A novel cytochrome P450 from *Campylobacter jejuni 11168*

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Thesis presented for the degree of Doctor of Philosophy

The University of Edinburgh

2005

Declaration

I declare that this thesis was composed by myself and the work presented herein is my own other than where referenced to others.

Nicolae Corcionivoschi

2005

For Duncan and Adriana

Acknowledgements

A very special thank you to my supervisor Prof. Graeme Reid, for being there for me and encouraging me when I need it most. Thank you, Dr. Caroline Miles and Dr. Carsten Schwalb for all your help during my PhD, and to Laurie and Lorna for teaching me English. A big thank you to Prof. S.K. Chapman, and to all the people from his group, for teaching me about P450s. Thank you Dr. Bruce Ward for helping me in culturing *Campylobacter* and for having your door opened for me all the time. A special thank you to my wife, Adriana who always believed in me and for being the supporting shoulder. I want to mention here Duncan who by his presence made the last part of my PhD easier and for giving me a smile when sometimes I lost mine.

Thank you Prof. Kenneth Murray and Darwin Trust of Edinburgh for giving me this chance.

Mulţumiri

Aş vrea sa mulţumesc mamei mele fară ajutorul cărieia acum poate nu aş fi fost aici. Aş vrea să mulţumesc părinţilor soţiei mele, Maria şi Adrian, pentru ajutorul acordat pe parcursul acestor trei ani, ajutor fără de care totul ar fi fost imposibil şi pentru că mi-au dat o soţie minunată. Aş vrea să mulţumesc în mod special D-lui Prof. Dr. Dan Drinceanu pentru că ma îndrumat spre performanţă şi care mi-a spus mereu „ *nu face totul după ureche, citeşte*". Mulţumesc D-lui Prof. Dr. Ioan Vintilă pentru că a crezut în mine şi mi-a acordat această şansă.

Abstract

After isolation in 1970s, *Campylobacter jejuni* become the most commonly recognized cause of bacterial gastroenteritis in man. In animals is frequently found in bovines on ovines. Publishing of the genome sequence of *Campylobacter jejuni 11168* (Parkhill, 2000) revealed the presence of only one cytochrome P450 in an operon involved in sugar and cell surface biosynthesis. The gene name is Cj1411c, is 1359 bp long and encodes 453 aa. The sequence is strictly conserved in *Campylobacter jejuni* RM221. Similarities with two cytochrome P450s, one form *Silicobacter* sp. and one form *Poloromonas* sp., were identified. These two enzymes are known to be involved in ascorbate and aldarate metabolism.

The recombinant construct allowed the expression of active P450 enzyme with a 450 nm peak when binds CO. The protein was purified in proportion of ~ 70 %.

By deleting the P450 gene from the *Campylobacter jejuni 11168* genome clear changes in cell morphology were identified cells becoming wider and shorter. The capsular sugar profile of the NCI strain reveals the presence of arabinose which was not found in the wild type strain. The arabinose was identified by both HPLC and NMR.

The phenotype studies showed clear differences between NCI and WT cells : NCI cells are less resistant to starvation in the stationary phase; by exposure to the atmospheric oxygen 36.47 % of the wild type cells survived after 24 hours and only 16.61 % of the NC1 cells survived; by growing the NCI cells in competition with the Wt cells the growth rate of NCI cells approximately 10X lower than the Wt; NCI cells were proved to be less resistant to high temperature, more resistant to low temperatures and pH. By analysing the results obtained with NaCl and glycerol we have determined that is not the osmotic pressure that is affecting the growth of *Campylobacter* and the differences between the Wt and NCI strain might be related with changes in cell surface components.

Abbreviations

Nucleotides:

A - Adenine
C – Cytosine
G – Guanine
T – Thymine

Amino acids:

Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acids	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Standard units:

°C	degree Celsius
Da	Daltons
g	gram
l	litre
m	metre
M	molar
s	second
V	volt
ppm	parts per million

Textual abbreviations:

ADP	adenosine diphosphate
Amp	ampicillin
ATP	adenosine triphosphate
dH_2O	distilled water
EDTA	ethylenediaminetetraaccetic acid
FAD	flavin adenine nucleotide
FMN	flavin mononucleotide
HPLC	high performance liquid chromatography
IPTG	isopropyl thiogalactoside
Kan	kanamycin
LB	Luria-Bertani broth
NADH	nicotinamide adenine dinucleotide
NCI	knockout strain
NMR	nuclear magnetic resonance
MH	Mueller-Hinton broth
OD	optical density
PAGE	polyacrylamide gel elctrophoresis
PCR	polymerase chain reaction

SDS	sodium dodecyl sulfate
TEMED	N, N, N', N'-tetramethylene diamine
Tris	tris(hydroxymethyl)-aminomethane
UV	ultra-violet
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
Wt	wild type

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Chapter 1
INTRODUCTION

1.1. Biology

The discovery in 1970s of the *Campylobacter* enteritis finalised a century of research in trying to describe the spiral bacteria in the colons of children who had died of "cholera infantum" (Escherich 1886). *Campylobacter* (fig. 1.1) was first described in 1913 in England by McFadyean and Stockman who implicated these organisms as casual agents of abortion in sheep. Just five years later Smith (1918) reported on the association of similar organisms with bovine abortions in the United States. *Campylobacter* was first identified as a foodborne gastrointestinal bacterium in 1977 (Skirrow, 1977) and is at this moment, the leading cause of bacterial gastroenteritis in the western world (Skirrow *et.al*, 1993).

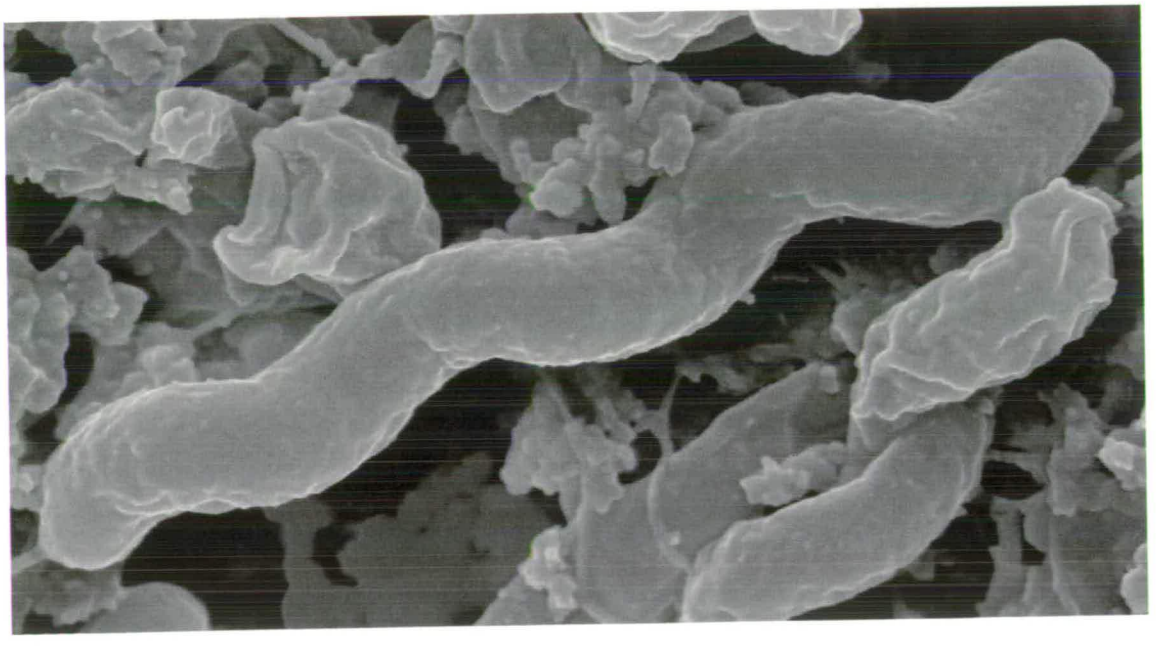


Fig 1.1. *Campylobacter jejuni* NCTC 11168 visualised by scanning electron microscopy (personal, unpublished)

Solomon (1999) described over 20 species and subspecies of *Campylobacter* from which 8 species have been identified as agents of human gastrointestinal disease (table 1.1). They are members of the family Campylobactereaceae together with *Helicobacter* and *Arcobacter*.

Table 1.1

Human pathogenic *Campylobacters*

Organism	Disease	Reservoirs
Campylobacter jejuni ssp. jejuni	Enteritis, systemic illness Guilian-Barré syndrome	Human, domestic pets, rodents, birds
Campylobacter coli	Enteritis	Pigs, birds
Campylobacter lari	Enteritis	Birds
Campylobacter hyointestinalis	Enteritis	Cattle, pigs
Campylobacter upsaliensis	Enteritis	Domestic pets
Campylobacter fetus ssp. fetus	Enteritis, systemic illness	Cattle, sheep
Campylobacter cinaedi	Enteritis	Hamsters

Genus *Campylobacter* – Bacterial Nomenclature Up-to-Date

(http://www.dsmz.de/bactnom/bactname.htm)

Campylobacter butzleri
Campylobacter cinaedi
Campylobacter coli
Campylobacter concisus
Campylobacter cryaerophilus
Campylobacter curvus
Campylobacter fennelliae
Campylobacter fetus subsp. *fetus*
Campylobacter fetus subsp. *venerealis*
Campylobacter gracilis
Campylobacter helveticus
Campylobacter hominis
Campylobacter hyoilei
Campylobacter hyointestinalis subsp. *hyointestinalis*
Campylobacter hyointestinalis subsp. *lawsonii*
Campylobacter jejuni subsp. *doylei*
Campylobacter jejuni subsp. *jejuni*
Campylobacter lanienae
Campylobacter lari
Campylobacter laridis
Campylobacter mucosalis
Campylobacter mustelae
Campylobacter nitrofigilis
Campylobacter pylori
Campylobacter pylori subsp. *mustelae*
Campylobacter rectus
Campylobacter showae
Campylobacter sputorum subsp. *bubulus*
Campylobacter sputorum subsp. *mucosalis*
Campylobacter sputorum subsp. *sputorum*
Campylobacter upsaliensis

All members of *Campylobacter* genus are small, nonsporeforming, Gram-negative bacteria that have a characteristic curved, S-shaped or spiral morphology (1.5-6.0 μm long and 0.2-0.5 μm wide). They are motile microorganisms with a polar flagellum at one or both ends of the cell. *Campylobacter jejuni* is a microaerophilic organism which requires an atmosphere containing 5 % O_2, 10 % CO_2 and 85 % N_2. Growth is not supported by atmospheric levels of oxygen but it is also unable to grow under completely anaerobic conditions. According to Solomon (1999) *Campylobacter jejuni* is able to grow at temperatures between 30-47 ^{0}C, at a pH not lower than 4.9 and at maximum concentration of NaCl of 1.5 % (Griffiths and Park, 1990).

A likely explanation for the 5 % O_2 required is the existence of one or more essential oxygen-requiring metabolic reactions in this microorganism – e.g. the oxidative decarboxylation of coproporphrinogen III to protoporphyrin IX which is an essential step in heme biosynthesis and the reduction of ribonucleotides by ribonucleotide reductase (RNR) to provide 2`-deoxyribonucleotides for DNA synthesis and repair. In *Campylobacter*, compared with other bacteria, we find only class I of RNR which is oxygen dependent (Sellars *et al*, 2002).

Aeration rapidly promotes transformation to the coccoid form according to Moran and Upton (1986). Interestingly the same authors mention in 1987 that cultures aerated in the presence of light transform to the coccoid state more rapidly than those aerated in dark. Oxidative stress increases invasiveness and intracellular survival (Harvey *et al.*, 1996).

Exposure to oxygen is inevitable for bacterial pathogens and, as in all other microorganisms, this leads to the formation of ROS (reactive oxygen species) (Jones *et al*, 1993). There are three enzymes identified in *Campylobacter* which are involved in oxidative stress defence in campylobacters:

- SOD (superoxide dismutase) plays an active role in the defence against oxidative stress and aerotolerance and is an important factor for survival of *campylobacter* in food (Park, 2002);
- Ahp (alkyl hydroperoxide reductase)
- KatA (catalase)

The bacterium can grow on a wide variety of media characteristic to each species of *Campylobacter* (eg. Mueller-Hinton). However, most researchers have used basal media developed for more fastidious pathogenic microorganisms. In the last few years isolating *Campylobacter* has become an easy procedure since its resistance to various antibiotics has been discovered: rifampicin, vancomycin, trimethoprim, polymyxin B, amphotericin B and cycloheximide (Karmali *et.al*, 1981).
In normal growth conditions various morphological changes are observed. According to Griffiths, 1993, Ng *et al.*, 1985 and Leach *et al.*, 1997 after 12 hours (mid-log phase) incubation all the cells were short spirals and after 24 hours all cells were

plateable spirals. After 48 hours (late stationary phase) the culture was a heterogeneous population of coccoid cells and spirals (Thomas *et al.*, 1999).
Morphologically, campylobacters are heterogeneous with two stages predominating: in younger cultures the rapidly motile, spiral forms predominate, whilst in older/stressed cultures, rapid rounding of the cell occurs, producing predominantly coccoid cultures.

The coccoid cells are about 0.2-1.2 μm in diameter (Pead, 1979), are heterogenous in diameter falling into two populations, small (0.2-0.4) and large (> 0.6 μm), on the evidence of filtering (Koike & Shizmazki, 1982) – fig 1.2.

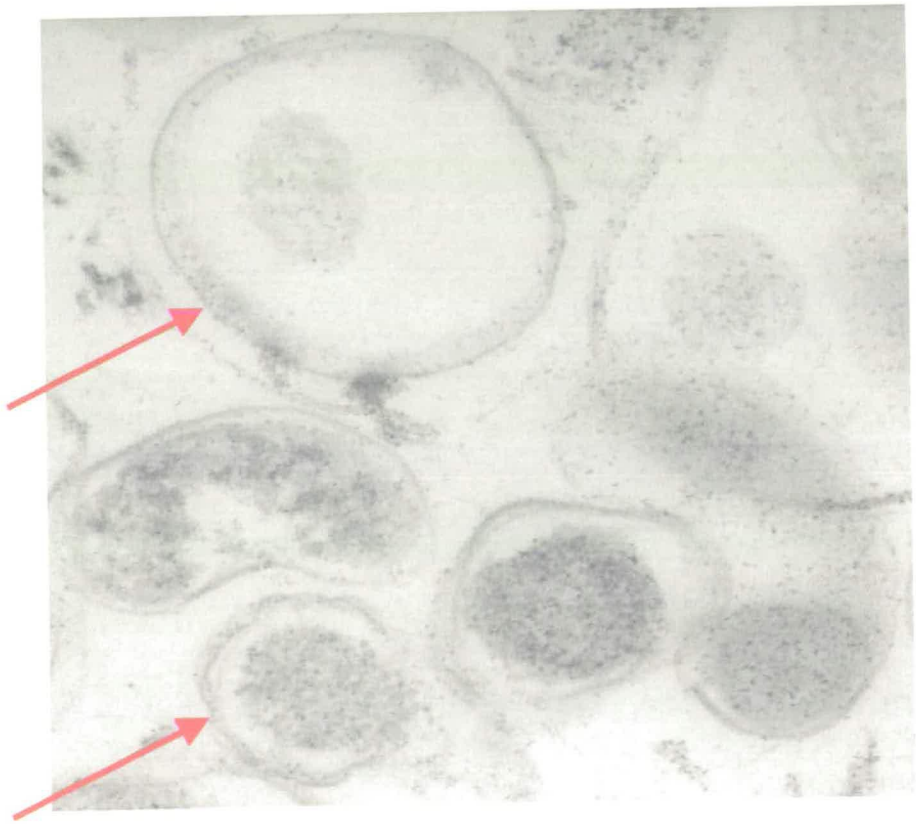


Fig 1.2. Indicated by arrows coccoid campylobacters visualised by Transmission Electron Microscopy (personal, unpublished)

Electron and light microscopy indicate morphological differences between spiral and coccoid cells. In spiral cells the cytoplasm is closely attached to the cell wall (Ng *et al.*, 1985) with most spirals possessing bipolar flagella, attached to a concave depression (Pead, 1979, Brook and Murray, 1987).

In coccoid cells the cytoplasm is detached from the cell wall (Ng *et al.*, 1985) whilst the outer membrane is enlarged in relation to the rest of the cell, but is thinner than the outer membrane of spirals (Moran and Upton, 1987). Coccoid cells retain the normal length flagella (Moran and Upton, 1987), forming a bundle in the coccoid cell (Jones *et al.*, 1991). Coccoid cells are extremely sticky forming large, irregular clumps (Rollins *et al.*, 1983).

The membrane fatty acid profiles of coccoid cells formed at 4-12 ^{0}C were similar to spirals, hence *Campylobacter jejuni* does not adapt the fatty acid content of the membrane during transition to the coccoid form at low temperatures (Haezeleger *et al.*, 1995), whilst *Campylobacter coli* cells incubated at low temperatures show an increase in long chain and cyclopropyl derivatives (Höller *et al.*, 1998).

Aged, coccoid *Campylobacter jejuni* cultures have decreased amounts of 28 and 33 kDa proteins compared to spiral cultures (Shibata *et al.*, 1991).

1.1.2. Incidence

Campylobacter spp. continue to be highly important human pathogens. The most effective forms of control at present are the pasteurisation of milk and the proper handling and cooking of 'high-risk' foods such as poultry meat. Given the difficulties of handling contaminated products in domestic kitchens a longer term aim must be to work towards producing *Campylobacter*-free poultry.

Examples of cases reported around the world reported at the *International Scientific Forum on Home Hygiene* in January 2004:

- England & Wales: > 55,000 reported cases per year
- Scotland: > 5000 reported cases per year
- Netherlands: > 3300 cases in 2000
- Czech Republic: 17,000 cases in 2000
- United States: > 70,000 cases per year
- Australia: > 10,000 reported cases per year
- Japan: 5000 cases per year

1.1.3. Infections

Over 95 % of campylobacteriosis cases are endemic, the rest attributable to outbreaks, usually due to contaminated private water supplies or unpasteurised milk (Blaser, 1997). The infections dose ranges from 500 to 10^6 cells, dependent upon strain and transmission medium (Robinson, 1981 and Saleha *et al.*, 1998).

1.1.3.1. Factors involved in Campylobacter infection development

In enteric pathogenic bacteria the factors involved in pathogenesis are adhesion, invasion, toxin production and subversion of the host cell process (table 2.1; Crushell *et.al,* 2004).

Table 1.2

Campylobacter virulence factors

Bacterial factors
- *Motility/chemotaxis*
- *Adhesion*
  - PEB 1
  - CadF
  - jipA
  - MOMP
  - LOS
  - CPS
  - Flagellin
- *Invasion*
  - Flagellum
  - pVIR (type IV secretion apparatus)
- *Cytholethal distending toxin*
- *Secreted effector proteins (CIA proteins)*

Analysing the genome of *Campylobacter jejuni 11168* described by Parkhill *et al.*, (2000) we discover that not all the classical virulence mechanisms are present in this strain. This is because 11168 does not contain the genetic resources for adhesins, invasins, protein secretion systems or pathogenicity islands. There are no pilus structures encoded on the chromosome and the only toxin genes identifiable are the *cdt* genes. The cytolethal distending toxin (CDT) affects epithelial cell morphology causing distension and eventually cell death in *in vitro* models. The CDT is expressed in humans during colonization and it is highly immunogenic.

Campylobacter cell-associated polysaccharide structures LPS and LOS are very important virulence factors for several stages of the gastrointestinal disease. The LOS and LPS ganglioside-like epitopes are proved to be associated with neuropathies (Fry, 2000).

1.1.3.2. Gastro-enteritis

Campylobacter jejuni gastrointestinal infections are clinically presented as inflammatory enteritis, with an incubation period of 24-27 hours (Blaser, 1997).The illness is usually self-limiting, lasting up to a week (Wallis, 1994 and Allos & Blaser, 1995). The mean excretion time of *Campylobacter jejuni* in stools is 16 days (Allos, 1997), extending up to 6 weeks (Nachamkin *at al.*, 1998). Treatment with erythromycin or ciprofloxacin is seldom required (Skirrow, 1990).

1.1.3.3. Guillain-Barré Syndrome (GBS)

The GBS defines a clinical entity that is characterised by rapidly progressing limb weakness and the loss of tendon reflexes. The disorder affects children and adults of all ages and both sexes, although men are more frequently affected than woman (Kuwabara, 2004).

Campylobacter jejuni is the most frequently isolated cause of GBS (41 %) particularly from those patients with severe disease and prolonged disability (Huges & Rees, 1997). One in every 1000 *Campylobacter* infections results in GBS, and one of every 160 *Campylobacter jejuni* type O:19 infections (Allos, 1997). Evidence suggests that *Campylobacter* LPS molecules bear a striking resemblance to host neurone (ganglioside) molecules, causing the host immune system to inadvertently attack its own cells (Nachamkin *at al.*, 1998).

1.1.3.4. Miller-Fisher syndrome

In 1956, Charles Miller Fisher, a Canadian whose specialisation was stroke, described three patients with acute external ophthalmoplegia (eye paralysis), sluggish pupil reflexes, ataxia (lack of balance) and areflexia (absent tendon reflexes). Two patients had no weakness; the other had a facial palsy and possible weakness. All three recovered spontaneously. He described this syndrome as a variant of Guillain-Barre syndrome.

1.1.3.5. Reiter syndrome

In 1916 Hans Reiter, a German military physician, described the disease in a World War I soldier who had recovered from a bout of diarrhea. Dr. Reiter described three characteristic features of the disease: inflammation of the joints, urinary tract, and eyes. More recently, doctors have recognized a fourth major feature: ulcerations of the skin and mouth.

When a preceding infection is recognized, symptoms of Reiter's syndrome appear about one to three weeks after the infection. *Chlamydia trachomatis* is the bacterium most often associated with Reiter's syndrome acquired through sexual contact. Several different bacteria are associated with Reiter's syndrome acquired through the digestive tract, including *Salmonella, Shigella, Yersinia,* and *Campylobacter*. People may become infected with these bacteria after eating or handling improperly prepared food, such as meats that are not stored at the correct temperature.

1.1.3.6. Reactive arthritis and others

Reactive arthritis is a sterile joint inflammation developed during or on after an infection elsewhere in the body, occasionally causing disability (Lichtman, 1996). Other postinfections complications include haemolytic anemia, carditis and encephalopathy (Allos & Blaser, 1995).

1.1.4. Epidemiology

Campylobacter enteritis is considered to be a food-borne disease rather than food poisoning, with infection often being derived from a range of foods and also water-based environmental sources. Campylobacters are a part of the natural intestinal flora of a wide range of domestic and wild birds and animals (Ketley, 1997).

1.1.4.1. Poultry

Most commercially raised poultry have campylobacters in their intestinal flora. The high body temperature (42 ^{0}C) of birds may explain the high optimal growth temperature of *Campylobacter jejuni* (Skirrow, 1990). Thermophilic campylobacters have been isolated from many species of bird, including, gulls, crows, puffins, owls, pigeons (Kapperud & Rosef, 1983), starlings, sparrows, blackbirds (Smibert, 1978), quails (Minakshi & Ayyagari, 1988), turkeys (Wallace *et al.*, 1998), ducks (Saleha *et al.*, 1998) and ostriches (Stephens *et al.*, 1998).

Luechtefeld (1980) reported that wild birds including ducks and geese may be excretors of *Campylobacter spp.* Some serotypes common in wild animals are those associated with infections in humans.

1.1.4.2. Cattle

In the UK, all cattle are assessed for visible cleanliness before slaughter using a 5-point scoring system (Meat Hygiene Service), with lower scores (1–2) given to visibly clean/dry animals and the higher scores (4–5) given to excessively dirty (mud, faeces) and wet animals (Reid, 2001).

The hide of cattle is known to be a source for the microbial contamination of beef, with microorganisms transferred onto the carcass from the hide, during the slaughter and dressing processes. The results of this study indicate that the brisket area on the hide of cattle most frequently carries food-borne pathogens and is therefore most likely to lead to cross-contamination of beef during the de-hiding process (Reid, 2001).

Typical prevalence rates for *Campylobacter* spp. are 5.0–53.0% (Fedorka-Cray *et al.*, 1998; Wesley *et al.*, 2000). Prevalence rates of these pathogens, however, are subject to influences such as seasonal variation (Hancock *et al.*, 1997; Mechie *et al.*, 1997; Wesley *et al.*, 2000).

1.1.4.3. Swine

Studies in the United States and Netherlands shown that more than half of commercially raised pigs excrete the organism. Washing and treating the intestines with salt diminishes but does not eliminate the contamination (Butzler, 1984).

1.1.4.4. Sheep

Campylobacter is an important cause of epizootic infections in sheep. In a recent paper published in 2004 by Zweifel from 653 sheep slaughtered 114 species of *Campylobacter* were isolated from which 64.9 % were shown to be *Campylobacter jejuni*.

1.1.4.5. Dogs and cats

In the United States have been reported an increased risk of *Campylobacter jejuni* and *Campylobacter coli* infection in humans after direct contact with diarrhoeic animals during the week before the onset of their illnesses (Saeed *et al.*, 1993). Results from case–control studies in humans with a confirmed *Campylobacter jejuni* diagnosis

conducted in Norway in 1991–1992 pointed to drinking untreated water, consumption of poultry and having contact with a dog or cat as the most-important risk factors (Kapperud, 1995). There are thus clear indications that dogs and cats might play an important role in the epidemiology of *Campylobacter* infections in humans (Sandberg, 2002).

1.1.4.6. Other reservoirs

Humans can be considered minor reservoirs for *Campylobacter jejuni*. In developing countries humans can play an important role in transmission of infection.

Water can constitute a reservoir because of its fecal contamination by wild or domestic animals as well as soil by contamination form the same source.

From all these reservoirs there are few modes of transmission of *Campylobacter* from its animal reservoir to humans:

- transmission following direct animal contact
- ingestion of contaminated foods
- milk-borne transmission
- transmission by other foods
- ingestion of contaminated water
- person-to-person transmission
- perinatal transmission
- transmission during childhood

1.1.5. Why a food-borne illness?

Doyle and Roman (1981) discovered that the bacterium is able to multiply in food at temperatures between 32-47 ^{0}C similar to the normal temperatures required for growth. Harris *et al.* (1986) suggested that the campylobacters can be transmitted from the following foods: raw and cooked chicken, game birds, undercooked fish and shellfish. To this categories raw milk can be added and other categories of raw meat. Unlike *Salmonella, Campylobacter* is not found in eggs, but is found in unpasteurised milk, contaminated water and feces of infected cats or dogs.

Poultry products are a major source of campylobacters with 70-90 % of chickens carrying the bacteria according with the last report of the Minnesota Health Department. The contamination of the products occurs by defeathering, evisceration and dipping during slaughtering (Saleha *et al.,* 1998).

One of the most common ways of *Campylobacter* transmission is via milk and diary products, especially if they are unpasteurised or if contaminated birds gain access through the sealing (Humphrey and Hart, 1998).

Presence of *Campylobacter jejuni* in raw meat is most commonly associated with porcine carcasses but the bacterium is also associated with beef and lamb at a level of 1.4 % (Skirrow, 1990).

1.1.6. Campylobacter lipopolysaccharide

In Gram-negative bacteria the outer membrane serves as an interface between the bacterium and its surrounding environment. One of the most important roles is involvement in the host-parasite relationships. The components of the outer membrane can participate in adherence of the pathogen to the host cells, invasion of the host cells, serum resistance and resistance to phagocytosis. The lipopolysaccharide (LPS) can also contribute to the toxicity of Gram-negative cells. How the LPSs are bound to the bacterial membrane is presented in figure 1.3.

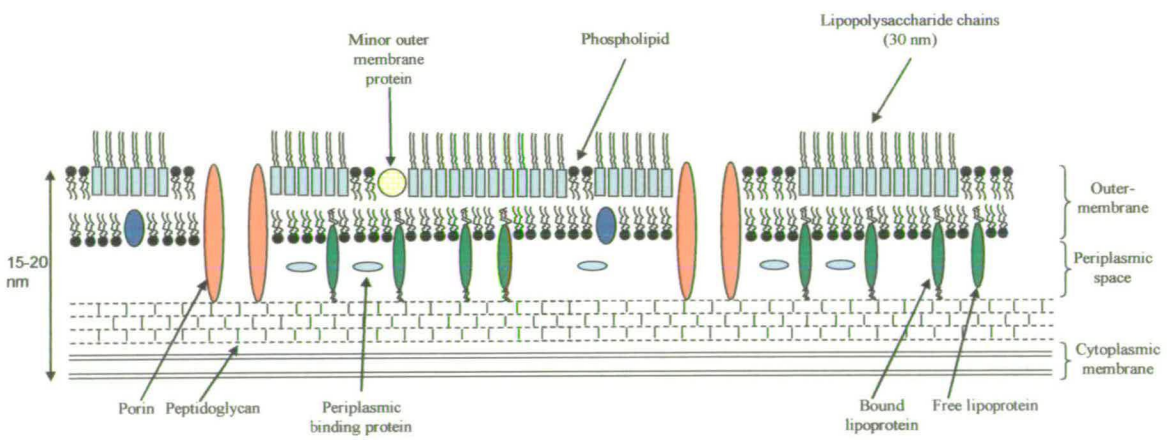


Fig 1.3. Envelope of Gram negative bacteria

1.1.6.1. Structure of Campylobacter lipopolysaccharide

LPSs of various bacterial species share a common architecture of polysaccharide or oligosaccharide (OS) covalently bound to the lipid component, lipid A. The enterobacterial LPS consists of three domains, each domain having different structural and functional characteristics - fig 1.4 (Moran, 1997).

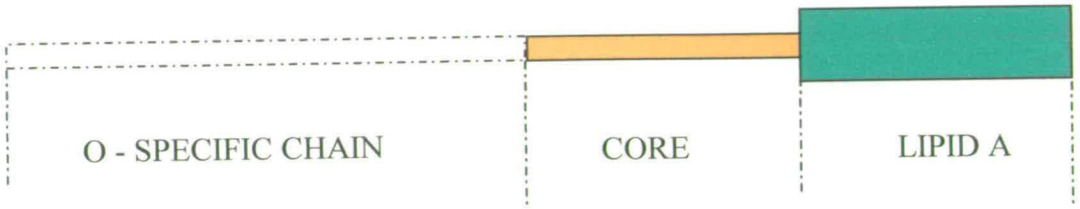


Fig. 1.4. Representation of the three region of enterobacterial lipolysaccharide

O-specific chain – contributes to the antigenicity and serospecificity of the native molecule and is a polymer of identical repeating units that may contain up to seven different sugars. This region is characteristic for each parental bacterial strain in terms of nature, ring form and anomeric configuration.

Core OS – is composed of a 10-15 series of sugars, mediates binding of T lymphocytes, is involved in immunomodulation, and is essential for the permeation of the bacterial outer membrane. The core of enterobacterial LPS can be subdivided into inner-core and the outer-core. The most common sugar constituents of the core regions of LPS are: D-glucose, D-galactose, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, L, D-Heptose and ketodeoxyoctulosonate (Kdo), (Naess, 1982, 1984, 1984 and Beer, 1986). Examples of the LPS core structures are presented in figure 1.5 (Moran, 1997).

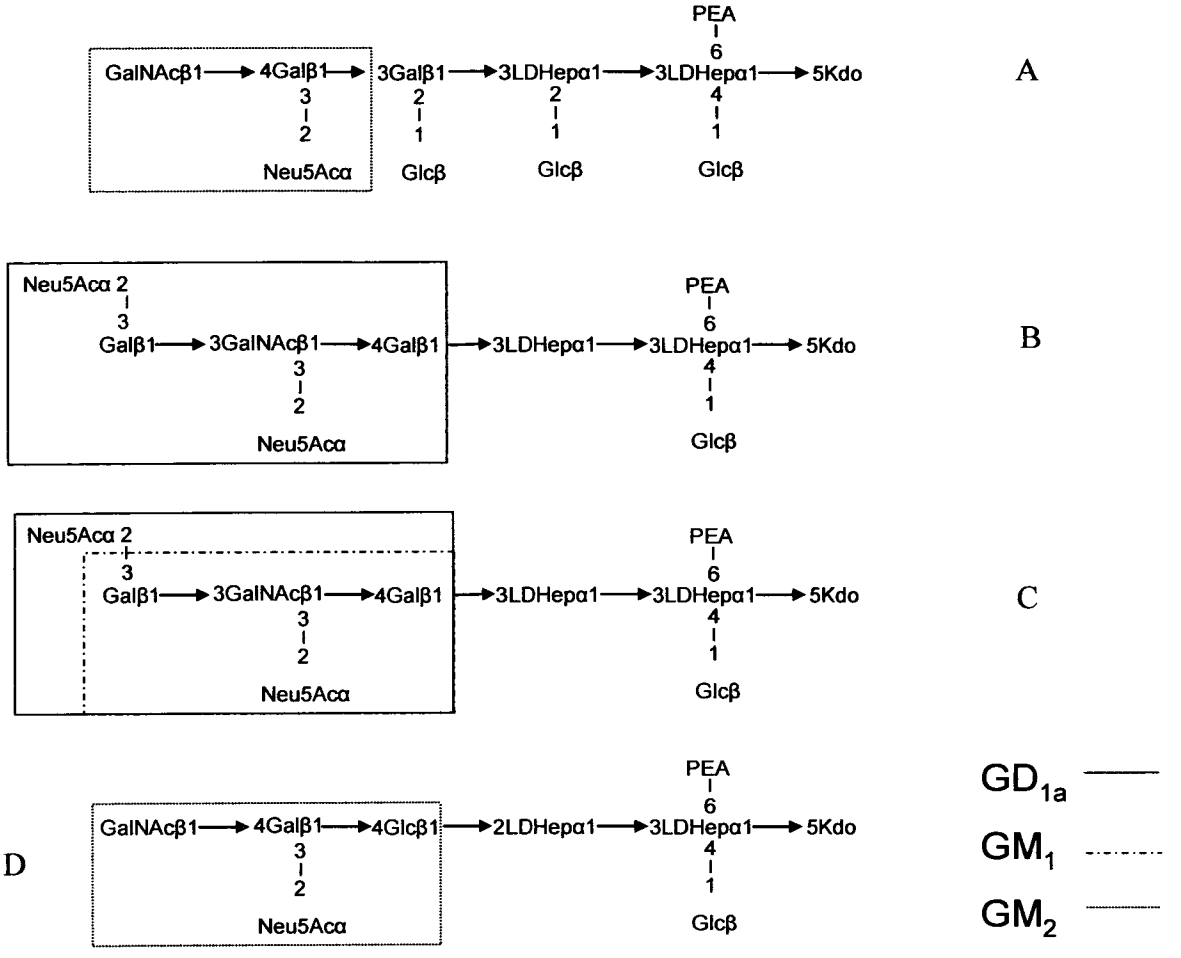


Fig. 1.5. Molecular mimicry of saccharide moieties of gangliosides by core oligosaccharides of *Campylobacter jejuni* serostrains O:1 (A), O:4 (B), O:19 (C), and O:23 and O:36 (D). PEA, O-phosphoetanolamine; Kdo, 3-deoxy-D-manno-2-octulosonic acid; LDHep, L-glycero-D-manno-heptose; Glc, D-glucose; Gal, D-galactose; GalNAc, N-acetyl-D-galactosamine; Neu5Ac, N-acetylneuraminic acid (Aspinall, 1993 and 1994). GD_{1a}, GM_1 and GM_2 are ganglioside like structures which includes molecules composed of ceramide linked by a glycosidic bond to an oligosaccharide chain containing hexose and **N-acetylneuraminic** acid (NANA, acidic sugar known also as **sialic acid**) units.

Lipid A – (fig 1.6) represents the innermost component which anchors LPS in the outer membrane. The structure of lipid A is highly conserved whereas that the core OS is more variable and the O-specific chain is highly variable. For lipid A variations in structure are given by the type of hexosamine present, the degree of phosphorylation and the presence of the phosphate substituents. The features which are mostly responsible for the variation in structure are: the nature, chain length, number and location of the fatty acyl chains (Moran, 1995 and Zähringer, 1994).

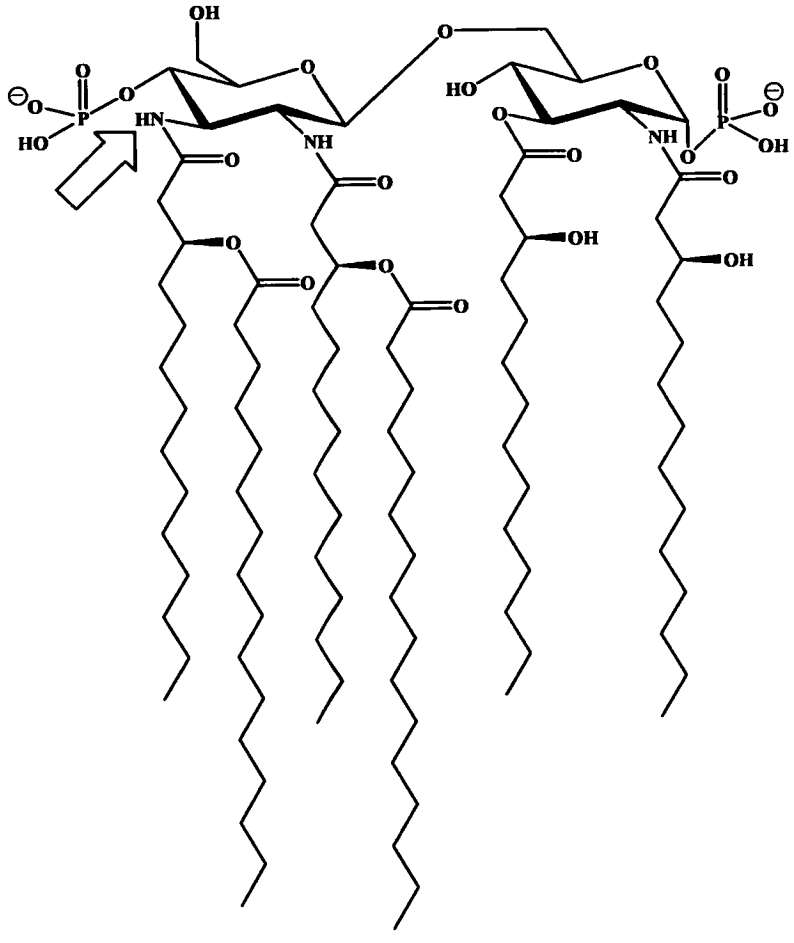


Fig. 1.6. Chemical structure of major component of lipid A of *Campylobacter jejuni* O:2. This figure shows that the major major molecular species is a hybrid backbone of a β (1`-6)-linked GlcN3N-GlcN disaccharide carrying phosphate groups at the position 1 and 4`and substituted with six fatty acids, similar with the one in other enterobacterial lipid A.
The 3` amide group is indicated with an arrow.

Given the importance of the lipooligosaccharides (those are responsible for adherence and invasion, colonisation and disease, maintainance of cell surface charge, serum resistance) mainly in the development of Guillain-Barré syndrome those structures became one of the most important features in *Campylobacter* pathogenesis. In *C. jejuni 11168* the genes involved in the biosynthesis of oligosaccharides are clustered in a single locus. Concerning the capsular polysaccharide of *C. jejuni 11168,* analysis

of the genome sequence identified a group of genes with significant similarity to the *kps* (involved in capsular polysaccharide biosynthesis) genes form *E. coli* and *Neisseria* spp which possess a type II/III capsule locus (Linton *et.al,* 2001). Karlyshev *et.al,* (2000) and Bacon *et. al,* (2001) concluded that the high molecular lipopolysaccharides (LPS) was actually a capsule. Figure 1.7 presents the complete structure of *Campylobacter jejuni 11168* LOS published by Michael in 2002.

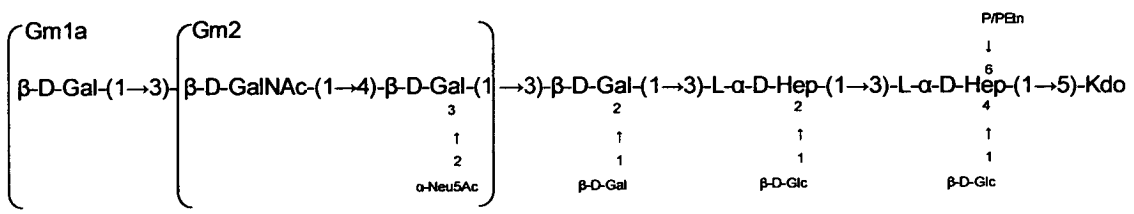


Fig. 1.7. The complete structure of *Campylobacter jejuni* NCTC 11168 LOS (Michael *et.al,* 2002) The GM2 and GM1a ganglioside mimics are covalently attached to the inner core region composed of basal core OS units, Gal, LD-Hep and Glc.

1.1.7. Genetics of Campylobacter lipolysaccharide

The aim of this part of the chapter is to make a review over the genes identified in the *Campylobacter jejuni* genome as being involved in the biosynthesis of LPS.

waaC

The deduced partial protein sequence has similarity to the amino-terminal regions of lipopolysaccharide heptosyltransferases I, which transfer heptose residue to the Kdo molecule, initiating the synthesis of the inner core. In strain 81116 at least four genes adjacent to *waaC* seem to be involved in polysaccharide biosynthesis (Benjamin *et.al,* 2000).

Orf1

Fry *et.al,* 1998 identified that in NCTC 11168 is a deduced ORF between *waaC* and *gal*E that encodes 269 residues, whereas in strain 81116 minor sequence differences disturb the ORF. *Orf1* shows no similarity to any known LPS biosynthesis gene.

galE

The protein encoded *gal*E has sequence similarity to UDP-Glc and UDP-Gal. All known *Campylobacter* LPS structures contain both Gal and Glc (Fry *et.al,* 1998).

wlaB

Higgins *et.al,* 1990 suggested that the *wla*B gene contains an ATP binding site at the signature for ABC transporters suggesting that that the protein suggesting that this protein is involved in transport across the cytoplasmic membrane.

wlaC, wlaD and wlaE

Skurnik *et.al,* 1995 identified that the protein products of these three genes possess one or two putative transmembrane segments which are similar to putative glycosyltransferases. For examples *Yersinia enterocolica* contains two genes *Trs*D and *Trs*E, which are similar to *wla*C and *wla*E, are galactosaminyltransferases involved in outer core synthesis.

wlaF

Yoshida *et.al,* 1995 suggested that this is the largest gene found in the cluster and encodes a predicted protein of 82.2 kDa. This protein is similar transmembrane

oligosaccharyltransferases from eukaryotes and the O-chain polymerase from *E. coli* probably involved in transfer of an oligosaccharide from a lipid precursor to a protein.

***wla*G**

The *wbb*P (RfpB) protein of *Shigella dysenteriae* transfers a single Gal residue to a GlcNAc-containing lipid (Gohmann *et.al,* 1994). Unlike other proteins the WlaG protein of *Campylobacter jejuni* is probably not membrane bound, since it has no significant hydrophobic regions (Gygi *et.al,* 1995).

***wla*H**

*wla*H shows similarity to enzymes (*wba*P) involved in transferring the first sugar of the subunits of O-chain to the lipid precursor undecaprenol phosphate. This first sugar differs between bacterial species and strains, but in most cases Gal is the first sugar with witch O-chain synthesis is initiated (Wang *et.al,* 1996 and Wang *et.al,* 1994).

***wla*J**

The 203-amino-acid product of *wlaI* is most similar to the 207-amino-acid NeuD protein from *E. coli,* an enzyme involved in the synthesis of the K1 polysaccharide (Annunizato *et.al,* 1995), a β-2,8-linked linear polymer of about 200 sialic acid (NeuNAc) residues. The *neu*D protein is suggested to be involved in NeuNAc transfer (Annunizato *et.al,* 1995).

***wla*J**

The protein does not have significant similarity to any other protein, and thus the function of *wla*J in *C. jejuni* remains unclear. Further analysis of the gene indicates that its presence may affect the production of the O-chain ladder (Wang *et.al,* 1996).

***wla*K**

*wla*K shows the highest similarity to the *Per* (*rfb*E) protein from *Vibrio cholerae,* a putative perosamine synthetase (Manning *et.al,* 1995) Similarity to *deg*T from *Bacillus stearothermophilus* was also found. This protein is required in the pathway to synthesize 2,6-3,6-and 4,6-dideoxyhexoses and is thought to be an enzyme for transmaminations leading to amino sugars (Thorson *et.al,* 1993). *wlaK* mutant did, however, demonstrate an altered cell morphology compared with the wild type. Wlak may be involved in core biosynthesis, perhaps affecting the sialyation of the core due to lack of synthesis of NeuNAc.

***wal*L**

Burrows *et.al,* 1996 suggested that the *wla*L protein is homologous throughout the entire protein to the *cap*D, *wbp*M, and *trs*G proteins from *Staphylococcus aureus, Pseudomonas aeruginosa,* and *Yersinia enterocolitica,* respectively

***wla*M**

Everiss *et.al,* 1994 suggested that the *wla*M gene product shows a low level of similarity to an accessory colonization factor, *acf*B, which contains a methyl-accepting chemotaxis motif.

1.2. Cytochromes P450

The Cytochromes P450 were first discovered by Martin Klingenberg (1958) who was studying the spectrophotometric proprieties of pigments in microsomal fraction from rat livers. When the reducing agent was added to microsomes and CO was bubbled in the solution a complex with an unusual Soret absorbance band was observed at 450 nm (fig. 1.8).

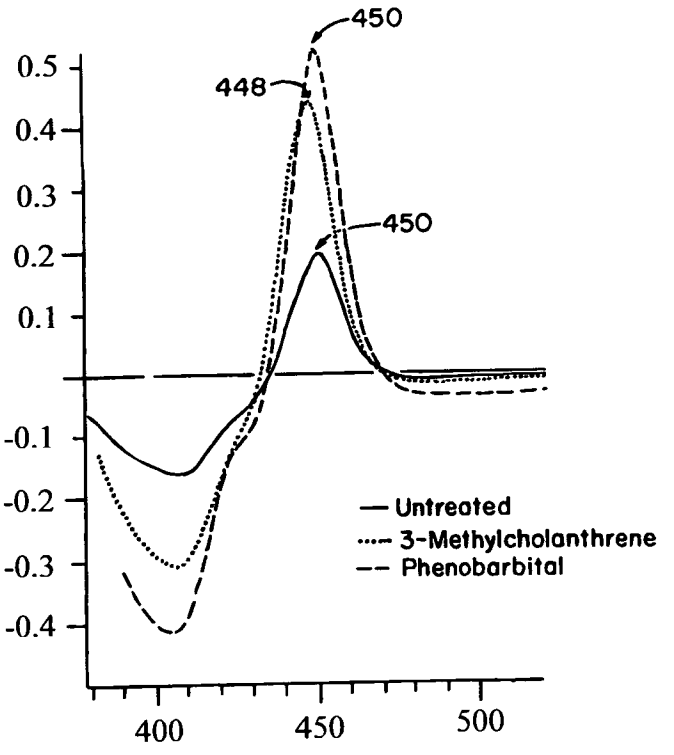


Fig. 1.8. Spectrophotometric identification of P450s from rat liver microsomes

The identification of this *red pigment* remained a curiosity until in early 1960s Omura and Sato characterised the pigment as a hemoprotein. In 1963 Estabrook demonstrated the role of adrenal cortex P450 in the hydroxylation of progesterone. In 1965 the P450s from liver microsomes were found to have a role in the metabolism of drugs and other xenobiotics.

1.2.1. What do P450s do?

P450s represent one of the most diverse families of monooxygenases being present in all domains of life: bacteria, eukarya and archaea. In the last few years, through genome sequencing projects, a large number of P450s have been identified. Tijet (2001) identified in *Drosophila melanogaster* ~ 90 sequences and over 275 P450 genes in *Arabidopsis thaliana*. In *Mycobacterium tuberculosis* 20 genes responsible for encoding P450 monooxygenases have been identified and 18 in *Streptomyces coelicolor* (Lamb, *et.al,* 2002). In other bacteria like *Mycobacterium leprae* or *Campylobacter jejuni 11168* the genome encodes for only one cytochrome P450. On the other hand the number of the identified P450s significantly increased when the human genome was characterised as having 40 cytochromes or in the rice genomes over 400 P450s have been identified.

In biochemical terms P450s are *b*-type heme containing (fig. 1.9) NAD(P)H-dependent monooxygenases. The heme iron is coordinated by the sulphur atom of a cysteine residue and is able to bind and activate molecular oxygen. P450s catalyse the stereo- and region-specific insertion of an oxygen atom, from molecular oxygen, into a wide range of organic substrates:

$$RH + NAD(P)H + O_2 + 2H^+ \rightarrow ROH + NAD(P)^+ + H_2O$$

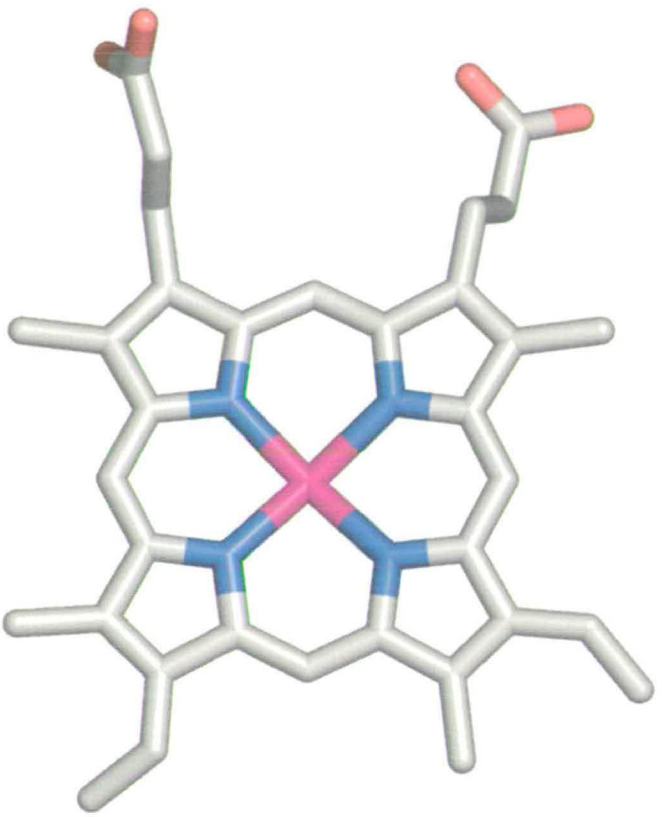


Fig. 1.9. Heme b (protoheme IX) forms the prosthetic group in different enzymes like *b*-type cytochromes and cytochrome P450, haemoglobin and myohemoglobin, catalase and most peroxidases

Electrons are shuttled from NADPH to the heme via one or two redox proteins according to the class of P450.

P450s are involved in a wide range of metabolic processes, both anabolic and catabolic. In insects for example they are involved in growth, development, feeding and protection against xenobiotics (Anzenbacher and Domanski 2001), including resistance to pesticides and tolerance to plant toxins (Scott *et.al,* 2001). Fatty acids, lignin, hormones, alkaloids, flavanoids and terpenoids are some of the most common targets for P450s in plants (Schuler, 1996). For mammals the best example might be CYP11A1 which converts cholesterol to pregnenolone. In fungi one of the most known is represented by CYP51 a P450 involved in fungal sterol biosynthesis which was described by Luppety *et.al,* (2002). Another fungal P450 is CYP107A1 which is

required to hydroxylate an important precursor in erythromycin biosynthesis (Andersen *et. al,* 1993 and Healy *et. al,* 2002). Hiroshi *et. al,* (2004) mentions fungal P450s as being involved in hydroxylation of substituted toluene to form their hydroxymethyl derivatives.

As examples from bacterial systems CYP 101 is known to metabolise camphor as an unusual energy source for its bacterial host *Pseudomonas putida*. In addition many of the bacterial P450s are known to be involved in oxidative biotransformation of natural compounds such as herbicides (Patel *et. al,* 1992 and Nagy *et. al,* 1995).

1.2.2. Classification

P450s are classified into four main classes according with their redox cofactor: (fig. 1.10)

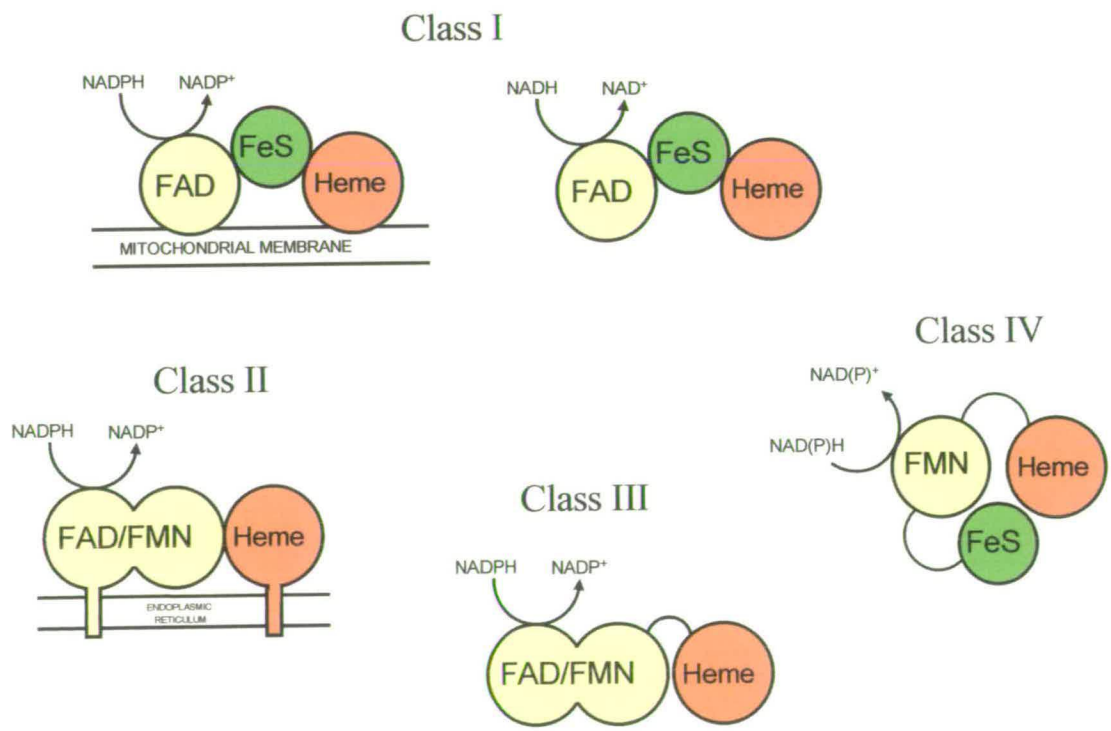


Fig. 1.10. Schematic representation of the fourth different classes of cytochrome P450 systems (personal, unpublished).

Class I which is characteristic for mitochondrial and bacterial P450s it has as components a flavodoxin reductase, an iron-sulfur protein and the cytochrome. In this class the electrons are passed to the heme via FAD and the iron-sulfur cluster containing ferredoxin. The bacterial P450s from this class are soluble proteins comparing with the microsomal P450s which are associated with the mitochondrial membrane (eg. P450cam from *Pseudomonas putida*).

Class II, characteristic for mammalian systems, has as components an FAD/FMN diflavin reductase and the cytochrome which bound together to the endoplasmic reticulum. In this class the electrons are passed form NADPH to the heme via the diflavin reductase (fig.10).

Class III is similar to class II in terms of electron transfer with the characteristic that the diflavin reductase and the heme are fused in a single polypeptide by a short hydrophilic polypeptide linker.

Class IV is characteristic for soluble bacterial P450s. Electrons from NADPH are passed to the heme via a flavodoxin reductase (FMN). One good example for this new class is P450Rhf from *Rhodococcus* sp. (Rene De Mot *et.al,* 2002).

1.2.3. Structure

Cytochromes P450 are structurally divided into two domains, the alpha helical domain that contains the heme binding site and the substrate binding site. The beta sheet domain is situated near the N-terminus that in some cases interacts with the membrane.

In primary structure P450s have, in general, 450 ± 100 amino acids with the protoporphyrin IX binding motif near the C-terminal:

FXXGXXXCXG

The structural feature common to all P450s is the presence of a phylogenetically conserved residue which provides a thiolate as a fifth ligand to the heme iron.

The fifth ligand to the heme iron is represented by a conserved cysteine residue. Except this cysteine, which is highly conserved through the P450 species there are few other conserved residues:

- a threonine residue distal to the heme ligand (eg. Thr268 in P450 BM3) (Turan *et.al,* 1998)
- a tryptophan π-stacking with the heme – important in heme binding (eg. Trp96 in P450 BM3) (Munro, *et.al,* 1994)
- a phenylalanine (eg. Phe393 in P450 BM3) (Ost *et.al,* 2001)

In their secondary and tertiary structure all the bacterial P450s have structural elements in common (fig. 1.11). In their tertiary structure they all have both the α-helixes and regions β-sheets, having an approximate trigonal prism arrangement Helices dominate the structure, with the majority of these lying parallel to the heme plan.

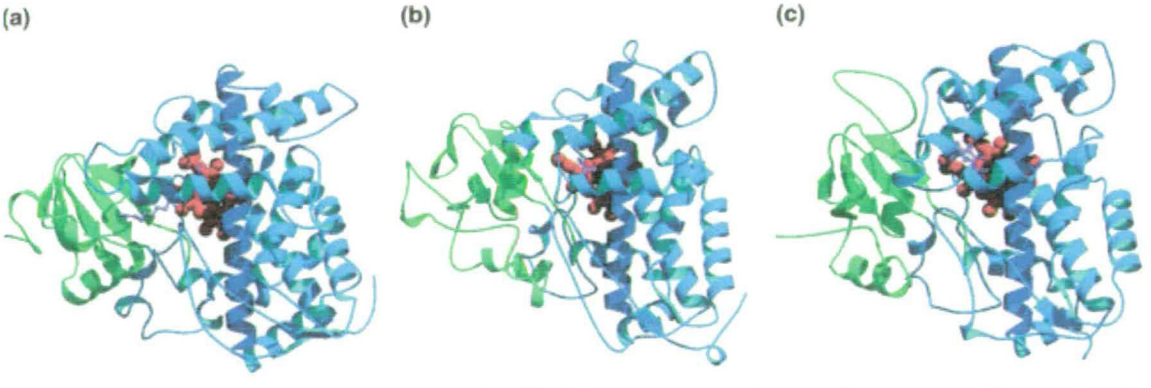


Fig. 1.11. The atomic structure of three similar P450s. (a) P450 BM3, (b) P450cam, (c) P450 eryF. The α-helix in presented in blue and the β-sheet domain in green. The heme cofactor is presented in red with the substrate in each case in purple (Munro *et.al,* 2002).

1.2.4. The Catalytic cycle

The mechanistic information is mainly provided by the studies conducted on P450cam which is considered to be similar for all P450s (Miles *et.al,* 2000). Being a bacterial P450 the nature of the reactive species and intermediates are believed to be applicable to all members of the P450 family by analogy.

In its resting state the P450 heme iron is ferric low-spin with an octahedral geometry, having a 5^{th} ligated cysteine and with water as the sixth ligand.

Step 1-2 of the catalytic cycle (fig. 1.12) corresponds to substrate binding to the active site of the P450. Binding of the substrate determines a change in geometry from octahedral to square base pyramidal and a transition from low spin (S=1/2) to high spin (S=5/2) at the heme iron.
The spin-state shift (acting as a thermodynamic switch) is essential for the P450 catalytic cycle, as the substrate binding is accompanied by a large, positive increase (~ ±100 mV) in the heme reduction potential. The positive increase of the heme reduction potential (~ ±100 mV) allows the first electron transfer to occur. In the absence of substrate the heme reduction potential is to low to allow the first electron transfer from the reductase. This step is essential because molecular oxygen has strict selectivity for ferrous-heme, dioxygen does not bind to the ferric heme.

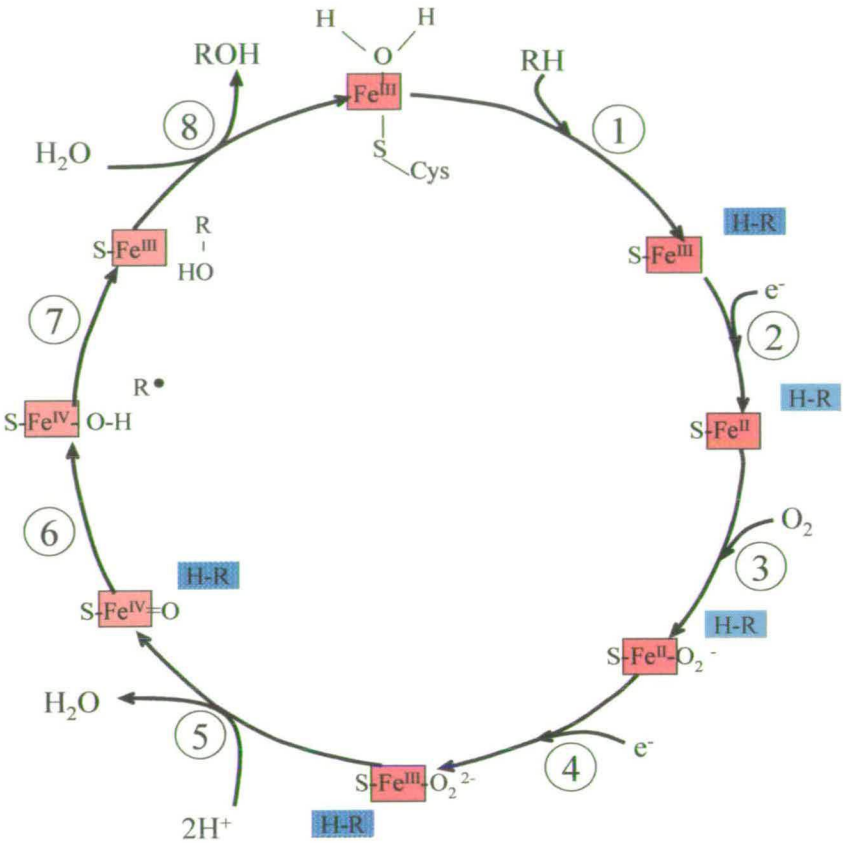


Fig. 1.12. The P450 catalytic cycle. The individual steps are detailed in text.

The reaction between organic substrates and molecular oxygen is spin forbidden and in order for reaction to occur the oxygen must be activated. Step 3 represents the first step in the process of oxygen activation. Binding of oxygen leads to the formation of super-oxy complex.

A second electron transfer is required by the stoichiometry of the reaction (step4) leading to the formation of the per-oxy species.

In step 5 the O_2^{2-} reacts with two protons from the surrounding solvent breaking the O-O bond. Consequently an O_2 molecule is forming by protonation a water molecule and is leaving the complex. A highly reactive oxy-ferryl specie is formed.

In the followings steps of the catalytic cycle the oxygen reacts with substrate which in the step 7 is hydroxylated and the enzyme returns to its initial step.

1.2.5. Reaction types

P450s can catalyse a variety of reactions despite their conformity to the same catalytic cycle. The general reactions are:

- carbon hydroxylation (hydroxylation of fatty acids, 11β-hydroxylation of steroids);
- aromatic hydroxylation (characteristic to P450s from hepatic microsomes);
- heteroatom dealkylation and oxygenation (the reaction is limited to compounds containing N, S and P and halogens as heteroatoms);
- oxidative bound cleavage (conversion of cholesterol to pregnenolone by P450scc);

In humans P450s play a central role in cellular metabolism and the maintenance of cellular homeostasis. In table 3.1 are included few examples of reactions in which P450 might be involved.

Table 1.3

Examples and types of reactions catalysed in humans and their tissue localization

Tissue	Mitochondria	Microsomes
Adrenal Placenta Ovary Testis	Choloesterol Side Chain Cleavage 11β-hydroxilation of DOC	21-OH of progesterone 17α-OH of Pregnenolone Aromatase
Kidney	11α-OH of Vitamin D	ω hydroxylations of fatty acids
Liver and Intestine	27-OH of cholesterol	Bile acid formation Polyunsaturated Fatty acid epoxidation <u>Xenobiotic metabolism</u> N and O dealkylations Alcohol oxidation Alkane and arene oxygenation Aromatic hydroxylations

Project aim

Recently, the complete genome sequence of *Campylobacter jejuni NCTC 11168* was published revealing the presence of only one open reading frame (Cj1411c) encoding for a cytochrome P450, in contrast to 20 found in *M. tuberculosis*. The gene Cj1411c encodes for a soluble 52.6 kDa protein with a predicted isoelectric point of 9.3.

The P450 gene is part of an operon which hosts genes involved in the synthesis of cell surface components (capsula). *Campylobacter* capsule are important in adherence, invasion and colonisation of host cells (Bacon *et.al,* 2001 and Guerry *et.al,* 2002) and for maintenance of cell surface charge and serum resistance. These capsule are thought to cause autoimmunity leading to Guillan-Barre and Miller-Fischer syndromes (Jacobs *et.al*, 1995).

The structure of the lipoolygosaccharides and capsule polysaccharide was published last year (Michael *et.al*, 2002) revealing that the strain possessed a type II/III capsule locus found in other microorganisms such *Nisseria meningitidis.*
This project focuses on the cloning and characterisation of the only P450 enzyme of the human pathogen *Campylobacter jejuni NCTC 11168.* We aim to understand the metabolic role of this P450 cytochrome in order to elucidate its possible use as a new target for drug design.

Chapter 2
Materials and methods

2.1. Bacteria related

2.1.1. Sterilisation

Solution and media were typically sterilised by autoclaving for 15 minutes at 121 ^{0}C and 15 lb/inch^2. Alternatively, solutions were sterilised by filtration. Small volumes were filtered through acrodisc syringe filters (0.20 μm and 0.45 μm, Sartorius AG). Large volumes of solutions were filtered through 250 ml and 500 ml filter units (0.2 μm and 0.45 μm, Nalgene). Ordinary glassware were dry sterilised by baking in an oven at 180 ^{0}C for 2 hours such and glass pipettes for 4 hours.

2.1.2. Media

2.1.2.1. *E. coli*

All media were stored at room temperature after autoclaving. For solid media 1.5 % agar was added (w/v) prior to autoclaving.

Media	Per litre							
	Bacto-tryptone	Bacto-yeast extract	KCl	$MgSO_4$	$MgCl_2$	NaCl	Glycerol	Glucose
Luria-Broth	10 g	5 g	-	-	-	10 g	-	-
Terrific Broth	12 g	24 g	-	-	-	-	4 ml	-
SOC	20 g	5 g	0.186 g	2.47 g	2.03	0.58 g	-	3.60 g
pH adjusted to 7.2 with 5 M NaOH								

2.1.2.2. *Campylobacter jejuni NCTC 11168*

Muller-Hinton Broth (OXOID, UK) was prepared by adding 22 g to 1 l of distilled water. For plates 1.5 % agar was added and FBP (which is responsible for the elimination of the oxygen from the media – table 2.1).

Table 2.1

FBP composition

FBP strength	Plate	Broth
Sodium pyruvate	2.5 g	0.025 g
Sodium metabisulphite	2.5 g	0.025 g
Iron (II) sulphate	2.5 g	0.025 g
Distilled water	50 ml	5 ml
Per 100 ml medium	1 ml	3 ml

2.1.3. Growth conditions

2.1.3.1. *E. coli*

The *E. coli* strains were grown in Luria-Bertani media at 37 ^{0}C supplemented with appropriate antibiotics with shaking at 200 rpm. The expression strain was grown at 18 ^{0}C with shaking at 230 rpm.

2.1.3.2. *Campylobacter jejuni NCTC 11168*

The target strain was cultured in Mueller-Hinton Broth supplemented with FBP. The purpose of FBP is to scavenge free radicals from the media. All cultures were incubated at 42 ^{0}C for 42 hours. In order to obtain a pure culture antibiotics were added to the medium (Skirrow, 1979). Both FBP and Skirrow antibiotics (table 2) were filter sterilised.

2.1.4. Antibiotics

2.1.4.1. *E. coli*

The antibiotics were used according with the following concentrations:

Antibiotic	Concentration
Ampicilin	100 µg / ml
Chloramphenicol	34 µg / ml
Tetracycline	15 µg / ml
Carbenicilin	50 µg / ml
Kanamycin	50 µg / ml

2.1.4.2. *Campylobacter jejuni NCTC 11168*

Composition of Skirrow antibiotics

Antibiotic	Amount added to 1 l medium
Vancomycin	10 mg
Trimethoprim	5 mg
Polymyxin B	2500 IU
Distilled water	4 ml

2.1.5. Strains used

2.1.5.1. *E. coli*

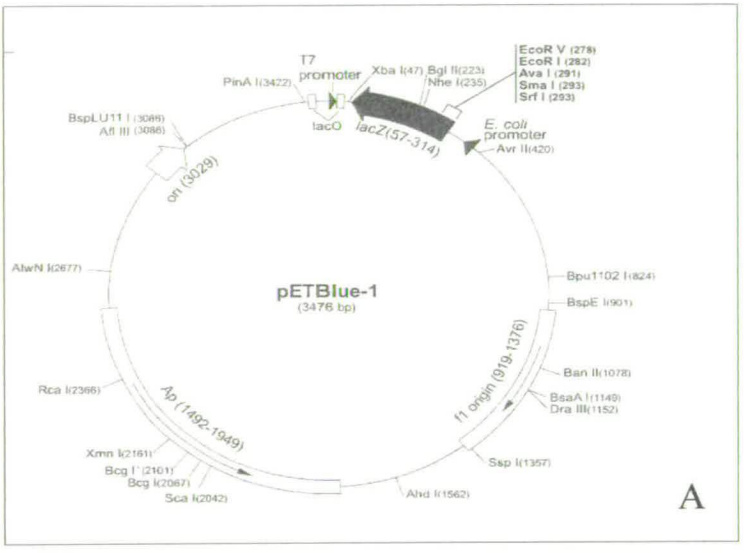
Strain	Genotype	Source
TG 1	*supE hsdΔ5 thi (Δlac-proAB) F' [traΔpro AB$^+$ lacI^q lacZΔM15]*	Lab stock
NOVA BLUE	*endA1 hsdR17 (r_{K12}-m_{K12}+) supE44 thi-1 recA1 gyrA96 relA1 lac [F' proA$^+$B$^+$ lac^{q}ZΔM15::Tn10(Tc^R)]*	NOVAGEN
TUNER (DE3) placI	*F$^-$ ompT hsdS$_B$ ($r_B^-m_B^-$) gal dcm lacY1 (DE3) placI(Cm^R)*	NOVAGEN

E. coli strains were stored for up to two weeks at 4°C. To preserve strains indefinitely, bacteria were grown in appropriate medium to stationary phase. One millilitre of culture was mixed with 77 µl DMSO and frizzed at –80 ^{0}C

2.1.5.2. *Campylobacter NCTC 11168*

The strain was donated by Professor David Kelly from the University of Sheffield. Stocks were made in 15 % glycerol plus Mueller-Hinton Broth in 1 ml plastic tubes and frizzed at –80 ^{0}C.

2.1.6. Plasmids used



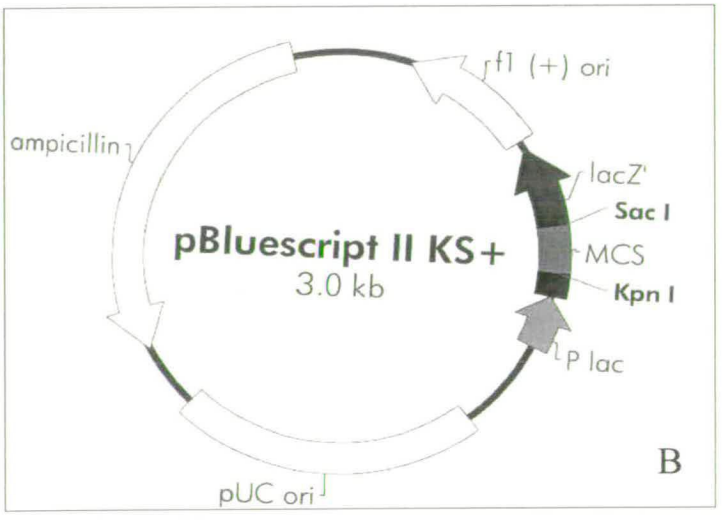


Figure 2.1 Vectors used.
A. pETBlue1 Blunt vector from NOVAGEN
B. pBlueScript vector from STRATAGENE

Other plasmids:

Plasmid	Structure	Used for:
pBP450Cj	P450 gene in EcoRV site of pBlue1 Blunt Vector	This study
pBSF3	The F3 fragment into the SmaI site of pBlueScript KS+	This study
pBSF3-Km^+	Km cassette into SmaI site of pBSF3	This study
pSUP10121	Containing a 900 bp Km^R gene in EcoRV site	Lab stock
pBP450CjC HIS	Containing the 6 HIS Tag at the C terminus of the protein sequence	This study

2.1.7. Primers used

Name	Structure (5'-3')
Cj1411cFOR	ATGAGTGAATGCCCCTTTTTTCCAAAAC
Cj1411cREV	GTCATAGCTTTCTTTTGCTAAATTTTATC
Cj1411cREVHIS	CGTCAGTGGTGGTGGTGGTGGTGTAGCTTTCTT TTGC
F300	TACAGAGCTTTTGCGTCCAAG
F800	GCAGGACATGAAACCACAGCAAG
pETBlueUP #70604-3	TCACGACGTTGTAAAACGA
pETBlueDOWN #70603-3	GTTAAATTGCTAAGGCAGTCA
F1FOR	TGCTATAGCCATAGTGGC
F1REV	AAAGGTAAAAATCCCGGGTGAAAAGAGTTAG AACTAAAATTCGC
F2FOR	TAACTCTTTTCACCCGGGATTTTTACCTTTTCTT CTAA
F2REV	GAATTTGTGCTTTTTGTG
F2REV2	GAATTTGTGCTTTTTGTGCTACTTGG
F1FOR2	TGCTATAGCCATAGTGGCTGTAGTTTG
Tn5Km(L)	CACGATATCCCGGAATTGCCA
Tn5Km(R)	CACGATATCTCAGAAGAACTC

2.1.8. Preparation of $CaCl_2$ competent cells

The desired *E. coli* strain was cultured in LB at 37 ^{0}C for 18 hours. 200 µl of the o/n culture was used to inoculate 5 ml of LB and cells were grown until O.D 0.5. Cells were than harvested at 5000 rpm for 3 minutes and resuspended in 2 ml of 0.1 M ice cold $CaCl_2$. After 40 minutes cells were harvested like before and resuspended in 1 ml of 0.1 M ice cold $CaCl_2$ and kept in ice for 45 min – 1 hour.

2.1.9. Transformation

2.1.9.1. *E. coli*

In 1.5 ml ice cold eppendorfs were added 1 or 2 µl plasmid DNA (depending of concentration) and 200 µl of $CaCl_2$ competent cells were added to the tube. Samples were kept on ice for 45 minutes. In each transformation controls were prepared with a test plasmid and a no DNA control (10 µl of water + 200 µl competent cells). A heat shock was than applied at 42 ^{0}C for 105 seconds. Tubes were than kept on ice for 5 minutes and at the end 800 µl of LB was added to each tube. Cells were incubated for 60 minutes at 37 ^{0}C (shaking) and plated on LB plus the appropriate antibiotics and incubated over night at 37 ^{0}C.

2.1.9.2. *Campylobacter NCTC 11168*

a) Natural transformation

For natural transformation *C. jejuni 11168* was cultured on Muller-Hinton agar plates o/n at 42 ^{0}C. The cells were harvested and diluted to a concentration of 10^8 cfu / ml. To induce competence, 200 µl cells were incubated for 3 hours at 37 ^{0}C on 1 ml Muller-Hinton Broth in 2 ml micro test tube. Then the DNA was added and incubated for another 3 hours at 37 ^{0}C. Cells were harvested and plated on Muller-Hinton plates

supplemented with Skirrow antibiotics and incubated at 42 ^{0}C for 48 hours (Wang, 1990).

b) Electroporation

For transformation by electroporation *C. jejuni 11168* was cultured on Muller-Hinton agar plates o/n at 42 ^{0}C. Cells were harvested in 1 ml EBP (272 mM sucrose, 15 % glycerol, 2.43 mM K_2HPO_4, 0.57 mM KH_2PO_4 at pH 7.4). Cells were harvested again and washed twice in EBP. 1μg DNA was dissolved in water and mixed with 50 μl electrocompetent cells and in a pre-chilled 0.56 mm cuvette were pulsed at 0.7 kV, 25 μF and 600 Ω. The content of the cuvette was mixed with 0.2 ml Muller-Hinton Broth and plated on Muller-Hinton agar plates for 5 hours at 37 ^{0}C. At the end of incubation the cells were harvested and plated on Muller-Hinton agar plates supplemented with Skirrow antibiotics and 300 μg Km / ml to select transformants (Miller 1988).

2.1.10. Blue / white screening

In order to identify the recombinant vectors from non-recombinant vectors the plasmid (pETBlue1 Blunt Vector) encode a functional *lacZ* α-peptide that complements the *lacZ* ω-fragment expressed by the host strain. The resulting β-galactosidase can cleave the chromogenic substrate X-gal to yield a blue colony phenotype. The white colonies phenotype are characteristic for the cells which contain the recombinant vectors because the inserts are cloned within the α-peptide open reading frame.

For blue / white screening of recombinants IPTG and X-gal are necessary. 35 μl of 50 mg / ml X-gal in dimethyl formamide and 20 μl of 100 mM IPTG were spread on the plates before plating the cells. The resulting white colonies were used in the following cloning steps.

2.1.11. Capsular sugar extraction

After centrifugation *Campylobacter* cells were resuspended in 70 % ethanol and were stirred for few minutes. Cells were harvested again and resuspended in acetone and air dried after collection. For hydrolysation was used 2 M trifluoroacetic acid (TFA) and samples were heated at 120 ^{0}C for 1 hour (10 mg acetone powder take 1 ml TFA), and dried in a speed vacuum. The resulted pellet was resuspended in a small amount of water (Karlyshev, 2001)

2.1.12. Capsular polysaccharides preparation

Capsular polysaccharides were prepared using a method described by Hitchcock (1983). Cells from 42 hours MH agar plates were resuspended after centrifugation in 100 µl lysis buffer containing 31.25 mM Tris-HCl (pH 6.8), 4 % SDS, 0.025 % bromphenol blue and 20 % glycerol. Samples were heated at 100 ^{0}C for 5 minutes. 20 µl of this was transferred in a fresh tube and 1 µl of 20 % proteinase K was added to the solution. The reactions were incubated for 1 hour at 50 ^{0}C.

2.1.13. Silver staining of the capsular polysaccharides

For detection the capsular polysaccharides were loaded into a 10 % polyacrylamide gel following a method described by Chao-Ming (1982) in seven steps. In step 1 the gel is fixed o/n in 40 % ethanol and 5 % acetic acid. In the second step the gel was washed for 5 minutes in a solution containing 0.7 % periodic acid, 40 % ethanol and 5 % acetic acid. The gel was washed for 15 minutes, in the third step, in a second dish with water this stept being repeated 3 times. Staining was performed in the forth step: 2 ml of ammonium hydroxide was added to 28 ml of 0.1 N sodium hydroxide, 5 ml of silver nitrate (20 %) is added and the solution is vigorously mixed. At the end 115 ml water is added to make a total volume of 150 ml. The gel is kept in this solution for 10 minutes. After this step the gel is washed again like in step 3. In step 6 the gel is kept for 2-5 minutes in 200 ml of 50 mg citric acid and 0.5 ml of 37 % formaldehyde per litre. At the end the gel is washed and stored in water.

2.1.14. Lipopolysaccharide preparation

After 42 hours growth both the wild type and the knockout strain were collected by centrifugation and freeze dried. Cells were washed with a wash buffer (20 mM Tris pH 7.4, 0.15 M NaCl, 10 mM $MgCl_2$) and re-centrifuged. The pellet was then diluted with water to a concentration of 10 % and heated to 65 ^{0}C. A similar volume of phenol has been heated to the same temperature and mixed with the pellet by stirring for 10 minutes followed by cooling to 2-4 ^{0}C on ice. The mixture has been centrifuged than at 5000 g for 15 minutes at 0 ^{0}C and the upper layer was dialysed against water for 16-40 hours. The volume was reduced to half by evaporation and centrifuged for 4 hours at 100000 g. A gel layer was formed after centrifugation and it contained the LPS which was resuspended in water and freeze dried.

2.1.15. Isolation of cell envelope

For isolation of cell envelopes from *campylobacter jejuni 11168* Blaser method was used (Blaser, 1997). This method involved resuspension of the washed pellet in 10 mM Tris-HCl (pH 7.4) followed by sonication on ice four times for 30 seconds with 30 seconds rest. The lysate was then centrifuged twice for 20 minutes at 5000 x g to remove whole cell. The supernatant containing the cell envelopes was retained.

2.1.16. Paper chromatography

Paper chromatography performed using Whatman paper no 1 and using the solvent butanol / acetic acid / water. The samples were loaded as small spots (1-1.5 cm in diameter) and spaced at least 2.5 cm apart. The sugars were identified by silver staining of the chromatogram.

2.1.17. Silver stain of the chromatogram

The paper chromatogram was silver stained by the following method: the paper was dipped in solution A and dried for 3-5 minutes and next in solution B two times and dried for 10-20 minutes accordingly with the sensitivity required. The chromatogram was dipped in solution C and then put in distilled water in a basin and washed thoroughly o/n and dried flat, this being the last step of the procedure.

Solution A: dissolve 0.2 g $AgNO_3$ in 0.4 ml H_2O and add 26 ml acetone with rapid stirring.

Solution B: 25 ml of 10 M NaOH were mixed with 100 ml absolute ethanol

Solution C: 10 % sodium thiosulphate in distilled water

2.1.18. HPLC

The hydrolysed sugars were analysed by HPLC. Samples were filtered in 0.45 μm filters and eluents filtered degassed. Water has not been filtered because ultra pure water was used instead.
The HPLC was performed in a Dionex HPLC with a CarboPacA1 column using the following program for NaOH concentrations: 0-5 min, 10 mM, 5-30 minutes, 0 mM, 30-70 min, 0-800 mM (linear gradient), 70-75 min, 800 mM, 75-76 min, 800-10 mM, 76-85 min, 10 mM.

2.1.19. Nuclear Magnetic Resonance (NMR)

Sugars were prepared as described in chapter 2 and analysed on a Dionex HPLS with a CarboPac PA1 column. The eluent flow-rate was 1 ml/min (0.5 M, 0.6 ml/min) was added post column, and sugars were quantified with a pulsed amperometric detector (with gold electrode).

The 1 Dimensional (1D) ^1H spectra were acquired on a 600 MHz Bruker Avance spectrometer equipped with a cryoprobe. The purified peak 2 from the NC1 spectra

was dissolved in D_2O and spectra collected at 25 ^{0}C (16 scans were used to acquire the data). The HOD signal (4.78 ppm) was used as an internal standard to reference the spectra. By comparison of the standard probe spectrum (L – Arabinose from Sigma-UK) with the sample spectrum a perfect identity in profile was identified. The peak 2 from the NCI strain was identified to be arabinose.

All NMR experiments were performed on a 600 MHz BRUKER Avance spectrometer equipped with a cryoprobe. 1D ^{1}H NMR spectrum of wild type was acquired using 512 scans.

The 2D gradient-selected COSY experiment was acquired in the magnitude mode using 32 scans per each of 1408 increments. The relaxation delay was 1.5 s and the acquisition times in the t_2 and t_1 were 340 and 140 ms, respectively. The total acquisition time was 25 hour. The 2D TOCSY experiment was acquired in the phase-sensitive mode using 80 scans per each of 512 increments. The relaxation delay was 1.5 s and the acquisition times in the t_2 and t_1 were 340 and 50 ms, respectively. The total acquisition time was 24 hours. A 140 ms DIPIS-2 spin-lock was used at 8.3 kHz B_1 field strength.

2.1.20. Viable counts

Viable counts method was used to count the viable cells. *Campylobacter* strain was grown in liquid media for 42 hours at 42 ^{0}C. Then aliquots of culture were used to inoculate pre-warmed medium. Samples were taken out at 12, 15, 18, 24, 30, 36, 42 hours an plated on solid media after appropriate dilution. Only the plates with 100-300 colonies were counted. The concentration of bacteria in the original sample is calculated as:

$$\text{CFU ml}^{-1} = \text{colonies on plate / final plate dilution}$$

2.1.21. Motility

The motility experiments were done according with the method described by Martinez (2004). Cells were placed in the middle of the plates of Mueller-Hinton Agar (0.4 %)

which were incubated for 42 hours at 42 ^{0}C. Motility was characterised like a area of growth around the central point of the plate and measured in millimetres (Martinez-Rodriguez, 2004).

2.1.22. Competition

Fresh Mueller-Hinton broth was inoculated with fresh 11168 wild type culture and knockout and incubated as described above. After 42 hours growth total viable counts were determined on Mueller-Hinton Agar. Muller-Hinton Agar plates supplemented with kanamycin were used to determined the viable counts for the KO. The WT cells were calculated by difference between the total viable counts and the KO viable counts (Martinez-Rodriguez, 2004).

2.1.23. Transmission electron microscopy - negative stain

To perform this the cells were deposited dropwise on a 3 mm carbon grid (Agar Scientific) and the negative stain was performed with uranyl acetate aqueous 4 %. Samples were imaged in Philips Biotwin CM120 and all pictures were taken with Kodak electron image film SO-163.

2.1.24. Transmission electron microscopy - thin section and positive stain

The cells were gently centrifuged and the supernatant was removed and replaced with fixative (derivative of Karnovsky):

i. 2 % paraformaldehyde
ii. 2.5 % glutar aldehyde (EM Grade)
iii. sodium cacodylate buffer 0.1 M pH 7.4

The material has been left for 1 hour in fixative and it had three changes of fresh buffer after that. This was then embedded in low melting point agar (Sigma) and re-fixed as above. The material was post fixed in osmium tetroxide 1 % aqueous for 1 hour. The following steps were dehydration in an alcohol series and infiltration with

Spurrs resin using propylen oxide as linking solvent (Agar Scientific). The polymerisation was realised by incubation of the samples at 70 ^{0}C in the oven.

Sections were made on a Leica UCT Ultramicrotome. All sections were 90 nm thick (gold) and were collected on HEX 400 and 200 MESH grids (Agar Scientific).

Staining was achieved by means of a triple stain method:

- lead citrate (CO_2 free environment) for 5 minutes
- uranyl acetate (4 % aqueous in dark) for 1 hour
- lead citrate (CO_2 free environment) for 10 minutes

Grids were examined and photographed as for negative staining procedure.

2.1.25. Scanning electron microscopy

For scanning electron microscopy the cells were fixed in Karnovsky's as described for transmission electron microscopy on a millipore-type filter of nylon (0.2 µm) construction (Sigma). Cells were post fixed with osmium tetroxide 1 % aqueous for 1 hour washed and then dehydrated in acetone series. Cells were then critically point dried in Polaron E 3000.

The dried cells specimens were mounted on pin stubs (Agar) 12.5 µm aluminium with epoxy (Araldite Quick Dry - Agar Scientific) and coated with gold palladium on a gatan alto cryo unit. Imaging was performed on a Hitachi 4700 SEM. Digital captures were used.

2.2. DNA related

2.2.1. Genomic DNA extraction

Genomic DNA extraction was performed using a Wizard® Genomic DNA Purification Kit from Promega UK. The genomic DNA was extracted from 80 ml culture in case of *Campylobacter jejuni 11168.*

2.2.2. Preparation of plasmid DNA

Plasmid DNA from *E.coli* was prepared using the QIAprep kit (Quiagen) following the manufacturer's instructions. DNA was extracted from 5 ml of *E. coli* cell culture grown to stationary phase.

2.2.3. Agarose Gel-Electrophoresis

Agarose gel electrophoresis of DNA fragments produced by restriction endonuclease digest or generated by PCR was typically performed in 0.8-1% (w/v) agarose gels. Gels were prepared by melting the appropriate amount of agarose in 1X TAE buffer and adding ethidium bromide to a final concentration of 0.1 μg/ml. DNA samples to be analysed were mixed with 1X Agarose gel loading buffer and water up to 14 μl and loaded directly onto the gel. The gel was placed in 1X TAE buffer and run at a constant voltage of 45 mA for 1 hour. To estimate the size of DNA fragments, 1Kb Plus DNA Ladder (Gibco BRL) was also loaded on the gel. The DNA could be visualised on a UV transilluminator.

1 X TAE Buffer (Tris-acetate):

0.04 M Tris – acetate

0.001 M EDTA

2.2.4. Purification of DNA from agarose gels

As an alternative to the previous protocol, the DNA fragments produced by PCR or by restriction endonuclease digestion were purified after separation on an agarose gel. Using a clean razor blade, the band of interest was excised from the gel and further purified using the QIAquick gel extraction kit (Qiagen), following the manufacturer's guidelines. DNA was typically eluted in 20 µl of elution buffer (supplied with the kit) and stored at -20°C.

2.2.5. Ligation of DNA fragments

Ligations were typically performed in a total volume of 15 µl, containing 20-30 ng of vector DNA, approximately 8 times this amount of insert DNA, 1X ligation buffer and 2 units of T4 DNA ligase (Promega or Novagen). The reactions were incubated at 16 ^{0}C overnight.

2.2.6. Restriction digests

Restriction endonuclease digestion of DNA was typically performed in volumes of 15-50 µl. These volumes contain the required quantity of DNA (usually 2-5 µg) and the appropriate restriction buffer (as supplied by the manufacturer) at 1X concentration. The restriction enzyme (typically 2-5 units) was added the last to the reaction taking care to keep the restriction enzyme volume below 10 % of the total volume. The restriction digest was incubated at the temperature recommended by the supplier for a period of 2-10 hours. For the double digest reaction, both enzymes were added concurrently providing that both would retain full activity in a suitable buffer. The products of the digestion were analysed by agarose gel electrophoresis and isolated from the gel and purified.

2.2.7. Automated sequencing

A typical reaction mix was set up as follows:

Template DNA	200 ng of plasmid DNA or 100-120 ng of PCR product
Primer	1.6 pmol
Terminator mix	4 µl
Sterile, distilled water	up to 10 µl

The following sequencing program was run for 25 cycles:

Step1: 96°C for 30 seconds
Step2: 50°C for 15 seconds
Step3: 60°C for 4 minutes

When was necessary DNA was precipitated from the reaction mix by adding 25 µl of ice-cold ethanol (abs.) and 1 µl of 3 M NaOAc, pH 5.2, incubating on ice for 20 minutes followed by centrifugation (13,000 rpm, 30 minutes, 4°C). The pellet was washed with 250 µl of ice-cold 70% (v/v) ethanol and dried on bench for 1 hour. Samples were processed by the ICMB Automated Sequencing service on an ABI377 instrument.

2.2.8. Polymerase chain reaction (PCR)

Specific regions of DNA were amplified using the polymerase chain reaction (PCR). A PCR reaction mix was set-up in a 0.5 ml Eppendorf tube as follows:

10X Polymerase Buffer	1X
Template DNA	10-500 ng of genomic DNA10-30 ng of plasmid DNA
Oligonucleotid primer 1	0.5 µM
Oligonucleotid primer 2	0.5 µM
dNTPs (dATP, dCTP, dGTP, dTTP)	200 µM each

$MgCl_2$ (when necessary)	1.5-6 mM
DNA polymerase[a]	1.25 units per 50 µl
Sterile distilled water	up to 50 µl

PCR reactions were carried in a PHC - 2 cycler from TECHNE programmed depending on the length of the desired product and the annealing temperature of oligonucleotide primers used.

A typical program contained the following steps:

Step 1 – Denaturation:	94°C	3 minutes
Step 2 – Denaturation:	94°C	1 minute
Step 3 – Annealing:	T_d°C[b]	1 minute
Step 4 – Extension:	72°C	1 minute[c]
32 cycles of Step 2 – Step 4		
Step 5 – Final extension	72°C	3 minute

[a]for most applications, *Pfu* DNA polymerase was used.

[b]T_d°C is the annealing temperature calculated for oligonucleotides up to 20 bases long with the formula:

$$T_d°C = \{4(G+C) +2(A+T)\} - 4$$

[c]One minute extension time per every kb of the length of the desired product.

2.2.9. Colony PCR

One bacterial colony was resuspended in 50 µl dH_2O and boiled for 5 minutes. 5 µl of the lysate was used to perform PCR reaction under the conditions described above.

2.3. Protein related

2.3.1. Spectrophotometric quantitation of proteins

Protein concentrations were determined by the Bradford method using the Bio-Rad Protein Assay and following the manufacturer's instructions. Briefly, a series of BSA standards were prepared by diluting 10 mg/ml BSA (NEB) in sterile, distilled water to give four to five dilutions between 0.2 and 0.9 mg/ml. One hundred microliters of each standard solution was mixed with 5 ml diluted (1:5) Bradford Dye Reagent (Bio-Rad), vortexed and allowed to stand for 5 minutes at room temperature. The OD_{595} of each sample was measured (with a 0 mg/ml solution as a blanc) and the values used to plot a graph of BSA concentration versus OD_{595}. The protein sample of unknown concentration was simultaneously treated in the same manner and the concentration was extrapolated from the standard plot. Each protein concentration was assayed in triplicate.

2.3.2. Haem staining

For haem staining, after electrophoresis the haem stain method followed four steps:

1. Was the gel in solution A for 5 minutes
2. Was in solution B for 5 minutes
3. Leave on dark for 15 minutes
4. Add 1 ml of hydrogen peroxide

When sufficient colour developed the reaction was stopped by rinsing with dH_2O

Solution A (100 ml in total volume)

- 30 ml methanol
- 10 ml NaOAc pH 5.2, 2.5 M

- 60 ml H_2O

Solution B

- 100 ml – As for solution A
- 0.04 g tetramethylbenzidine

2.3.3. SDS-PAGE

It is generally used to separate proteins according to their molecular weight. Two layers of polyacrylamide gels were used 12 % resolving gel and 5 % staking gel.

Components	Resolving gel (ml)	Staking gel (ml)
40 % acrylamide	3	1
1.5 M Tris pH 8.8	2.5	-
1 M Tris pH 6.8	-	1
10 SDS	0.1	0.08
APS	0.1	0.08
TEMED	0.005	0.01
EDTA	0.1	0.08
dH_2O	4.2	5.66

The resolving gel is poured into the 1 mm gap between the glass plates leaving sufficient space for the staking gel. After polymerization is complete (30 minutes) the covering water was discarded and the staking gel and the comb are fixed in the remaining space. After 20 minutes the comb in easily removed and water was used take out the air bubbles.

Samples were loaded in volumes of 15 µl (a mixture of protein solution, 5 µl of loading buffer and water up to 15 µl). Protein concentration was determined using Bradford method. Samples were migrated in the gel for 1 hour (or until the DYE is coming out of the gel) and a voltage of 40 mA.

For protein identification a molecular weight marker P77085 from NEW ENGLAND Bio-Labs was used.

Loading buffer:

- 50 mM Tris - Cl (pH 6.8)
- 100 mM dithiothreitol
- 2 % SDS
- 0.1 % bromphenol blue
- 10 % glycerol

Electrophoresis buffer: (1 X solution)

- 25 mM Tris
- 192 mM glycine
- 0.1 % (w/v) SDS
- pH 8.3

2.3.4. Coomassie staining

Polypeptides separated by SDS - polyacrylamide gels have been stained with Coomassie Brilliant Blue R250.

Gels were immersed for 1 hour and 30 minutes in solution A and placed on a rotating platform at room temperature. The staining solution was than discarded and gel destained in solution B until clear protein bands appear in the gel. Solution B was changed 3 or 4 times during the process.

Solution A: 0.25 g Coomassie Brilliant R250 was dissolved in 90 ml of methanol : water (1 : 1 v/v) and 10 ml glacial acetic acid. Solution was filtered through a Watman No 1 filter.

Solution B: 30 % methanol and 10 % acetic acid

2.3.5. Electrophoretic Transfer of Proteins to PVDF Membrane

Proteins were transferred electrophoretically from SDS polyacrylamide gel to Hybond-P membrane (Amersham Pharmacia) using the Bio-Rad transfer system and following the manufacturers' protocol. The transfer was performed in 1X Western Transfer Buffer at 100V for 2 hours or at 20V over-night. Completion of transfer was assessed by the pre-stained marker.

10X Western Transfer Buffer: 1.5 M Glycine
200 mM Tris-HCl, pH 8.3

2.3.6. Purification of P450 Cj1411c protein

2.3.6.1. Bacterial cultures

Eight of 2 litre flasks containing 1.5 l LB, containing 1 % glucose, were inoculate with o/n culture of *E. coli* TUNER (DE3) placI containing pBP450Cj plasmid. Cells were grown at 37 ^{0}C until they reached $O.D_{600\ nm}$ 0.5. Cultures were than inoculated with 1 mM IPTG and growth continued for another 24 hours at 18 ^{0}C for protein expression.

2.3.6.2. Preparation of bacterial lysate

After 24 hour growth at 18 ^{0}C cells were harvested by centrifugation at 9000 rpm and 4 ^{0}C temperature and the pellet was resuspended in 50 mM Triethanolamine. The cell membrane was disrupted by sonication for 1 minute per gram pellet and centrifuged at 15000 rpm for 30 minutes. The supernatant containing the P450 protein was ready now to be used in next purification steps.

2.3.6.3. Purification on DE52 anion exchange column

Cells expressing the P450s were washed by resuspension in ice-cold 50 mM Triethanolamine (pH 8). Protease inhibitors [benzamidine hydrochloride and phenylmethanesulfonyl chloride (PMSF) at 1 mM] were also added. Cells were broken by sonication with an MSE Soniprep sonicator on 80% power. Ten bursts of sonication for 15 s were given, with 1 min intervals between bursts. Cells were kept on ice throughout to minimize proteolysis. Following sonication, the extract was separated from cellular debris by centrifugation at 18000*g* for 45 min. The soluble extract was removed and dialyzed extensively into 50 mM Triethanolamine (pH 8), prior to loading onto a DE 52 (Pharmacia) column (5 cm × 40 cm) preequilibrated in the same buffer

The soluble extract was removed and dialyzed extensively into 50 mM Triethanolamine (pH 8), prior to loading onto a DE 52 (Pharmacia) column (5 cm × 40 cm) preequilibrated in the same buffer. The P450s red band came out through the column without binding the DE 52 resin. A linear gradient of potassium chloride in 50 mM Triethanolamine (pH 8) was applied to the column and fractions were collected (0-500 mM over 500 mL). The reddest colored fractions containing the highest quantities of P450 was collected immediately and kept at –20^{0}C before use. The column was washed extensively (10 column volumes) with buffer A,

2.3.6.4. Purification on Ni^{2+} column

In order to simplify the purification of the P450 protein an C terminus six hystidine construct was done.

For the His-tagged protein the pellet from 1 l culture was resuspended in 1 X binding buffer pH 8 and sonicated 1 min / g cells. The cells were centrifuged for 30 minutes at 15000 rpm and the protein lysate was filtered using a 0.2 μm sterile filter and loaded onto a Ni^{2+} affinity column equilibrated in 1 X Binding Buffer.

8 X Binding buffer

40 mM imidazole
4 M NaCl
160 mM Tris
pH 8

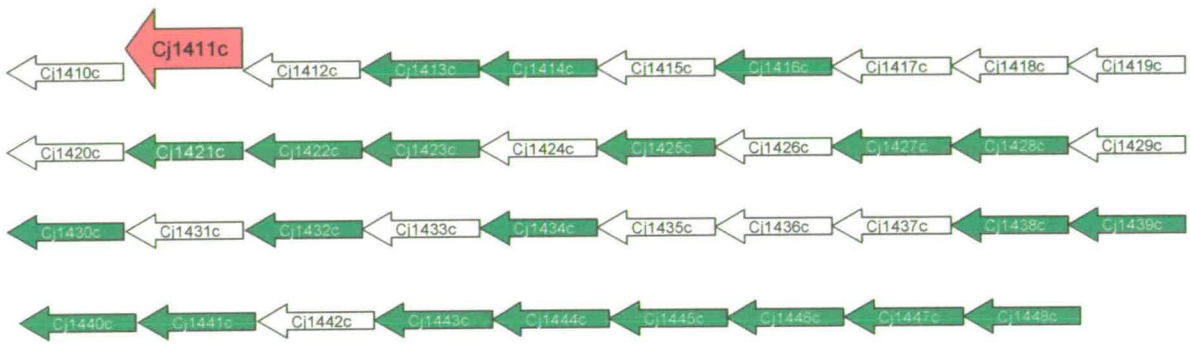
4 X Elution Buffer

4 M Imidazole
2 M NaCl
80 mM Tris-Cl
pH 8

Chapter 3
CLONING EXPRESSION AND PURIFICATION

3.1. Introduction

The cytochrome P450 coding sequence (Cj1411c) of *Campylobacter jejuni 11168* is 1359 bp long. Is located in a region with an unusual number of genes (40 genes), transcribed in the same direction, from which 55 % of the genes involved in sugar or polysaccharide biosynthesis. The remaining 45 % is represented by unknown proteins and few amino acid transferases. The structure of the operon is presented in figure 3.1.



Cj1413c	Polysaccharide modification protein
Cj1414c	Polysaccharide modification protein
Cj1416c	Putative sugar nucleotidyltransferase
Cj1421c	Possible sugar transferase
Cj1422c	Possible sugar transferase
Cj1423c	Putative sugar-phosphate nucleotidyltransferase
Cj1425c	Putative sugar kianase
Cj1427c	Putative sugar-nucleotide epimerase / dehydratase
Cj1428c	Putative fucose synthetase
Cj1430c	Putative sugar-nucleotide epimerase / dehydratase
Cj1432c	Putative sugar transferase
Cj1434c	Putative sugar transferase
Cj1438c	Putative sugar transferase
Cj1439c	UDP-galactopyranose mutase
Cj1440c	Putative sugar transferase
Cj1441c	Putative UDP-glucose 6-dehydrogenase
Cj1443c	KpsF protein
Cj1444c	Putative capsule polysaccharide export system periplasmic protein
Cj1445c	Putative capsule polysaccharide export system inner membrane
Cj1446c	KpsT protein
Cj1447c	Putative capsule polysaccharide export ATP-binding protein
Cj1448c	Putative capsule polysaccharide export system inner membrane

Fig. 3.1. The diagram of the P450 genome region. Each gene is represented by an arrow. In green are marked genes involved either in sugar metabolism or polysaccharide biosynthesis. The P450 gene is marked in red (Cj1411c) and located at the begging of the operon. The predicted gene function in presented for the genes involved in sugar or polysaccharide biosynthesis.

In the primary structure the protein contains 453 amino acids having at the C-terminal the characteristic protoporpyrin IX binding motif (fig. 3.2). In secondary structure the protein is dominated by helixces with few coils and strands (fig. 3.3).

MSECPFFPKPYKNKASTLLTFLLKRRSWLDGLYERSYKMQTGYVKMPNFDLYVINDTKEVKR
MMVDEVREFPKSAFLHELLSPLLGESIFTTNGEVWKKQRELLRPSFEMTRINKVFNLMSEAVA
DMMDRFSKYPNHAVIEVDEAMTFITADVIFRTIMSSKLDEEKGKKILNAFVTFQEQSVHTAMR
RMFRFPKWLSYVLGDCKRAKAGDVIRQVLSDIIKPRYDMADNAEFEDILGSLLLVVDADTNK
RFSFEEILDQVAMLFLAGHETTASSLTWTLYLLSLYPKEQEKAYEEITQVLQGGVIEISHLRQFK
YLTNIFKESLRLYPPVGFFAREAKKDTQVRDKLIKKGSGVVIAPWLIHRHEEFWTNPHGFNPSR
FEGEYKKDAYLPFGVGERICIGQGFAMQEAILILANILKTYKLELEEGFVPDVVGRLTVRSANG
MRIKFSKRKL

Fig.3.2. The protein sequence of *Campylobacter jejuni 11168* P450. The conserved heme binding sequence is shown in red

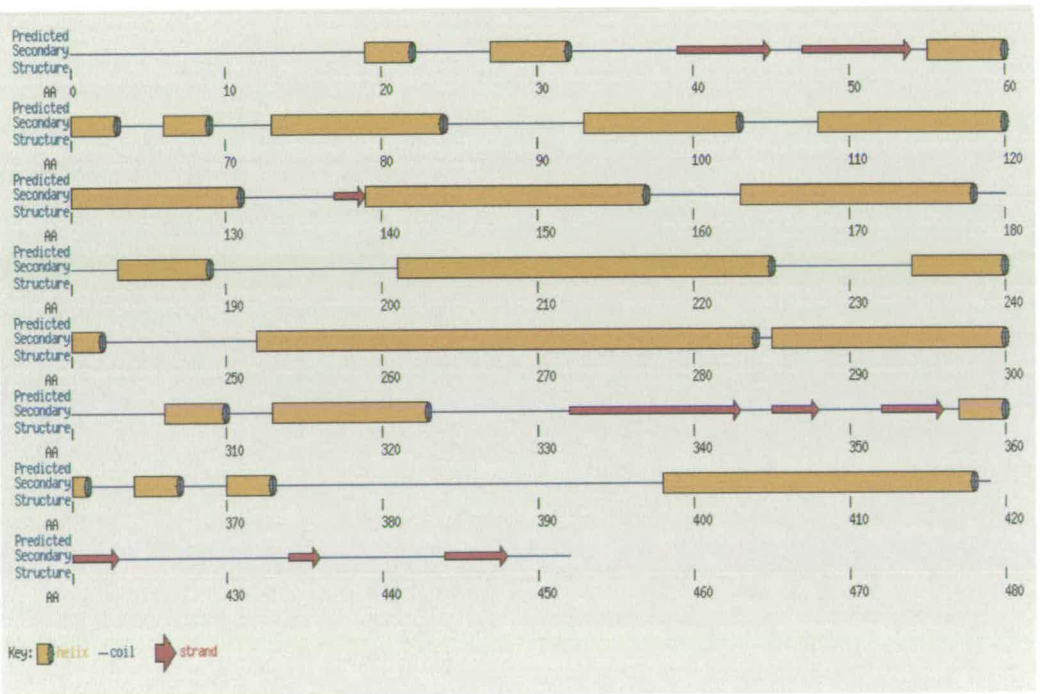


Fig.3.3. Secondary structure of Cj1411c predicted using PSIPREDwhich is a secondary structure prediction method, incorporating two feed-forward neural networks which perform an analysis on output obtained from PSI-BLAST (Position Specific Iterated - BLAST) (Altschul et al., 1997).

The *Campylobacter jejuni 11168* P450 is unique among the P450 enzymes by its unusual location, no other enzymes are known to be located in an operon involved in cell surface biosynthesis. His closest homologues were identified in *Polaromonas* sp. (38 % identity) and *Silicobacter* sp.(34 % identitiy). *Silicibacter* sp. TM1040 is a

member of the *Roseobacter* of the alpha-proteobacteria, which is among the most abundant and ecologically relevant marine bacterial groups. *Polaromonas* strain JS666 (ATCC No. BAA-500), a member of the family *Comamonadaceae* in the beta-proteobacteria, is a novel, aerobic, *cis*-dichloroethene (cDCE)-assimilating organism with optimum growth at 20-25 ^{0}C. Strain JS666 is closely related to the Antarctic marine isolate *Polaromonas vacuolata*. Both of these genes are involved in carbohydrate metabolism (ascorbate and aldarate).The sequence alignment of these two enzymes with the *Campylobacter jejuni* P450 is presented in figure 3.4.

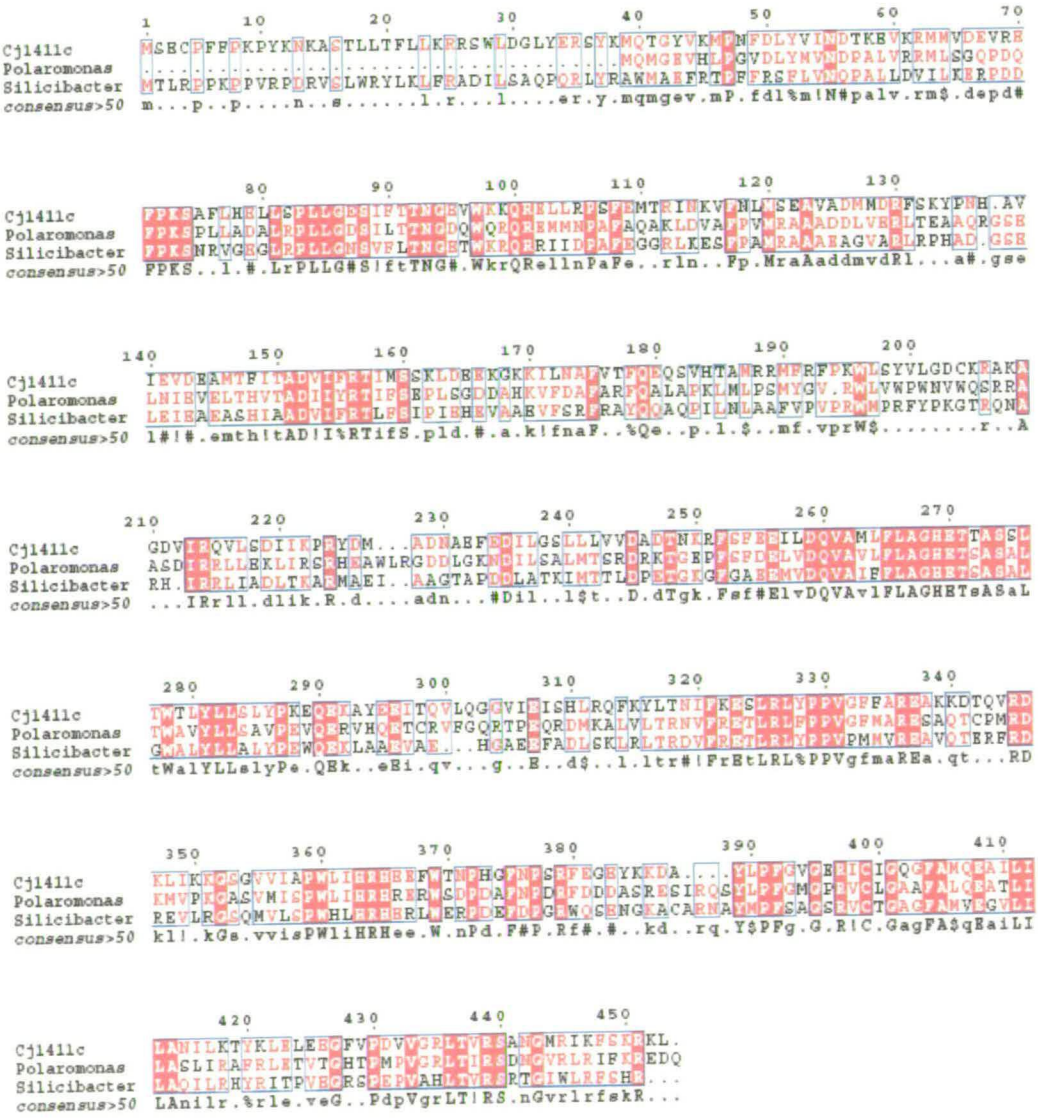


Fig. 3.4. Sequence alignment of cytochrome P450 from *Campylobacter jejuni 11168* with other two cytochromes P450 from *Polaromonas* sp. and *Silicibacter* sp. (conserved amino acids are marked in red).

As described in chapter I mitochondrial and bacterial P450 systems have three components: and FAD-containing flavoprotein (NADPH or NADH-dependant reductase), an iron-sulfur protein and P450. By comparison with the P450 Rhf isolated from *Rhodococcus* sp. NCIMB 9784, were the reductase domain is an FMN-FeS domain fused to the N-terminal heme-containing oxygenase, in *Campylobacter jejuni 11168* the reductase components are not fused in a single polypeptide. The FMN was identified under Cj1382c and Fe-S under Cj1377c. Having all the components separate the *Campylobacter* P450 reductase is represented under a new class (class V – figure 3.5).

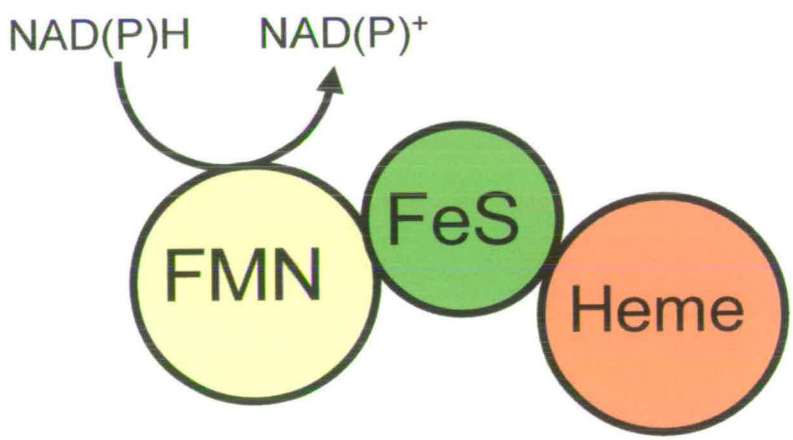


Fig. 3.5. Diagram of the possible class V of P450 reductases (personal, unpublished).

For cloning and expression of the cytochrome P450 a system constructed by Novagen UK called Perfectly Blunt Cloning Kits (technical details presented across the chapter) which are designed for cloning of any DNA fragment which has 5' overhangs and 3' overhangs was used. The DNA fragments are made blunt-ended by a single step reaction followed by a heat inactivation step and then the fragments are ligated in to the dephosphorylated vector.

The recombinant plasmid is transformed in *E. coli* NOVA BLUE and positive colonies are identified by blue / white screening (pETBlue-1 Blunt vector Manual, NOVAGEN). This approach enables the use of high-fidelity proofreading enzymes for amplification, thus decreasing the probability of generating mutations in the target sequence. I addition, under many conditions blunt cloning is more efficient than T-cloning, most likely due to the observation that the efficiency of a single dA addition by *Taq* DNA polymerase varies significantly depending on the sequence context of the DNA ends, and even the number of PCR cycles performed.

The cloning strategy follows accordingly with the NOVAGEN Manual the following steps (figure 3.6):

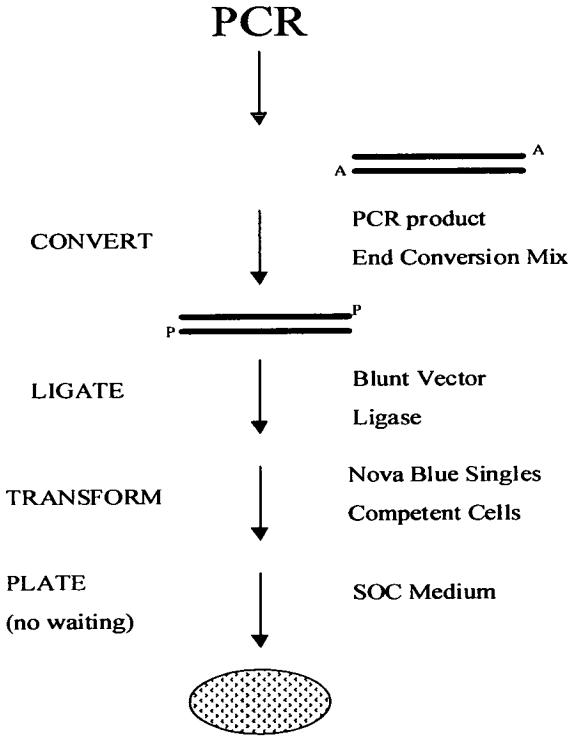


Fig. 3.6. Cloning protocol with Perfectly Blunt Cloning Kit from NOVAGEN. The DNA fragments are made blunt-ended by a single step reaction followed by a heat inactivation step and then the fragments are ligated in to the dephosphorylated vector

The pETBlue™ Perfectly Blunt Cloning Kits include *Tuner*™(DE3)placI competent cells for expression. This strain carries a chromosomal copy of the T7 RNA

polymerase gene. The expression of the target proteins is controlled by the T7*lac* promoter which is IPTG inducible. The strain NOVA BLUE is used like a mother strain which allows us to check the constructs. The system is suitable for inserts which have an ATG start codon.

3.2. Cloning of Cj1411c

3.2.1. Genomic DNA preparation

For genomic DNA preparation *Campylobacter jejunui NCTC 11168* strain was grown on Mueller-Hinton Broth for 42 hours in an atmosphere containing 5 % O_2, 10 % CO_2 and 85 % N_2 in a microaerobic box (fig. 3.7). Cells were harvested by centrifugation when they reached the stationary phase and genomic DNA was extracted using Wizard Genomic Kit from Promega (figure 3.8).

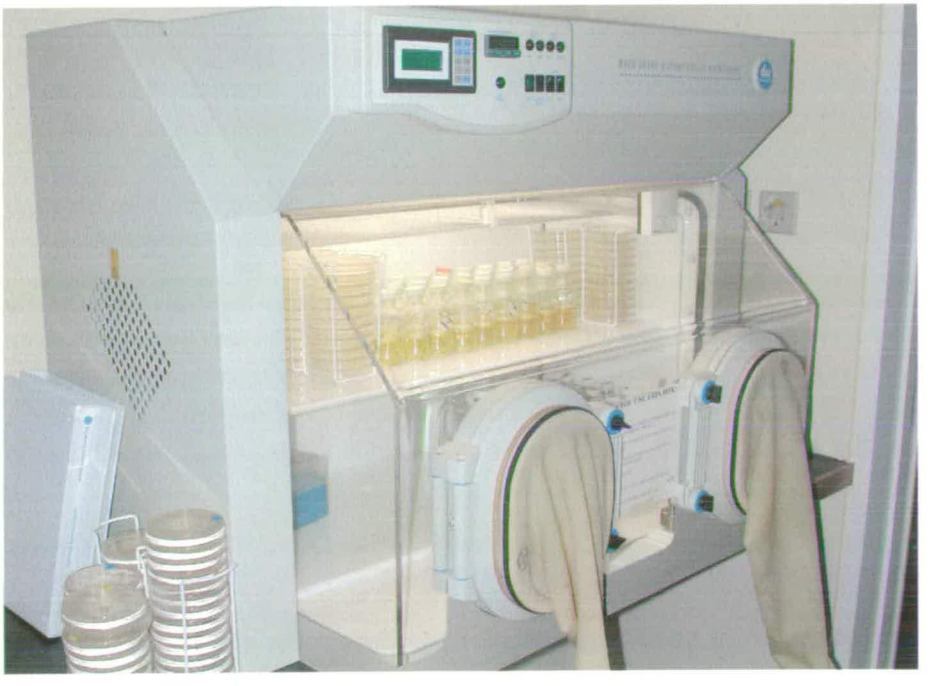


Fig. 3.7. Microaerophilic box used for *Campylobacter jejuni 11168* growth

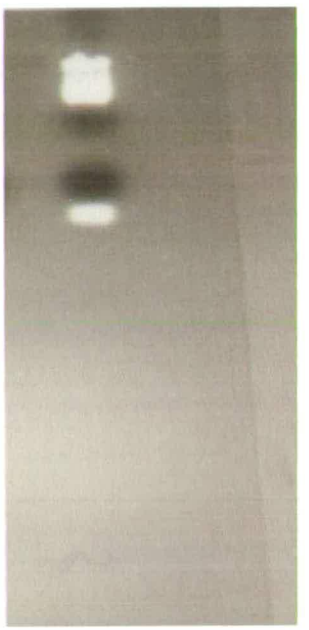


Fig. 3.8. Genomic DNA extracted from *Campylobacter jejuni 11168* (2 µl DNA used to be identified on 0.8 % agarose gel)

3.2.2. PCR

P450 gene was amplified from genomic DNA by PCR using the primers Cj1411cFOR and Cj1411cREV. Amplification was made using Promega *PFU* polymerase to avoid any possible mutation during PCR. The PCR reaction was performed using 5pmol of each primer and 1.25 U of *PFU* polymerase. The total volume of reaction was 50 µl

PCR steps:

Step I: 95 ^{0}C for 1 minute - 1 cycle

Step II: 95 ^{0}C for 30 seconds
53.2 ^{0}C for 30 seconds } 32 cycles
72 ^{0}C for 2 minutes

Step III: 72 ^{0}C for 5 minutes - 1 cycle

After amplification each reaction mix was checked in 0.8 % agarose gel and in each case a fragment of the expected size (1359 bp) was observed (fig. 3.9). PCR reactions were stored at -20 ^{0}C until the next cloning step was performed.

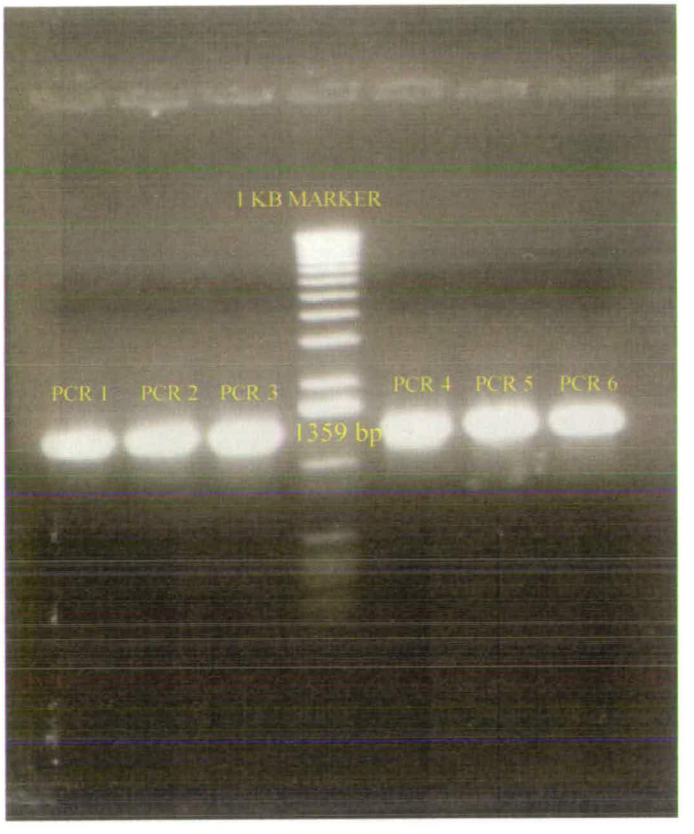


Fig. 3.9. Visualisation of PCR results in agarose gel. PCR 1,2 and 3 have be done by using 1 µl genomic DNA and 0.5 µl for PCR 4, 5, and 6. 3 µl from each reaction have been used in the gel.

3.2.3. Ligation of Cj1411c in pETBlue-1 Blunt vector

After gel purification the fragment was ligated in pETBlue-1 Blunt vector. The purified insert was cloned into the *EcoRV* site which is located such that an insert will specify the N terminus of the expressed protein. The sense primer starts with an ATG initiation codon at the 5' end to allow the expression of the protein.

For ligation the fragment was phosphorylated in two steps: 0.05 pmol amplified product was mixed with 5 µl End Conversion Mix and nuclease free water up to 10 µl in a 0.5 ml tube. Tubes were than incubated for 15 minutes at 22 ^{0}C and the reaction was inactivated by heating at 75 ^{0}C for 5 minutes and kept on ice for 2 minutes. In the second step 1 µl of dephosphorylated vector and 1 µl of T4 DNA ligase were added and the reaction was incubated for 15 minutes at 22 ^{0}C.

3.2.4. Initial transformation in *E. coli* Nova Blue

After ligation the transformation was performed with E. coli Nova Blue competent cells. After transformation, cells were plated on LB solid media supplemented with carbenicilin (50 µg / ml) and tetracycline (15 µg / ml). Transformation was performed as described in material and methods (2.2.8). For blue/white screening X-gal and IPTG were added to the media, prior to plating the cells and the plates were incubated overnight at 37 ^{0}C. The plasmid DNA from the white colonies was extracted as described in materials and methods (2.3.2) and the constructs were checked with restriction enzymes. The plasmids were cut with *EcoRI* and *Bsu36I* which should give after restriction two fragments one of 4335 bp and one of 500 bp in the case of double digest. Cleavage with *EcoRI* gave the expected fragment of 4835 bp (fig. 3.10). The recombinant plasmids which had the expected digestion products were used for sequencing. The inserted gene was sequenced by automatic sequencing using the primers Cj1411CFOR and Cj1411CREV which are the amplification primers, pETBlueUP #70604-3 and pETBlueDOWN #70603-3 which are primers that anneal in the vector sequence, F300 which starts to read at with the base number 300 and F800 which starts with the base 800. There were no mutations detected in the sequence.

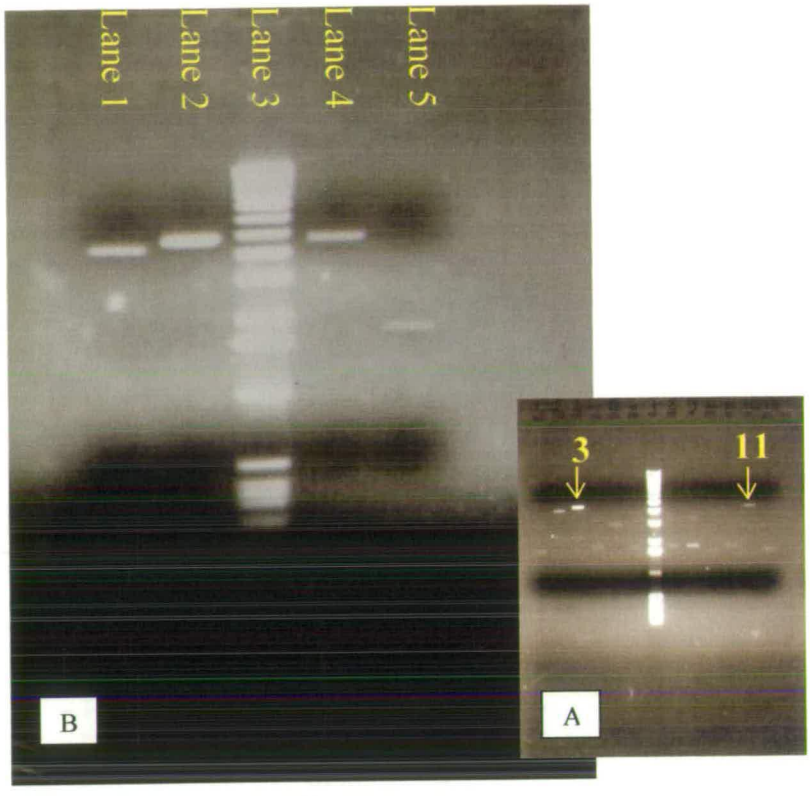


Fig. 3.10. Visualisation of the restriction digests in the agarose gels. Gel A – After restriction with *EcoRI* two plasmids were identified as having 4835 bp in length (plasmids 3 and 11). Gel B: Lane 1 – restriction of the plasmid 3 with *EcoRI* and *Bsu36I*, Lane 2 – restriction of plasmid 3 with *EcoRI,* Lane 3 – 1 kb marker, Lane 4 - restriction of plasmid 11 with *EcoRI*, Lane 5 - restriction of the plasmid 3 with *EcoRI* and *Bsu36I.* Only plasmid 3 has been proved to have the expected orientation. The position on the restriction map for each enzyme and the size of the fragments obtained is described at subchapter 3.2.4.

Using the same protocol, but using the primers Cj1411cFOR and Cj1411cREVHIS, we also constructed a gene encoding a recombinant protein with a 6 HIS Tag at the C terminus of the sequence. The construct was cloned into the pETBlue1 Blunt vector and expressed in *E. coli Tuner (DE3) placI.* The plasmid has been named pBP450CjCHIS.

3.3. Expression and purification

For expression the recombinant plasmid pBP450Cj was used to transformed Tuner (DE3) placI. Cells resistant to carbenicilin (50 µg / ml) and chloramphenicol (34 µg / ml) have been used for protein expression.

Expression trials were performed in 100 ml flasks containing LB + 1 % glucose and the appropriate antibiotics which were inoculated with 1 ml of o/n culture. Cultures were than incubated at 37 ^{0}C by shaking at 200 rpm until the cultures has reached 0.5 O.D. For expression cells were induced with 1 mM IPTG and grown for another 24 hours at 18 ^{0}C and samples were taken at different intervals for testing the protein expression.

For protein expression tests 1 ml of cell culture was centrifuged and the pellet diluted in 50 µl water. The pellet was boiled for 5 minutes and 2 µl of solution was mixed with 5 µl SDS PAGE loading buffer and water up to 15 µl. Samples were run in 15 % SDS PAGE gel and the proteins visualised by coomassie staining (fig. 3.11). The first 5 amino acids of the protein indicated in lane 5 of the SDS Page gel was N-terminal sequenced. The obtained sequenced matched perfectly with the P450 protein sequence. The red coloured pellet characteristic of P450 expression appeared after 24 hours (fig. 3.12). The native enzyme was identified by spectral characterisation and CO binding reaction. A 450 nm peak was observed in whole cell extracts (fig. 3.13).

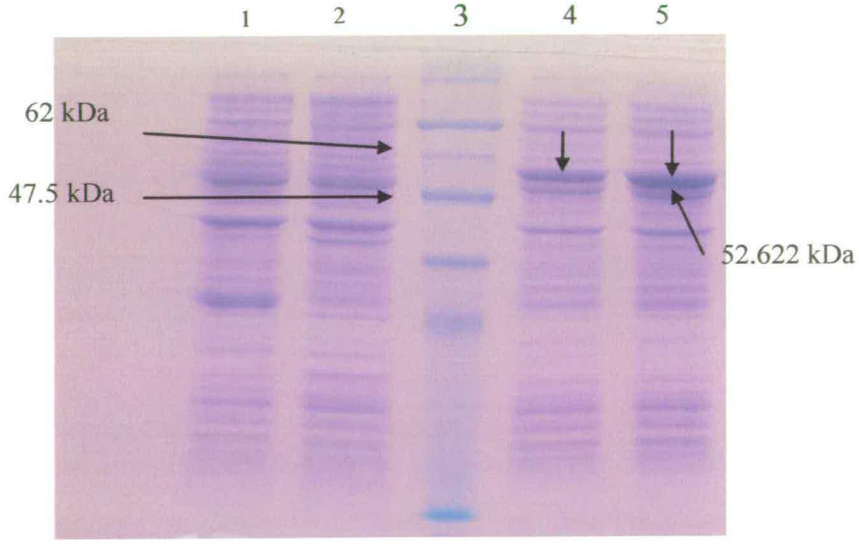


Fig. 3.11. Visualisation of protein expression using 15 % SDS-PAGE and stained with Coomassie Blue. Lane 1 – cells only (no plasmid), Lane 2 – uninduced (no IPTG), Lane 3 – marker, Lane 4 – expression after 8 hours, Lane 5 – expression after 24 hours

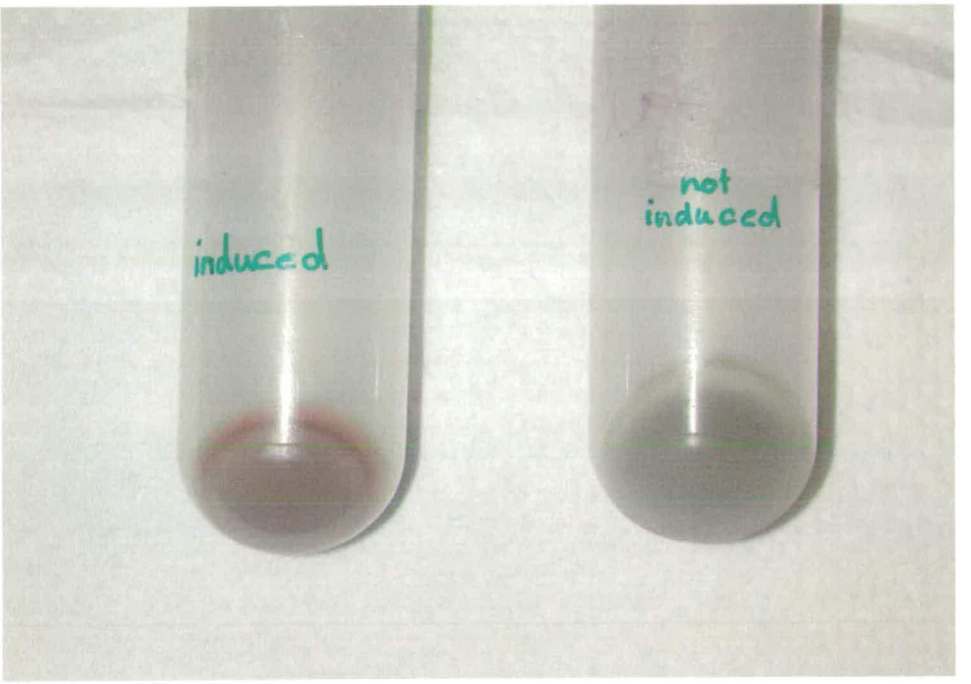


Fig.3.12. Expression in *E. coli* Tuner (DE3) placI is identified by the changing of the cell pellet colour to red (cells were grown 24 hours at 18 ^{0}C).

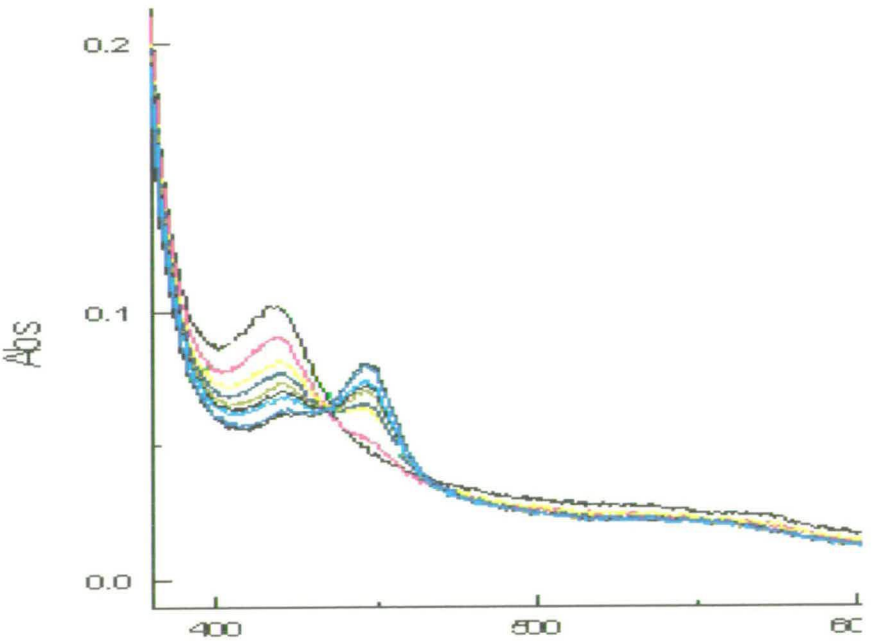


Fig. 3.13. Expression identified by 450 nm absorption in SORET band with CO bound. Sample preparation: cell membranes were disrupted by sonication, centrifuged and supernatant collected. To reduce the protein sodium dithionite was used and CO was bubbled into the sample. Successive spectra shows the transition of the 420 nm peak, of the oxidised protein, to 450 nm peak by exposure of the protein to the CO.

The initial purification trials were started with the anion exchange resins DEAE Sephacel and Q sepharose and with hydroxyapatite, using TRIS-HCl buffer, HEPES (pH 6.8-8.2), Phosphate buffer (pH 6-8) and CAPS (pH 9.7-11). Unfortunately the protein did not bind to the resins in any of this conditions. The same problems were faced with the cation exchangers C25 and CM (carboxymethyl cellulose) at pH 7 the protein came through the column without any binding.

The next attempts were to try the hydrophobic resins:

- phenyl sepharose
- octyl sepharose
- decyl sepharose
- butyl sepharose

None of these columns provided a good purification. The protein was bound to the phenyl sepharose column but it could not be eluted.

DE52 purification:

Cells expressing the P450s were washed by resuspension in ice-cold 50 mM Triethanolamine (pH 8). Protease inhibitors [benzamidine hydrochloride and phenylmethanesulfonyl chloride (PMSF) at 1 mM] were also added. Cells were broken by sonication with an MSE Soniprep sonicator on 80% power. Ten bursts of sonication for 15 s were given, with 1 min intervals between bursts. Cells were kept on ice throughout to minimize proteolysis. Following sonication, the extract was separated from cellular debris by centrifugation at 18000*g* for 45 min. The soluble extract was removed and dialyzed extensively into 50 mM Triethanolamine (pH 8), prior to loading onto a DE 52 (Pharmacia) column (5 cm × 40 cm) preequilibrated in the same buffer

The soluble extract was removed and dialyzed extensively into 50 mM Triethanolamine (pH 8), prior to loading onto a DE 52 (Pharmacia) column (5 cm ×

40 cm) preequilibrated in the same buffer. The P450s red band came out through the column without binding the DE 52 resin. A linear gradient of potassium chloride in 50 mM Triethanolamine (pH 8) was applied to the column and fractions were collected (0-500 mM over 500 mL). The reddest colored fractions containing the highest quantities of P450 was collected immediately and kept at -20^0C before use. The column was washed extensively (10 column volumes) with 50 mM Triethanolamine (pH 8).

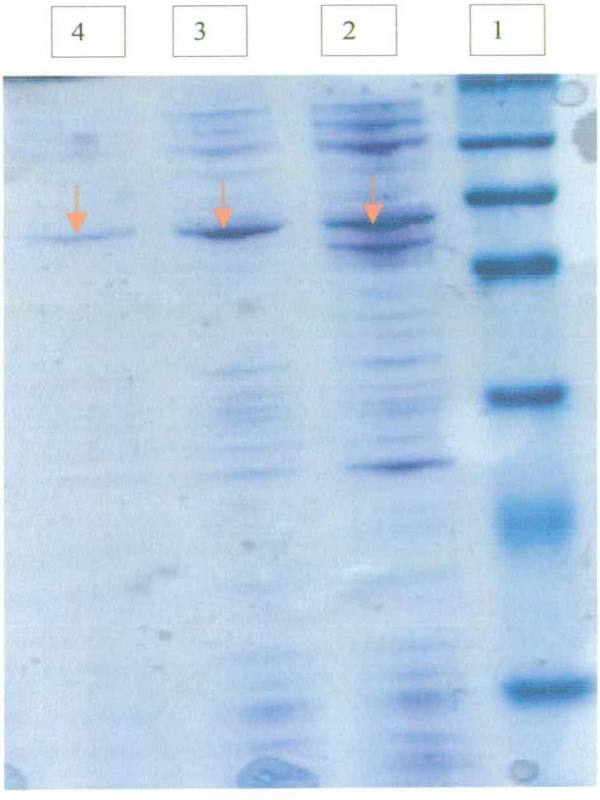


Fig. 3.14. Purification on DE52 – fractions visualisation on 15 % SDS-PAGE and Coomassie Blue Staining:

1. Marker
2. First DE52 after RNaseA, DNaseI and protamine sulphate treatment
3. Second DE52
4. Third DE52

The protein concentration was approximated to 12.5 mg / ml and was calculated using the SORET absorption. The concentrated protein was frozen at –80 ^{0}C until further experiments were proceeded.

3.4. Conclusions

The recombinant protein was successfully over-expressed using pETBlue1 Blunt as vector and *E. coli* Tuner (DE3) placI as expression strain, this study being a very useful tool for expression of recombinant P450s.

Purification of this cytochrome P450 was not completely successful but 70 % purity was achieved using DE52 anion-exchange and triethanolamine as a buffer at pH 8. Purification was done in reverse way by collecting the red fraction which ran through the column. The red fraction collected was concentrated up to 12 mg/ml but at this concentration the protein proved to be cloudy and very viscous.

To improve the purity of the protein to HIS-Tagged constructs were prepared (one with the 6HIS at the N terminus and one at the C terminus) but the purification trials failed as the protein was not bound to the nickel column.

Chapter 4
P450 GENE KNOCKOUT

4.1. Introduction

In the last few years research on pathogenic bacteria increasingly involves genetic manipulation. Tompinks (1992) revealed that for pathogenic bacteria it is important to identify the potential virulence factors and especially to identify their precise role by using isogenic mutants.

At the moment it is very common to produce mutants in *Campylobacter* either by transposom mutagenesis or by targeted gene inactivation. Introduction of the DNA into the organism may be achieved either by natural transformation or electro-transformation.

Spontaneous mutations that cause genetic change are naturally occurring in all cells. They may arise from errors in DNA replication resulting from tautomeric shifts that lead to mispairing of bases in duplex DNA. DNA replication errors can also lead to frameshift mutations that commonly occur at hot spot regions of repeated DNA sequence. In addition to DNA replication errors, spontaneous mutations can arise from naturally occurring DNA damage. Hydrogen peroxide generated as a by-product of aerobic respiration can cause oxidative damage to DNA that leads to base mispairing. Spontaneous mutations also arise from the ability of transposable genetic elements such as transposons to move from one plasmid to another plasmid or from a plasmid to a bacterial chromosome. The genetic change can be a result of conservative or replicative transposition that gives rise to one or two copies of the transposable element, respectively. The target DNA is further modified to contain a short repeated sequence of the target gene upon integration of the transposable genetic element.

Our protocol to generate a cytochrome P450 null mutant involved the replacement of the cytochrome P450 coding sequence with an antibiotic resistance gene (kanamycin) by homologous recombination. Two fragments, one upstream (F1 – 447 bp) and one downstream (F2 – 589 bp) of the P450 coding sequence were amplified separately by PCR and combined during the second PCR reaction giving a *SmaI* site at the junction point of the two DNA fragments. All the PCR reactions were performed using the

proofreading polymerase for amplification (*PFU*). The final DNA fragment obtained (F3 – 1036 bp) was cloned into the *SmaI* site of the pBluescript SK⁻ vector. The strategy for construction of the DNA fragments is presented in figure 4.1. The structure of F1REV and F2FOR primers is presented in figure 4.2.

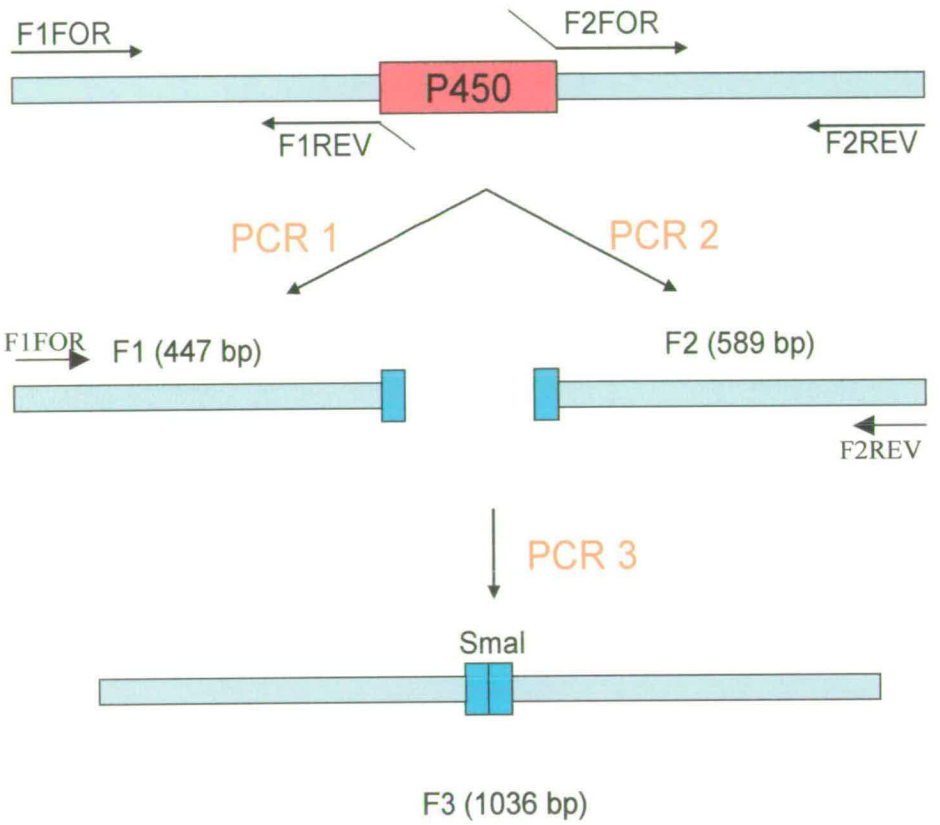


Fig. 4.1. Molecular strategy for P450 gene knockout construction
Two fragments one upstream (F1) and one downstream (F2), were amplified separately by PCR 1 for F1 and PCR 2 for F2. In PCR 3 fragments F1 and F2 were combined forming the F3 fragment which has the *SmaI* restriction site at the junction between the two fragments.

```
F2FOR                    TAACTCTTTTCACCCGGGATTTTTACCTTTTCTTCTAA
F1REV      CGCTTAAAATCAAGATTGAGAAAAGTGGGCCCTAAAAATGGAAA
```

Fig. 4.2. Design of the proximal primers used to achieve the fusion PCR. The 30 nucleotide overlap is presented with the *SmaI* cleavage site in blue and the rest of the DNA sequence in black

4.2. Primer design and PCR amplification of fragment F1

The F1 fragment (447 bp long) was amplified by PCR using as a template genomic DNA. The fragment was amplified using primers F1FOR (1 pmol / µl) and F1REV (1 pmol / µl) in reaction in which 0.5 µl genomic DNA was used. For a better amplification and to avoid the unexpected mutations *PFU* polymerase was used (0.5 µl / reaction). The thermocycle was programmed to work by the following steps:

a) 95 ^{0}C for 1 minute — 1 cycle

b) 95 ^{0}C for 30 seconds
48.5 ^{0}C for 30 seconds } 32 cycles
72 ^{0}C for 2 minutes

c) 72 ^{0}C for 2 minutes — 1 cycle
Hold at 4 ^{0}C over night

A ≈ 447 bp long fragment was identified in agarose gel using a molecular weight marker and was sequenced by automated sequencing to identify possible inserted mutations which can inserted during PCR reaction. The 447 bp long fragment (fig.4.3) was purified from agarose gel and used as a template in the fusion PCR.

Fig.4.3. F1 PCR. Primers F1FOR and F1REV successfully initiated PCR amplification of *C. jejuni 11168* genomic DNA upstream of Cj1411c (P450 gene). The results of PCR were analysed using agarose gel electrophoresis that revealed the specific amplification of the expected 447 bp fragment (lane 1). 1 kb ladder (lane 2) was used to indicate the size of the PCR amplification products.

4.3. Primer design and PCR amplification of fragment F2

Using a similar protocol, as for F1 fragment, the F2 fragment (589 bp) was amplified by PCR using genomic DNA as template (fig. 4.4). In this case the primers used were F2FOR (1 pmol / µl of reaction) and F2REV (1 pmol / µl of reaction). The fragment was successfully amplified using *PFU* polymerase and purified from agarose gel. The purified DNA was the second template used in the fusion PCR. To identify possible mutations F2 was sequenced by automated sequencing with two point mutations detected. The thermocycle was programmed as with fragment 1.

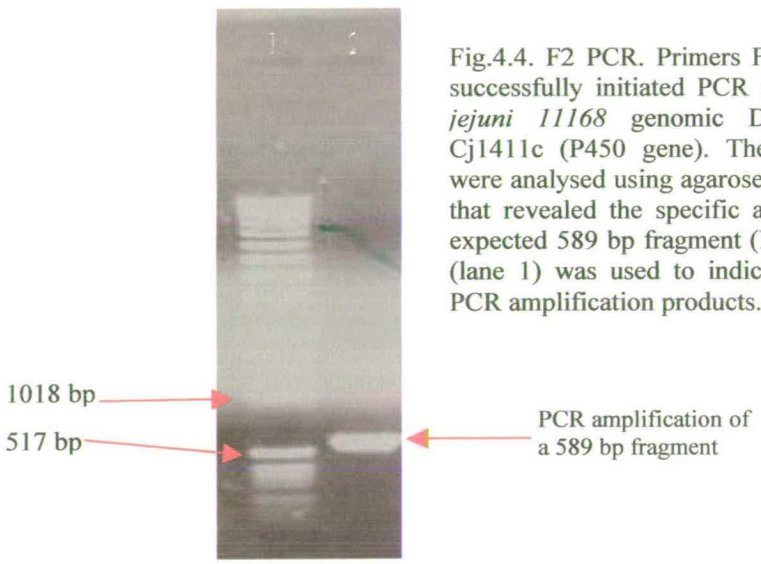


Fig.4.4. F2 PCR. Primers F2FOR and F2REV successfully initiated PCR amplification of *C. jejuni 11168* genomic DNA upstream of Cj1411c (P450 gene). The results of PCR were analysed using agarose gel electrophoresis that revealed the specific amplification of the expected 589 bp fragment (lane 2). 1 kb ladder (lane 1) was used to indicate the size of the PCR amplification products.

To eliminate amplification of erroneous products during the final fusion PCR step, the desired 589 bp product was excised from a preparative agarose gel and purified. A high yield of the 589 bp PCR product was recovered to provide a suitable template for fusion PCR.

4.4. Amplification of fragment F3

The PCR products of the first two primary amplifications were used as a template DNA for the final fusion PCR amplification. The distal primers F1FOR and F2REV were used to amplify the gene-flanking DNA as a continuous sequence containing an *SmaI* cleavage site.

Thermocycle steps:

c) 95 ^{0}C for 1 minute 1 cycle

d) 95 ^{0}C for 30 seconds
45 ^{0}C for 30 seconds } 32 cycles
72 ^{0}C for 2 minutes

c) 72 ^{0}C for 5 minutes 1 cycle
Hold at 4 ^{0}C over night

High levels of the fusion PCR fragment at a size of 1036 bp were obtained for each of the reactions with no secondary PCR products amplified (fig.4.5).

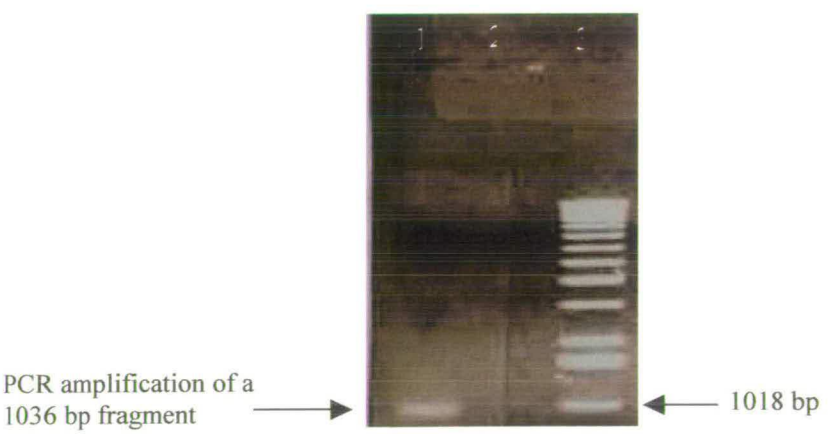


Fig. 4.5. F3 PCR. The distal primers F1FOR and F2REV initiated the fusion PCR of the gene-flanking genomic DNA sequences as a continuous fragment containing an *SmaI* cleavage site.

4.5. Cloning of the F3 fragment into pBlueScript II SK⁻ plasmid

The PCR template DNA generated by fusion PCR was inserted into pBlueScript II SK⁻ (figure 4.7). This vector was chosen because of its previous use as a suicide vector to generate gene replacement in *Campylobacter* and because provides a unique *SmaI* cleavage site suitable for insertion of the insertion of the fusion PCR product by blunt-ended cloning necessitated by *PFU* amplification.

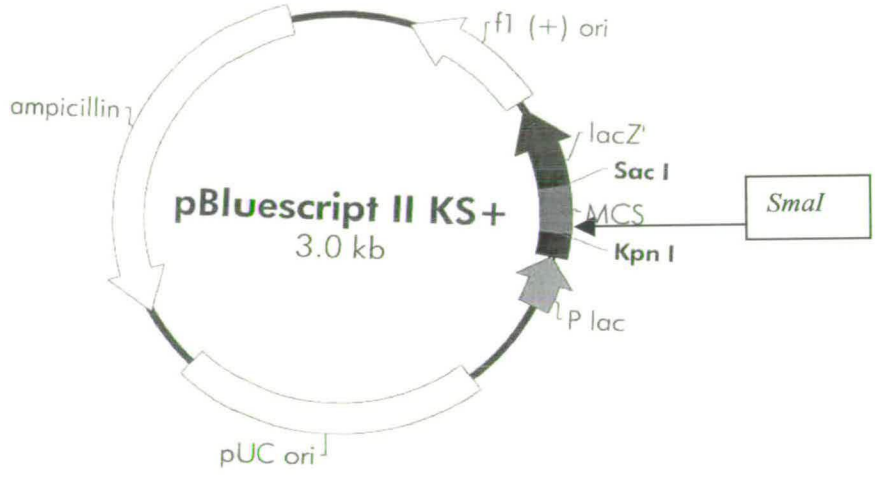


Fig.4.7. The map of pBlueScript II SK⁻

The *SmaI* site that was used for insertion of the homologues template DNA PCR product is shown.

A sample of pBlueScript II SK⁻ plasmid DNA was cut with *SmaI* to generate blunt ends that were ligated to blunt ends of the fusion PCR amplified using *PFU* polymerase (fig. 4.9). Recombinant plasmid pBlueScript II SK⁻ containing the homologous template DNA fusion PCR product was transformed into *E. coli* TG1. The transformants were screened for ampicilin resistance encoded on the plasmid DNA. On the *cells only* control plate no colonies were found which indicated that the colonies on the transformation plates would all contains the pBlueScript II SK⁻ plasmid. The recombinant plasmid has been called pBSF3 (fig. 4.8). Plasmid DNA was isolated from 7 single ampicillin resistant colonies and *SmaI* restriction digests were performed to identify those with the recombinant pBlueScript II SK⁻plasmid. Cutting with *SmaI* indicated the presence of the *SmaI* restriction site inside the F3

fragment. Two of the colonies screened had the inserted fusion PCR into the *SmaI* site.

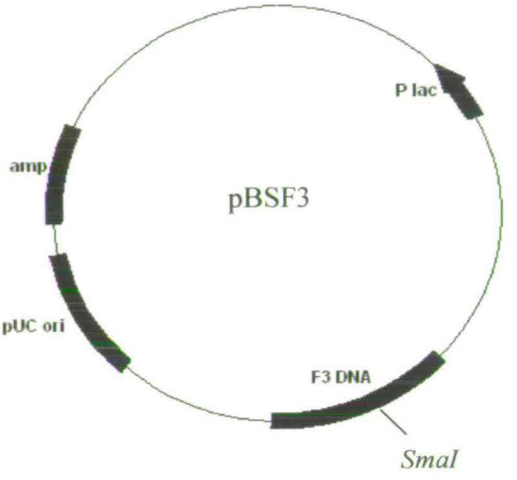


Fig. 4.8. Schematic representation of the pBSF3 plasmid

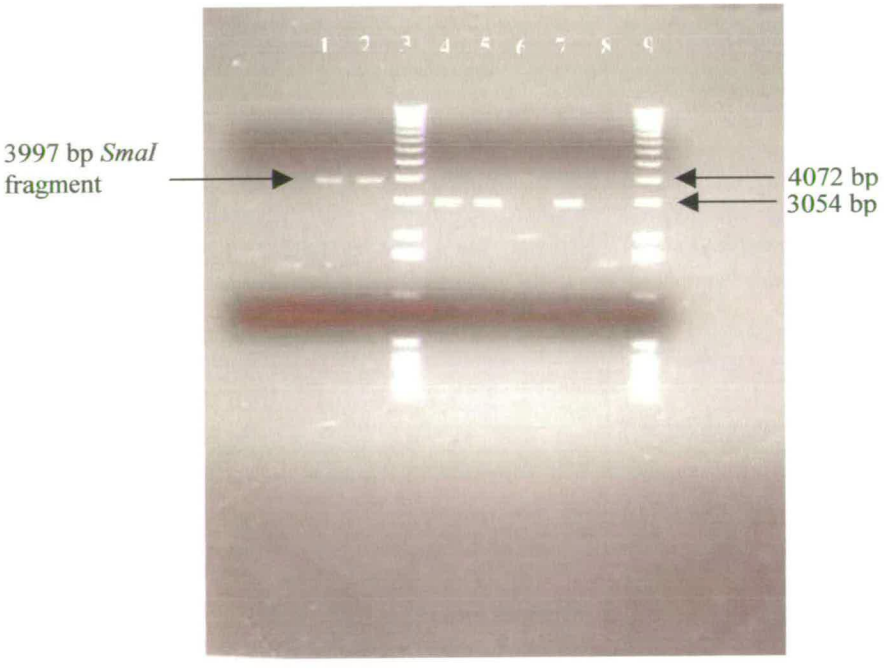


Fig. 4.9. Restriction digests to identify the recombinant pBlueScript II SK⁻ plasmid
The homologous template DNA fragment was inserted in pBlueScript II SK⁻ using blunt-end cloning at a *SmaI* cleavage site. Recombinant pBlueScript II SK⁻ plasmid, named pBSF3, was identified using *SmaI* that cut the inserted homologous template DNA to give a single restriction fragment at 3997 bp (lanes 1 and 2). The presence of a single restriction band suggests that the F3 fragment has been inserted into the *SmaI* site of the pBlueScript II SK⁻ plasmid with the designed *SmaI* site present in the middle of the F3 fragment. Lanes 4, 5, 6, 7 and 8 shows that this colonies had transformed only the pBlueScript II SK⁻ plasmid without the fusion PCR product. 1kb ladder was loaded into lanes 3 and 9.

The gene replacement fragment was a kanamycin cassette from *E. coli* transposom Tn5 provided as a 939 bp fragment from pSUP10121 plasmid. The *EcoRV* cleavage sites flanking the 5` and 3` termini of the cassette were digested to release the *EcoRV* kanamycin fragment from pSUP10121 plasmid. pBSF3 plasmid was digested with *SmaI* to generate blunt ended termini in order to ligate the kanamycin cassette. The recombinant plasmid was transformed into *E. coli* TG1 and transformants were screened for ampicillin and kanamycin resistance. There were no colonies on the *E. coli* TG1 *cells only* control indicating good antibiotic selection. The number of colonies obtained from cells transformed with recombinant plasmid generated from blunt-ended ligation was sufficient in order to perform the restriction digests for identification of insertion of the kanamycin cassette into the *SmaI* site of the pBSF3 plasmid. Following antibiotic selection, plasmid DNA was isolated from 3 single colonies and *EcoRI* restriction digests were used to verify insertion of the kanamycin cassette (fig.4.10).

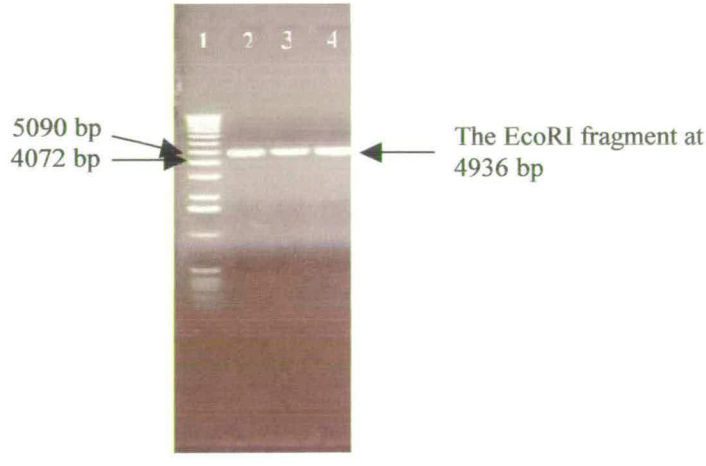


Fig. 4.10. Restriction digests to identify the insertion of the kanamycin cassette
The blunt-ended kanamycin cassette was inserted in the homologous template DNA of pBSF3 to form a kanamycin resistant gene replacement fragment. The new plasmid was named pBSF3-Km^+. Insertion of the kanamycin cassette into the *SmaI* site of the pBSF3 plasmid was verified using *EcoRI* restriction digests which cuts the plasmid in a single position without cutting into the homologous template DNA or inside the kanamycin cassette. The new plasmid (pBSF3-Km^+) has a size of 4936 bp. The restriction fragments of pBSF3-Km^+ plasmid DNA isolated from colonies 1-3 are shown in lanes 2-4. 1kb DNA ladder (lanes 1) was used to indicate that the EcoRI restriction fragments were of the correct size.

4.6. DNA transfer into *Campylobacter jejuni 11168* wild type

The pBSF3-Km$^+$ plasmid carrying the gene replacement fragment was transferred by electroporation and natural transformation into the wild type strain following the protocols described in material and methods. The pBSF3-Km$^+$ plasmid was introduced into *Campylobacter jejuni 11168* to allow homologous recombination to occur between the cloned gene replacement fragment and homologous DNA of the bacterial chromosome.

Following the transformation the transformants were screened for kanamycin. No colonies were identified on *Campylobacter* only control indicating effective kanamycin selection. All the colonies resistant to kanamycin were than subjected to PCR screening to identify the replacement of the P450 gene sequence from the genome.

4.7. PCR screening to identify the replacement of the P450 gene

PCR screening was used to scan the transformants genomic DNA for the correct replacement of the P450 gene. Three types of colony PCR were performed:

PCR 1 – using Cj1411cFOR and Cj1411c REV primers on transformants genomic DNA and on wild type strain (fig.4.11, A and B)

PCR 2 – using Tn5Km(L) and Tn5Km(R) which will amplify the Km cassette on transformants genomic DNA (fig.4.11 C)

PCR 3 – using primers F1FOR and F2REV which will amplify the homologous template DNA (1962 bp fragment) from the transformants genomic DNA (fig 4.11 D)

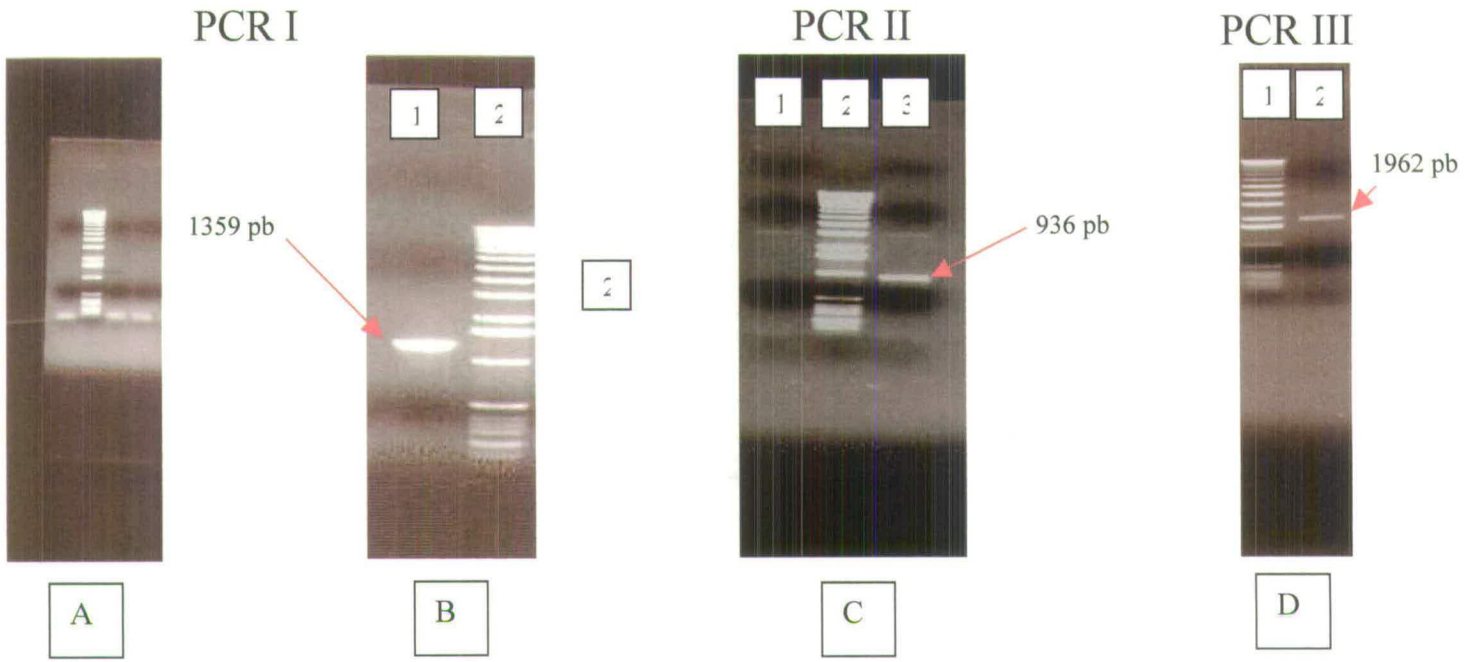


Fig. 4.11.PCR screening of transformants

A – PCR was performed using Cj1411cFOR and Cj1411cREV with transformants colonies and no amplification was detected by visualisation on agarose gel.
B – this second PCR result was performed in conditions similar to PCR A but instead of transformants colonies the wild type colonies were used and 1359 bp fragment was identified in agarose gel which corresponds to the P450 gene (indicated by arrow in lane 1). In lane 2, 1 kb ladder is present.
C – in order to detect the presence of the kanamycin cassette on the knockout strain chromosome Tn5Km(L) and Tn5Km(R) were used. Lane 1 shows that with the wild type colonies no amplification was detected and when the knockout colonies are used a band corresponding to 939 bp is amplified (lane 3). This PCR proves that the kanamycin cassette was inserted into the wild type chromosome.
D – the third PCR was performed to identify the presence of the homologous template DNA (1962 bp) on the knockout chromosome. The same transformant colony used in PCR II was used for amplification of the desired fragment (1962 bp), in PCR III, which suggests that the homologous recombination was successfully performed (lanes 2). In lane one, 1 kb ladder is present.

In order to compare P450 expression into the wild type strain with the NCI strain the cells were sonicated and the supernatant was tested for P450 expression. As presented in figure 4.13, there was no P450 expression detected in the NC1 strain while the wild type shows that the P450 expression is present (fig. 4.12).

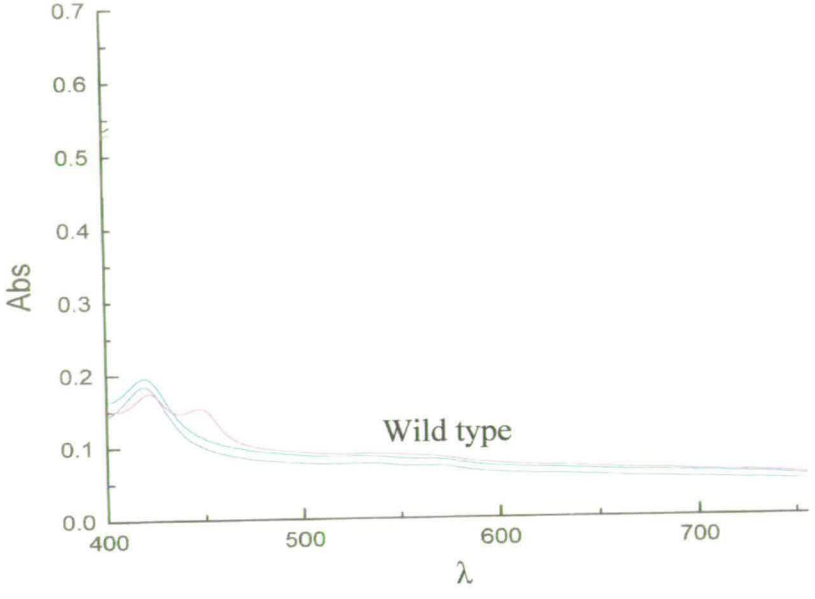


Fig. 4.12. Wild type supernatant spectra for detection of the P450 expression. To detect the P450 expression sodium dithionite was added to the supernatant and the CO was added to detetect de P450 peak. (green line represents the supernatant without sodium dithionite, blue line with sodium dithionite and red line added CO with the detection of the P450 peak).

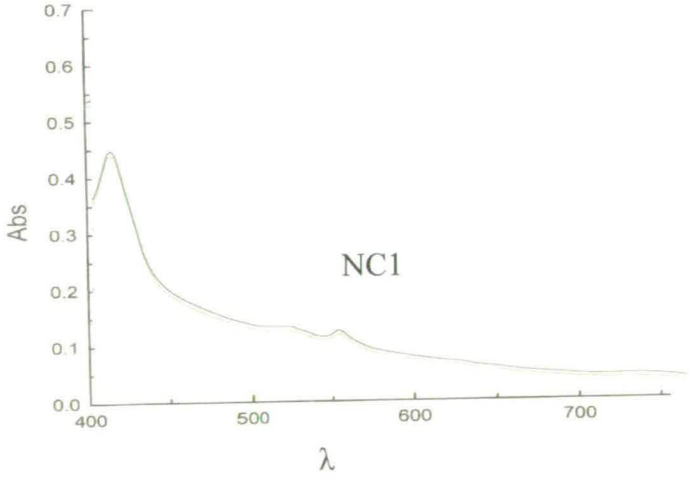


Fig. 4.13. NC1 supernatant spectra for detection of the P450 expression. To detect the P450 expression sodium dithionite was added to the supernatant and the CO was added to detetect de P450 peak. (brown line represents the supernatant without sodium dithionite, green line with sodium dithionite and red line added CO with no detection of the P450 peak).

4.8. Conclusions

This chapter provides very useful information for knockout construction in *Campylobacter spp.*. The pBlueScript II SK$^-$ (figure 4.7) was proved to be a suitable vector for replacing the P450 sequence with a kanamicin resistance gene from *E. coli* Tn5.

The successful deletion of the P450 gene from the *Campylobacter jejuni* 11168 genome, which is proved in figures 4.12 and 4.13 offered the possibility to study the phenotypic differences between the NC1 and Wt strain.

Chapter 5
PHENOTYPIC CHARACTERISATION

5.1. Introduction

The survival of *Campylobacter* in foods and the environment is influenced by physiological mechanisms. It is important to know how the cells will respond to conditions that are used to preserve and store foods, such as poultry, that are commonly contaminated by *Campylobacter* and ultimately consumed. Temperature, pH, and salting, are all measures incorporated by food processors to reduce the number of viable pathogenic micro-organisms in foods.

The purpose of the work described in this chapter was to determine what effect different growth conditions or different preservations condition have upon the NCI and wild type cells and especially to determine whether deleting the P450 gene from the genome affects in any way the life cycle of this enteric pathogen. All the experiments presented in this chapter were reproducible and statistically significant.

5.2. Growth curve of *Campylobacter jejuni* WT and NCI strain

The aim of this experiment was to check whether deletion of the P450 gene affects the life cycle of the NCI strain. Cells of both strains were grown for 42 hours at 42 ^{0}C and sampled at 12, 15, 18, 24, 30, 36 and 42 hours (growth conditions described in materials and methods). Both strains had an ascendant growth with the main difference that the NCI strain reaches the growth peak after ~ 24 hours and the WT strain after ~ 30 hours growth. At that stage the viable counts were 5.43 (Stdv ±0.62) x 10^{11} for NCI and 6.96 (Stdv ±0.46) x 10^{11} for WT (table 5.1 and figure 5.1).

Table 5.1

Viable counts of the WT and NCI strain during 42 hours growth period

Time (hours)	WT	NCI
12	4.4 (±0.6) x 10^9	4.3 (±0.69) x 10^8
15	1.5 (±0.8) x 10^{10}	5.8 (±0.75) x 10^9
18	3.9 (±0.32) x 10^{10}	3.7 (±0.23) x 10^{10}
24	4.4 (±1.2) x 10^{11}	5.43 (±0.62) x 10^{11}
30	6.96 (±0.46) x 10^{11}	2 (±0.42) x 10^{11}
36	8.96 (±0.52) x 10^{10}	6.5 (±0.2) x 10^{10}
42	4.36 (±0.26) x 10^{10}	5.26 (±0.91) x 10^{10}

± Standard deviation

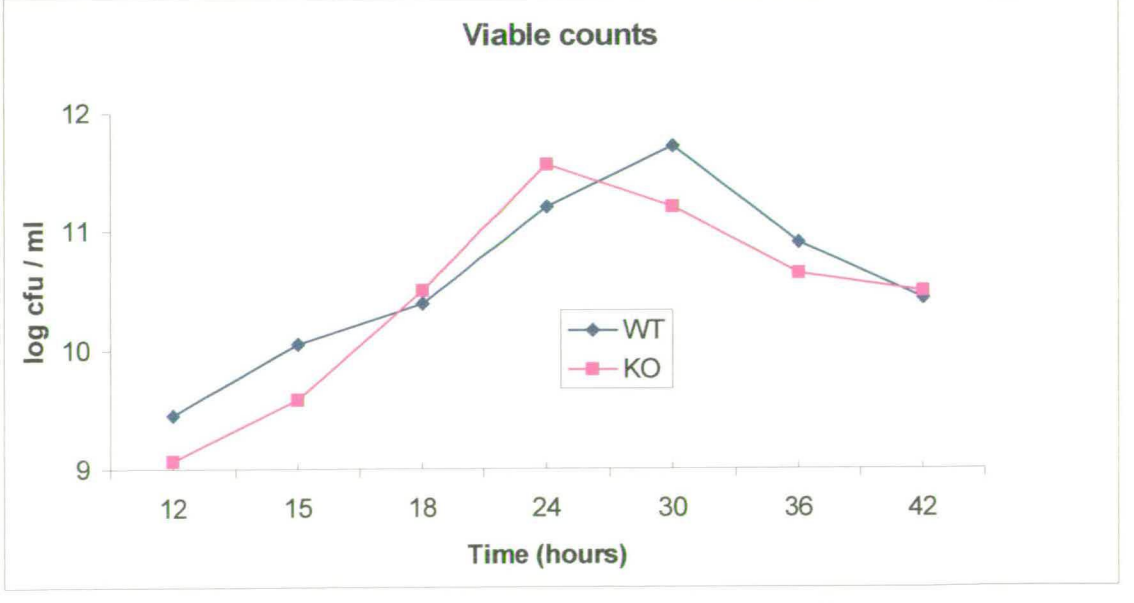


Fig. 5.1. Viable counts during the growth period. Cells were grown at 42 ^{0}C and samples were taken during an interval of 42 hours and viable counts were determined. The experiments were repeated three times, they showed similar pattern; only the results from one experiment are presented.

5.3. Stationary phase behaviour

Previously it was reported that during the stationary phase *Campylobacter jejuni* viable counts follow an unusual pattern (Kelly *et al*, 2001). When the stationary phase is reached the organism shows a rapid loss of viability which is followed later by an increase in viable counts.

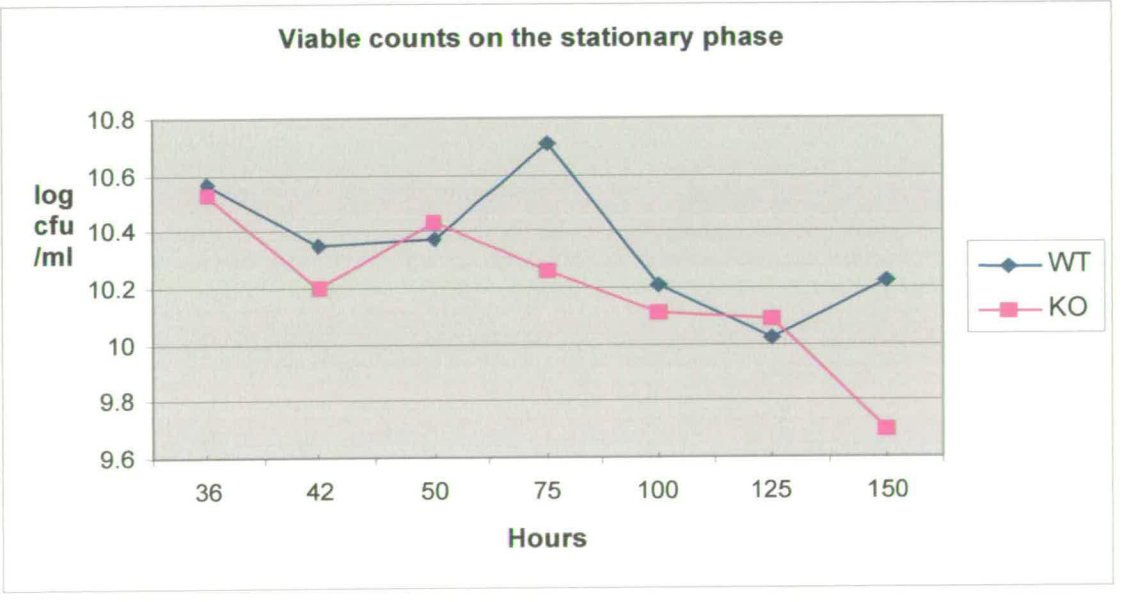
In the stationary phase the cell counts were 10^{10} CFU/ml with the maximum cell count at 75 hours when the Wt reached 7.1 (Stdv ±1.9) x 10^{10} CFU/ml. The NC1 counts were 5.3 x 10^{10} CFU / ml at 36 hours and 2.6 x 10^{10} at 75 hours growth.

Table 5.2

Viable counts on the stationary phase

± Standard deviation

Strain	Time (hours)						
	36	42	50	75	100	125	150
WT	5.7 (±0.21) x 10^{10}	3.5 (±0.2) x10^{10}	3.7(±0.9) x 10^{10}	7.1(±1.9) x 10^{10}	2.1(±0.2) x 10^{10}	0.2(±0.3) x 10^{10}	2.2(±0.6) x 10^{10}
NC1	5.3 (±0.41) x 10^{10}	2.1(±0.7) x 10^{10}	4.3(±1.5) x 10^{10}	2.6(±0.8) x 10^{10}	1.1(±0.4) x 10^{10}	0.9(±0.8) x 10^{10}	7.3(±1.2) x 10^{9}



5.4. Rate of survival under aeration conditions

Campylobacter in known as a bacterium sensitive to atmospheric oxygen at a concentration over 5 %, especially to ROS (se chapter I). Conical flasks containing 50 ml PBS were inoculated with 100 µl of fresh culture at a concentration of ~ 10^8 CFU/ml. The flasks were than incubated in air at 37^0C and 90 rpm. Samples were removed after 24 and 42 hours exposure to oxygen and viable counts determined. Cells were plated on MH only for the WT strain and MH + Km^+ for the NCI strain. From the initial cell count (42 hours growth), 16.61 % NCI colonies and 36.47 % WT colonies survived. After 42 hours both strains showed less than 1 % survival rate by exposure to atmospheric oxygen.

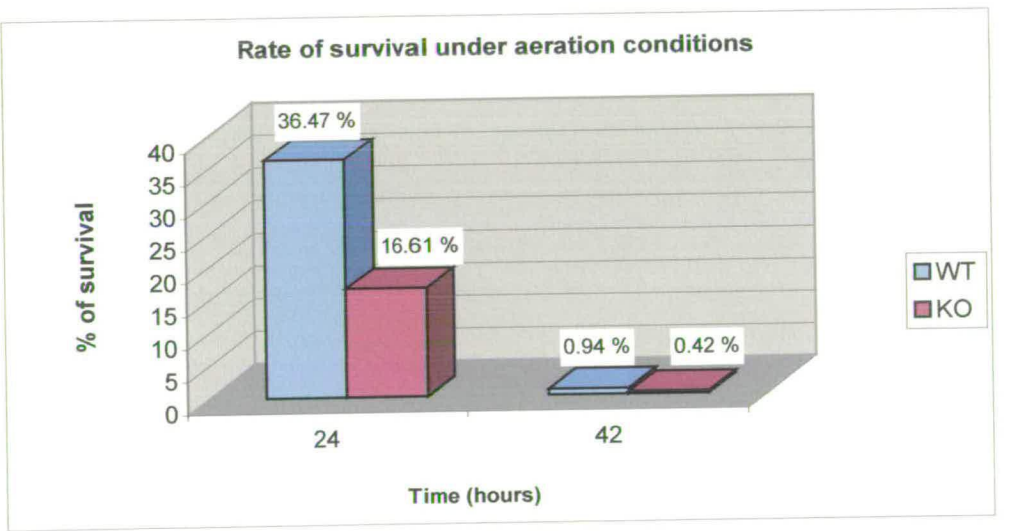


Fig. 5.3. NCI and Wt cells were exposed to atmospheric levels of oxygen. Viable counts were determined after 24 and 42 hours growth. The experiments were repeated three times, they showed similar pattern; only the results from one experiment are presented.

5.5. Competition

To test whether the wild type strain has a growth advantage during the growth period, competition experiments were performed over a 42 hour growth period. Samples were taken at different time points (12, 18, 30, 42 hours) and the viable cells were counted by counting the colonies on the plates. Cells were plated on MH only and MH + Km^+ and by difference the WT colonies were identified.

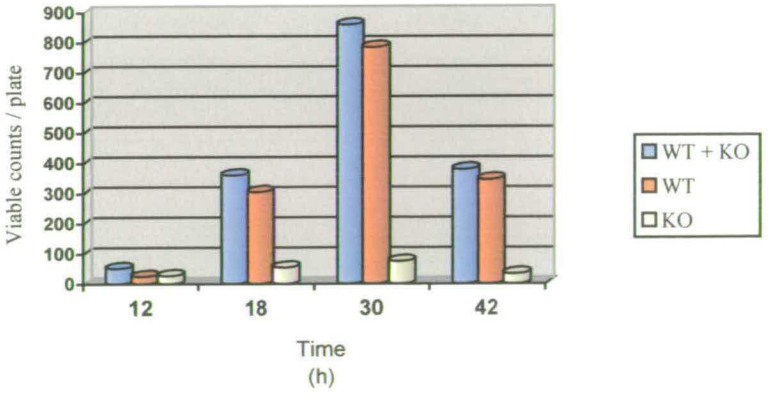
As shown in table 5.3 and figure 5.4 the WT cells have a significant growth advantage against the NCI strain. The experiment was repeated three times and an average between plates was made. In any case, they showed similar pattern; only the results from one experiment are presented.

Table 5.3

Number of colonies counted on plates at dilution 10^{-8}

Time (hours)	WT+NCI	WT	NCI
12	52	25	27
18	360	305	55
30	980	785	75
42	380	346	34

Fig. 5.4. Competition during growth between NCI and WT strains



5.6. Survival of cells under heat stress

Campylobacter jejuni grows optimally at 42°C, they are also sensitive to heat stress and are readily inactivated by standard cooking processes and pasteurisation. NCI and WT cells grown previously at 42°C were subjected to temperatures of 50°C, 53°C, and 55°C for ten minutes. Other studies have shown that most strains of *C. jejuni* are unable to grow at 47°C and above.

Studies were carried out in a series of eppendorf tubes and for each treatment duplicate determinations were done. Each tube contained 1mL of either NCI or WT cells and was heated for a period of ten minutes at 50°C, 53°C, or 55°C in a water bath. Dilutions of the samples were prepared using 100µl of distilled water and 20µl of the heat stressed cells. An inoculum of 20µl of each diluted sample containing the NCI strain was spread onto a MH agar plate supplemented with kanamycin and 20µl of the diluted heat stressed WT cells were plated on MH agar. Three plates for each dilution were prepared and incubated in the optimum growth conditions for 42 hours. After this period, the plates were removed and a viable cell count for each plate was obtained and the average for each sample was recorded.

At 50°C, both WT and NCI cells survived relatively well with a viable cell count (CFU) between 1 (Stdv ±0.2) x 10^5 and 1 (Stdv ±0.31) x 10^6 cells/ml. However, the survival of *Campylobacter* was still markedly decreased when compared to survival at the optimum growth temperature of 42°C at which temperature the NC1 counts were 5.26 x 10^{10} with a standard deviation of ±0.91 and 4.36 x 10^{10}, with a standard deviation of ±0.26 for the wild type.

As the temperature to which the NCI and WT cells were subjected to increases, the CFU of WT shows a rapid decline in survival by approximately two log units at each temperature. For example, between 50°C and 53°C the CFU of WT decreases from 1.75 (Stdv ±0.61) x 10^5 to 1.75 (Stdv ±0.26) x 10^3 cells/ml.

On the other hand, the survival rate of NCI *Campylobacter* remains relatively constant over all three temperatures. In comparison to the WT cells, NCI shows a

more gradual decline in survival at the highest temperature. Therefore, NCI cells demonstrate a greater resistance and ability to survive under heat stress whereas WT cells begin to die as the temperature moves further away from 42°C (fig. 5.5).

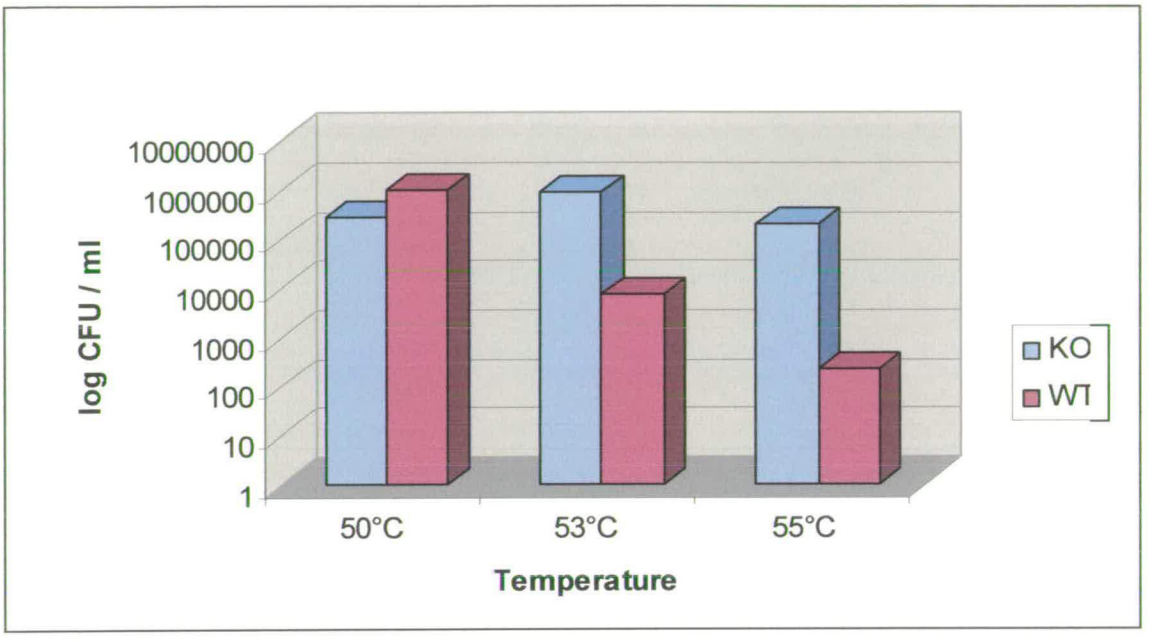


Fig. 5.5. Heat Stress.
NCI and WT pre-cultured cells grown at 42°C were subjected to 50°C, 53°C, and 55°C for 10 minutes. The samples were diluted then plated and left for 42 hours at optimum conditions after which a viable cell count was obtained. The experiments were repeated three times, they showed similar pattern; only the results from one experiment are presented.

5.7. Rate of survival at low temperatures

Freezing is a food preservation method used by both manufacturers and consumers and has multiple effects on living cells. Ultimately, freezing is subjecting the cells to drying as the amount of free water available to support growth is reduced. As water freezes, the solutes present in the media tend to concentrate in the unfrozen portion of the water where, consequently, bacteria will also accumulate. As a result, water will diffuse out of the cell to try and dilute the higher external solute concentration. Secondly, irreversible physical damage to the bacterial cell can occur due to the conversion of water into uneven ice crystals that can puncture the cell membrane. Oxidative damage has also been suggested as a mechanism by which freezing injures micro-organisms sensitive to oxygen, such as members of the *Campylobacter* genus.

Recent studies (Holler *et.al*, 1998) have shown that the resistance to chilling at 4°C and freeze-thawing at -20°C varies among *C. jejuni* strains, which implies a genetic basis for tolerance to low temperatures and susceptibility to cold injury.

Further studies could explore the effect of extracellular solutes such as NaCl on the susceptibility or resistance to low temperatures. Other modifications to the freezing or cooling medium, including pH, may also affect the ability of NCI and WT cells to survive.

Samples were prepared by transferring 1mL of either WT or NCI *Campylobacter* inoculum to sterile eppendorf tubes. These tubes containing the cultures were then kept at 4°C, or frozen at -20°C and -80°C, and samples were removed for plating at one-week intervals over a three weeks period. Once again, the NCI strain was plated onto MH agar containing kanamycin antibiotic and the WT cells on MH agar. Each sample was plated in triplicate and once the plates had been incubated for 42 hours in the optimum atmosphere, a viable cell count for each plate was determined and the average for each sample was calculated.

Over a period of three weeks, samples were obtained for NCI and WT at one-week intervals from various temperatures (4°C, -20°C, and -80°C). Similar results to the heat stress studies were obtained for this experiment in which the NCI cells, once again, seemed better adapted than the WT to survive at low temperatures.

The rate of survival reflected the time it took for the sample to freeze. Freezing will take longer to occur at -20°C than -80°C and will not occur at 4°C. This is demonstrated in figure 5.6, where at -80°C the CFU for both NCI and WT remains between $1x10^5$ and $1x10^6$ cells/ml due to the sustainability of the cells at such a low temperature. However, at -20°C and 4°C WT does not survive after one week For NCI, the rate of survival shows a more gradual and slower decline as, even in week three, there is still a substantial amount of growth on the plates at all three temperatures.

The cause of the sudden decline in the survival rate of wild type *Campylobacter* at low temperatures is not known at present. Many bacteria, including *E.coli*, produce cold shock proteins in response to temperatures below the optimum growth temperature, which enable the cells to continue replicating. However, *Campylobacter* is unable to replicate at temperatures as low as 4°C.The ability to carry out respiration, generate ATP, and motility are all maintained at temperatures as low as 4°C, which may have an implication in food storage.

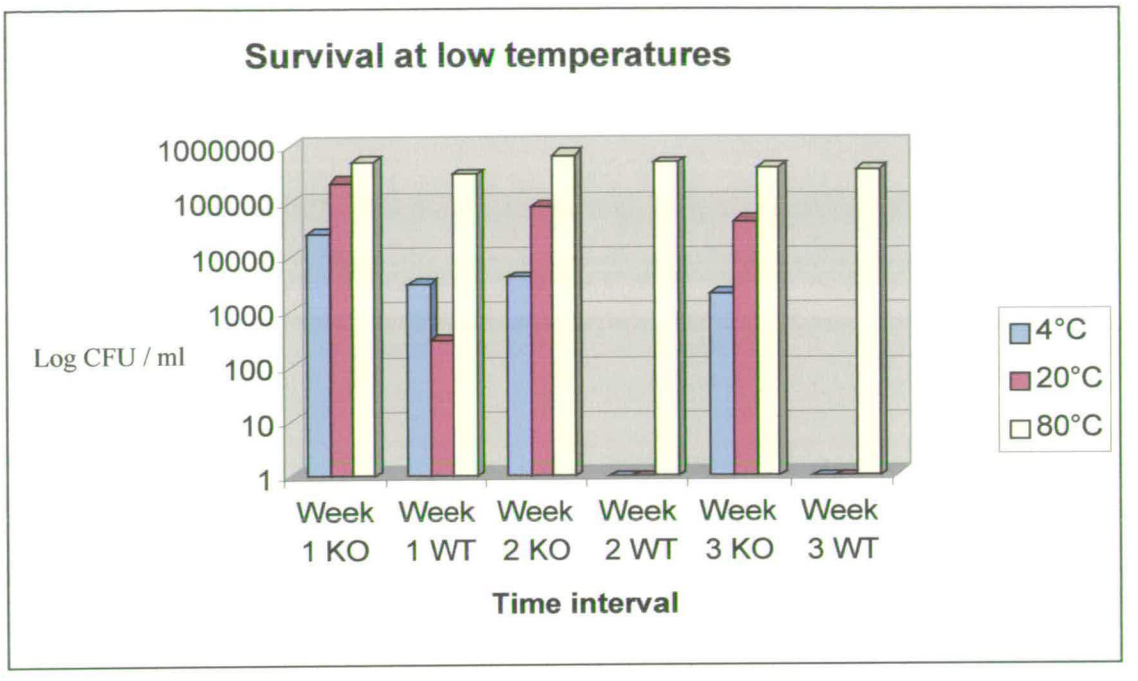


Fig. 5.6. Pre-cultured NCI and WT cells grown at 42°C were stored at 4°C, -20°C, and -80°C over a period of three weeks. At one week intervals, samples were removed and diluted for each temperature, which were then plated and grown in the optimum conditions for 42 h. A viable cell count was determined after the full incubation time. For statistical significance technical replicates were used for reproducibility with a Stdv between ±0.5 and ±0.1.

5.8. pH resistance

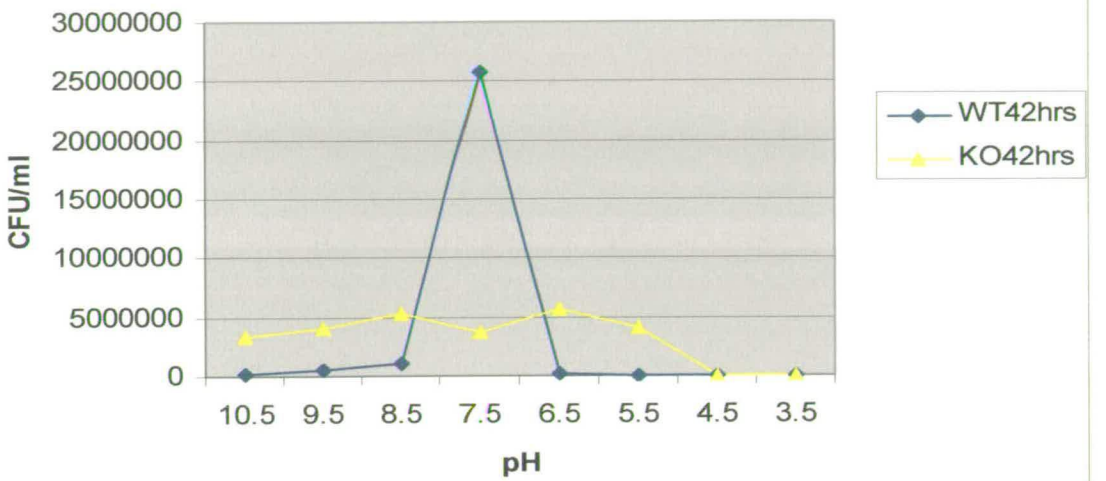
Most bacteria are unable to grow in an environment with a pH below four. Taking this into account, many food manufacturers add vinegar, which is essentially diluted acetic acid, to the food to lower the pH and inhibit microbial growth (Doyle, 1981).

MH medium was prepared as stated above and the pH was altered by the addition of either NaOH, which increased the alkalinity of the media, or HCl, which made the medium more acidic. The pH of the medium was adjusted to 3.5, 4.5, 5.5, 6.5, 7.5, 8.5, 9.5, and 10.5. For each pH value, the medium was divided up into three 50mL bottles for the WT cells and three 50mL bottles for the NCI strain. Before the bottles were inoculated with *Campylobacter* cells, the medium was autoclaved and then allowed to cool before testing the pH again (Doyle, 1981).

Each bottle was inoculated with 1mL of either NCI or WT *Campylobacter* and the experiment was carried out in triplicate. The bottles were left for 24 hours at 42°C in the optimum gas mixture for *Campylobacter*, after which time 20µl from each sample was removed and down using 100µl of distilled water. These samples were then plated following the procedure used in the previous experiments. After 42 hours of incubation, a second sample was removed from each bottle, dilutions were prepared in a similar manner, and again plated. All plates were left for 42 hours in the optimum conditions for *Campylobacter*. At the end of incubation period, the survivors were counted and the average for each sample was determined.

This study determined the effect that pH had on the ability of *Campylobacter* to survive in a medium supportive of its growth. Previous studies (Doyle, 1981) have shown that the optimum pH for growth of *Campylobacter* is in the range of 6.5 to 7.5. This is demonstrated in figure 5.7 and 5.8, where WT survives best at a pH value of 7.5. The WT cells otherwise fail to survive at pH above or below 7.5 after 42 hours. The low growth for the knockout might be explained by the fact that the inocullum was kept for about a week at 4 ^{0}C before inocullation.
NCI manages to sustain a relatively constant survival rate over a pH range of 10.5 to 5.5, after which the cells begin to die and no growth occurs on the plates.

Fig. 5.7. **Survival of *Campylobacter* at various pH units**



The pH of MH medium was altered using either NaOH to increase the pH or HCl to decrease the pH. Pre-cultured NCI and WT cells were transferred into 50mL of media with the altered pH (ranging from 3.5 to 10.5) and the growth was monitored over a period of 42h. After 42h, samples were removed, diluted, and plated to obtain a viable cell count following 42h of incubation.

Fig. 5.8. Rate of growth in 50 ml Mueller-Hinton

A – NCI strain growth at various pH values

B – WT strain growth at various pH values

5.9. Solute effects on the survival rate of *Campylobacter jejuni* wild type and NC1 strain

The cell membrane is selectively permeable, allowing for the passage of only certain molecules. NaCl is practically non-penetrant (Doyle, 1982), whereas glycerol pass through the lipid bilayer surrounding the cell. Initially, this experiment was carried out using only NaCl. However, to determine whether it was the change in the ionic strength or osmotic pressure of the cell's environment affecting the survival of the cell, an additional experiment was carried out using an uncharged compound, such as glycerol.

When the solute concentration outside the cell is higher than the solute concentration inside the cell the water activity, which describes the amount of water available to support cell growth, is reduced. When salt is added to the cell's environment, water will begin to leave the cell to balance the external and internal solute concentration and establish equilibrium. So essentially, the cell is subjected to drying and unless it is able to increase its internal solute concentration by either producing compatible solutes or accumulating them from the environment, it is unable to survive.

In bacteria the cytoplasmic levels of glutamate increases after exposure to media of high osmolarity, and especially in Gram-negative bacteria can elicit greater then 10-fold increases in the levels of glutamate. This amino-acid represents more then 90 % of the free amino-acids in some organisms grown in media of elevated osmotic strength. The intracellular levels of proline in enteric bacteria are also very high during the osmotic stress. The addition of proline into the growth medium may reduce the effect of osmotic stress on growth inhibition by increasing the uptake of proline inside the cell (Csonka, 1989).

Water activity also influences the storage stability and preservation of foods, such as meat, and so has a direct implication on the microbiological safety of food.

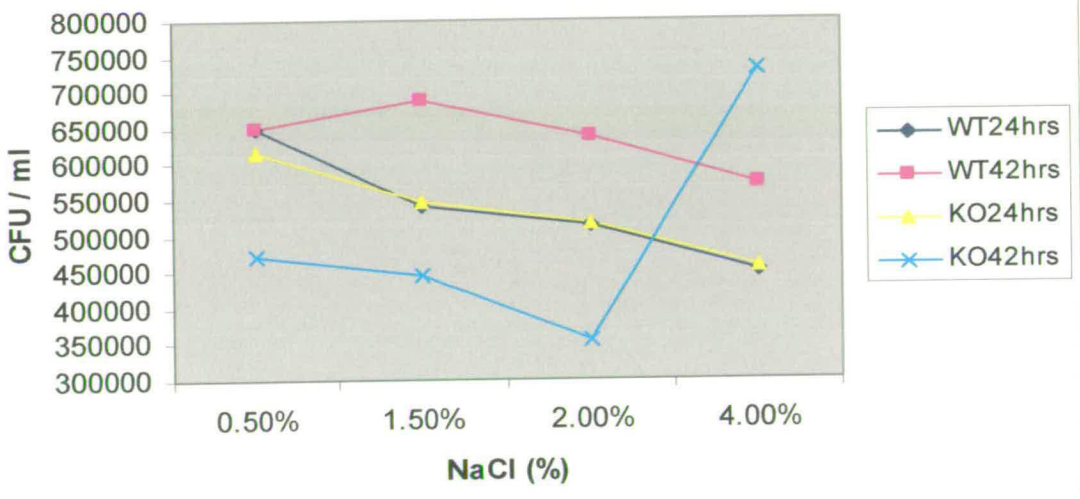
The studies were done using a series of 100mL bottles containing 50mL of MH broth supplemented with 0.5%, 1.5%, 2.0%, or 4.0% (wt/vol) sodium chloride. The medium was then autoclaved before inoculation with 1mL of either NC1 or WT *Campylobacter* cells. Following inoculation, the bottles were left in optimum growth conditions. Triplicate determinations were done for each treatment studied. After 24 hours, a 20µl sample was removed and diluted down using 100µl of distilled water, which was then plated according to the above procedure. A further 20µl was obtained from each sample after 42 hours of incubation, diluted, and then plated. All plates were left for 42 hours under optimum growth conditions after which the viable cell count was enumerated.

This same experiment was repeated using glycerol. The molar concentration of sodium chloride in the 0.5%, 1.5%, 2.0%, 4.0% solutions had to be calculated in order to ensure that the same molar concentration of glycerol was used. This is in order to determine whether it is the osmotic pressure that is affecting the growth of *Campylobacter*. The same protocol was followed using glycerol instead of sodium chloride (Doyle, 1982).

5.9.1 NaCl

The survival rate for WT and the survival rate for NC1 *Campylobacter* at different concentrations of NaCl follow a very similar pattern. Initially, wild type is able to grow at 1.5% NaCl after 24 hours, at time 0 (at the inoculation) the Wt counts were 3.6 x 10^4. At 0.5%, the viable cell count for WT cells at 24 hours remains the same even after the full 42 hour incubation period. With every other concentration used, the CFU of WT *Campylobacter* after 24 hours increased by approximately 150000 cells/ml (figure 5.9). Again, this level of change is expected from plating variation. Overall however, it can be seen that WT survival declines as the osmolarity is increased.

Fig. 5.9. **Solute effects on *Campylobacter* rate of survival using NaCl**



The concentration of NaCl in the MH medium was altered to 0.5%, 1.5%, 2.0%, 4.0% (wt/vol). The pre-cultured NC1 and WT cells were inoculated into the media and left for 42h. Samples were removed at 24h and again at 42h for plating and the viable cell counts were recorded after 42h incubation of the plates. The experiments were repeated three times, they showed similar pattern; only the results fromone experiment are presented.

NCI *Campylobacter* demonstrates a reduced survival at each concentration of NaCl after 0.5%. At 4.0%, after 42 hours of incubation there is a sudden and rapid increase in the CFU from 350000 to 750000 viable cells/ml. From previous experiments (Doyle *et.al,* 1982), it has been shown that WT *Campylobacter* has a reduced ability

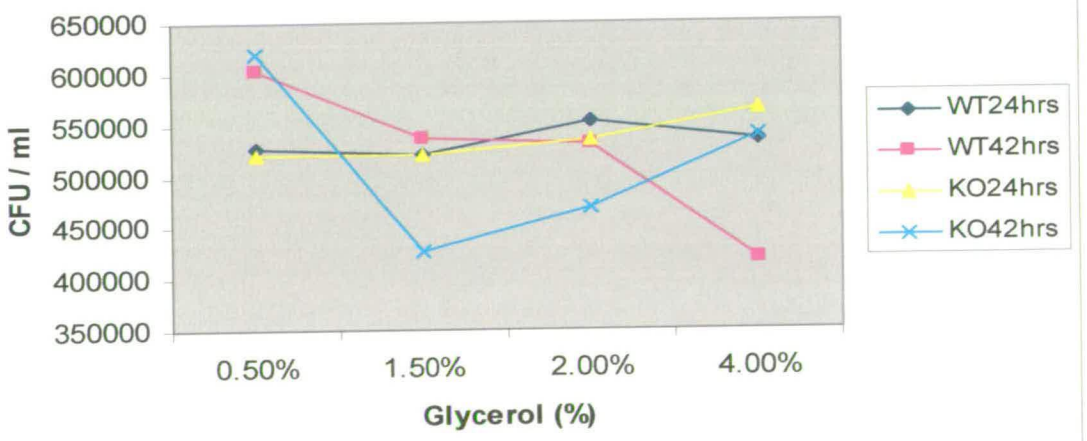
to grow in a salt concentration above 2.0% so this result from NCI at 4.0% where the survival rate increases quite dramatically is unexpected.

5.9.2. Glycerol

After 24 hours between 0.5 to 1.5%, the survival rate for both WT and NCI remains unchanged. Therefore, the increase in external solute concentration have a small effect on the number of viable cells and there is almost no difference between the responses of NC1 and Wt cells. At 2.0%, the CFU of both NCI and WT increases slightly and only at 4.0% do the cells of NCI and WT *Campylobacter* reacts differently.

At 42 hours, the pattern of survival is slightly different. With WT, there is a step-like reduction in CFU at each concentration of glycerol from 6 (Stdv ±0.36) $x10^5$ at 0.5% to 4 (Stdv ±0.43) $x10^5$cells/ml at 4.0% (figure 5.10). On the other hand, NCI has a similar survival level at 0.5% to wild type but is then significantly affected by the change in concentration to 1.5% with a reduction in the cell count by approximately 2 (±0.5) x 10^5 cells/ml. After 1.5%, the CFU for NCI begins to increase reaching 5.5 (±0.29) x 10^5 cells/ml at 4.0%. The variations observed in figure 5.10 reflect probably the plating variation.

Fig. 5.10. **Solute effects on *Campylobacter* rate of survival using glycerol**



Pre-cultured NCI and NCT1168 cells were transferred to 50ml of MH media. The glycerol concentration of the media had previously been altered to 0.5%, 1.5%, 2.0%, and 4.0% (wt/vol). At each concentration, the molarity of glycerol was similar to the molarity of NaCl for the corresponding concentration. The samples were removed after 24h and at 42h, which were then diluted and plated. Viable cell counts were determined after 42h incubation of the plates

5.10. The effect of bile salts on the survival rate of *Campylobacter*

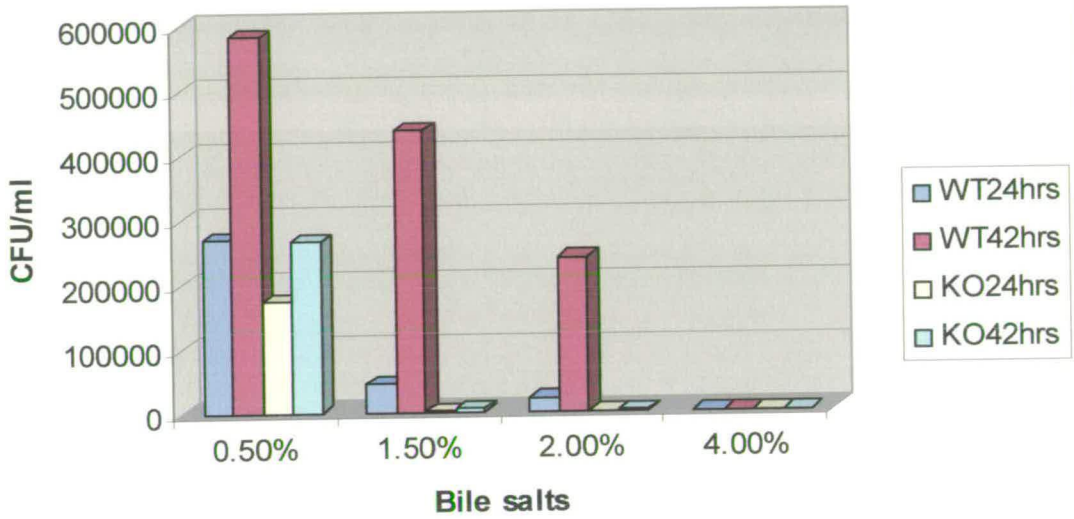
An adaptation that is vital to the survival of *Campylobacter* in the gut and, hence, its success causing infection, is its ability to withstand the presence of bile salts in the surrounding environment. Such an adaptation will serve as a competitive advantage over other bacteria colonising the gut. The volume of the bile salt pool in man has been estimated as either 4 g or 7.6 mmol was identified as 7.6 ± 0.9 mmol, which is secreted and reabsorbed at least twice during the digestion of a single meal (Jazrawi *et al*, 1986 and Van Deest *et.al,* 1968).

This experiment aimed to observe any differences between the response of wild type and knockout to elevated levels of bile salts in the media and, as a result, speculate on their behaviour within their normal habitat of the intestine (Van Deest, 1968).

MH broth was supplemented with 0.5%, 1.5%, 2.0%, 4.0% (wt/vol) bile salts (cholate and deoxycholate) and 50mL of media was transferred into 100mL bottles for each treatment for NCI and wild type. The medium was then autoclaved and, once cooled, 1mL of either NCI or WT *Campylobacter* cells were transferred to each bottle. This experiment was carried out in triplicate. The bottles were left in optimum growth conditions for 24 hours after which 20µl was removed and diluted with 100µl of water. The series of dilutions were then plated as before and a second set of samples were obtained and diluted after a further 18 hours. Viable cell counts for each plate were determined at the end of the incubation period.

Unlike previous experiments, WT is more resistant to the presence of bile salts than knockout, which failed to survive at any concentration above 0.5% of bile salts. WT survived particularly well at 0.5% reaching a CFU of 5.75 (Stdv ±0.6) x 10^5 cells/ml when left to grow for the optimum 42 hours, after which the CFU began to decrease to around 2.25 (Stdv ±0.53) x10^5 cells/ml at 2.0%. At 4.0%, neither wild type nor NC1 survived (figure 5.11).

Fig. 5.11. ***Campylobacter*** **survival at various concentrations of bile salts**



The ability of NCI and WT cells to survive various concentrations (0.5%, 1.5%, 2.0%, 4.0%) of bile salts in the MH medium was studied. Pre-cultured NCI and WT cells were transferred into 50mL of medium containing the bile salts. Samples were removed after 24h and at 42h, which were then diluted in preparation for plating. The surviving cells were then counted after 42h incubation of the plates. The experiments were repeated three times, they showed similar pattern; only the results from one experiment are presented.

5.11. Conclusions

Both strains had an ascendant growth curve. The growth peak was recorded at 24 hours for NC1 and at 30 hours for the Wt. At that stage the viable counts were 5.43 (Stdv±0.62) x 10^{11} for NCI and 6.96 (Stdv±0.46) x 10^{11} for Wt (table 5.1 and figure 5.1).

In the stationary phase at 75 hours the WT reached 7.1 (Stdv ±1.9) x 10^{10} CFU/ml. The NCI counts are decreasing constantly with the lowest cell count 2.1 (Stdv±0.7) x 10^{10} CFU/ml at 42 hours growth. These results show (table 5.2 and figure 5.2) that the rate of survival is similar for the NCI strain compared with the WT in the stationary phase.

Under aeration conditions the rate of survival for both strains was similar after 42 hours, only after 24 hours the rate of survival seems to be double for the Wt comparing with the NC1 (figure 5.3).

NCI cells demonstrate a greater resistance and ability to survive under heat stress whereas WT cells begin to die as the temperature moves further away from 42°C (fig. 5.5).

The survival rate for WT and the survival rate for NCI *Campylobacter* at different concentrations of NaCl follow a very similar pattern. Therefore, NCI is able to survive at higher concentrations of both glycerol and NaCl (above 2%).

WT is more resistant to the presence of bile salts than knockout, which failed to survive at any concentration above 0.5% of bile salts. At 4.0%, neither wild type nor NC1 survived (figure 5.11).

After a short period of storage at low temperature (4 ^{0}C) the NC1 cells had a very low growth even at normal pH values of the growth media.

Chapter 6
CAPSULAR SUGAR PROFILE

6.1. Introduction

The localization of the P450 gene in an operon in which most gene products are apparently involved either in biosynthesis of cell surface components or in sugar biosynthesis was the starting point in identification of the P450 enzyme function. Marsh (1986) identified the cytochrome P450 system more likely to be involved than a "glucuronolactone dehydrogenase" to be responsible D-glucaric acid *in vivo*. There is no other published evidence about any other P450s being involved in carbohydrate metabolism.

6.2. CPS and LOS composition

The reason for this experiment was to determine whether deleting the P450 gene from *Campylobacter jejuni 11168* genome has any effect on the carbohydrate content of lipooligosaccharide. Capsular polysaccharide (CPS) and LOS were prepared as described in chapter 2 and identified on a 15 % SDS-PAGE gel and silver stained for identification. One sample of the wild type strain (20 µl) and two samples of the NC1 strain (20 µl each) were used. The gel was migrated for 8 hours at 180 V and silver stained (see chapter 2). As seen in figure 6.1 no differences were identified in profile between wild type and NC1.

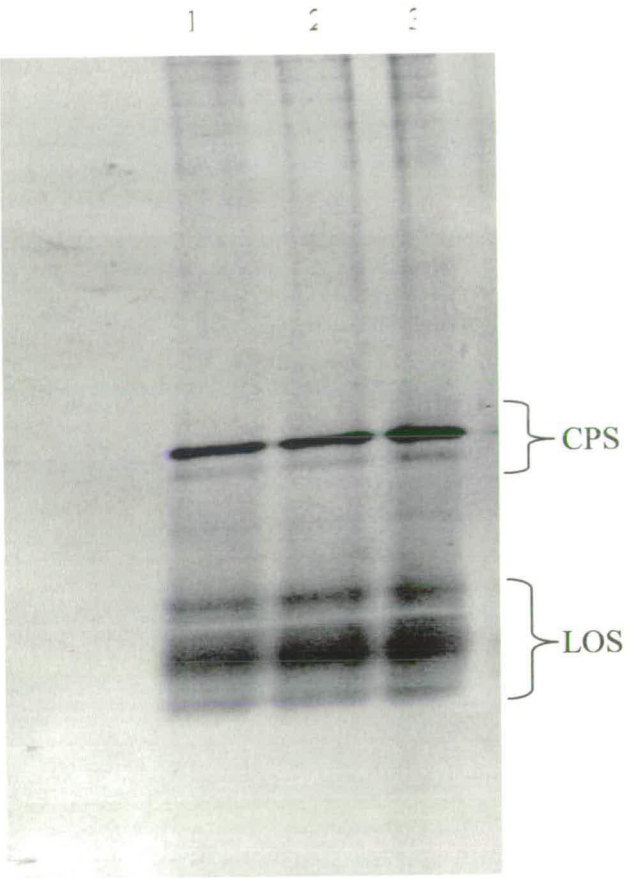


Fig. 6.1. CPS detection, in 15 % SDS-PAGE, of the wild type and NCI strain visualised by silver staining.

Lane1 – wild type (20 µl), Lane 2 and 3 NCI (20 µl)

6.3. Paper chromatography

Chromatography is a method commonly used for analyzing complex mixtures by separating them in the component chemicals. In order to determine whether the sugar profile inside the core polysaccharide was changed, by deleting of the P450 gene, polysaccharides were hydrolysed with TFA (as described in materials and methods) and samples were loaded on Whatman paper and silver stained for sugar profile identification. The main differences (figure 6.2) have been identified. Two extra bands are present in the NCI lane (2) which shows clearly that 2 new sugars are

formed by deleting the P450 gene. That was the first direct indication that the P450 enzyme may be involved in carbohydrate metabolsim.

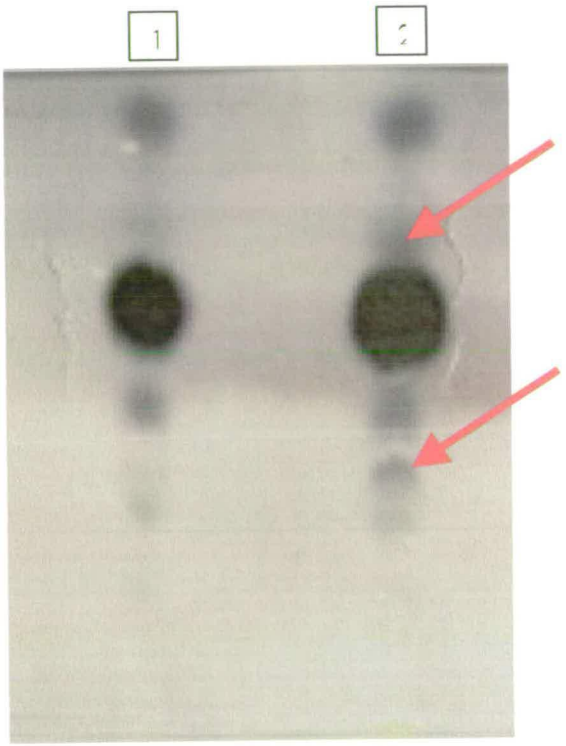


Fig. 6.2. Paper chromatography using Butanol/Acetic acid/water as a liquid phase. A sensitive silver stain was used to stain for sugars. Lane 1 – wild type, Lane 2 - NCI (indicated by arrows differences in sugar profiles identified by silver stain).

6.4. HPLC analysis of sugar profile

Sugars were prepared as described in chapter 2 and analysed on a Dionex HPLS with a CarboPac PA1 column. The eluent flow-rate was 1 ml/min (0.5 M, 0.6 ml/min) was added post column, and sugars were quantified with a pulsed amperometric detector (with gold electrode).

Analysing the capsular sugar profile by HPLC two main differences were identified between the wild type and NCI (mutant strain). Rhamnose, arabinose, galactose and glucose were used as components in the marker mix sample (fig. 6.3). The wild type and NC1 show similar elution profiles with two peaks making the difference between strains. Figure 6.4 shows that the wild type contains a sugar which is eluted after 13 minutes (peak 2) which was not identified in the NCI strain. On the other hand the NCI strain produce a sugar, eluted at 11.5 minutes, close to the elution times of the

rhamnose and arabinose markers (fig. 6.5). In order to identify peak 2 from the NCI spectrum, arabinose and rhamnose were injected into the samples and subjected again to analysis by HPLC. As shown in figure 6.6, the rhamnose peak does not match the NCI peak 2. When arabinose was injected into the sample (fig. 6.7) these peak match perfectly the NCI peak 2. It was clear from this experiment that the mutant produces arabinose which does not appear to accumulate in the wild type strain. We have tested whether the peak 2 from the wild type is arabinose by injecting arabinose into the Wt sample and found an extra peak which is eluted at the same time as the arabinose marker (fig. 6.8 – rhamnose and fig. 6.9 arabinose) i.e. peak 2 in Wt is not arabinose.

6.5. NMR analysis of sugar peaks

6.5.1. NMR on NC1 peak 2

The 1 Dimensional (1D) ^{1}H spectra were acquired on a 600 MHz Bruker Avance spectrometer equipped with a cryoprobe. The purified peak 2 from the NC1 spectra was dissolved in D_2O and spectra collected at 25 ^{0}C (16 scans were used to acquire the data). The HOD signal (4.78 ppm) was used as an internal standard to reference the spectra. By comparison of the standard probe spectrum (L – Arabinose from Sigma-UK) with the sample spectrum a perfect identity in profile was identified. The peak 2 from the NCI strain was identified to be arabinose (fig. 6.10).

6.5.2. NMR on wild type peak 2

All NMR experiments were performed on a 600 MHz BRUKER Avance spectrometer equipped with a cryoprobe. 1D ^{1}H NMR spectrum of wild type was acquired using 512 scans.

The 2D gradient-selected COSY experiment was acquired in the magnitude mode using 32 scans per each of 1408 increments. The relaxation delay was 1.5 s and the acquisition times in the t_2 and t_1 were 340 and 140 ms, respectively. The total acquisition time was 25 hour. The 2D TOCSY experiment was acquired in the phase-sensitive mode using 80 scans per each of 512 increments. The relaxation delay was 1.5 s and the acquisition times in the t_2 and t_1 were 340 and 50 ms, respectively. The

total acquisition time was 24 hours. A 140 ms DIPIS-2 spin-lock was used at 8.3 kHz B_1 field strength.

1D ^1H NMR spectrum shows signals in the region 2-6 ppm that likely originate in carbohydrate protons. Two signals at 5.22 (d, 3.7Hz) ppm and 4.49 (d, 8.3 Hz) ppm can be assigned to α- and β-anomeric protons based on their chemical shifts and H_1H_2 coupling constants. The sample contained many impurities; therefore it was possible, from the analysis of 2D NMR spectra discussed below, to determine only the type of carbohydrate without establishing if any non-carbohydrate substituents are attached to this ring (referred to below as compound I).

No transfer of magnetization was observed in the 2D TOCSY of compound I beyond proton H-4 despite using a long-mixing time of 140 ms. This indicates that this sugar residue is likely a hexopyranose in the galacto configuration. The H_2H_3 coupling constants of 11.2(α) Hz and 10.1(β) Hz that were determined from the 1D spectrum and the inspection of traces from 2D TOCSY spectrum confirm this hypothesis. Chemical shift of H1-H4 of I are summarized in table 6.1. For comparison, also the chemical shifts of Galp and GalpNAc are given (Jansson, 1989). It is evident that the H-2 protons in both anomeric forms of I have much lower chemicals shifts than those of Galp or GalpNAc. This suggests that the compound I has a free amino group attached at position C-2. Although we could not find data of such compound in the literature, GlcpN data (Vinogradov, 1998) (3.05 and 2.86 ppm for α and β anomers of H2 protons, respectively) are in a very good agreement with the observed chemical shifts of compound I. Compound I therefore has GalpN skeleton (figures 6.11 and 6.12).

Table 6.1

	αGal	αGal*p*NAc	αI	βGal	βGal*p*NAc	βI
H1	5.22	5.28	5.22	4.53	4.68	4.49
H2	3.78	4.19	2.95	3.45	3.9	2.81
H3	3.81	3.95	3.68	3.59	3.77	3.52
H4	3.95	4.05	3.93	3.89	3.98	3.85
H5	4.03	4.13	?	3.65	3.72	?

^1H Chemical shifts, ppm

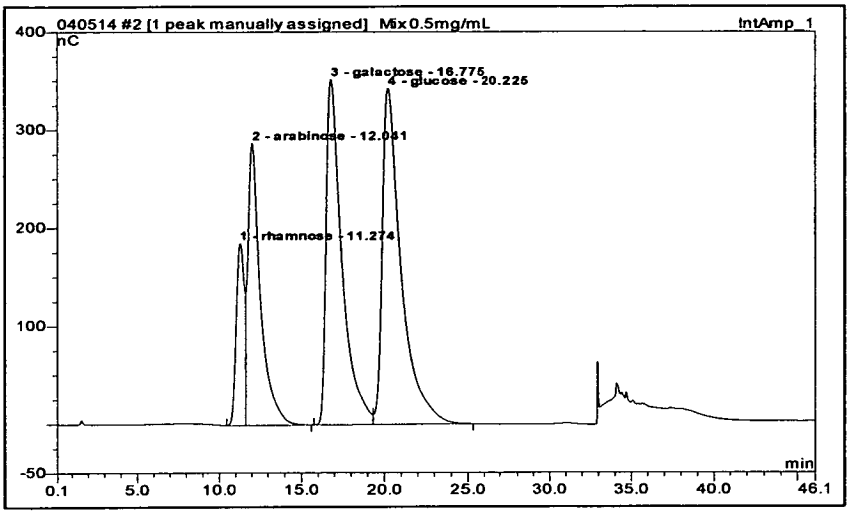


Fig. 6.3. HPLC spectra of the marker mix sample. Rhamnose, arabinose, galactose and glucose were used as markers. (individual sugar peaks are labelled, elution time being indicated)

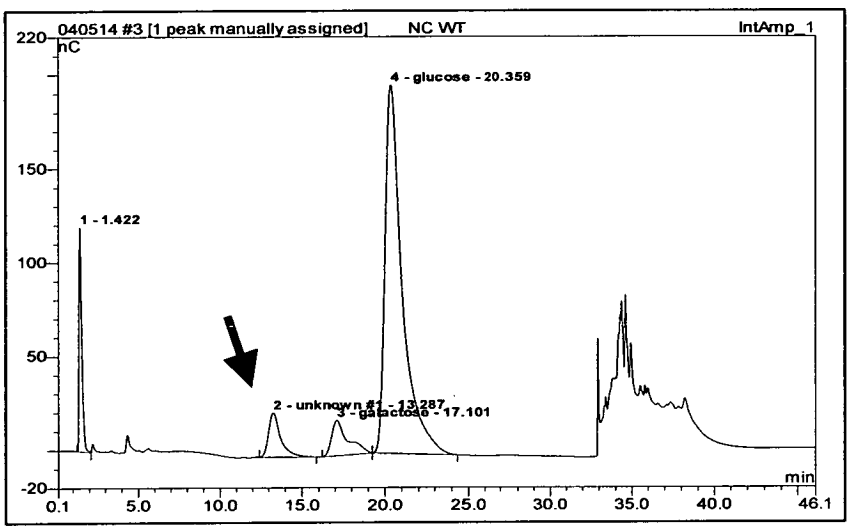


Fig. 6.4 . HPLC spectra of the wild type sugar profile. Arrow indicates the extra sugar present in to wild type profile. (individual sugar peaks are labelled, elution time being indicated)

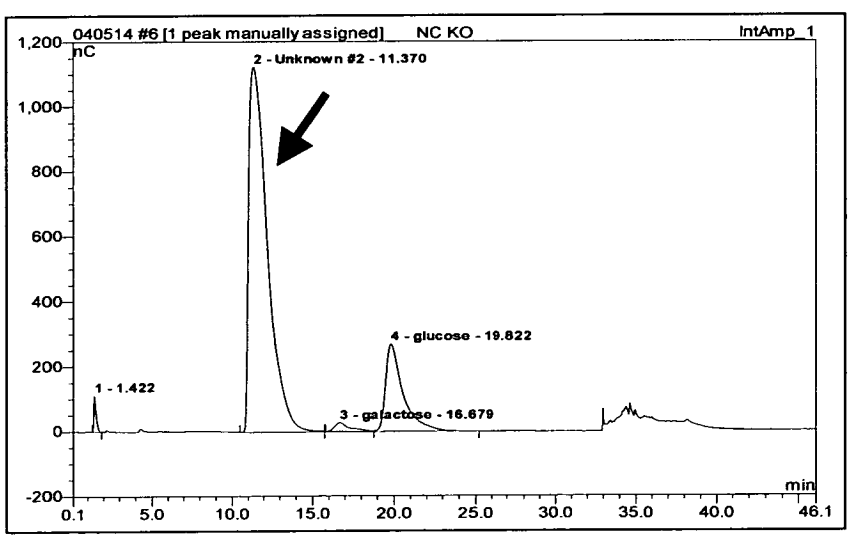


Fig. 6.5 . HPLC spectra of the NCI sugar profile. Arrow indicates the extra sugar present in to NCI profile. (individual sugar peaks are labelled, elution time being indicated)

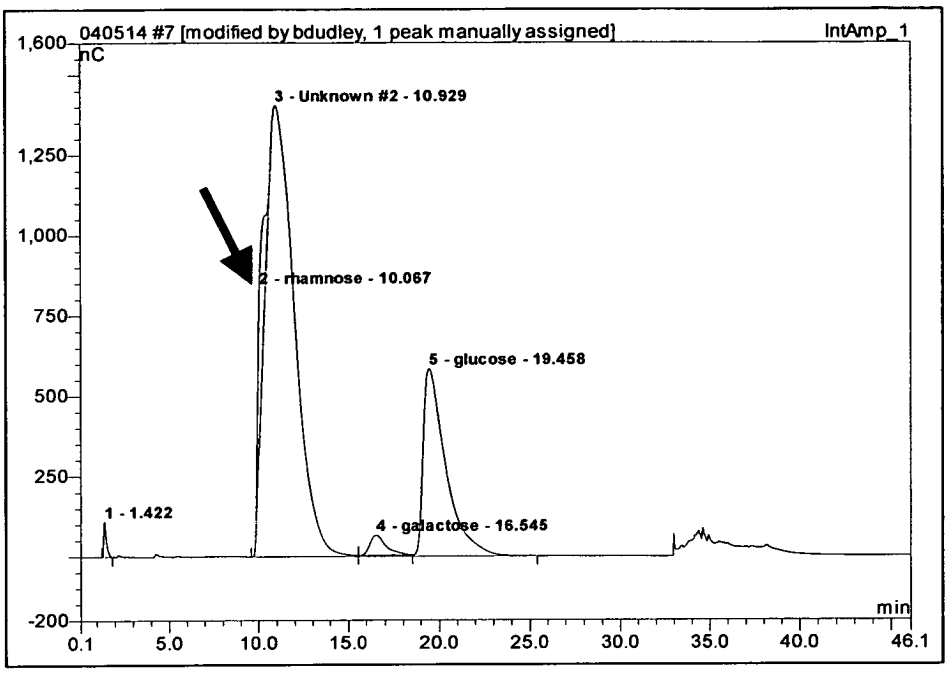


Fig. 6.6. HPLC spectra of the NCI sugar profile with injected rhamnose. Arrow indicates the rhamnose peak. 50 mg / ml rhamnose were injected into the sample (individual sugar peaks are labelled, elution time being indicated)

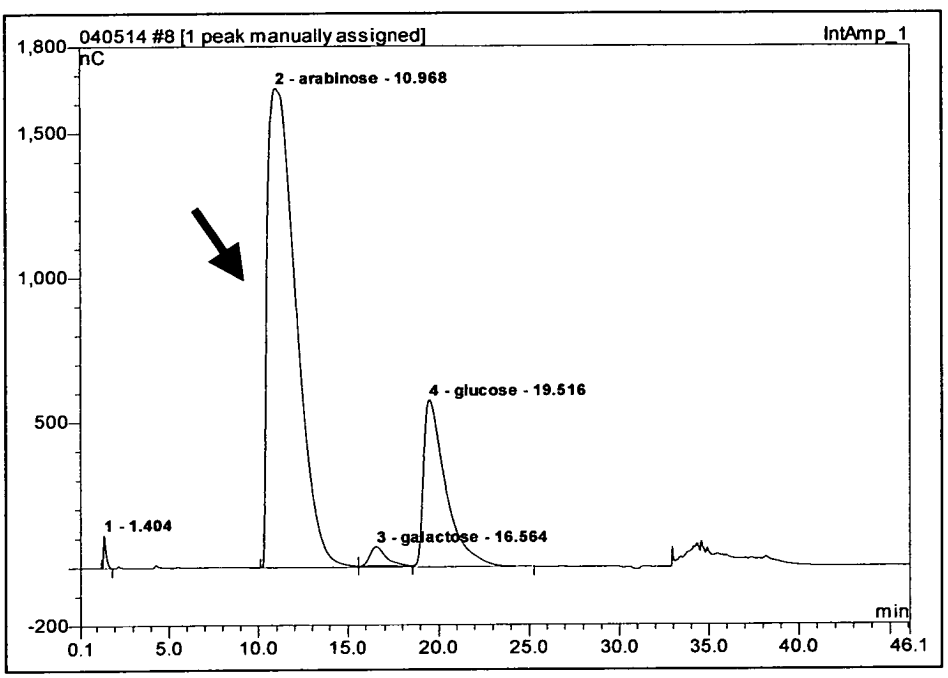


Fig. 6.7. HPLC spectra of the NCI sugar profile with injected arabinose. Arrow indicates the arabinose peak. 50 mg / ml arabinose were injected into the sample. The arabinose peak matches perfectly the NCI peak 2. (individual sugar peaks are labelled, elution time being indicated)

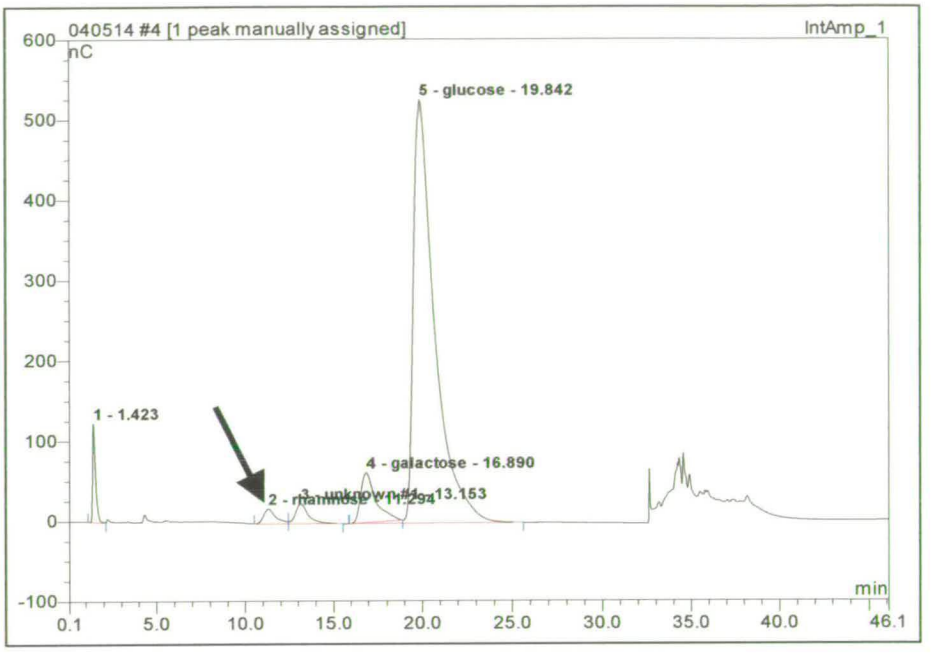


Fig. 6.8. HPLC spectra of the wild type sugar profile with injected rhamnose. Arrow indicates the rhamnose peak. 50 mg / ml rhamnose were injected into the sample. HPLC spectrum indicates that the rhamnose is eluted separately and did not match any of the wild type peaks (individual sugar peaks are labelled, elution time being indicated)

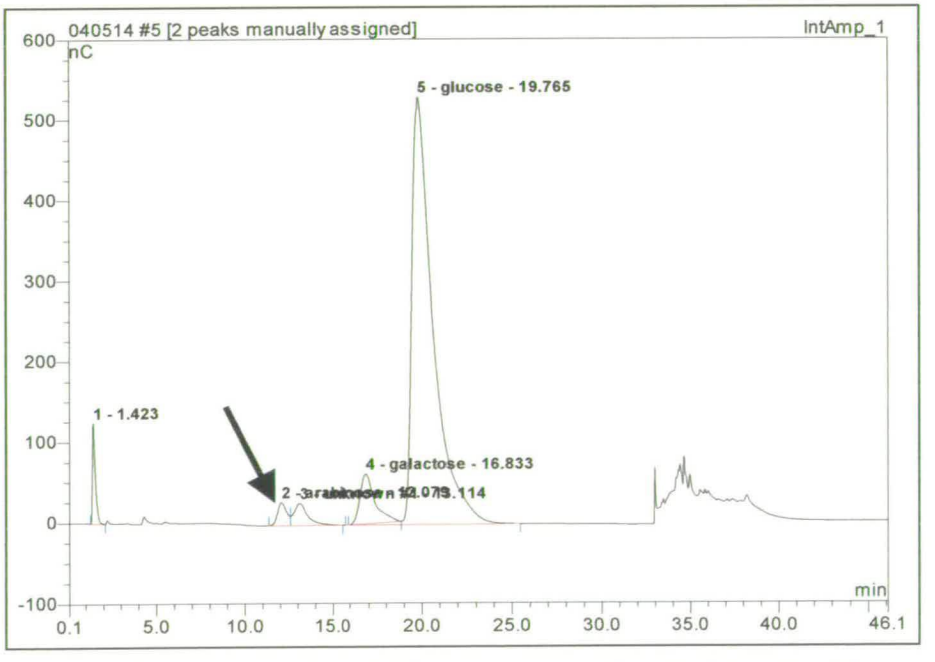


Fig. 6.9. HPLC spectra of the wild type sugar profile with injected arabinose. Arrow indicates the arabinose peak. 50 mg / ml arabinose were injected into the sample. HPLC spectrum indicates that the arabinose is eluted separately and did not match any of the wild type peaks (individual sugar peaks are labelled, elution time being indicated)

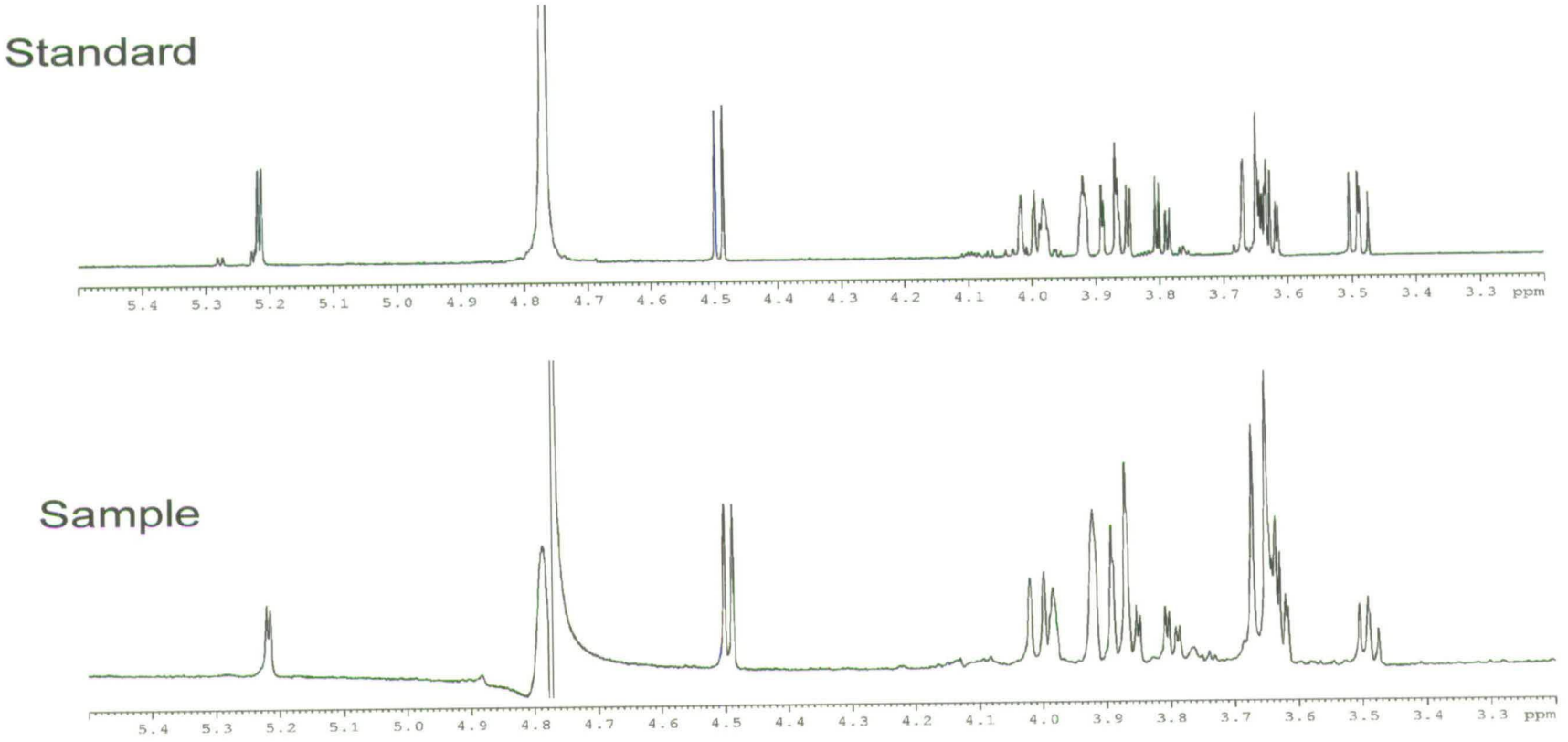


Fig. 6.10. 1D 1H spectrum of the NCI peak 2. Standard arabinose was used to compare the NMR spectrum of the peak 2. The two spectra mach perfectly arabinose being identified as a new sugar produced by the NCI strain.

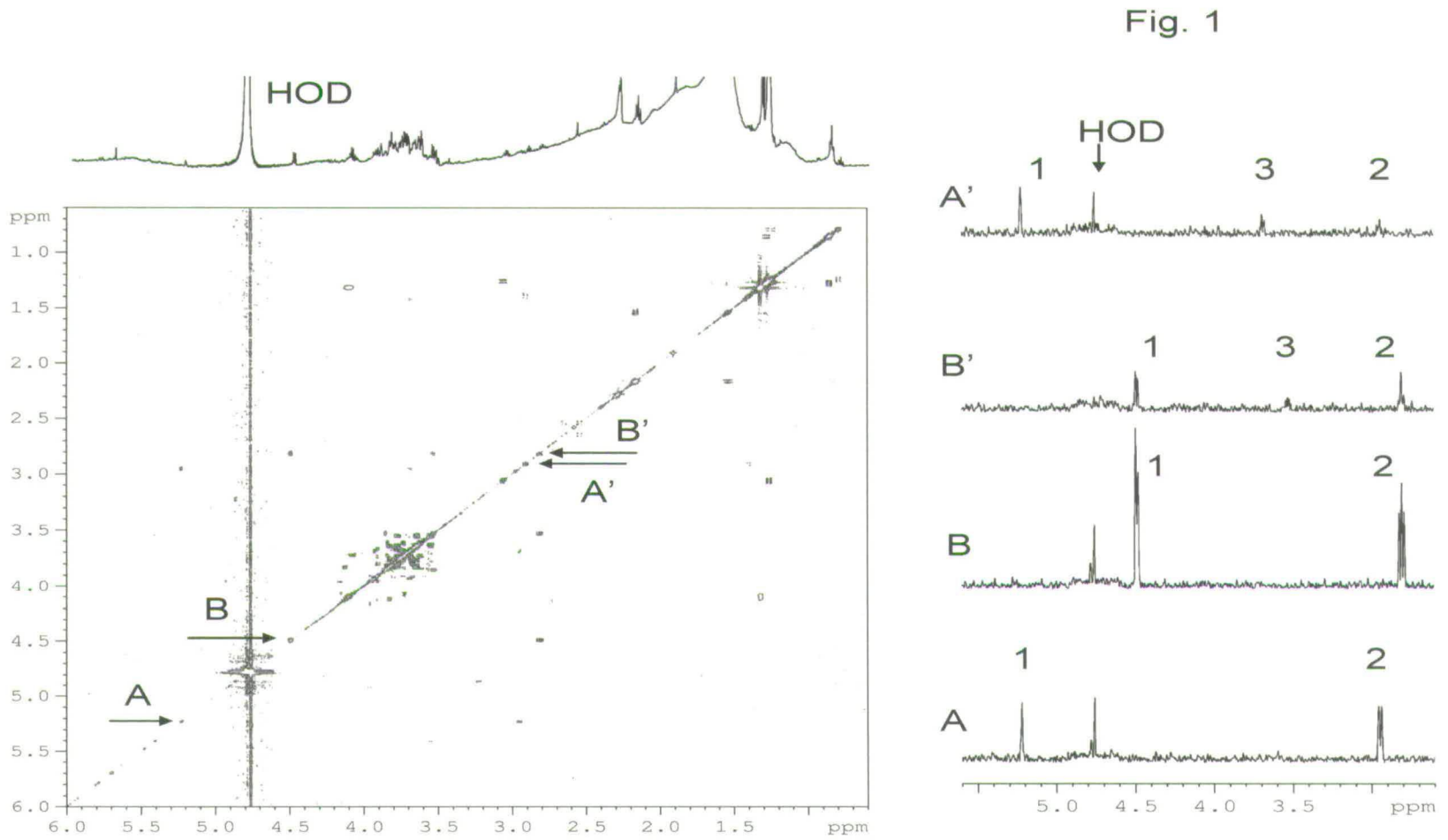


Fig.6.11. 2D COSY of wild type. 1D ^{1}H NMR spectrum is shown at the top of the 2D spectrum.
1D traces shown on the right were taken at positions indicated by arrows in the 2D spectrum. The A and A' traces were taken at chemical shifts of H1α and H2α protons, respectively, while the B and B' show traces taken at chemical shifts of H1β and H2β protons, respectively. This experiment established the chemical shifts of protons 1-3 in both α- and β-anomeric forms. This sugar was identified by HPLC and is presented as unknown in figure 6.4.

Fig. 2

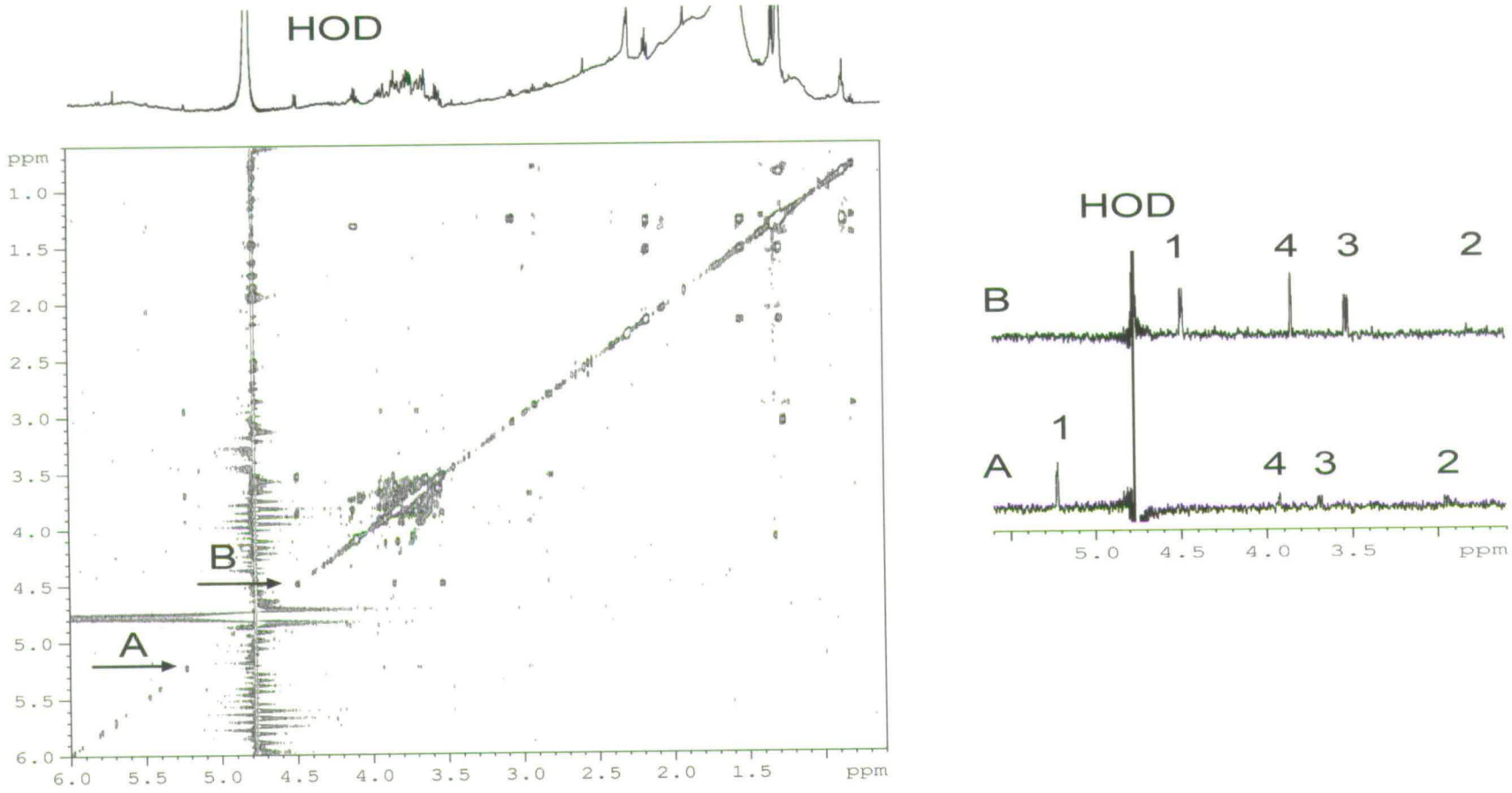


Fig. 6.12. 2D TOCSY of wild type. . 1D ^{1}H NMR spectrum is shown at the top of the 2D spectrum.
1D traces shown on the right were taken at positions indicated by arrows in the 2D spectrum. The A and B traces were taken at chemical shifts of H1α and H1β protons, respectively. This experiment confirmed the assignment of proton resonances 1-3 from the COSY experiment and also established the chemical shifts of H-4 protons.

6.6. Conclusions

This chapter shows that *Campylobacter jejuni 11168* P450 is involved in cell surface carbohydrate biosynthesis.

There was no difference detected in the overall profile of LPS but the HPLC on hydrolysed sugars detected a difference in sugar profile between the NC1 and Wt strain.

Arabinose was identified by HPLC and NMR to be the monosaccharide produced by the NC1 strain but undetected in the wild type.

As previously stated the only P450 enzyme previously known to be involved in sugar metabolism was identified in mammalian system and seems to be involved in conversion of the glucuronic acid to glucaric acid.

Under the same mechanism the arabinose might be changed to arabinonic acid (figure 8.1) which might be the explanation for the absence of arabinose in the Wt strain. The wild type extra peak, in sugar profile, was partially characterised by NMR.

Chapter 7
ELECTRON MICROSCOPY

7.1. Introduction

Electron microscopy is commonly used to identify differences in morphology between different species of bacteria and has been shown to be a very useful tool to identify morphological changes between wild type and a knockout strains in *Campylobacter jejuni 11168* and *11828*. Wood *et.al,* (1999) discovered that deleting genes involved in LPS or LOS biosynthesis resulted in altered cellular morphology in the knockouts (wider and shorter cells) by comparison with the wild type (fig 7.1).

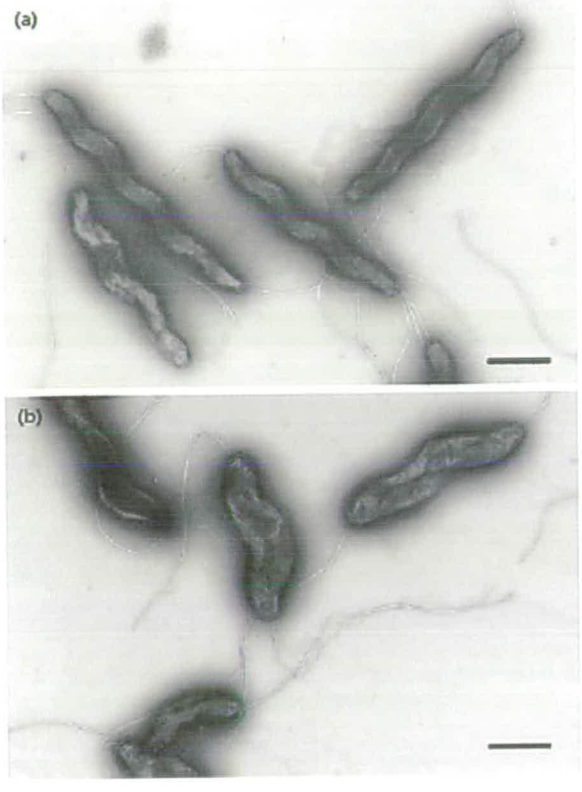


Fig. 7.1. Electron micrograph of negatively stained NCTC 11168 (a) and *orfF* mutant (b) illustrating that the mutation results in shorter and wider cells (Wood, 1999)
(Bars - 1 μm)

7.2. Visualisation of the wild type and knockout strain by Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM)

First indication of modified cell morphology came from the size of colonies, the NCI cells being almost half in diameter comparing with the wild type (fig 7.2).

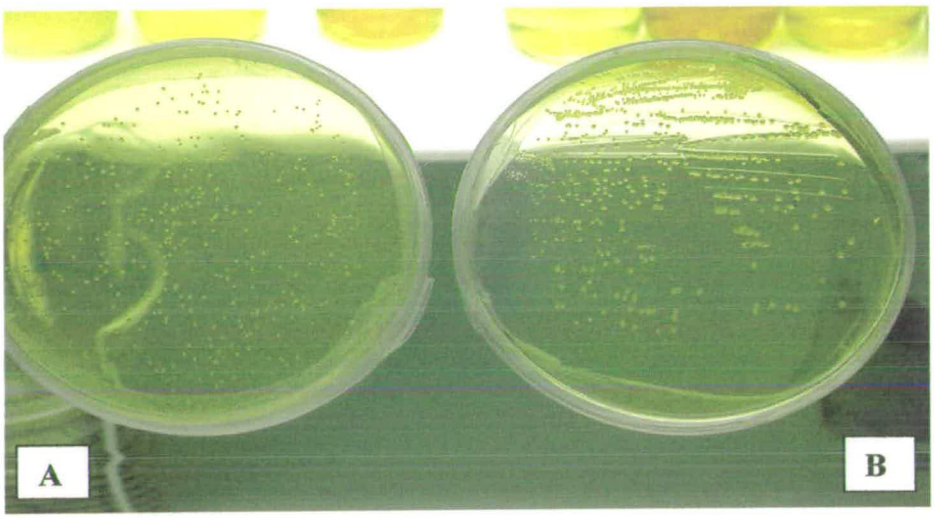


Fig. 7.2. *Campylobacter jejuni 11168* wild type and knockout (NCI) on Mueller-Hinton agar plates after 42 hours growth (A-NCI and B – wild type)

Further experiments were conducted using electron microscopy. As in the literature clear changes in cell morphology were identified by SEM (figure 7.3 – wild type and 7.4 – NCI) and TEM (figure 7.5 – wild type and 7.6 – NCI) wild type cells being shorter and wider than the knockout cells. Measurements were done, as described in materials and methods. The length of the wild type cells was 2.050 ± 0.61 μm and 1.828 ± 0.30 μm for the NCI. The breadth of the cells was 0.495 ± 0.04 μm for the wild type and 0.726 ± 0.26 μm for the NCI. Calculating the volume of the cells, with 0.524 ± 0.23 μm^3 the volume of the wild type was identified to be half of the NCI volume which was 1.095 ± 0.87 μm^3. The area and perimeter was also determined results being presented in table 7.1. The measurements were done using a computer software named Optimas 6. The cells were outlined, then thresholded to give the area and circumference. The length was obtained from this by obtaining the shortest width

measurement and measuring at 90 degrees to that, otherwise a measurement of the longest length would be diagonal and too long.

Table 7.1

Results on cell dimensions

Strain	Area	Perimeter	Length	Breadth	Volume
	μm^2	μm^2	μm	μm	μm^3
Wild type 11168	**0.703 ± 0.19**	**5.408 ± 1.31**	**2.050 ± 0.61**	**0.495 ± 0.04**	**0.524 ± 0.23**
NCI	**0.811 ± 0.34**	**4.826 ± 0.97**	**1.828 ± 0.30**	**0.726 ± 0.26**	**1.095 ± 0.87**

Abnormal cells were identified in both cases, ~ 40 μm in length (figure 7.7 and 7.8). Wild type cells have the characteristic spiral shape during the growth period as presented in figure 7.9 (A, B, C and D). Cells in all life stages are present as in figure A8 coccoid forms can be identified (indicated by arrow). The same normal growth characteristics have been observed by visualising the wild type cells under SEM in 3D view (figure 7.10 A and 7.10 B).

The NCI cells were dramatically affected by the replacement of the P450 gene. Figures 7.11A, 7.11B, 7.11C and 7.11D shows clear disturbed cell morphology, with a high number of coccoid cells present in the culture, in the middle of growth period. To see whether the cell wall was affected, cells were sliced and visualised by TEM. Clear disturbed cell surface is observed for NCI cells whether the surface of the wild type cells is smooth and compact (figure 7.12 and 7.13).

These changes in morphology had no effect on motility since no significant differences were identified between the NCI and wild type *Campylobacter jejuni 11168* (0.88 ± 0.39 cm for wild type and 0.69 ± 0.25 cm for the NCI strain).

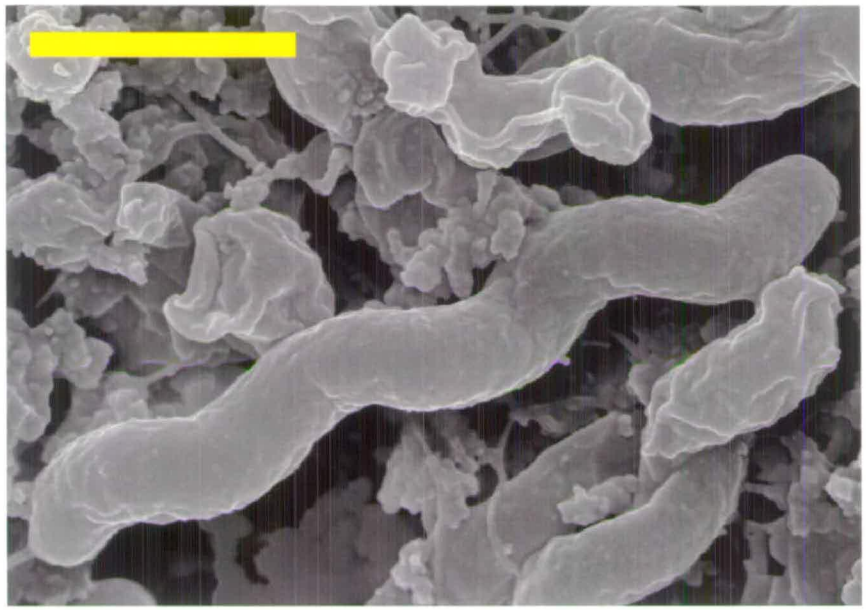


Fig.7.3. Visualisation, on SEM, of the
wild type cells
(Bars – 1 μm)
S4700, 5.0 kV, 2.6 mm x 6.00 k SE (U)

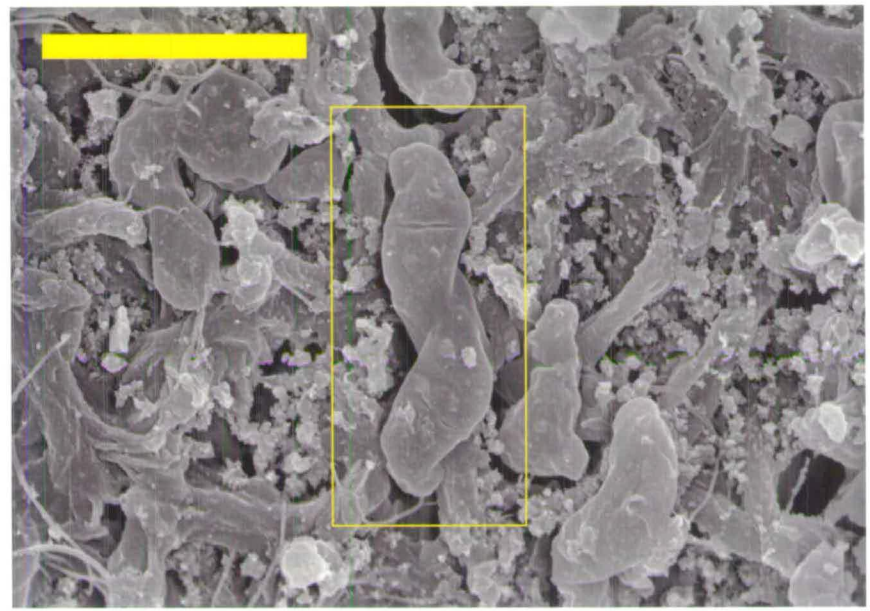


Fig.7.4. Visualisation, on SEM, of the
NC1 cells (yellow square)
(Bars – 1 μm)
S4700, 5.0 kV, 2.6 mm x 6.00 k SE (U)

Fig.7.5. Visualisation, on TEM, of the wild type cells (8.40 k)

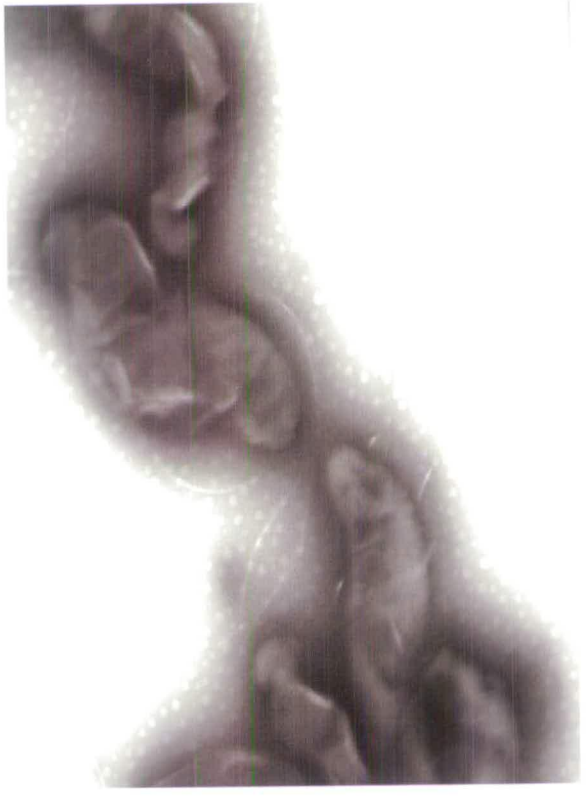


Fig.7.6. Visualisation, on TEM, of the wild type cells (27.5 k)

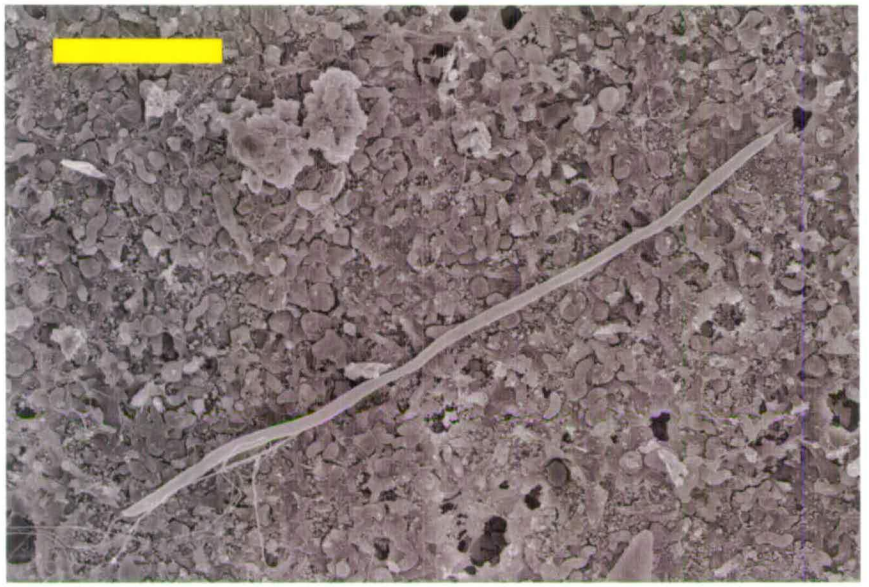


Fig. 7.7. Visualisation, on SEM, of a 40 μm long cell of *Campylobacter jejuni 11168* knockout strain (NC1)
(Bars – 5 μm)
S4700, 5.0 kV, 2.6 mm x 6.00 k SE (U)

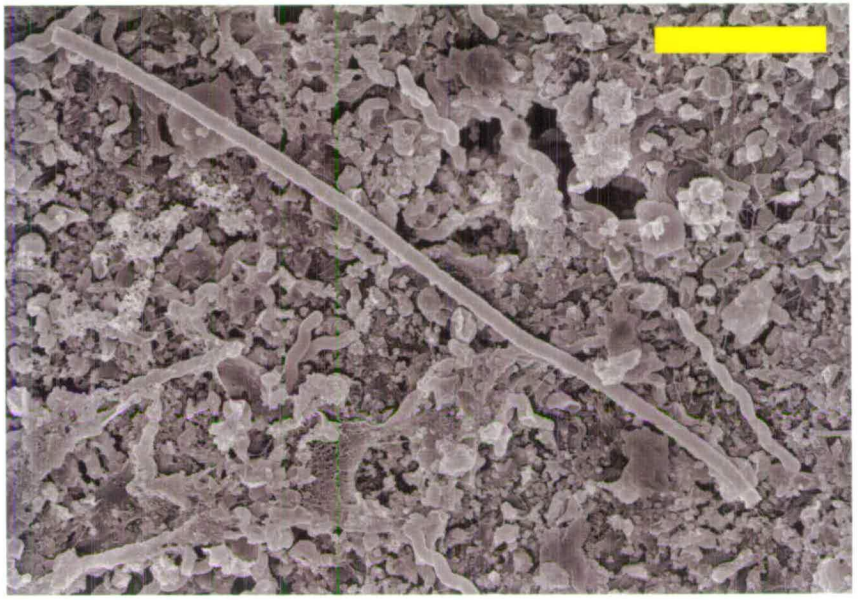


Fig. 7.8. Visualisation, on SEM, of a 40 μm long cell of *Campylobacter jejuni 11168* wild type
(Bars – 5 μm)
S4700, 5.0 kV, 2.6 mm x 6.00 k SE (U)

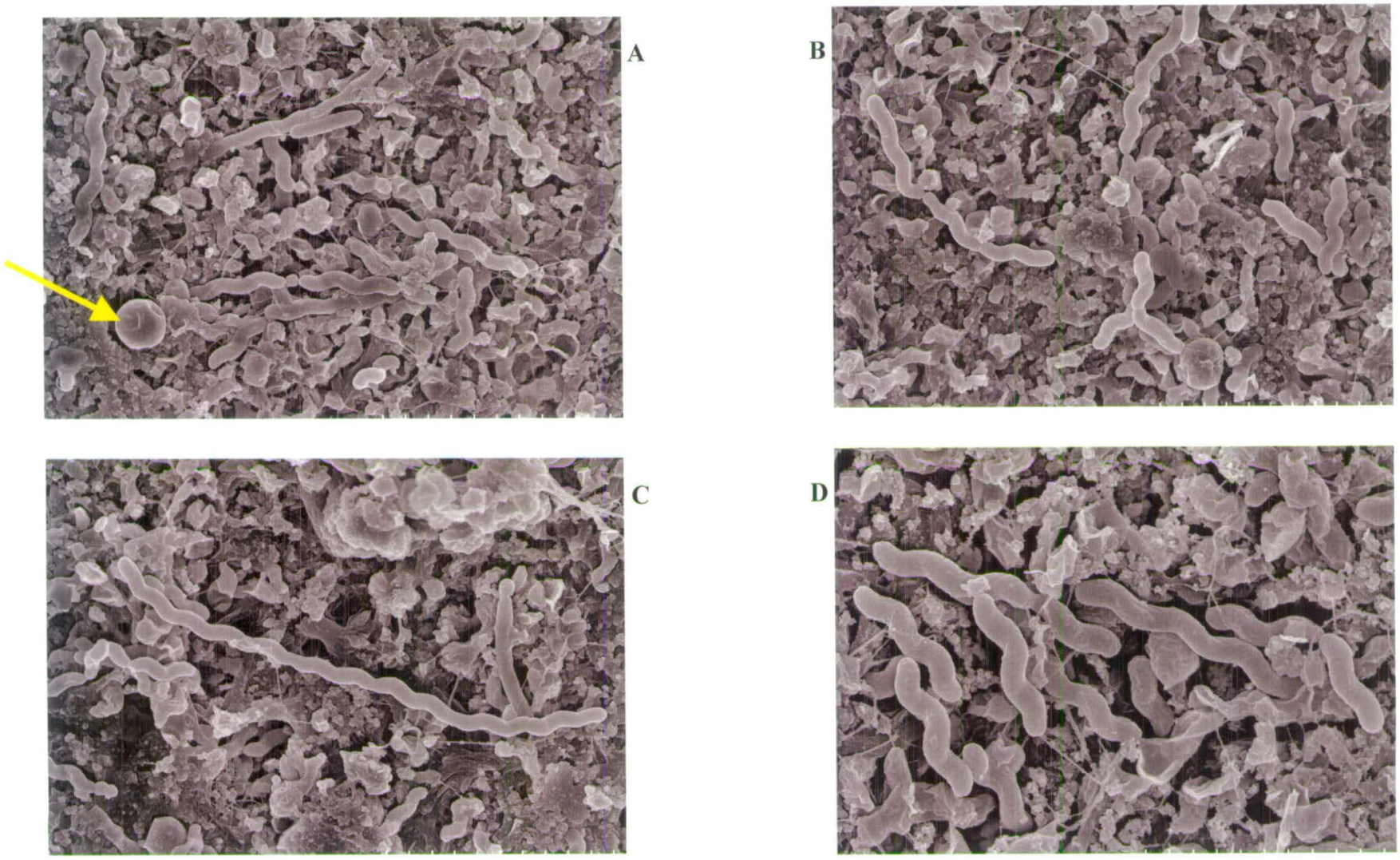


Fig.7.9 . Visualisation, on SEM, of the wild type cells at different scales
S4700, 5.0 kV, 2.6 mm x 10 k SE (U) for figures A, B and C
and S4700, 5.0 kV, 2.6 mm x 18 k SE (U) for figure A11

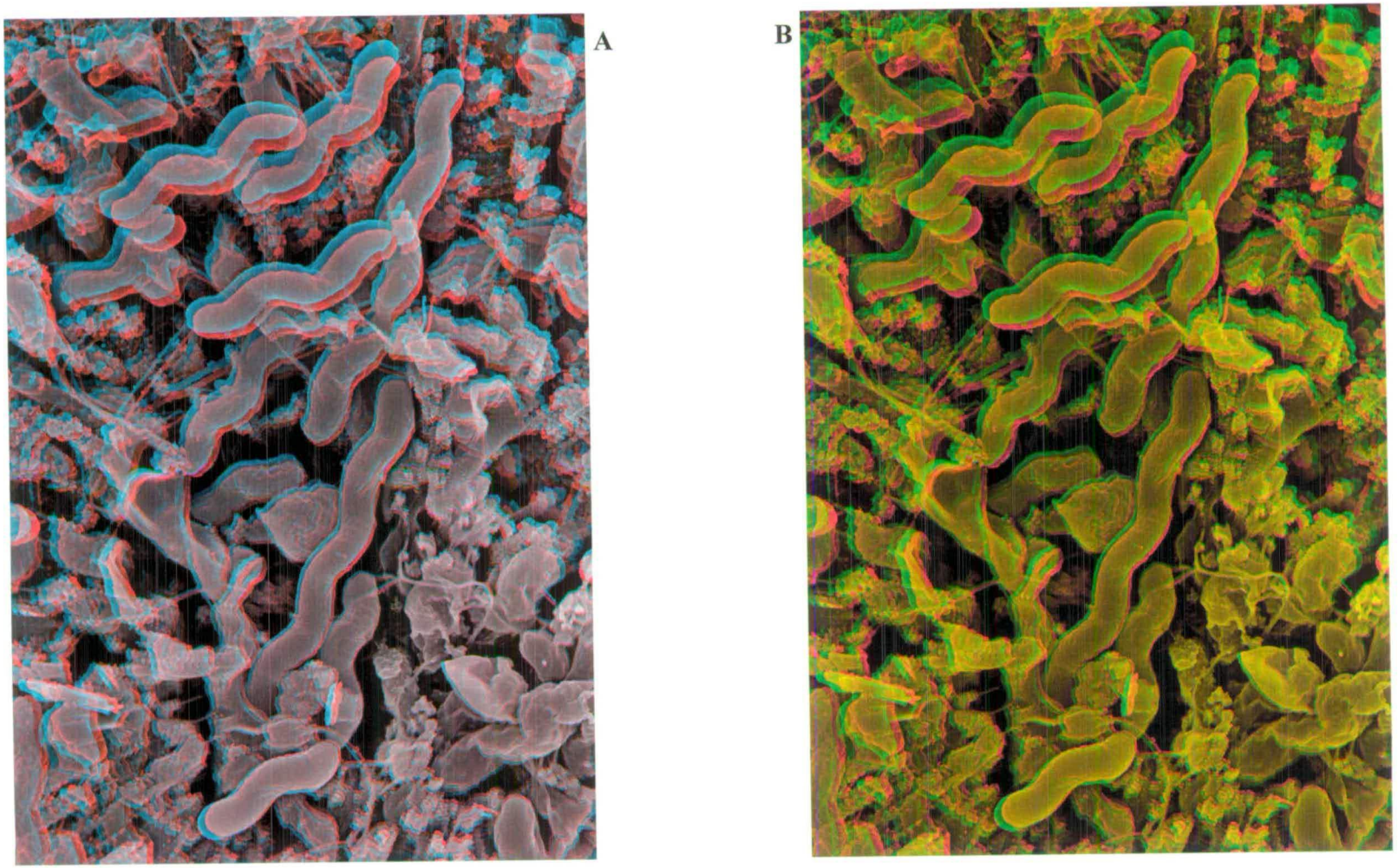


Fig.7.10 . 3D view over the wild type cells
S4700, 5.0 kV, 2.6 mm x 18 k SE (U) for figures A and B

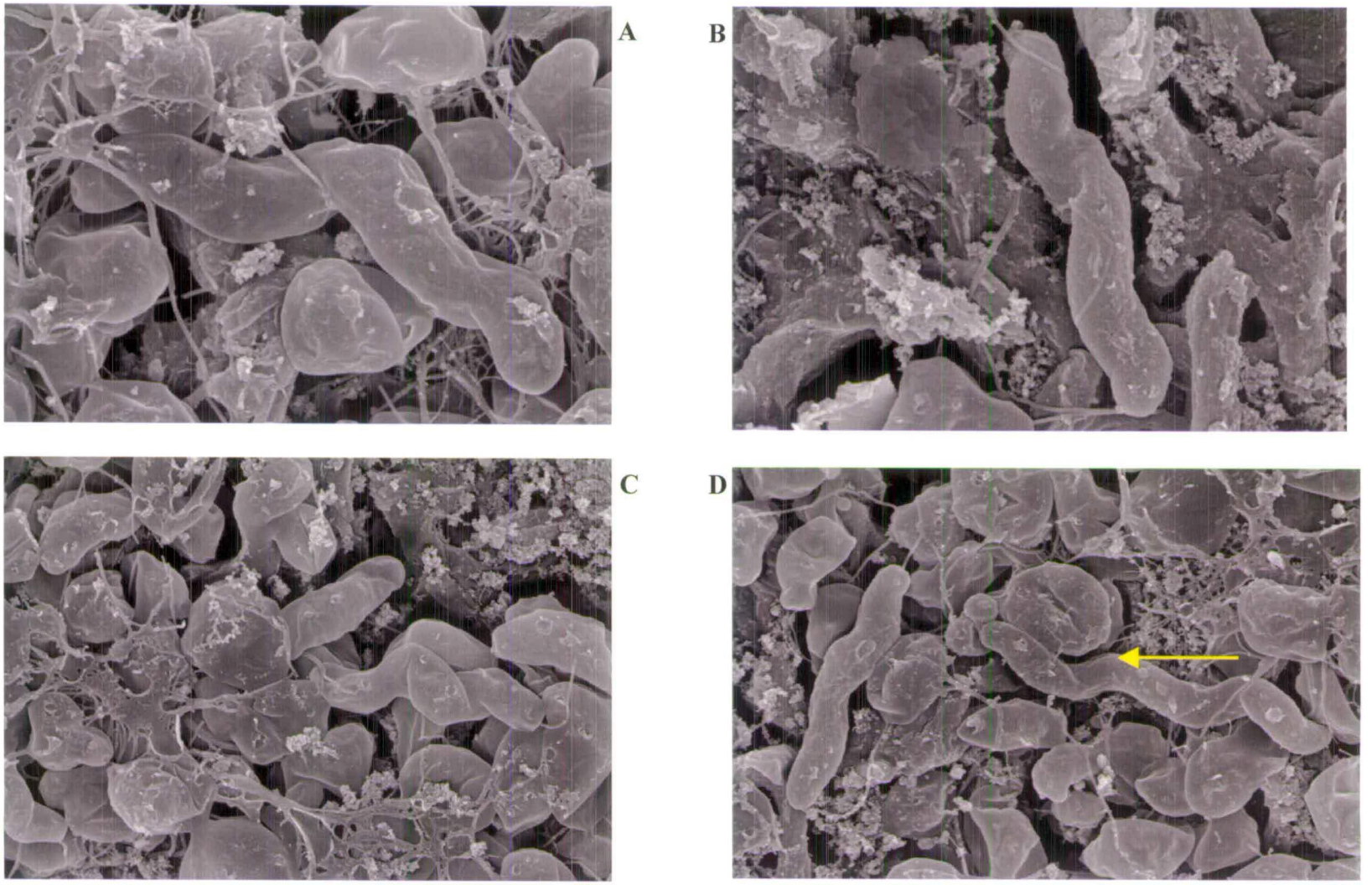


Fig.7.11. Visualisation on SEM, of the NCI cells at different scales
S4700, 5.0 kV, 2.6 mm x 35 k SE (U) for figure A; S4700, 5.0 kV, 2.6 mm x 40 k SE (U) for figure B; S4700, 5.0 kV, 2.6 mm x 25 k SE (U) for figure C and D (coccoid form indicated by arrow)

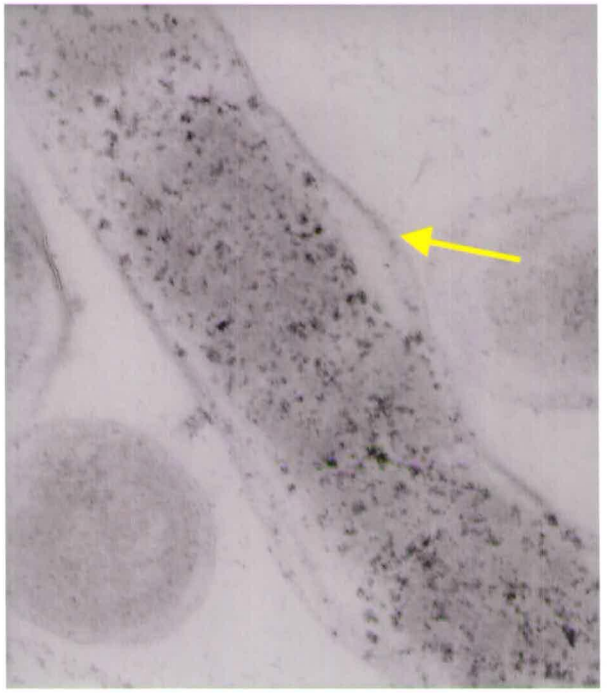


Fig.7.12. Visualisation, on TEM, of the sliced NCI cells

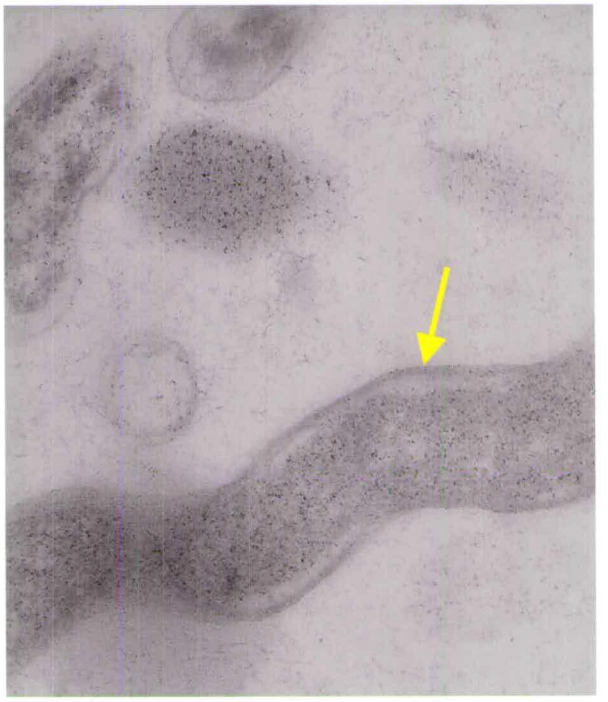


Fig.7.13. Visualisation, on TEM, of the sliced wild type cells

7.3. Conclusions

This chapter shows that the NC1 cells were affected by the replacement of the P450 gene. Figures 7.11A, 7.11B, 7.11C and 7.11D shows evidence for disturbed cell morphology, with a high number of coccoid cells present in the culture, in the mid-log growth stage. These changes were described before in the literature by Wood (1999) when genes located in similar clusters were deleted.

Changes in cell morphology were observed by TEM and SEM, with the NC1 cells becoming shorter and wider than the Wt.

A clear disturbed cell surface was observed for NCI cells whereas the surface of the wild type cells was smooth and compact (figure 7.12 and 7.13).

Chapter 8
CONCLUSIONS AND FUTURE WORK

This thesis describes the work carried out on the cytochrome P450 enzyme from *Campylobacter jejuni 11168*. Sequencing in 2000 of the *Campylobacter jejuni 11168* genome revealed the presence of a cytochrome P450 coding sequence which was proved to be unique by its localisation in an operon involved in cell surface biosynthesis. This cytochrome P450 was identified to be conserved among the *Campylobacter jejuni* strains by its presence in *Campylobacter jejuni RM1221.*

The recombinant protein was successfully over-expressed using pETBlue1 Blunt as vector and *E. coli* Tuner (DE3) placI as expression strain, this study being a very useful tool for expression of recombinant P450s. Purification of this cytochrome P450 was not completely successful but 70 % purity was achieved using DE52 anion-exchange and triethanolamine as a buffer at pH 8. Purification was done in reverse way by collecting the red fraction which ran through the column. The red fraction collected was concentrated up to 12 mg/ml but at this concentration the protein proved to be cloudy and very viscous. To improve the purity of the protein to HIS-Tagged constructs were prepared (one with the 6HIS at the N terminus and one at the C terminus) but the purification trials failed as the protein was not bound to the nickel column.

Chapter 4 describe a very useful protocol for gene replacement in *Campylobacter*. In a P450 knockout mutant (NC1) the stationary phase (chapter 5) was reached about 6 hours earlier than with the wild type strain. The NC1 cells are less resistant to starvation in the stationary phase having a faster rate of death comparing with the wild type. By exposure to air 36 % of the wild type cells survived after 24 hours and only 16 % of the NC1 cells survived. After 24 hours the rate of survival for both of the strains decreases dramatically (0.94 % Wt and 0.42 % NC1). The growth disadvantage of the mutant strain was confirmed in competition experiments. NC1 cells were proved to be less resistant to high temperatures. In comparison to the WT cells, NC1 shows a more gradual decline in survival at the highest temperature. Therefore, NC1 cells demonstrate a greater resistance and ability to survive under heat stress whereas WT cells begin to die as the temperature moves further away from 42°C.

The -80^{0}C proved to be the only temperature value at which the Wt strain survives by exposure over a three weeks period. The NC1 strain seems to have a good rate of survival at 4^0, -20^0 and -80^{0}C during three weeks exposure to low temperature (see chapter V) results which makes the NC1 strain more resistant to low temperatures. Testing the resistance of both strains to different pH values the NC1 strain grew at a higher pH range between 5.5-10.5, comparing with the Wt which recorded growth at pH 7.5. By analysing the results obtained with NaCl and glycerol we have determined that is not the osmotic pressure that is affecting the growth of *Campylobacter* and the differences between the Wt and NC1 strain might be related with changes in cell surface components.

To directly test the idea that the *Campylobacter jejuni 11168* P450 is involved in cell surface carbohydrate biosynthesis, the sugar content of surface polymers was analysed. There was no difference detected in the overall profile of LPS but the HPLC on hydrolysed sugars detected a difference in sugar profile between the NC1 and Wt strain. Arabinose was identified by HPLC and NMR to be the monosaccharide produced by the NC1 strain but undetected in the wild type. As previously stated the only P450 enzyme previously known to be involved in sugar metabolism was identified in mammalian system and seems to be involved in conversion of the glucuronic acid to glucaric acid. Under the same mechanism the arabinose might be changed to arabinonic acid (figure 8.1) which might be the explanation for the absence of arabinose in the Wt strain. The wild type extra peak, in sugar profile, was partially characterised by NMR (see chapter 6).

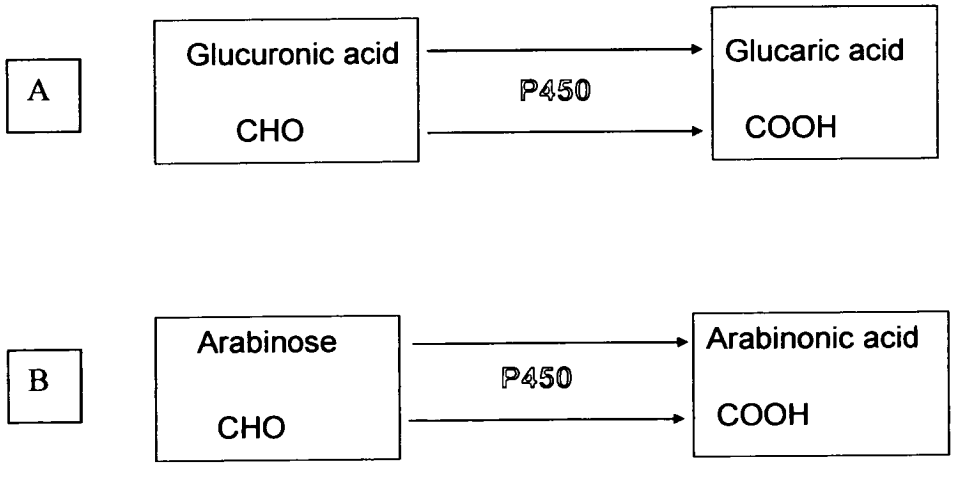


Fig. 8.1. Proposed reaction type.
A – mammalian system, B – bacterial system

It has previously been described in the literature (Wood, 1999 and Benjamin, 2000) that mutations performed inside the LPS and LOS cluster induced changes in cell morphology. The electron microscopy studies performed by us showed clear changes in cell morphology (by TEM and SEM), the NC1 cells becoming shorter and wider than the Wt.

By comparison with the P450 Rhf isolated from *Rhodococcus* sp. NCIMB 9784, were the reductase domain is an FMN-FeS domain fused to the N-terminal heme-containing oxygenase, in *Campylobacter jejuni 11168* the reductase components are not fused in a single polypeptide. The FMN was identified under Cj1382c and Fe-S under Cj1377c. Having all the components separate the *Campylobacter* P450 reductase is represented under a new class (see figure 3.5 in chapter III).

Being the only cytochrome P450 known to be involved in sugar metabolism future work must be done on purification and in trying to obtain a crystal structure which would be a major step in trying to understand the interaction with electron transfer partners. Biochemical studies must be developed on kinetics of the enzyme helping to understand the function of this novel enzyme and to identify the substrate and the product. On the biological side further experiments must be conducted on studying the difference in adhesion and infectivity between the Wt and NC1 strain. The cytochrome P450 could then be considered as a possible drug target for *Campylobacter* infection.

Chapter 9
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Chapter 10
MEETINGS

10.1 Meetings

06-08/06/02 6th Firbush Redox Enzymes Meeting (Speaker)

12-14/01/03 Enzyme mechanism: A structural perspective, St. Andrews, UK

04-06/06/03 7th Firbush Redox Enzymes Meeting

02-04/06/04 8th Firbush Redox Enzymes Meeting