms (both enteric and nonenteric) to be essential in lipid A biosynthesis (Galloway and Raetz 1990). A conditional-lethal mutant in UDP-*N*-acetylglucosamine acyltransferase has previously been generated in *E. coli* K12 using localised chemical mutagenesis. The mutant KLC 4177 (SM101) contains no measurable residual LpxA activity at 42°C, is supersensitive to hydrophobic and some peptide antibiotics, has a low spontaneous reversion frequency at 42°C, and is strikingly defective in lipid A biosynthesis *in vivo* at 42°C. Coleman and Raetz (1988) have demonstrated that all the biochemical and phenotypic alterations of KLC 4177 are corrected by *E. coli* plasmids bearing *lpxA* alone. This *E. coli lpxA*$^-$ mutant provided us with a model with which to study the effect of acyltransferase deficiency on cell viability and allowed us to identify a gene from *C.*

*jejuni* whose product is likely to be involved in the synthesis of at least one species of lipid A found in the LPS of *C. jejuni*.

### 4.1.1 COMPLEMENTATION OF KLC 4177 WITH *C. jejuni* PLASMID EXPRESSION LIBRARY.

Growth of the temperature-sensitive *E. coli* K12 mutant strain, KLC 4177 containing a *C. jejuni* F38011 plasmid expression library at the non-permissive temperature, indicated that a gene (or genes) from this library was able to restore the *lpxA*⁻ defect of KLC 4177. It was concluded initially from growth at 42°C that complementing activity was present in plasmids from *C. jejuni* F38011 which were able to complement the *lpxA*⁻ defect in KLC 4177.

KLC 4177 cells were transformed a second time with the complementing plasmids from *C. jejuni* F38011 to confirm that the *C. jejuni* expression plasmids were responsible for this cell viability. Control plates were incubated at 30°C to rule out spontaneous reversion. Reversion of the original *lpxA*⁻ mutant is unlikely based on: 1) the *lpxA* allele in KLC 4177 has a low spontaneous reversion frequency, 2) KLC 4177 transformed with pBIISK(+) failed to grow at the non-permissive temperature, and 3) secondary transformation showed the growth of all transformants at 42°C, (a result unexpected if KLC 4177 had reverted). As the KLC 4177 cells used for pBIISK(+) and pCI1-5 transformations were used from the same source, it is unlikely that a reversion event occurred during cell competence stages.

The fact that the plasmid pCI1 corrects the *lpxA*⁻ defect in KLC 4177 as seen by growth at the previously non-permissive temperature of 42°C, suggests that it contains a gene whose protein has an homologous function to that of LpxA, the protein which performs the same function in the model organism, *E. coli*. Thus it was expected that this gene would also encode the enzyme UDP-GlcNAc acyltransferase in *C. jejuni* F38011.

Four other plasmids were identified in this initial screen for *C. jejuni lpxA*. No common restriction enzyme fragment was seen in all these plasmids, however pCI4 had several *Hind*III fragments in common with pCI1, suggesting a common DNA fragment

between the two plasmids. It was also determined that pCI4 contains the *lpxA* analog as determined by PCR analysis.

Plasmids pCI2, pCI3 and pCI5 were not worked with to any great extent due to time constraints, however these plasmids have not been dismissed as they all show one important feature - the ability to complement the temperature-sensitive defect of KLC 4177. One suggestion for the absence of common restriction enzyme fragments is that the *lpxA* gene is not present in pCI2, pCI3 and pCI5 and the complementing activity is the result of a gene product other than LpxA. Another possibility for these three plasmids is that there could be more sequence present downstream of *lpxA* and no sequence present in the *fabZ* region of the insert for these plasmids. Sequence generated from the inserts within these plasmids would provide a great deal of information about whether the *lpxA* gene is present within these three clones, or whether another gene is present that is performing a similar function. One can speculate as to whether the production of another acyltransferase, such as the *lpxD* acyltransferase, is encoded within the inserts of pCI2, pCI3 and pCI5. This acyltransferase, similar in structure and function to *lpxA* may be able to complement the *lpxA* defect if overexpressed. It is known however, that death of cells occurs in *lpxA*$^-$ *lpxD*$^+$ *E. coli* strains.

A reduced rate of growth was observed for one of the transformants growing at 42°C compared to that of the other four transformants. This transformant contained the plasmid pCI3. One suggestion for the difference in growth rate may be due to provision of an alternative pathway for lipid A biosynthesis by this transformant. Bypass mutations for UDP-GlcNAc acyltransferase requirement could possibly be achieved by activation of a gene that codes for an isoenzyme, or by alteration of the regulation of the *lpx/dnaE* operon. Perhaps a bypass pathway allows a secondary protein to modify glucosamine by upregulating another acyltransferase. Another suggestion is that perhaps instead of altering a glucosamine molecule as in *E. coli* one is in fact altering a glucose as is the case for a lipid A species in *C. jejuni* and the glucose is serving instead as a substrate for LPS biosynthesis. More than one gene may be necessary for this step, and the insert in pCI3 is large enough to accommodate more than one gene.

Due to time constraints, further work was not carried out on this plasmid. To determine the differences between all the plasmids, biochemical analysis, for example thin layer chromatography (TLC), would need to be carried out. Also a larger number of complementing clones containing a plasmid could be analysed in order to ascertain whether or not extragenic bypass mutations of *lpxA2* can be isolated.

## 4.2 Transcription and Translation of *C. jejuni lpxA*.

Restriction enzyme mapping of one of the *lpxA* complementing plasmids, pCI1, revealed that it contained a 1.5 kb insert from the *C. jejuni* F38011 genome. Sanger dideoxy sequencing revealed the presence of four open reading frames; three partial ORFs and one complete ORF. It appears that almost the entire *lpxA* gene has been sequenced in *C. jejuni* F38011, however most of the nucleotide sequence remains to be confirmed (figure 18).

At the nucleotide level, $ORF_3$ (*lpxA*), showed no identity to any genes found in Genbank. This is not unusual given its strong AT-rich nature (70%). However at the polypeptide level it was shown that $ORF_3$ displayed strong amino acid identity to the UDP-*N*-acetylglucosamine acyltransferase protein from the *lpxA* gene of *E. coli, Haemophilus influenzae, Rickettsia rickettsii, S. typhimurium* and a number of other Gram-negative bacteria (probability of $3.8 \times 10^{-44}$; this is essentially the probability that this sequence has arisen by chance, therefore the further away from 1, the more likely the sequence is). This homology extended throughout the polypeptide sequence. An important identifying feature of the protein was the conservation of the 'isoleucine patch', a hexapeptide repeat motif that has been observed in all other LpxA protein sequences. This repeating hexapeptide motif consists of a conserved isoleucine, leucine, valine (or their equivalent methionine) residue being consistently repeated every six residues in a polypeptide sequence. This repeat is conserved at the polypeptide level for LpxA and other acyl and acetyl transferases and has also been termed 'the acetyltransferase motif'.

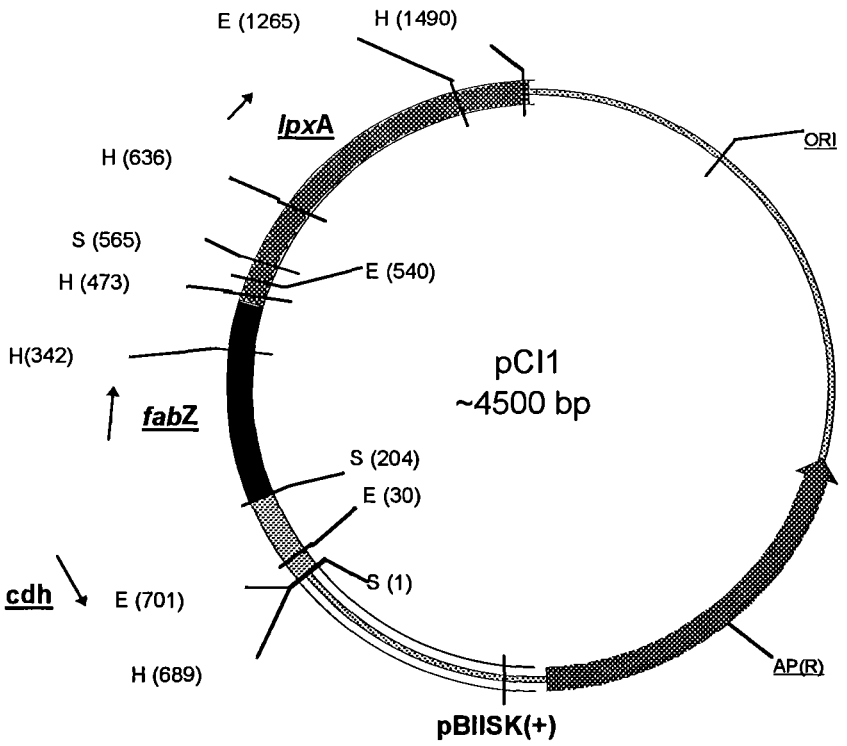



**Figure 18.** The restriction map of pCI1, estimated to be 4.5 kb, showing the orientation of the 1.5 kb *Sau*3A-*Sau*3A *C. jejuni* F38011 insert in pBIISK(+). Sequencing of the 1.5 kb *C. jejuni* insert revealed three ORFs - cdh, citrate synthase gene; *fabZ;* and *lpxA*. The Ap resistance gene (Ap(R)), encoded on pBIISK(+) is also shown. Restriction endonuclease sites are shown as deduced from sequencing and restriction enzyme mapping studies. E = *Eco*RI; H = *Hind*III; and S = *Sau*3A.

Although there is conservation at the amino acid level between alleles of *lpxA* from various Gram-negative bacteria, the amino acids present in the hexapeptide repeat differ in their identities compared to those from *E. coli.* In *C. jejuni* it was observed that of the conserved amino acids in this motif, the predominant amino acid is isoleucine. This may be due to the greater AT content of *C. jejuni* genomic DNA. Isoleucine is an AT-rich amino acid whereas valine and glycine are GC-rich amino acids. A second *lpxA* allele from *C. jejuni* strain NZRM 1958 has revealed considerable nucleotide sequence differences between both alleles, however on an amino acid level, the sequences are conserved (54% similarity). This suggests that considerable nucleotide changes are acceptable as long as primary amino acid sequence does not change. There is 62% amino acid homology up to the first large patch of nucleotide non-homology.

The *lpxA* gene in *E. coli* is part of a complex operon that also includes *firA/lpxD* (encodes an acetyltransferase involved in step 4 of lipid A biosynthesis), *fabZ* (encodes a dehydrase involved in saturated fatty acid biosynthesis), *lpxB*, *dnaE* (the catalytic subunit of DNA polymerase III), as well as at least 3 other additional open reading frames of unknown function. The entire operon has been sequenced in *E. coli* and *S. typhimurium*, but its natural regulation remains uncertain. Both *lpxA* and *lpxB* gene products have been overexpressed and purified to homogeneity in *E. coli*. $NH_2$-terminal protein sequencing has confirmed that *lpxA* and *lpxB* are structural genes in *E. coli*.

The nucleotide sequence and subsequent translation revealed the presence of the *fabZ* gene upstream of *lpxA* in *C. jejuni* F38011, suggesting that the order of genes is the same as that in *E. coli*. No putative promoter was identified from the *lpxA* sequence data at our disposal for *C. jejuni* F38011. It is likely that *lpxA* is transcribed together with *fabZ* as is thought in *E. coli* (Coleman and Raetz 1988), and that the promoter is found upstream of *fabZ*. Tomasiewicz and McHenry (1987) have found a functional promoter just upstream of *dnaE*, in $ORF_{23}$. It has yet to be determined whether this promoter is active *in vivo* or functions in concert with other promoters. In *E. coli* it has been reported that by clustering certain genes into operons, growth-rate-dependent functions will be coordinately expressed (Burton *et al.* 1983). It is thought that *fabZ*, *lpxA*, *lpxB*, and *dnaE* promoters may be part of such an operon, with possible internal promoters to conditionally decouple expression of certain genes (Coleman and Raetz 1988). This may well be the case in *C. jejuni*.

The initiation codon of the *lpxA* allele in *C. jejuni*, methionine, appears to be the same as that of *E. coli*. It also appears that the 3' end of *fabZ* overlaps the 5' initiation codon of *lpxA*, a similar situation that the two genes occupy in *E. coli*. It would be interesting to determine whether these two genes and others present in the operon are coupled transcriptionally or translationally in *C. jejuni* as suspected in *E. coli*. The *fabZ* and *lpxA* genes do not have to overlap as seen in *R. rickettsii*, where there are 6 nucleotides separating the rickettsial $ORF_{16}$ and *lpxA* genes (Shaw and Wood 1994).

Sequence downstream of *lpxA* in the *C. jejuni* F38011 insert in pCI1 did not show signs of homology to the *lpxB* gene or any other gene in the BLAST database across

the nearly 60 amino acids present. It would appear, from physical mapping with restriction endonucleases that plasmid pCI4 may contain enough sequence to determine whether it is present.

It would be of interest to determine whether *lpxB* is present downstream of *lpxA* as in *E. coli* and whether *fabZ/lpxA* are part of a larger operon, or if they are present independent of other essential LPS/DNA replication genes.

Translated, $ORF_2$ revealed amino acid similarity with the *fabZ* gene present in many Gram-negative organisms. The whole gene is not present upon the insert within pCI1, but we are able to speculate about whether this indicates that the order of genes is conserved for *fabZ* and *lpxA*. Evidence for this comes from several sources; 1) sequence of $ORF_2$ is similar to *fabZ* as indicated by BLAST; 2) PCR amplicon generation with the primers 96-24/96-25 (96-24 is found in the putative *fabZ* sequence); 3) PCR amplicon generation and sequence suggests *fabZ* is also present in *C. jejuni* NZRM 1958 and linked to *lpxA*; and 4) Southern data suggested genetic linkages.

In pCI1, the *fabZ* sequence is preceded by a *Sau*3A/*Sau*3A nucleotide fragment that, when translated had amino acid similarity to the citrate synthase gene from many organisms. This fragment of DNA is in the wrong orientation relative to *fabZ*, and it also encodes an internal portion of the citrate synthase enzyme. From this we conclude that the citrate synthase gene is unlikely to be positioned in the genome upstream of *fabZ*. From Southern blot analysis of the plasmid pCI4, NZRM 1958 genomic DNA and physical mapping comparisons with pCI4 it has been suggested that this gene is an artifact of construction of the plasmid expression library and does not belong in the *lpx* operon. It is unknown whether the citrate synthase gene is essential for *C. jejuni* survival. However it is unlikely that a partial gene sequence will be of use to the organism. Also the portion of citrate synthase gene sequence that was generated is contained on a *Sau*3A/*Sau*3A fragment only. This does not produce a contiguous part of the gene, only the internal portion of citrate synthase is revealed. We would have expected to see the 3' end of the gene before the start of *fabZ*. This was not observed from sequence data generated.

## 4.3 Protein Analysis.

Protein expression analysis from pCI1 was attempted using maxicells, but no unique polypeptides were revealed due to insufficiently radiolabelled polypeptides. We can only speculate on the level of protein expected. In *E. coli* K12 the *lpxA* gene encodes a 28.0-kDa protein (Coleman and Raetz 1988). We would expect a similar sized protein from *C. jejuni*, based on conservation of amino acid sequence within these organisms.

The enzyme UDP-GlcNAc acyltransferase is very specific for *R*-3-hydroxymyristoyl-acyl carrier protein (ACP) as the acyl donor (Williamson *et al.* 1991). Several fatty acyl transferases with an absolute specificity for acyl-ACP are capable of converting UDP-GlcNAc to UDP-2,3-diacylglucosamine (Anderson *et al.* 1985; Crowell *et al.* 1986). This suggests that *lpxA* cannot be replaced in these organisms (figure 19).

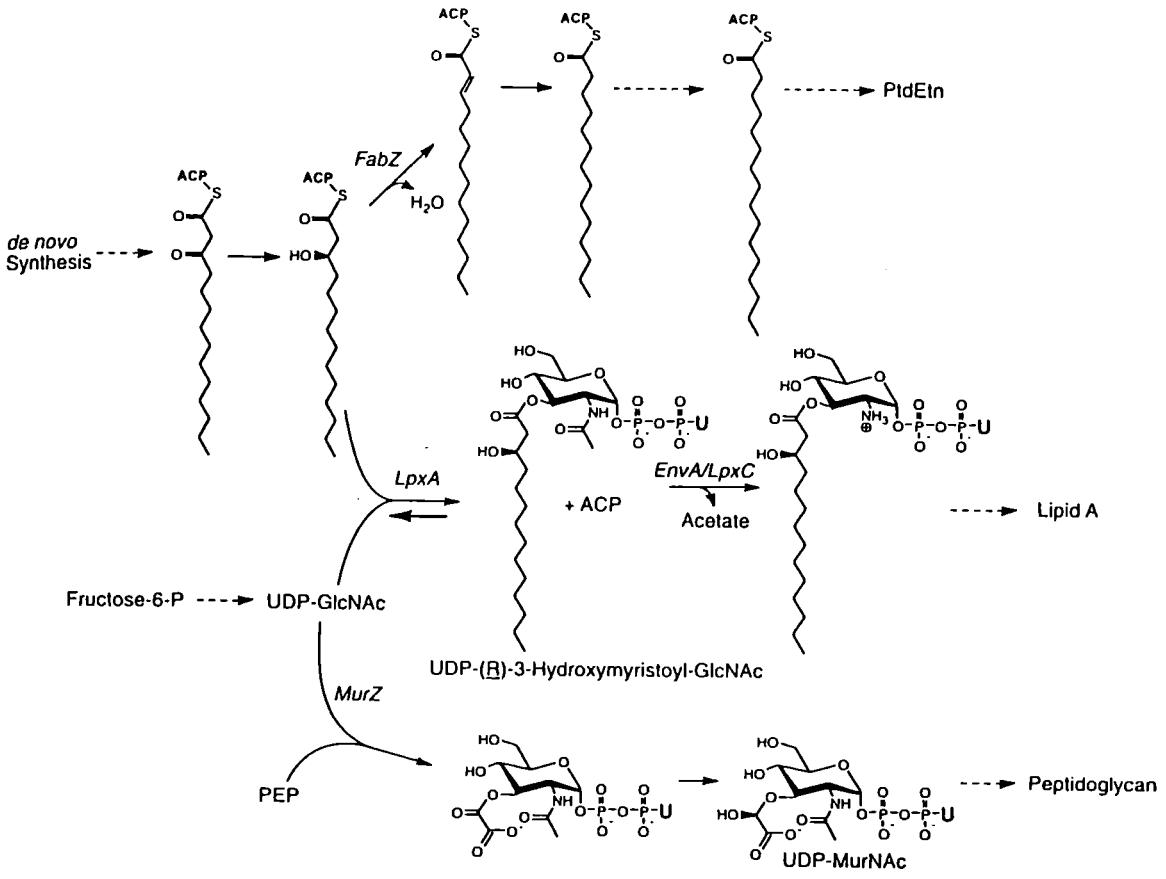



**Figure 19.** Relationship of key precursors of fatty acyl, lipid A, and peptidoglycan biosynthesis. Three major cell envelope components arise from two key precursors of *E. coli* metabolism. UDP-GlcNAc serves as the glucosamine source in both peptidoglycan and lipid A biosynthesis. The lipid A pathway begins with the acylation at the 3-OH moiety of the glucosamine ring of UDP-GlcNAc with *R*-3-hydroxymyristoyl derived from *R*-3-hydroxymyristoyl acyl carrier protein. The latter is also the precursor of palmitate residues found in membrane glycerophospholipids. Since the equilibrium constant for UDP-GlcNAc acylation is unfavourable, the second reaction of the lipid A pathway (the deacetylase) appears to function as the first committed step, and it may be regulated. Abbreviations: U, uridine; ACP, acyl carrier protein; PtdEtn, phosphatidylethanolamine (Adapted from Young *et al.* 1995).

## 4.4 Bacterial Growth Curve Data.

pCI1 containing a putative *lpxA* gene was capable of restoration of viability for KLC 4177 at 42°C (construct KLC 4178). However this restoration is less complete when compared to KLC 4176. This could be due to low expression of *lpxA* from a pBIISK(+) promoter as *lpxA* appears not to have an endogenous *C. jejuni* promoter. Equally, the low expression may be due to lack of regulation of the protein or some other factor, as *E. coli* and *Campylobacter* are different organisms with different genetic histories. A third possibility is that pCI1 "*lpxA*" is not *lpxA*, but a gene whose product may acylate GlcNAc at a low, but measurable rate. This last hypothesis would mean that, although the *C. jejuni* F38011 insert in pCI1 contains a gene whose product has the characteristic hexapeptide motif and amino acid sequence extremely similar to LpxA, presence of this is not enough to qualify the gene as *lpxA*.

An interesting observation was made in that for the last couple of hours of sampling a slight increase in optical density measurements was seen for KLC 4177 at 42°C. Perhaps this is an indication of KLC 4177 *lpxA*$^-$ revertants regaining acyltransferase activity. However, there was no increase in optical density comparable to that of the wild-type strain as observations past the 10 hour recording period revealed (data not shown). The growth curve would need to be performed again over a longer time interval to see if there is any significant change in the absorbance of KLC 4177 with an increased amount of time, or if the changes observed previously were just coincidental. Assays measuring lipid A synthesis *in vivo*, and UDP-*N*-acetylglucosamine acyltransferase activity *in vitro* could also be tested.

The fact that only conditional-lethal mutations of *lpxA* may be obtained suggests that the lipid A present in *E. coli* is essential for growth and replication, probably because it is required for outer membrane assembly. Whether an *lpxA*$^-$ defect in *C. jejuni* would have the same effect upon the viability of the organism remains to be seen as three different lipid A molecules are proposed to be present in *C. jejuni*. However, perhaps like *Rhizobium*, the first steps of lipid A biosynthesis are identical while the remaining steps vary (eg. result in GlcNAc modification to 2-deoxy-2-aminogluconic acid).

## 4.5 Future aims.

Future aims include the creation of a null mutation in the *lpxA* gene in *C. jejuni* by insertion of a kanamycin resistance cassette into the *lpxA* allele from *C. jejuni* strain NZRM 1958 and gene replacement onto the NZRM 1958 chromosome by homologous recombination. Viability of *C. jejuni* would be assessed to determine if the *lpxA* gene is essential. Thin-layer chromatography would be used to assess the production of all three lipid A species in *C. jejuni*, or if only a subset of lipid A molecules are produced. These experiments would allow testing of the essential nature of *lpxA* in *C. jejuni*, the function of LpxA (assay for acyltransferase activity), and provide insights into lipid A biosynthesis in *C. jejuni*.

In summary, the goals of this study were to identify whether an *lpxA* analog existed in *C. jejuni* by functional analysis. Additionally, once identified, characterisation of the complementing activity was to be undertaken. Clearly these objectives have been achieved - pCI1 (and 4 other plasmids) allow growth of the temperature-sensitive *lpxA*$^-$ strain KLC 4177 at the non-permissive temperature. The *C. jejuni* nucleotide insert of pCI1 was sequenced and a putative *lpxA* gene was identified. The *lpxA* gene appears to be transcriptionally coupled to *fabZ*, the gene upstream of *lpxA*. Southern analysis revealed the presence of this transcriptional unit as a single copy on the *C. jejuni* genome. Future lipid A genetic and biochemical studies will benefit from the information contained in this thesis.

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# APPENDIX I. Media.

## I. i. General Media.

Unless otherwise stated, all media are sterilised by autoclaving for 20 minutes at 120 kPa at 121°C. Solutions that were unstable at these high temperatures were filter sterilised through a 0.22 µm filter and added to media after autoclaving.

| **Luria Bertani Media (LB)** | per litre |
|---|---|
| 1.0% w/v bactotryptone | 10 g |
| 0.5% w/v yeast extract | 5 g |
| 0.5% w/v NaCl | 5 g |

Made up in $dH_2O$, pH adjusted to 7.4 with NaOH before autoclaving.

| **LB Agar (LBA)** | per litre |
|---|---|
| LB media with addition of 1.5% agar. | 15 g |

## I. ii. Specialised Media.

| **M63 Medium.** | per litre |
|---|---|
| 1.36% w/v $KH_2PO_4$ | 13.6 g |
| 0.2% w/v $(NH_4)_2SO_4$ | 2 g |
| 5 X $10^{-4}$ % w/v $FeSO_4.7H_2O$ | 0.5 mg |

Made up in $dH_2O$. pH was adjusted to 7.0 with KOH. After autoclaving, 10 ml of 20 % glucose was added as the carbon source. Also added was 1 ml of $MgSO_4.7H_2O$, vitamins eg. thiamine (B1) in the final concentration of 1µg/ml and amino acids were added at a concentration of 1x in the L-form (see 100x stock concentration below).

| **M9 Minimal Salts Media (MM).** | per litre |
|---|---|
| 0.6% w/v $Na_2HPO_4$ | 6 g |
| 0.3% w/v $KH_2PO_4$ | 3 g |
| 0.05% w/v NaCl | 0.5 g |
| 0.1% w/v $NH_4Cl$ | 1 g |

Made up in $dH_2O$. After autoclaving, 10 ml 0.01% $CaCl_2$ and 1 ml 1M $MgSO_4.7H_2O$ were added as well as 10 ml of 20% glucose as the carbon source. Amino acids were added at a concentration of 1x (see 100x stock concentration below (L-form)).

**Table 5: Amino acid supplements for minimal medium (100x stock solutions).**

| Amino Acids | mg/mL |
|---|---|
| L-alanine | 2 |
| L-arginine | 2 |
| L-asparagine | 2* |
| L-aspartic acid | 10* |
| L-cysteine | 2* |
| glycine | 2 |
| L-glutamine | 2 |
| L-glutamic acid | 10 |
| L-histidine (free base) | 2 |
| L-isoleucine | 3 |
| L-leucine | 3 |
| L-lysine | 3 |
| L-methionine | 2 |
| L-phenylalanine | 5 |
| L-proline | 2 |
| L-serine | 37.5 |
| L-threonine | 20 |
| L-tryptophane | 2* |
| L-tyrosine | 3* |
| L-valine | 10 |

* = filter sterilised. All other amino acids were autoclaved. Amino acids were dissolved in $dH_2O$ unless otherwise stated.

**Mueller-Hinton Agar**

300 g beef infusion

17.5 g acid hydrolysate of casein

1.5 g starch

17 g bacto-agar

Made up to 1 litre in $dH_2O$. pH 7.4 +/- 0.2.

**Campylobacter Blood-Free Selective Agar Base (Modified CCDA-Preston)**

| | per litre |
|---|---|
| 2.5% w/v Oxoid Nutrient broth no. 2 | 25 g |
| 0.4% w/v bacterial charcoal | 4 g |
| 0.3% w/v casein hydrolysate | 3 g |
| 0.1% w/v sodium desoxycholate | 1 g |
| 0.025% w/v ferrous sulphate | 0.25 g |
| 0.025% w/v sodium pyruvate | 0.25 g |
| 1.2% w/v agar | 12 g |

In 1 litre $dH_2O$, pH adjusted to 7.4 before autoclaving. After autoclaving 1 ml of cefoperazone solution (3.2g/L) was added.

**Casein Hydrolysate (ACID) Vitamin Free (Casamino Acid Substitute).**

7.6 % w/w total nitrogen

4.9 % w/w amino nitrogen

28.3% w/w NaCl

<0.1% w/w tryptophan

Made up as a 1% solution in $dH_2O$. pH adjusted to 7.0.

# APPENDIX II. Buffers and Solutions.

## II. i. Common Buffers.

Solutions requiring sterilisation were either autoclaved for 20 minutes at 120 kPa at 121°C or filter sterilised through a 0.22 µm filter.

**TE**

10 mM Tris-HCl

1 mM EDTA

Made up in $dH_2O$; pH adjusted to 8.0 and the solution filter sterilised or autoclaved.

**50 x TAE**

50 mM Tris base

0.11% v/v glacial acetic acid

1 mM EDTA (pH 8.0)

Made up in $dH_2O$ to 1 L and the pH adjusted to 8.0.

**1 x TAE**

20 ml of 50 x TAE made up to 1 litre with $dH_2O$.

**10 x TBE**

50 mM Tris base

50 mM Boric acid

1 mM EDTA

Made up in $dH_2O$ to 1L; pH adjusted to 8.0.

**DNA Loading Dye for Agarose Gel Electrophoresis**

30% Glycerol

0.25% Bromophenol blue

0.25% Xylene cyanol FF

Made up in $dH_2O$.

## II. ii. Specific Buffers and Solutions.

**II. ii. a.** ALKALINE LYSIS OF BACTERIAL CELLS; EXTRACTION OF PLASMID DNA.

The following solutions were filter sterilised, as necessary.

**Solution 1** (stored at 4°C)

50 mM glucose

25 mM Tris-HCl (pH 8.0)

10 mM EDTA (pH 8.0)

+ sterile $dH_2O$ to 20 ml.

**Solution II**

1% SDS

0.2 N NaOH

+ sterile $dH_2O$ to 20 ml.

**Solution III** (stored at 4°C)

12 ml of 5 M potassium acetate

8.7 % glacial acetic acid

+ sterile $dH_2O$ to 20 ml.

Resulting solution is 3 M with respect to potassium and 5 M with respect to acetate.

## II. ii. b. CHROMOSOMAL DNA PREPARATION SOLUTIONS.

The following solutions were filter sterilised.

**Solution I**

50 mM glucose

50 mM Tris-HCl (pH 8.0)

50 mM EDTA

10 mg/ml lysozyme (added freshly before use)

$dH_2O$ was added to 20 ml.

**Solution II**

50 mM Tris-HCl (pH 8.0)

50 mM EDTA

1% SDS

50 µg/ml proteinase K (added fresh before each use)

$dH_2O$ was added to 20 ml.

## II. ii. c. SEQUENCING POLYACRYLAMIDE GEL SOLUTIONS.

**Sequagel Concentrate**

| | |
|---|---|
| 23.75% acrylamide | 237.5 g |
| 1.25% methylene bisacrylamide | 12.5 g |
| (ratio of acrylamide to bisacrylamide is 19:1) | |
| 50% urea (8.3 M) | 500 g |

Made up to 1 L in $dH_2O$.

**Sequagel Buffer**

| | |
|---|---|
| 50% urea (8.3 M) | 500 g |

Made up to 1 L in 10x TBE.

**Sequagel Diluent**

| | |
|---|---|
| 50% urea (8.3 M) | 500 g |

Made up to 1 L in $dH_2O$.

**Sequencing Gel**

5% acrylamide sequencing gels were usually used for DNA sequence analysis. The following volumes were used:

| | |
|---|---|
| Sequagel concentrate | 20 ml |
| Sequagel diluent | 70 ml |
| Sequagel buffer | 10 ml |
| 10% APS - freshly made in $dH_2O$ | 800 µl |
| TEMED | 40 µl |
| Final volume | 100 ml |

**Dye for sequencing gels**

98% deionized formamide

10 mM EDTA (pH 8.0)

0.025% xylene cyanol FF

0.025% bromophenol blue

**Sequencing Gel Fixer**

10% acetic acid

10% isopropanol

In $dH_2O$.

## II. ii. d. SDS-PAGE GEL ELECTROPHORESIS FOR PROTEINS.

**Separating Gel**

To make a 12% acrylamide separating gel, the following volumes of solutions were mixed to a total volume of 40 ml.

| | |
|---|---|
| 4 x lower buffer | 10.0 ml |
| acrylamide stock* | 16.0 ml |
| $H_2O$ | 13.8 ml |
| 10% ammonium persulphate (fresh in $dH_2O$) | 0.2 ml |
| TEMED | 10 µl |

30:0.8 acrylamide:bis-acrylamide

**Stacking Gel**

To make a 3% acrylamide stacking gel, the following volumes of solutions were mixed to a total volume of 10 ml.

| | |
|---|---|
| 4 x upper buffer | 2.5 ml |
| acrylamide stock | 1.0 ml |
| $H_2O$ | 6.4 ml |
| 10% ammonium persulphate (prepared fresh) | 0.2 ml |
| TEMED | 4.5 µl |

**Acrylamide stock**

| | |
|---|---|
| acrylamide | 300 g |
| bisacrylamide | 8 g |

$dH_2O$ up to 1 litre. Filter through a 0.45 µm filter. Store at 4°C.

**10% SDS**

| | |
|---|---|
| sodium dodecyl sulphate (SDS) | 10 g |

$dH_2O$ up to 100 ml. Store at room temperature.

**4 x Lower Buffer** (1.5 M Tris-HCl, pH 8.8, 0.4% SDS)

| | |
|---|---|
| Tris base | 181.7 g |
| 10% SDS | 40 ml |

$dH_2O$ up to 1 litre. Adjust the pH to 8.8 with HCl. Add 1 ml of TEMED.

**4 x Upper Buffer** (0.5 M Tris-HCl, pH 6.8, 0.4% SDS)

| | |
|---|---|
| Tris base | 60.6 g |
| 10% SDS | 40 ml |

$dH_2O$ up to 1 litre. Adjust pH to 6.8 with HCl. Add 2 ml of TEMED.

**0.1% BpB**

| | |
|---|---|
| bromophenol blue | 10 mg |
| $H_2O$ | 10 ml |

Store at room temperature.

**2 x Sample Buffer (SB)**

| | |
|---|---|
| 4 x upper buffer | 12.5 ml |
| glycerol | 20.0 ml |

$dH_2O$ up to 60 ml.

**2 x Loading Buffer (LB)**

| | |
|---|---|
| β-mercaptoethanol | 0.5 ml |
| 0.1% BpB | 0.25 ml |
| 10% SDS | 4.0 ml |
| 2 x SB | 5.3 ml |

**4 x Running Buffer Stock (RBS)**

| | |
|---|---|
| Tris base | 60.0 g |
| glycine | 288 g |

$dH_2O$ up to 5 litres.

**1 x Running Buffer (1 x RB)**

| | |
|---|---|
| 4 x RB | 1 litre |
| $H_2O$ | 3 litres |
| 10% SDS | 40 ml |

**Stain**

| | |
|---|---|
| isopropanol | 125 ml |
| acetic acid | 50 ml |
| $H_2O$ | 325 ml |
| Coomassie brilliant blue (R250) | 1.25 g |

**Destain**

| | |
|---|---|
| methanol | 1 litre |
| acetic acid | 1.4 litres |

$dH_2O$ added to 20 litres.

## II. ii. e. SOUTHERN HYBRIDISATION SOLUTIONS.

**Depurination Solution**

0.2 M HCl

**Denaturation Solution**

0.5 M NaOH

1.5 M NaCl

**Neutralisation Solution**

1 M Tris-HCl, pH 7.5

1.5 M NaCl

**20 x SSC**

3 M NaCl

0.3 M Trisodium citrate.

In $dH_2O$ and pH adjusted to 7.2 with citric acid. This solution served as a stock of SSC and thus subsequent dilutions were made using this stock and $dH_2O$.

**Transfer Solution**

20 x SSC, pH 7.0

**Maleic Acid Buffer**

0.1 M maleic acid

0.15 M NaCl

pH adjusted to 7.5.

**10 x Blocking Solution**

10% blocking reagent

90% maleic acid buffer

Stored at 4°C.

**Detection Buffer**

0.1 M Tris-HCl

0.1 M NaCl

50 mM $MgCl_2$

**Standard Hybridisation Buffer**

5 x SSC

0.1% N-lauroylsarcosine

0.02% SDS

1% 10 x blocking reagent

**Post-Hybridisation Wash I**

2 x SSC

0.1% SDS

**Post-Hybridisation Wash II**

0.1 SSC

0.1% SDS

# Appendix III. Blast Sequence

```
>sp|P10440|LPXA_ECOLI ACYL-[ACYL-CARRIER-PROTEIN]--UDP-N-ACETYLGLUCOSAMINE
            O-ACYLTRANSFERASE (UDP-N-ACETYLGLUCOSAMINE ACYLTRANSFERASE)
            >pir||XUECDP acyl-[acyl-carrier-protein]--UDP-N-acetylglucosamine
            O-acyltransferase (EC 2.3.1.129) - Escherichia coli >gi|146661
            (M19334) acyl-[acyl carrier protein]--UDP-N -acetylglucosamine
            O-acyltransferase [Escherichia coli] >gnl|PID|d1012622 (D83536)
            O-acyltransferase [Escherichia coli]
            Length = 262

  Plus Strand HSPs:

 Score = 243 (111.8 bits), Expect = 3.8e-44, Sum P(3) = 3.8e-44
 Identities = 48/122 (39%), Positives = 70/122 (57%), Frame = +1

Query:   646 VIIGKKQLIENIRVINSGTVKGRCFTSIGGNAFIMDYCHIAHDCILGNKYYFRNNATLAG 825
             V IG + .I     I+ GTV+G   T +G +  +M   HIA DC +GN+    NNATLAG
Sbjct:    84 VEIGDRNRIRESVTIHRGTVQGGGLTKVGSDNLLMINAHIADDCTVGNRCILANNATLAG 143

Query:   826 HVEVGDFTVVGGLTPIHQFVKVGEGCMIAGASALYSGYRTVLFSRGNRASIRSLNLVGTR 1005
             HV V DF ++GG+T +HQF  +G   M+ G S +       + ++GN A+   +N+ G +
Sbjct:   144 HVSVDDFAIIGGMTAVHQFCIIGAHVMVGGCSGVAQDVPPYVIAQGNHATPFGVNIEGLK 203

Query:  1006 RR 1011
             RR
Sbjct:   204 RR 205

 Score = 115 (52.9 bits), Expect = 3.8e-44, Sum P(3) = 3.8e-44
 Identities = 20/68 (29%), Positives = 38/68 (55%), Frame = +3

Query:   414 IHPSAVIEHGAQLGDHVVLKAYAYVSKDAKIGNNVVIKQGARILSDTTIGDHSRVFSYAI 593
             +HP+A++E GA +G +  +  +  V    +IG   V+K    +  T IG  + ++S A
Sbjct:     8 VHPTAIVEEGASIGANAHIGPFCIVGPHVEIGEGTVLKSHVVVNGHTKIGRDNEIYSVAS 67

Query:   594 VGDIPQDI 617
             +G++ QD+
Sbjct:    68 IGEVNQDL 75

 Score = 58 (26.7 bits), Expect = 2.6, Sum P(2) = 0.92
 Identities = 11/39 (28%), Positives = 20/39 (51%), Frame = +1

Query:   724 SIGGNAFIMDYCHIAHDCILGNKYYFRNNATLAGHVEVG 840
             SIG NA I  +C +     +G     +++  + GH ++G
Sbjct:    19 SIGANAHIGPFCIVGPHVEIGEGTVLKSHVVVNGHTKIG 57

 Score = 58 (26.7 bits), Expect = 2.6, Sum P(2) = 0.92
 Identities = 14/46 (30%), Positives = 18/46 (39%), Frame = +1
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Query:   760 HIAHDCILGNKYYFRNNATLAGHVEVGDFTVVGGLTPIHQFVKVGE 897
             HI   CI+G           L  HV V   T +G    I+    +GE
Sbjct:    25 HIGPFCIVGPHVEIGEGTVLKSHVVVNGHTKIGRDNEIYSVASIGE 70

 Score = 49 (22.5 bits), Expect = 3.8e-44, Sum P(3) = 3.8e-44
 Identities = 8/30 (26%), Positives = 16/30 (53%), Frame = +1

Query:  1003 RRRFDKDEVDRLSRAFKTLFRQGDLKENAK 1092
             RR F ++ +  +  A+K ++R G   +  K
Sbjct:   204 RRGFSREAITAIRNAYKLIYRSGKTLDEVK 233

 Score = 40 (18.4 bits), Expect = 7.3e-29, Sum P(3) = 7.3e-29
 Identities = 9/39 (23%), Positives = 20/39 (51%), Frame = +3

Query:   486 VSKDAKIGNNVVIKQGARILSDTTIGDHSRVFSYAIVGD 602
             + K A +    ++++GA I ++  IG    V  +  +G+
Sbjct:     2 IDKSAFVHPTAIVEEGASIGANAHIGPFCIVGPHVEIGE 40
```