Glucose metabolism via the EntnerDoudoroff Pathway in a select subgroup of *Campylobacter jejuni*

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Declaration

I can confirm that this is my own work and the use of all materials from other sources have been properly and fully acknowledged.

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Signed
Date. 17.9.2021

Abstract

The spiral Gram-negative bacterium *Campylobacter jejuni* is a microaerophile that lives in the intestinal tract of many farmed animals in a commensal-like association, but on infection of humans is pathogenic. It is the leading cause of bacterial gastroenteritis worldwide. Multi-locus sequence typing (MLST) has identified subgroups belonging to ST21CC21 and ST45CC45 clonal complexes (CC) as generalists colonising different animals and causing human disease, while others show a narrower host-specificity. *C. jejuni* depends on amino acids, short chain fatty acids and intermediates of the TCA cycle as primary energy and carbon sources and cannot utilise glucose as a growth substrate due to an incomplete Embden-Meyerhof Parnas (EMP) pathway. Recently, a *glc* locus encoding enzymes of the Entner-Doudoroff (ED) pathway plus *glcP,* encoding a sugar permease, and *glk,* encoding glucose kinase, were identified in the genome sequence of a small number of *C. jejuni* and *C. coli* strains and ability of selected strains to metabolise glucose demonstrated.

This study investigated the association of the *glc* locus with *C. jejuni* strains of farm-associated Norway rat origin. The typed ED pathway of most isolates fell within two broad groups, belonging to either EDMLST types 2, 3, 9 and 11 (including strains Dg275 and Dg95) or to EDMLST types 21 and 23 (including strain Dg43) and this division correlated with distinct phylogenetic branches based on cgMLST. Growth studies combined with DNA amplification and Sanger sequencing confirmed possession of a complete *glc* locus and ability to utilise glucose for all of these strains and absence of all *glc* genes from a couple of other distantly related strains belonging to clonal complexes 21 and 45 for which partial *glc* genes were listed in the annotated genomes. This is consistent with presence of the *glc* locus in *C. jejuni* strains primarily of environmental origin. The genetic organisation and insertion of the *glc* locus within each of the 3 *rrn* loci of Dg43 and within *rrn*A and *rrn*B of Dg95 was demonstrated by long range PCR and Sanger sequencing, followed by hybrid Illumina and Nanopore sequencing of the genome.

In a previous study that assessed colonisation of chickens by strains Dg95 and Dg275, all five recovered isolates from one chicken no longer utilised glucose (Mohammed, 2018). Here, inability to use glucose was explained by identification of a mutation in *glk* in both copies of the *glc* locus for two isolates. Interestingly, in one isolate, Ch95-95-1 the mutation (Gly167STOP) created a pseudogene, while in a second isolate the mutation (GluGlyTrpHis) was within the predicted sugar binding site (consensus GluXGlyHis).

As ED positive strains of C. *jejuni* are notably absent from chicken isolates in the PUBMLST /campylobacter database, these results might reflect some incompatibility of glucose metabolism and competitive colonisation of the chicken caecum by *C. jejuni*.

During this study, glucose was consistently shown to enhance survival of ED+ strains during the decline phase of growth whether in rich or in a minimal medium, under standard conditions (5% O_2,10% CO_2, 2% H_2). Under conditions of aerobic stress (15.9% O_2, 4.5% CO_2, 0% H_2) strains carrying the *glc* locus also showed a shorter lag phase, indicating that these strains are already primed to deal with aerobic stress. The mutant Ch95-95*glk* showed no glucose dependent benefit to survival in 5% or 15.9% oxygen. At suboptimal concentrations of oxygen (2% O_2, 10% CO_2, 2% H_2), addition of glucose had no apparent benefit, consistent with less benefit in the caecum. Neither maltose nor lactose could be metabolised, although *in vivo* access to glucose would likely occur via degradation of these and other sugars by members of the intestinal microbiota.

The metabolism of glucose was also shown to protect cells from the harmful effect of H_2O_2 in the decline phase from 48-72h. Dg275 and Dg95 cells grown in glucose exhibited ~4-8 fold higher cfu on exposure to H_2O_2 compared to cells grown without glucose. This correlated with the consistent observation that glucose metabolism by these strains delayed coccoid formation and extended survival of cells in the decline phase of growth. This study provides evidence that glucose metabolism by ED positive strains of *C. jejuni* extends survival under adverse conditions. It would be interesting to further explore the impact of glucose metabolism on survival in different environments, including in the chicken and Norway rat caecum and in environmental locations outside the host.

Dedication

- To my parents for supporting me.
- To my sisters and brothers; especially my little brother Saeed.
- To my friends and my colleagues in the lab for all their help and support especially Othman Mohammed from Iraq.
- To all my friends (Kholoud, Dareen, Abrar, Zahra, Lubna, Saya and her husband).

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List of Abbreviations

AGE	Agarose Gel Electrophoresis
Asp	Aspartate
BA, BA-2%	Blood Agar, Blood agar containing 2% agar
BHI	Brain heart infusion
BIGSdb	Bacterial Isolate Genome Sequence Database
bp	Base Pairs
CC	Clonal Complex
CDT	Cytolethal Distending Toxin
CFU	Colony Forming Unit
cgMLST	Core genome multilocus sequence type
CPS	Capsular Polysaccharide
DMEM	Dulbecco‟ Modified Eagle Medium
DMEMf	Dulbecco's Modified Eagle's Medium plus iron ascorbate (IA)
DNA	Deoxyribonucleic acid
Dpi	Days of Post Infection
DW	Distilled Water
ED	Entner-Doudoroff pathway
EDMLST	Entner-Doudoroff pathway in Multilocus Sequence Typing
EDTA	Ethylenediaminetetraacetic acid
EMP	Embden-Meyerhof-Parnas
FBS	Fetal Bovine Serum
gDNA	Genomic DNA
GGT	Glutamate by γ-Glutamyl Transpeptidase
Glc	Glucose
IA	Iron Ascorbate
ID	Identification Number
LOS	Lipooligosaccharide
MHA	Mueller Hinton Agar
MHB	Mueller Hinton Broth
MLST	Multilocus Sequence Typing
MRD	Maximum Recovery Diluent
nH2O	Nano pure water
OD	Optical Density
PBS	Phosphate Buffered Saline
PCR	Polymerase chain Reaction
Pro	Proline
RNA	Ribonucleic acid
rMLST	Ribosomal Multilocus Sequence Typing
rRNA	Ribosomal RNA
RG1	Rat group1
RG2	Rat group 2 , possess *glc* locus
RT	Room Temperature
Ser	Serine
spp.	Species
ST	Sequence Type
ST21CC21	Sequence Type 21 Clonal Complex 21
ST45CC45	Sequence Type 45 Clonal Complex 45
TAE	Tris-acetat EDTA buffer
wgMLST	Whole Genome Multi Locus Sequence
WGS	Whole Genome Sequence
w/v	Weight per Volume
v/v	Volume per Volume

Table of Contents

List of Figure

List of Tables

Chapter1
Literature Review

1.1 Discovery and historical overview

Campylobacter jejuni has become one of the most important foodborne pathogens in developed and developing countries (PHE, 2020). It is estimated that worldwide 550 million people fall ill yearly due to *Campylobacter* diarrhea, including 220 million children under the age of five years (WHO, 2020); (https://www.who.int/news-room/factsheets/detail/*Campylobacter*).

In 1886, a small organism from the colon of infants was detected and described (Kist, 1986). However, it was not until the 1960s and 1970s that significant progress was made in understanding, identifying the appropriate culture conditions and isolating this spiral bacterium, *Campylobacter* (Dekeyser *et al*., 1972). Dekeyser and co-workers used a method for isolating *Campylobacter* from human faeces by using a filtration technique (Dekeyser *et al*., 1972). Subsequently, the filtration process was abandoned and a selective culture media were developed, such as Skirrow medium (Skirrow,1977) which was made selective by the addition of vancomycin (10 mg/l), polymyxin B (2–5 IU/ml), and trimethoprim (5mg/l), This media includes horse blood and incubation was performed at 43°C in an atmosphere of 5% oxygen, 10% carbon dioxide, and 85% hydrogen.

In 1963, Sebald and Véron established the genus *Campylobacter* after separating it from vibrio (e.g., *V. cholerae* and *V. parahaemolyticus*) (On., 2001), but *Campylobacter* spp. were not recognised as a cause of human gastroenteritis until the late 1970s with the development of selective isolation (Skirrow, 1977). Table 1.1 provides a historical overview of the stages of discovery of these spiral bacteria, the establishment of the genus *Campylobacter*, and species and subspecies typing. The whole genome sequence of *C. jejuni* NCTC 11168 (11168-GS) was analysed in 2000 and contributed to understanding physiology of *C. jejuni* and to identification of *C. jejuni*specific colonisation and virulence factors (Parkhill *et al.*, 2000). A 15-year longitudinal survey of the molecular epidemiology of clinical *Campylobacter* isolates was Conducted in Oxfordshire between 2003 and 2018.

(https://pubmlst.org/campylobacter/projects/Oxfordshire_Human_Surveillance).

Between 2003 to 2009, 3300 isolates of *Campylobacter* were characterised using multilocus sequence typing (MLST) with data deposited on PubMLST in near real time (Cody *et al.*, 2012). Since 2010, whole-genome sequencing (WGS) has been performed to produce high-quality draft sequences that have also been deposited (Cody *et al.*, 2013). This extensive resource of sequence data was in agreement that *C. jejuni* lacks the ability to catabolism glucose via the Embden Meyerhof-Parnas (EMP) pathway and in general has an asaccharolytic metabolism (Parkhill *et al.*, 2000; Velayudhan and Kelly, 2002; Vegge *et al*., 2016). Annotation of sequences revealed that a small proportion of *C. coli* and *C. jejuni* strains encoded genes of the Entner-Doudoroff (ED) pathway and functionality of this *glc* locus was demonstrated for *C. coli* (Vegge *et al.*, 2016; Vorwerk *et al*., 2015).

Year	**Scientist (s) / Ref**	**Discovery**
1886	Theodor Escherich / (Kist, 1986)	First description of *Campylobacter* like spiral bacteria in the colons of infants with e enteric disease.
1910s	[1]McFaydean and Stockman. / (Skirrow, 2006). [2]Smith and Taylor / (Smith and Taylor, 1919).	[1]Isolate *Vibrio*-like organisms from aborted foetuses and which resembled a *Vibrio*. [2]Smith and Taylor isolated a spirilla bacterium and proposed the name '*Vibrio foetus'* with abortion in cattle.
1947 – 1959	[1]Vinzent, Dumas and Picard / (Vinzent *et al.* 1947). [2] Stegenga and Terpstra / (Stegenga and Terpstra, 1949) [3]King / (King ,1957). [4]Florent/ (Florent, 1959)	[1]Described *Vibrio* by different biochemical and antigenic characteristics. [2] Described pathogenic role of *V. fetus* venerealis in cows. [3]Described *Vibrio* with many features. [4]Distinguish two types of *V. foetus* by their biochemical and pathogenic characteristics, namely *V.foetus* venerealis and *V. foetus* intestinalis.
1963 – 1973	[1] Dekeyser / (Dekeyser *et al*.,1972) [2]Sebald and Véron / (Sebald, and Véron. 1963) [3]Véron and Chatelain/ (Véron and Chatelain, 1973).	[1]The isolation of *Campylobacter* from faeces at the National Institute for Veterinary Research, Brussels. [2]Establishment of genus *Campylobacter* with *C. foetus* formerly *V. foetus*), as type species, transfer of *Vibrio jejuni* and *V. coli* to the genus *Campylobacter*. [3] The genus *Campylobacter* has been reviewed.

1970s	[1]Skirrow/(Skirrow,1977) [2]Butzler /(Butzler,1978) [3] Butzler and Skirrow/ (Butzler and Skirrow,1979).	[1]Development a simple method for culturing *C. jejuni* and *C. coli* from stool specimens. [2]First account of *Campylobacter* enteritis in man, routine diagnostic isolation of *campylobacter* in Africa. [3]The first full report of *Campylobacter* enteritis in man was published.
1980s	[1]Penner and Hennesey (1980) [2]Lior / (Lior *et al.*, 1982) [3] Romaniuk / (Romaniuk *et al.,*1987) [4] Goodwin / (Goodwin *et al.,*1989b)	[1]The Penner scheme discovered method depend on a passive hemagglutination technique by soluble antigen extracts of *Campylobacter* isolates. [2]Serotyping schemes developed for subtyping *C. jejuni.* [3]16S rRNA sequence were used to show that *C. pylori* was in fact more closely related to *Wolinella succinogenes.* [4]The establishment of a new genus, transfer of *Campylobacter pylori* to *helicobacter pylori*.
1990s	[1] Vandamme and De Ley, 1991a [2]Maiden *et al.,*1998	[1]Reclassification of the *campylobacter* *Campylobacter* genus in the bacterial family C*ampylobacteraceae*, which includes the C*ampylobacter*, *Arcobacter* and *Sulfurospirillum.* [2] MLST typing established for *Neisseria meningitidis.*

2000 -	[1]Parkhill *et al*., 2000 [2]Dingle *et al.,* 2001 [3] Colles&Maiden,2012a, b [4]Cody, *et al.*,2017	[1]Deciphered whole genome sequence for *C. jejuni* NCTC11168. [2]MLST typing established for *C. jejuni/ C. coli.* [3]WGS established as a more discriminatory typing method for *C.jejuni* outbreak investigations. [4]cgMLST genotyping method used screen the the phylogenetic relationship of strains.

Table 1.1 History of identification and classification stages of discovery of *Campylobacter* spp.

1.2 *Campylobacter* classification

When the ability to selectively culture and isolate *Campylobacter* was established, scientists worked hard to classify them. Between 1974 and 1988, 12 new species and subspecies were identified and proposed A new approach to classification based on the natural evolution of the highly conserved gene for the macromolecule 16SrRNA (Goodwin *et al.*,1989a), led to comparative sequence analyses of this gene and identification of distinct clades within the *Campylobacter* genus, as it was then defined. In 1989 the new genus *Helicobacter* was proposed and the bacterium *C. pylori* was renamed *Helicobacter pylori* (Goodwin *et al*., 1989a).

Campylobacter was assigned to the rRNA superfamily VI, corresponding to the ε-division of *Proteobacteria*. The genera in this superfamily were identified as a diverse yet related group that was clearly phylogenetically distinct from other *Proteobacteria* (Vandamme *et al.*,1991). The groups sharing rRNA (VI) homology comprised groups I (*Campylobacter* and *Bacteroides ureolyticus* (now *C. ureolyticus*), II (*Arcobacter*), and III (*Helicobacter* and *Wolinella succinogenes*). Both *Campylobacter* and *Arcobacter* were classified as belonging to the family

Campylobacteriaceae (Vandamme and Ley, 1991) within the order *Campylobacterales*. A recently detailed phylogenetic analysis of members of ε*Proteobacteria* concluded that they should be removed from the phylum Proteobacteria and placed in the new phylum *Campylobacterota* (Waite *et al.,* 2017). The number of species of *Campylobacter* has continued to increase. The List of Prokaryotic names with Standing in Nomenclature (LPSN) now includes 32 named species of *Campylobacter* (Parte *et al.*, 2020).

Most reported isolates of *Campylobacter* causing human diseases are enteric pathogens that cause human gastroenteritis. These include *C. jejuni* spp. *jejuni* and subsp. *doylei*, *C. coli*, and less frequently, *C. upsaliensis* and *C. lari* (WHO, 2020). In addition to *C. jejuni*, *C. coli, C. upsaliensis,* and *C. lari,* many other *Campylobacter* species have been linked to human infections. These include *C. concisus, C. rectus, C. hyointestinalis, C. insulaenigrae, C. sputorum, C. helveticus, C. foetus, C. mucosalis* and *C. ureolyticus* (Heredia and García, 2018). *C. foetus* subsp. *venerealis* and *C. foetus* subsp. *foetus* are major veterinary pathogens (Rollins and Joseph, 2000). *C. jejuni* subsp. *jejuni* is the subspecies most commonly associated with human gastroenteritis, and it has accounted for more than 90% of all reported cases of campylobacteriosis.

The subspecies *C. jejuni* subsp. *doylei* has been less frequently isolated but has been associated with both enteritis and gastritis (Parker *et al.*, 2007). *C. coli* is another species that is commonly associated with human gastroenteritis. It is the second most frequently reported *Campylobacter* species that causes human infections (Crim *et al.*, 2015; Beier *et al.*, 2018). *C. coli* includes three clades. *C. coli* strains, which belong to clade 1, cause most human infections, while *C. coli* clades 2 and 3 are rare (Johansson *et al.*, 2018).

1.3 Disease, epidemiology, and source of infection

C. jejuni is the most common cause of gastroenteritis cases recorded in the world, and infections are mild to acute. Symptoms of *Campylobacter* infection are usually similar to those caused by other enteric pathogens, such as *Shigella* and *Salmonella*.

Diarrhoea is considered a typical symptom of *Campylobacter* infection. Bloody diarrhoea may lead to severe dehydration, emesis, abdominal pain, high temperature (fever), and headache. Symptoms present two to five days after infection by the bacteria, but the incubation period can range from one to ten days (WHO,2020). In addition, some complications may accompany *Campylobacter* infection, such as hepatitis, pancreatitis, and bacteremia (WHO,2020). The infection can be serious in immunocompromised hosts (CDC,2016) leading to reactive arthritis and Guillain -Barre syndrome (GBS) (Ang, 2002). Previous studies have shown that the prevalence of campylobacteriosis has increased in both developed and developing countries over the last 10 years. In Europe, more than 200,000 human cases have been reported annually since 2007 (EFSA, 2016). Moreover, 23 million people fall ill from ingesting unsafe food each year in Europe (WHO,2019). In some countries, the number of cases of campylobacteriosis is higher than that of salmonellosis. For example, 69,414 confirmed cases of *Campylobacter* were reported compared to 14,269 confirmed cases of salmonellosis in Germany (Robert Koch Institute, 2018).

In both Australia and New Zealand campylobacteriosis is the most commonly reported cause of bacterial gastroenteritis disease (Hansson *et al.*, 2018).

The total number of reported human cases of *Campylobacter* infection in England and Wales increased from 52.381 in 2016 to 56,729 in 2017, (PHE, 2020), indicated decrease cases compared to previous years, where the cases were 5964 in 2019, while were 4269 cases in 2020 (PHE, 2020; PHE, 2019).

In 2020, the reporting rates for *Campylobacter* were lower compared to the previous year, for example 5964 cases were reported in weeks 27-30 in 2019, 4269 cases were reported in the same period in 2020. This evidence of a possible small decline may be attributed to the coronavirus pandemic and related measures having an impact on rates of infections (FSN, 2020); https://www.foodsafetynews.com.

In 2017, 56% of *Campylobacter* laboratory-confirmed cases in England and Wales were in males. The section of the population with the highest number of laboratory reports was the 50–59-year age group and the peak month for *Campylobacter* reports was July (PHE, 2017). In Canada, the reported number of cases per 100,000 population decreased slightly from 30.2 in 2017 to 29.14 in 2018. The confirmed cases in males were higher than in females, and the number of *Campylobacter* cases also increased in the summer months (Kuhn *et al.,* 2018).

Although, as well as increased infection rates between June and November, others factors have been reported to influence the rates of infection, include geographical and seasonal variation, such as the temperature at slaughter (Jorgensen *et al*., 2011). The identification and understanding of the transmission pathways of *Campylobacter* infection is crucial in controlling its proliferation and preventing human disease (Newell *et al*., 2017)

The true incidence of campylobacteriosis worldwide is largely unknown for multiple reasons, such as underreporting, difficulties in diagnosis, as well as differences in reporting systems and in surveillance, especially in developing countries (WHO, 2013). For example, the absence of national surveillance programs for campylobacteriosis in some countries such as Arabian Gulf region makes it difficult to give an accurate picture about infection prevalence in the populations (Senok and Botta, 2009). The prevalence of campylobacteriosis differs in developed and developing countries. In the developing world, children under the age of five are frequently infected with this pathogen, particularly in hyper-exposed developing nations, sometimes resulting in death.

The prevalence of campylobacteriosis differs in developed and developing countries. In the developing world, children under the age of five are frequently infected with this pathogen, particularly in hyper-exposed developing nations, sometimes resulting in death. The epidemiological information provided on campylobacteriosis is insufficient to fully assess the extent of its prevalence and severity in developing countries such as Africa (Kaakoush *et al*., 2015). Some patients become asymptomatic, which may suggest some induced protective immunity.

In contrast, *C. jejuni* subsp. *doylei* (*Cjd*), which also plays a role in gastritis (Owen *et al*., 1985) and enteritis (Lastovica and Skirrow, 2000) is more commonly representative of *Campylobacter* in blood cultures than stool cultures (Lastovica and Skirrow, 2000), In one study *Cjd* represented only 9.7% of the *Campylobacter* isolates from stool cultures, while 24% of the *Campylobacter* blood cultures (Lastovica, 1996).

A link between *Campylobacter* and both Crohn's disease (CD) and ulcerative colitis (UC) has also been proposed. (Man *et al.*, 2011). *Campylobacter* spp. are considered normal flora in the intestines of a wide range of domestic animals, birds, and farm animals, such as cats, dogs, pigs, and chickens (Dearlove *et al.*, 2016). As shown in fig 1.1, animals are considered a primary reservoir of *Campylobacter,* which causes campylobacteriosis. Farm animals, particularly poultry, are considered a major reservoir of *Campylobacter* species and sources of human infection (Grant *et al*., 2018; EFSA. 2011). Wild birds can carry *Campylobacter* (Hald *et al.*, 2016), and it has been demonstrated that a high proportion of farm-associated rodents are infected with *Campylobacter* (Meerburg and Kijlstra, 2007; Mohammed, 2018). Both have been proposed as a potential reservoir contributing to the transfer of *Campylobacter* into and within the farm environment.

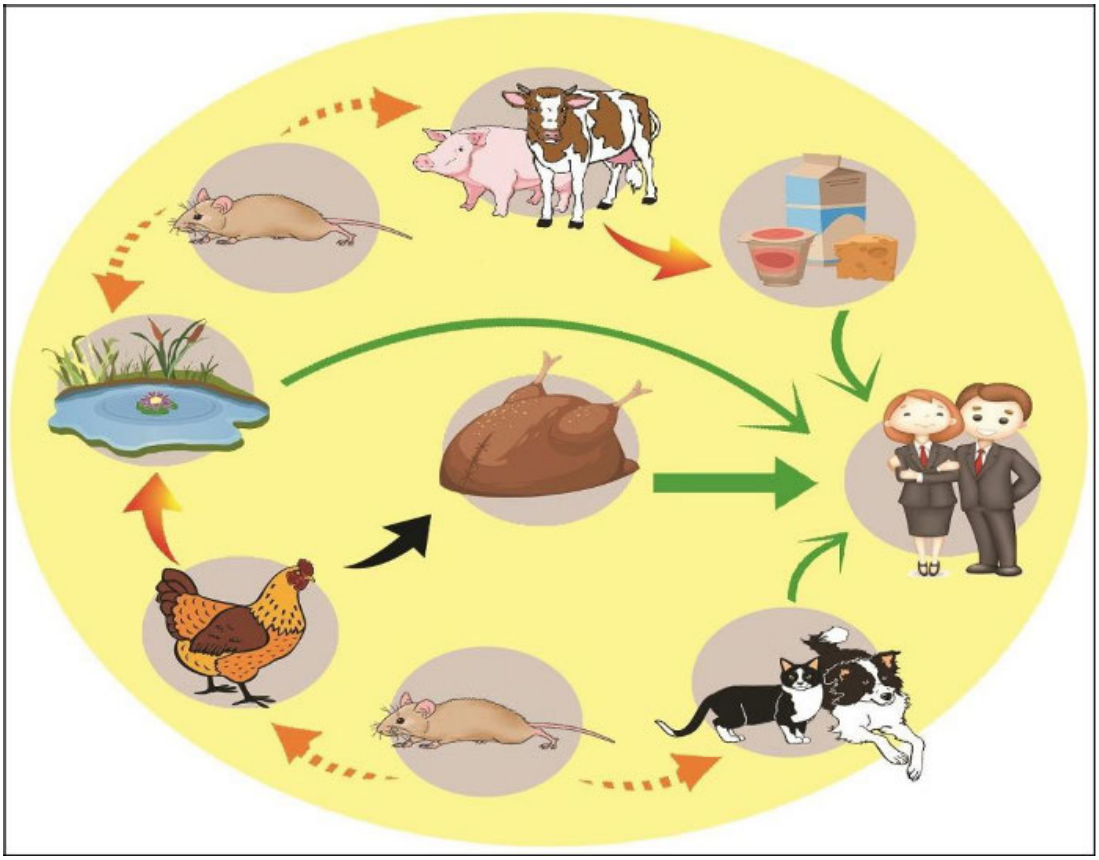


Figure 1.1 Transmission of *C. jejuni* between hosts. There are several different reservoirs for infectious *C. jejuni*, which is commonly found in the intestinal tracts of poultry. Prepared chicken meat is considered a primary source of *C. jejuni* infection in humans. Water sources, raw milk, and milk products can be polluted by the faeces of animals and wild birds. Humans are directly infected by ingesting polluted animal products, such as milk and meat, especially domestic fowls, and to a lesser extent by contaminated drinking water (Dasti *et al.*,2010; Johnson *et al.*, 2017; Young *et al.*, 2007). The green arrow in fig. 1.1 indicates proven sources of *C. jejuni* infection in humans. The thick green arrow indicates the main source; the black arrow refers to the carry- over of *Campylobacter* from infected chicken to contaminated meat; the orange arrowrefers to transmission among different reservoirs; and the dashed orange arrow (not yet proven) indicates potential transmission to pets and farmyard animals and chickens by rats (adapted from Young *et al.,*2007).

The transmission of *C. jejuni* and *C. coli* to humans may be by a direct mode or an indirect mode (García-Sánchez *et al.*, 2018). The major direct route of infection is through undercooked meat and cross contamination by contaminated meat, such as poultry (Zimmer *et al.*, 2003). However, the consumption of contaminated raw milk, dairy products, and water (Grzybowska Chlebowczyk *et al.*, 2013) is also considered an important source of *Campylobacter* infections. This is comparable to salmonellosis, which is acquired through the consumption of undercooked meat, although *Salmonella* gastroenteritis, but not campylobacteriosis, is also commonly associated with eggs. *C. jejuni* can infect humans through direct contact with animals such as pets (ESR,2016; Westermarck, 2016), farm animals (Hansson *et al.*, 2018), and in abattoirs when thorough handwashing and food safety procedures are not followed (Aung *et al.*, 2015). Indirect transfer occurs when animals shed faeces in the environment (Goni *et al.*, 2017). In some animals, *C. jejuni/coli* may occasionally cause gastroenteritis, which is indicated by diarrhoea. This has been reported in chickens (Humphrey *et al.*, 2014), dogs, cats, cows and pigs (Kaakoush *et al.*, 2015). Whether animals have symptoms of gastroenteritis or are asymptomatic and carry *Campylobacter* as a commensal, faeces in the farmyard and environment have the potential to spread to other animals and humans. *Campylobacter* in faeces can result in polluted water and transfer to animals or to humans who contact and drink this water (Domingues *et al.*, 2012).

1.4 Subtyping of *Campylobacter jejuni*

To a limited extent, subtyping bacterial species can be helpful in recognising subgroups of *Campylobacter* associated with human disease and *C. jejuni* transmission by specific animal hosts (Colles and Maiden, 2012b). To identify potentially important subgroups of *C. jejuni* and *C. coli,* many typing methods have been tested. Typing s systems can be classified into two

broads methods: phenotypic and genotypic. Both have been used to discriminate *C. jejuni* and *C. coli* at the species and subspecies levels (Wiedmann, 2002).

1.4.1 Phenotyping methods

Phenotyping methods used to type bacteria depend on the presence or absence of biological, serological, or metabolic activities (Arbeit, 1995). There are three phenotypic techniques: biotyping, serotyping, and multilocus enzyme electrophoresis. Biotyping utilises metabolic activities to identify bacterial isolates, such as colonial morphology, environmental tolerances, and biochemical reactions.

Regarding *C. jejuni*, biotyping methods have included metabolic activity (Analytic Profile Index [API]) and multilocus enzyme electrophoresis (MEE) (Wiedmann, 2002), while some surface structures used in subtyping include heat-stable antigens, such as capsular polysaccharides (Karlyshev *et al.,* 2000; Penner and Hennesey, 1980), as well as membrane proteins and flagella (Linton *et al*., 2001). API Campy and the Vitek 2 system are both commercially available methods that were developed for the identification of *C. jejuni/coli*. The API Campy system was reported to identify 94.4% of *C. jejuni* ssp. *jejuni* and 73.8% of *C. coli* in one survey, but it failed to correctly identify 52.3% of other *Epsilobacteria* (Martiny *et al.*, 2011). The Vitek 2 NH card, which is used for the identification of *Neisseria*, *Haemophilus,* and other fastidious bacteria, was used successfully in identifying most *C. jejuni* ssp. *jejuni* (89.6%) and *C. coli* (87.7%) strains isolated from humans (Martiny *et al.*, 2011). Matrix assisted laser desorption time of flight (MALDI-TOF) mass spectrometry is now an accurate method of identifying *C. jejuni* and *C. coli,* but like API, it does not differentiate greatly at the subspecies level, so it is used primarily in identifying *C. jejuni* and *C. coli* (Martiny *et al*., 2011).

Serotyping depends on variations in cellular surface structures anduse of specific antibodies and antisera to detect surface antigens present on bacteria, thus distinguishing strains depending on differences in key surface macromolecules (Arbeit, 1995; Wiedmann, 2002).

Early serotyping studies separated the *Campylobacter* group (*Vibrio foetus*) into three serotypes based on heat-stable antigens (Berg *et al.*, 1971). The Penner serotyping scheme is a serotyping method based on the capsule polysaccharide (CPS) of *Campylobacter* (Penner and Hennessy, 1980). Forty-seven antisera are used to differentiate *C. jejuni* serotypes based on CPS (Guerry *et al*., 2012). Eight serotypes of *C. jejuni* have been linked to diarrhoeal cases worldwide, among which HS4 complex, HS2, and HS1/44 are the most dominant (Pike *et al.*, 2013). *C. jejuni* serotypes have also been associated with clinical disease (Pike *et al.*, 2013). The Lior scheme uses bacterial agglutination of heat-labile antigens to identify *Campylobacter* strains (Lior *et al.*, 1982). Both methods combined provide the best differentiating results (Nielsen *et al.*, 2000; McKay *et al.*, 2001). A different method, multilocus enzyme electrophoresis (MEE), is based on variations in the electrophoretic mobility of different constitutive enzymes by electrophoresis under nondenaturing conditions (Wiedmann, 2002).

1.4.2. Genotypic methods

Genotyping schemes involve subtyping based on the properties of genetic material, such as the DNA or RNA of a microbe. Many techniques have been applied to subtype *C. jejuni* and *C. coli,* including pulsed-field gel electrophoresis (PFGE) (Aber, 1981), ribotyping (Williams *et al.*, 1998), restriction endonuclease analysis, a variety of polymerase chain reaction (PCR) based assays including random amplified polymorphic DNA typing (RAPD) (Fayos *et al.*, 1993), multilocus sequence typing (Kokotovic and On, 1999). Pulse-field gel electrophoresis (PFGE) emerged as an early successful molecular approach to reproducibly differentiate *C. jejuni* strains (Salipante *et al.*, 2014; Gibson *et al.*, 1995). This method involves the electrophoretic separation of restriction enzyme digested genomic DNA. One application, ribotyping, is based on the detection of the three copies of the *rrn* locus (encoding 5, 16 and

23SrRNA) located in different positions within the *Campylobacter* chromosome (Wassenaar and Newell, 2000). This method involves the electrophoretic separation of restriction enzyme digested genomic DNA, followed by the identification of all DNA fragments containing a *rrn* loci by Southern blotting (Denes *et al.*, 1997; Wassenaar and Newell, 2000).

Polymerase chain reactions PCR, multilocus sequencing typing MLST (deHaan *et al*., 2013) and whole genome sequencing WGS (Cody *et al*., 2013) have become frequently used because they are easier, faster, and more accurate in identifying and subtyping pathogenic *Campylobacter* (Zhang *et al.*, 2017).

1.4.2.1 Polymerase chain reaction (PCR)

PCR assays have been used extensively to sensitively detect and differentiate *C. jejuni* and *C. coli* to species and subspecies levels. PCR reactions use novel primers to bind to either unique genes carried by individual species or the sequences within a gene that show differences between species or strains. A commonly used example of targeting *Campylobacter* speciesspecific genes is the use of primers to target the hippuricase gene (*hipO*) of *C. jejuni* and the aspartate kinase gene (*aspK)* of C. *coli* for the differentiation of these two species (Banowary *et al.*, 2015). While the sequence of the 16S rDNA is considered a central target in identification of bacteria, it is not reliable in differentiating *C. jejuni* and *C. coli* (Huq *et al.,* 2014). PCR and sequence analysis have also been applied to flagellin gene typing (Owen *et al.*, 1990). Amplified fragment length polymorphisms (AFLP) (Kokotovic and On, 1999) and multiplex PCR assays have also been used to detect *Campylobacter* spp. (Zhao *et al*., 2001). Multiplex PCR assays are used to identify the presence or absence of multiple gene targets within a single sample. For example, genes targeted have included were 23S rRNA and *hipO* from *C. jejuni; sapB2* from *C. fetus* subsp. *fetus* and *glyA* from each of *C. coli, C. lari*, and *C. upsaliensis* (Wang *et al.,* 2002; Huq e*t al*., 2014).

1.4.2.2 Multilocus sequence typing (MLST)

The concept of multilocus sequence typing (MLST) arose from the established principles of MEE (Maiden *et al.*, 1998). In MEE, bacterial strains are distinguished by variations in the electrophoretic mobility of several constitutive enzymes assayed under nondenaturing conditions (Wiedmann, 2002). In 1998, MLST was first developed for *Neisseria meningitidis* to overcome the poor reproducibility between laboratories of older molecular typing schemes and subsequently applied to *C. jejuni/coli* (Maiden *et al.*, 1998). MLST became one of the main tools in the study and typing of *C. jejuni*. It has been used to study the population structure and epidemiology of this bacterium, particularly its association with different hosts and clinical diseases (Dingle *et al.*, 2005). The MLST scheme relies on the comparison of sequences from seven unlinked housekeeping genes: *uncA*, (ATP synthase_subunit) *glnA* (glutamine synthetase), *gltA* (citrate synthase), *glyA* (serine hydroxymethyltransferase), *pgm* (phosphoglucomutase) (recorded as *glmM*), *tkt* (transketolase), and *aspA* (aspartase A). Strains are defined as sequence types (ST) and arranged in clonal complexes (CC). Each different allele is assigned a number depending on its order of detection, and the collection of the seven allelic numbers defines its sequence type (ST). Clonal complexes (CC) are defined as a group of phylogenetically related STs that share at least four alleles (Griekspoor *et al.*, 2013; Sheppard *et al.*, 2013). The existence of ST numbers and metadata, including the source of *C. jejuni* and *C. coli* isolates on an online database has helped in studying the phylogenetic relationships of *C. jejuni* isolates and to some extent in correlating clonal complexes with source and disease (Colles and Maiden, 2012a). Clonal complexes have been grouped according to common sources, particularly in association with a specific host versus the ability to colonise many hosts. For example, ST21CC21 and ST45CC45 are two examples of clonal complexes defined as generalist, as they have been isolated from multiple host sources such as broiler flocks,

ruminants, and environmental strains (Wilson *et al.*, 2008) and they are frequently associated with human disease (Kwan *et al.*, 2008).

Examples of clonal complexes associated with specific hosts include the ST257CC, which is commonly associated with poultry in Europe; ST828CC, which is common in pigs; ST42CC and ST 61CC, found in ruminants in the UK; ST682 CC and ST177CC, which are mainly found in starlings in the UK and New Zealand (Wilson *et al.*, 2008). Several additional clonal complexes, ST137CC, ST583CC, -ST2354CC, and ST1324CC have also been frequently isolated from wild birds (French *et al.*, 2009).

C. jejuni strains are highly diverse clonal complexes commonly associated with human gastroenteritis ST21CC-21, ST45CC-45, and ST42CC-42 (Kwan *et al.*, 2008). ST21CC is the most common, largest, and widely distributed CC, representing 18.9% of all *C. jejuni* strains submitted to the PubMLST database (Rokney *et al.*, 2018). ST1359 has been recognised as a prominent ST in Israel (Rokney *et al.*, 2018). The second most prevalent worldwide was ST-45 (4.2%). Moreover, four major CCs were identified in Santiago and Chile including ST50 CC-21, ST-475 CC-48, ST 257 CC257 and ST-353CC-353 (Collado *et al.*, 2018; Levican *et al.*, 2019). *Campylobacter* MLST sequence data associated with relevant metadata are accessible via the PUBMLST database (www. PUBMLST.com/campylobacter). Although MLST sequence data were traditionally obtained by PCR amplification and sequencing segments of each of the seven genes, the seven MLST sequences are now usually extracted from WGSs to define ST and CC groups. Because of the availability of WGS data, MLST can be extended to subsets of selected gene sequences of choice. For example, MLST analysis has advanced to include ribosomal multilocus sequence typing (rMLST), which utilises the sequence data from 53 *rps* genes associated with the bacterial ribosome (Jolley *et al.*, 2012; Maiden *et al.*, 2013), core genome MLST (cgMLST) (Cody *et al*., 2017), and whole genome MLST (wgMLST), all of which provide fine-tuned phylogenetic analyses of strains.

1.4.2.3 Whole genome sequence technologies and typing (WGS)

New technology and reduced costs of WGS have revolutionised public health monitoring and control of infectious diseases. WGS has led to an increased understanding of pathogenic strains, and it has facilitated the monitoring and treatment of many pathogenic bacteria (Uelze *et al.*, 2020; Jagadeesan *et al.*, 2019). Using newer methods, large numbers of strains are rapidly sequenced (Loman and Pallen, 2015).

Concurrent advances in computer programmes have facilitated the analysis of large volumes of sequence data and supported the assembly of novel genomes in a short period. Large and small variants that might be missed in targeted approaches can now be identified. Thus, single nucleotide variants (SNP), insertions/deletions, copy number changes, and large structural variants can be readily identified. These detailed sequence data provide greater discriminative power and enable comparisons of genetic relatedness between bacteria, including the subspecies level (Uelze *et al.*, 2020; Jagadeesan *et al.*, 2019). WGS also aids the rapid identification of relevant genes (e.g., virulence factors, metabolic properties, antibiotic resistance, and vaccine status) and tracking of epidemiological strains has provided new opportunities to investigate the evolution of pathogens, even over short periods (Jagadeesan *et al.*, 2019).

First-generation DNA sequencing technology, that is, Sanger sequencing or the chain termination method, was developed by Frederick Sanger and his colleagues in 1977. Sanger sequencing depends on the detection of labelled chain-terminating nucleotides that are incorporated by a DNA polymerase during the replication of a template (Sanger *et al*., 1977), this method involves three main steps: chain termination synthesis of DNA; size separation of fluorescently labelled products by capillary electrophoresis; and the analysis of output to determine the DNA sequence.

Sanger sequencing is used in the highly accurate and fast, cost-effective sequencing of short segments of DNA. Sanger sequence data are highly accurate up to 700 base pairs. The sequence quality degrades between 700 and 900 bases.

Despite its accuracy, the sequencing of a whole genome sequence using Sanger sequencing requires the shotgun cloning of DNA fragments (Loman and Pallen, 2015). The rapid and efficient sequencing of many bacterial genomes was only possible following the development of new technologies and high-throughput sequencing (HTS) methods (Churko *et al.*, 2013). Next-generation sequencing (NGS) or "second generation" sequencing includes the technology used in the Illumina sequencing platform.

Illumina sequencing involves four steps: generating a genomic library (100–150bp) with ligated adapters; annealing fragments to flow cells via adapters at low concentrations, followed by amplification and cluster generation of each bound sequence; sequencing by synthesis using fluorescently tagged nucleotides and detection; and analysing the data of millions of clusters. Overlapping sequences within each short read permits their assembly into longer sequences or contigs to produce a draft genome sequence of a series of contigs that covers the genome but is not fully assembled. This is highly automated, efficient, and relatively cheap, permitting access to the sequence data of tens of thousands of isolates of *C. jejuni* and *C. coli*. For example, the PubMLST/Campylobacter database currently contains sequence data of >50,000 draft genomes.

The third most recent major advance, or "third generation" sequencing, is single molecule sequencing, which produces long reads (Heather and Chain, 2016). An example is nanopore sequencing, which was developed by Oxford Nanopore Technologies (Brown and Clarke, 2016). This technology uses minimally fragmented DNA. The nucleotide sequence is read without the need for active DNA synthesis.

A nanopore is established in an electrically resistant membrane, a voltage is set across the membrane, and the ionic current through the nanopore is monitored. As single strands of DNA are passed through the nanopore, interference in the current by each different nucleotide is recorded, which yields exceptionally long reads of DNA sequence (Wang *et al.*, 2015). Biological nanopores are typically made from pore-forming proteins, such as *Staphylococcus aureus* alpha-haemolysin.

Nanopore sequencing does not require any specialist imaging equipment to detect nucleotides, and it can be performed on a small handheld device in the field. Although one disadvantage of the current nanopore technology is the higher error rate compared with short Illumina and Sanger sequencing, an important application is its combined use with NGS sequencing to produce a final closed genome sequence.

1.4.3 Application of typing and sequence analysis to epidemiology of *Campylobacter*

Some typing methods rely on WGS, such as core genome MLST (cgMLST) (Jolley *et al.*, 2012), whole genome MLST (wgMLST) (Cody *et al.*, 2013), and ribosomal MLST, all of which allow for even finer typing than MLST. WgMLST acts to analyse all loci in a given isolate, including intergenic regions, if they are defined. It also enables the recognition of genetic relationships between epidemiologically associated isolates and isolates that potentially have the same infection source (Jolley and Maiden, 2010). The identification of genetically closely related isolates within the same ST from sporadic infections has provided evidence suggesting that sporadic infections may sometimes have a common infection source, such as ST50 (Revez *et al.*, 2014).

In addition, cgMLST analysis helped to show a high genetic diversity of *C. jejuni* including 13 novel STs and alleles in *C. jejuni* and *C. coli.* (Bravo *et al.*, 2020).

The pangenome is a new tool for redefining pathogenic species, it contains two parts, the core and the accessory (or dispensable) genome (Tettelin *et al.*, 2005). Core genome defines all genes common to all strains (Tettelin *et al.*, 2005), while the accessory genome is considered the subset of genes shared by some organisms but not present in all. Accessory genes are often associated with virulence, specific metabolism, or resistance to antibiotics (Mira *et al.*, 2010) and they are often acquired by horizontal gene transfer. The pangenome is defined *as* all genes identified in WGSs of a subset of strains (Alcaraz *et al.*, 2010; Tettelin *et al.*, 2005). The pangenome helped in investigating patterns of species and subspecies genetic variation among seven published strains of *C. coli* and *C. jejuni.* Several genes that were differentially present in these two species and subspecies were identified (Meric *et al*., 2014).

1.5 *Campylobacter jejuni* pathogen and commensal

1.5.1 The mucus layer and *C. jejuni*

C. jejuni lives in intestinal crypts of a host where there is a low level of oxygen (Looft *et al.*, 2019). They are adapted to colonise the mucus layer (Stahl and Vallance, 2015). *C. jejuni* possesses factors that contribute to colonisation, such as its spiral shape and motility via polar flagella, both of which help its movement through the thick mucus layer to reach the mucosal surface to achieve optimum growth (Stahl *et al.*, 2016). Different antimicrobials and immune response components, such as histatins, defensins, lysozyme, antibodies, and nitric oxide, are present within the mucin layer (Alemka *et al.*, 2012).

The ability of some *Campylobacter* spp. to resist the antimicrobial agents present in mucin and oxidative stress enhances bacterial survival and mucus penetration (Hofreuter, 2014). It is interesting that in avian species, disease due to *C. jejuni* is low and the bacterium is generally referred to as a commensal although some impact on weight gain in infected chickens has been reported (Hermans *et al.*, 2012a).

In chickens, *C. jejuni* does not appear to multiply intracellularly but lives and multiplies primarily within the mucus layer (Van Deun *et al.*, 2008). In contrast, there is evidence that *C. jejuni* has the ability to invade the epithelial cells during gastrointestinal disease in humans (Naughton *et al*., 2014). The reason for the difference is not yet understood. This could be related to differences in the host's body temperature or gut environment. Bird species have an interior body temperature of 42°C, while human gut temperature is 37°C. Moreover, food intake and the gut microbiome are distinctive in both. Each of these differences could affect the expression and stability of many key virulence factors (Van Putten *et al.*, 2009). In addition, the structure and epithelium of the gut itself differ in poultry and humans, which can affect the

sensitivity to virulence factors. Moreover, the interplay with the innate immune system is critical in controlling microbes in the gut (Zheng *et al*., 2020).

Some key factors required for colonisation and pathogenicity are described in the general overview shown in fig1.2.

Both pathogenic bacteria and commensals must survive and multiply in mucin. Pathogenic *C. jejuni* invades epithelial cells, while commensals appear to stay within the mucus (Naughton *et al.*, 2014). The gut mucus layer in both humans and chickens contains extensive O-glycosylated mucin proteins that are connected to epithelial surfaces or are released as secretory mucins to form the gel-like mucin layer. Mucins contain a high level of the amino acids such as, serine, proline and threonine (Moran *et al*., 2011).

These vary within and among species and between different tissues (Linden *et al.*, 2008). *C. jejuni* is thought to bind these terminal groups. However, *C. jejun*i receptors have not been identified, and the receptors HpaA and SabA, although recognised in the related pathogen, *H. pylori*, are not encoded in the *C. jejuni* genome (Day *et al*., 2012). There is preliminary evidence that chicken mucus, but not human mucus, inhibits *C. jejuni* from attacking epithelial cells *in vitro* (Alemka *et al.*, 2010). This concurs with the discovery that human mucin MUC2 upregulates the expression of *C. jejuni* NCTC11168 virulence genes, such as *cdt* (toxin); *fla* (motility); *jlpA* (adhesion) and the exceptionally compelling *ciaB*, which is known to be critical in attacking epithelial cells (Tu *et al*., 2008).

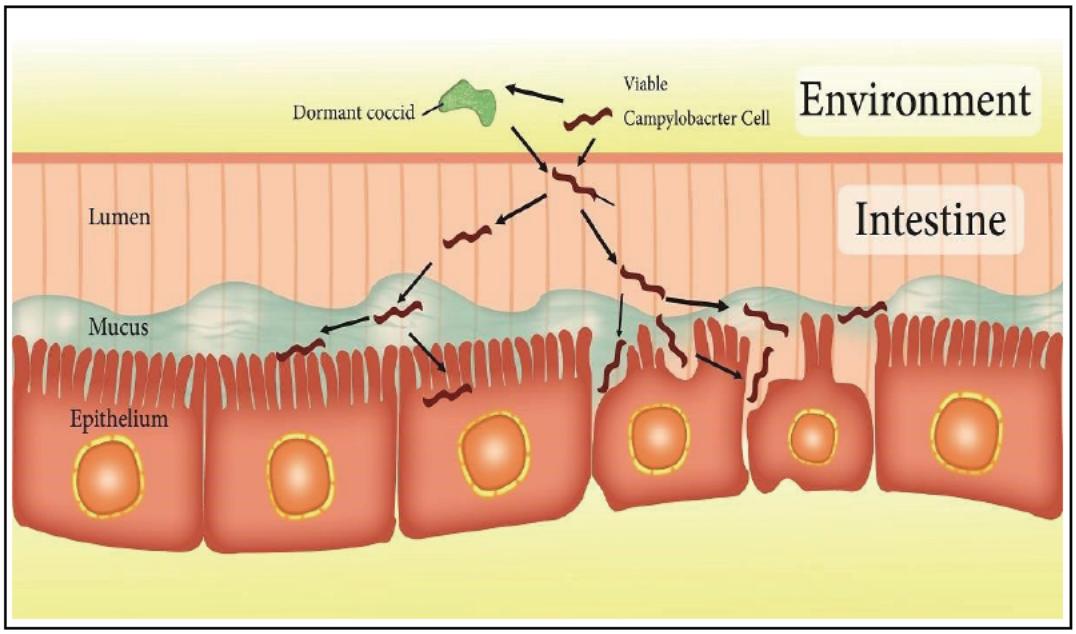


Figure 1.2 The different phases of *Campylobacter* colonisation in human intestines intestines. During disease in humans, *C. jejuni* invade the epithelial cells, resulting in a proinflammatory response, an increase in cytokine production, inflammation, and diarrhoea. Based on information (van Vliet and Ketley, 2001; Young *et al*., 2007).

1.5.2 Pathogenicity factors

1.5.2.1 Motility

C. jejuni has polar flagella and it is highly motile. Motility and chemotaxis are key in virulence and in host colonisation (Yao *et al.*, 1997; Gilbreath *et al.*, 2011). Colonisation requires the ability to move through the mucus layer that covers the intestinal epithelial cells. Motility of *Campylobacter*, especially in viscous substances, is unusual and corkscrew like because of the polar location of flagella and their helical cell shape (Ferrero and Lee, 1988). Mutants in *C. jejun*i flagellar genes showed reduced adherence and absence of invasion (Yao *et al.,* 1994; Guerry, 2007).

There are two flagellin subunits: a major flagellin (FlaA) and a minor flagellin (FlaB). These are immunogenic, but they are not recognised by the innate immune system (Ramos *et al.*, 2004). The main structural components of the flagellum (Guerry, 2007) are glycosylated, which is a key factor in virulence, as variable glycosylation leads to poor recognition by the immune system, thus enhancing immune evasion (Guerry *et al.*, 2006). In addition to its role in motility, flagella have been shown to promote the binding of *C. jejuni* to the host epithelium (Guerry *et al.*, 1991; Yao *et al.*, 1994). Interestingly, the *C. jejuni* flagellum has also been shown to mediate the secretion of non-flagellar proteins and thus contribute to the regulation of virulence by mediating the release of *Campylobacter* invasion antigens (Cia proteins) (Guerry, 2007).

1.5.2.2 Adhesion and invasion

C. jejuni interaction with host cells has been considered a potential target for intervention in illness in human hosts (Hu and Kopecko, 2008; Ketley, 1997). The intestinal mucosa is the specific area of colonisation by *C. jejuni*, and the subsequent invasion of epithelial cells leads to mucosal damage and inflammation, resulting in diarrhoea often associated with *Campylobacter* infection in humans (Fauchere *et al.*, 1986; Wooldridge *et al.*, 1996).

C. jejuni appears to prefer to adhere to epithelial cells in the human colon and chicken cecum rather than to other sites. (Everest *et al.*, 1992; Sylte *et al.*, 2019). Chickens are considered a natural host of *C. jejuni/coli* colonisation, and they present a significant source of infection in humans worldwide. Thus, the chicken model, as well as human tissue culture cells, has been extensively used to understand the factors involved in colonisation by *C. jejuni* (Bingham-Ramos & Hendrixson, 2008). In 2007, a study was conducted using cells grown from human and chicken intestinal biopsies. The cultured cells were infected by some strains of *C. jejuni*.

The bacteria invaded both the primary human intestinal cells and the chicken intestinal cells, but avian mucus decreased the *Campylobacter* interaction with epithelial cell surfaces, whereas human intestinal mucus did not (Byrne *et al*., 2007). These results are consistent with the roles of both the host mucus and the epithelial surface in contributing to susceptibility to adhesion and invasion.

Two proteins involved in the adhesion of *C. jejuni* to host cells are Cad F and FlpA. Both proteins bind the host extracellular matrix protein, fibronectin, but they appear to bind to different sites on fibronectin (Konkel *et al.*, 1997; Flanagan *et al.*, 2009). Mutants in *cadF* were unable to bind fibronectin and did not colonise newly hatched leghorn chickens (Ziprin *et al.*, 1999). Mutations in these genes resulted in a major decrease in the adhesion of *C. jejuni* to human intestinal epithelium cells (Ziprin *et al.*, 1999; Monteville *et al.*, 2003). Peb1A, a 28 kDa protein, was also reported to be important in the cell adherence of *C. jejuni* (Pei and Blaser, 1993). Mutations in *peb1A* led to significantly decreased adherence as well as the decreased invasion of HeLa cells (Pei *et al.,*1998).

CapA is an autotransporter lipoprotein (Jin *et al.*, 2001), which has been shown to be important for the adhesion and invasion of human epithelial cells (Ashgar *et al.*, 2007), whereas mutation of *capA* did not affect chicken colonisation (Flanagan *et al*., 2009). Another surface-exposed lipoprotein of 372 amino acids, J*lpA* was shown to enhance adhesion to host epithelial cells, as mutations in *jlpA* led to decreased adherence (Jin *et al*., 2001; Lehri *et al.,* 2015; Burnham and Hendrixson, 2018).

Following adhesion, epithelial cell invasion is a key step that leads to the induction of disease and gastroenteritis. *In vitro* and *in vivo* experiments have demonstrated that *C. jejuni* is capable of invading epithelial cells although the efficiency of invasion varies according to both bacterial strain and host cell type (Konkel *et al.*, 1992).

The co-cultivation of *C. jejuni* with INT-407 cells stimulates the production and secretion of more than eight Cia proteins (*Campylobacter* invasion antigens) (Konkel *et al.,* 1999). Some of these proteins are secreted through the flagellum type III secretion system (T3SS), and they are essential for efficient invasion (Konkel *et al.*, 2004; Christensen *et al.*, 2009). Interestingly, *C. jejuni* produces Cia proteins in response to bile salts and other host factors, and their secretion occurs after the adhesion of *C. jejuni* to the host's intestinal mucosa (Rivera-Amill *et al.*, 2001). CiaB, C, and D have been studied in detail. They are effector proteins that are injected into the host cell, and they appear to be involved in the stimulation of Interleukin-8 secretion and the induction of an inflammatory response. The deletion of any of these resulted in delayed disease in a pig model (Konkel *et al.*, 1999; Ó Cróinín and Backert, 2012; Buelow *et al.*, 2011; Christensen *et al.*, 2009; Neal-McKinney and Konkel, 2012; Samuelson and Konkel., 2013; Lehri *et al*., 2015).

1.5.2.3 Cytolethal distending toxin (CTD)

CDT is a common virulence factor secreted by several gram-negative bacteria (Gargi *et al*., 2012) and is involved in the pathogenesis of *C. jejuni* (Young *et al.*, 2007). It has also been identified in *C. lari*, *C. coli, C. foetus,* and *C. upsaliensis*. CDT inhibits the mitotic cell cycle of HeLa and Caco-2 cells at the G2 stage, which eventually leads to cell death (Whitehouse *et al.*, 1998; Johnson and Lior 1988).

It causes cell swelling, elongation, distention, and death in mammalian cells, such as HeLa cells and Chinese hamster ovary cells. It also damages the DNA and causes inflammation of the intestine (Whitehouse *et al.*, 1998; Pickett *et al.*, 1996; He *et al.*, 2019). CDT contains three subunits, Cdt A, B and C, all of which are required for toxicity (Pickett *et al.*, 1996; Lee *et al.*, 2003).

CdtB works as a nuclease (Lee *et al.*, 2003) and has structural similarities to the DNAase I of mammalian eukaryotic cells (Lara-Tejero and Galan, 2001). CdtA and CdtC, but not CdtB, bind to the HeLa cell surface (Lee *et al.*, 2003).

CDT is present in many but not all *C. jejuni* and *C. coli* strains. There is a large variation in CDT titres (Pickett *et al.* 1996; Eyigor *et al.*, 1999). Most *C. jejuni* strains have relatively high CDT activity, while *C. coli* strains show mainly low activity (Pickett *et al.,* 1996). The reason for this difference is not yet clear. (Pickett *et al.,* 1996). The analysis of the genome sequence of *C. jejuni* (NCTC11168) identified only *cdt* genes as clear toxins (Parkhill *et al*., 2000). Two additional genes were found to contain haemolysin domains, and a previously published report identified a phospholipase (PldA) with haemolytic activity (Grant *et al.,* 1997).

1.5.2.4 Capsular polysaccharide CPS

The first report linking CPS to virulence of C. *jejuni* was in 2001 (Karlyshev *et al*., 2001), CPS has been shown to be involved in *C. jejuni* induced diarrheal disease in ferrets, and in commensal colonization in chickens and mice (Keo *et al*., 2011). In 2017, the role of CPS in bacteremia in mice and abortion in pregnant pigs was demonstrated by gene-specific mutagenesis in *C. jejuni* IA3902 (Sahin *et al*., 2017). The loss of capsule production led to the complete abolishment of bacteremia and abortion. However, the exact role that CPS plays in the disease pathogenesis of *C. jejuni* still remains incompletely understood (Guerry *et al*., 2012).

1.6 *Campylobacter jejuni* physiology

1.6.1 General features of *Campylobacter jejuni*

The proposal that *Epsilonbacteria*, including the genus *Campylobacter,* be removed from the phylum Proteobacteria and reclassified as the new phylum *Campylobacterota* (Waite *et al.*, 2017) highlighted the phylogenetic distance of these bacteria from other well characterised

gram-negative bacteria. *C. jejuni* has a thin spiral or curve-shaped cell morphology that is about 0.2–0.8 μm wide and 0.5–4 μm long under optimal growth conditions with a polar flagellum at one or both ends.

It is non-spore forming, but under unfavourable conditions, including the stationary phase of growth, the morphology changes to a coccoid form. Coccoid cells were shown to be metabolically active (Hazeleger *et al*., 1998), but they cannot be cultured on laboratory media; hence, they have been referred to as a viable but nonculturable (VBNC) form (Ikeda and Karlyshev, 2012).

However, whether the coccoid form is an alternate morphology that can revert to spiral cells or whether they are simply dying cells remains unclear. Although protein synthesis is not required for coccoid formation (Hazeleger *et al.*, 1998), changes in peptidoglycan structure occurs, and this involves the activity of DL-carboxypeptidase Pgp1 and a putative N-acetylmuramoyl-L-alanyl amidase AmiA (Frirdich *et al.*, 2019). The activity of these enzymes results in the predominance of shorter (dipeptide) peptidoglycan-associated peptides. The VBNC state of bacteria is considered a strategy for coping with stressful environments. If coccoid *Campylobacter* could revert to the infective spiral form, it would be a significant threat to food safety and public health (Dong *et al*., 2020).

C. jejuni has been defined as "thermotolerant", as it requires temperatures between 34 and 44°C to grow and multiply. Most strains grow optimally between 37°C and 42°C (van Vliet and Ketley, 2001). *C. jejuni* is unable to grow at temperatures below 30°C, which correlates with the absence of genes coding for the cold-shock proteins involved in the adaptation to low temperatures (Hazeleger *et al.*, 1998). The optimal pH for the growth of *C. jejuni* is between pH6.5 and 7.5. The majority of *Campylobacter* species are microaerophilic, and they do not grow in the presence of atmospheric oxygen (Park, 2002). *C. jejuni* grows optimally in an atmosphere of 5–10% O_2 at a CO_2 concentration of 10%. (Kelly, 2001).

However, *C. jejuni* cannot grow under anaerobic conditions (Kelly, 2001) because it needs low levels of oxygen used in respiration and nucleic acid synthesis (Pittman and Kelly, 2005). *C. jejuni* possesses a complex respiratory chain that allows both aerobic and anaerobic respiration and it uses different electron acceptors and donors, such as nitrate, fumarate, and nitrite, to harmonise stress under both microaerobic and aerobic conditions (Sellars *et al.*, 2002). During respiratory metabolism, *C. jejuni* acts to oxidise succinate to fumarate by fumarate reductase Frd-ABC (Weingarten *et al*., 2009).

Fumarate is considered an important electron acceptor for the oxygen-independent respiration of *C. jejuni* (Sellars *et al.*, 2002). These features enable its survival in some environments, such as mammalian or avian gut. *C.jejuni* have several oxygen sensitive enzymes, which could be the single explanation for their oxygen sensitivity.

1.6.2 Oxidative stress in *C. jejuni*

C. jejuni faces a wide range of stress conditions during colonisation of farmed animals, food processing and transmission to humans. Included in this is exposure to levels of oxygen that are much higher than the optimal levels of 5 -10% oxygen observed in the laboratory.

As *C. jejuni* is an obligate microaerobic bacterium (Kaakoush *et al.,* 2007), acquiring energy through oxidative phosphorylation, it is exposed to low levels of the reactive oxygen species (ROS) superoxide radical (O^{-2}), hydrogen peroxide (H_2O_2), and hydroxyl (·OH), even during optimum growth conditions. On exposure to higher than optimal levels of oxygen, production of ROS is increased and accumulation of ROS inside *C. jejuni* cells can lead to damage to macromolecules such as DNA (Kim *et al*., 2015; Imlay, 2003).

In the presence of oxygen, the reactive oxygen species superoxide anion ($O^{\cdot-}_2$), hydrogen peroxide (H_2O_2) and hydroxyl radicals (·OH), are formed by the stepwise one-electron reduction of molecular oxygen (Imlay, 2003) fig1.3A.

Hydrogen peroxide (H_2O_2) and hydroxyl radicals are formed during aerobic metabolism, resulting from the autooxidation of respiratory dehydrogenases. Intracellular ferrous iron (Fe^{2+}) and H_2O_2 produce a highly reactive hydroxyl radical via the Fenton reaction (Imlay, 2003). fig 1.3B.

Hydroxyl radicals react with polypeptides and proteins by a hydrogen abstraction or oxygen addition, resulting in damage to protein structure and cleavage of nucleic acids by abstracting a hydrogen atom from the ribose (Tullius and Greenbaum, 2005). Although, full details of how hydrogen peroxide causes cell death is not completely clear (Marinho *et al*., 2013).

C. jejuni possesses many defence genes that detoxify ROS as covered below. In addition, NADPH one of the products of glucose metabolism in some strains C. *jejuni*. NADPH acts as the anti-oxidative defense mechanisms of most organisms (Nordberg and Arnér, 2000; Singh *et al*., 2008).

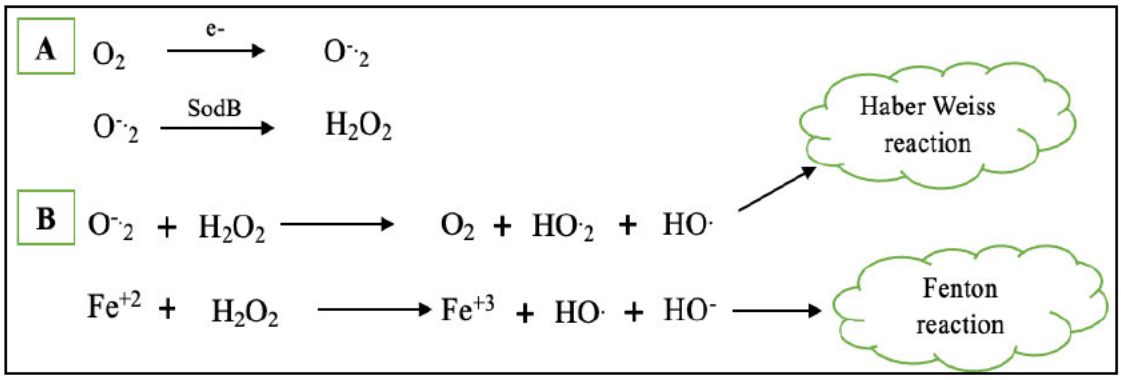


Figure 1.3 Schematic representation of generation of reactive oxygen species. (A) Superoxide radical is generated when molecular oxygen acquires an additional electron. Superoxide dismutase (SodB) converts a superoxide radical to H_2O_2. (B) Production of hydroxyl radical (OH•) from H_2O_2 via the Fenton reaction or via the Haber Weiss reaction and interaction with superoxide ion ($O2^{-}$). Figure modified from (Collin, 2019).

All these stresses could cause damage to in acellular macromolecules, such as DNA, proteins, and lipids, if the cell is unable to neutralise these toxic compounds (Storz and Imlay, 1999; Imlay, 2008).

In addition, *C. jejuni* has *sodB* gene which is involved in reducing the side effects of oxidative stress (Pesci *et al.*, 1994). A detailed discussion of genes encoding this ROS detoxifying enzymes is provided in subsection 1.6.2.2.

Several studies have highlighted the effect of H_2O_2 stress on *C. jejuni* growth for example, by adding H_2O_2 to cultures of *C.jejuni* 9131 at concentrations of 0mM –1.0mM for 15min (Yamasaki *et al.*, 2004). The results showed that the viability of *C. jejuni* 9131 rapidly decreased, also, when reference strain NCTC11168 and the *C .jejuni* Bf strain were exposed to oxidative stress reagents (hydrogen peroxide H_2O_2 and paraquat—PQ) in different concentrations, the Bf strain showed a higher survival rate in the presence of H_2O_2 and PQ than the *C. jejuni* NCTC 11168 strain. In 0.5 mM paraquat, the *C. jejuni* cultivated Bf strain showed a limited reduction, whereas the *C. jejuni* NCTC 11168 strain showed a decrease of 4.7log. Concentrations above 1mM PQ were lethal for the reference strain but not for *C. jejuni* Bf. Also, cells of *C. jejuni* Bf showed changes in morphology and expression of the gene *mreB*, which has a role in maintaining the bacillary cell morphology, was down-regulated (Rodrigues *et al*., 2015). Consequently, the relationship between resistance of Bf strain and other strains to oxidative stress and changes in cellular morphology and metabolism are areas requiring further study.

1.6.2.1. Enzymes involved in defence against ROS in *C. jejuni*

As described above, ROS products damage DNA and proteins. Hence, identifying ROS defence mechanisms is important in understanding how *C. jejuni* deals with this environmental stress during food processing and infection.

C. jejuni possesses many defence systems to detoxify and reduce the impact of ROS and also responds by regulating the expression of genes involved (Storz and Imlay, 1999; Imlay, 2008), as summarised in Table 1.2.

The catalase gene (*katA, cj1385*), which is one of the main contributors to redox stress resistance, catalyses the conversion of H_2O_2 to H_2O and O_2 (Imlay, 2008; Flint *et al*., 2016). Only this single gene encoding catalase is present in *C. jejuni.* A primary role of KatA in scavenging H_2O_2 under oxidative stress and in the survival of *C. jejuni* within macrophages has been demonstrated using a *C. jejuni* Δ *katA* knockout mutant (Grant and Park, 1995). The heme trafficking protein encoded by *cj1386*, located downstream of the *katA* gene in the *C. jejuni* genome, is a novel gene associated with oxidative stress defence. Construction of a Δ *cj1386* mutant led to increased sensitivity to hydrogen peroxide, similar to Δ *katA* (Flint *et al.,* 2012).

Double mutant *C. jejuni* Δ *katA* Δ *cj1386*, was created and the results were similar to that with the Δ *cj1386* and Δ*katA* single deletion mutants, (Flint *et al.,* 2012). The *cj1386* product was subsequently shown to bind heme in a 1:1 ratio and co-immunoprecipitation revealed a transient interaction with KatA, supporting a role of this product in specific trafficking of heme to KatA (Flint and Stinzi, 2015).

C. jejuni possesses three different antioxidant enzymes in the peroxiredoxin family, including Tpxs, Bcp and alkyl hydroperoxide reductase AhpC. AhpC converts H_2O_2 to H_2O plus O_2, *C. jejuni* also became highly sensitive to cumene hydroperoxide (an organic peroxide) when an *ahpC* knockout mutant was created (Baillon *et al.*, 1999). Table 1.5. Tpxs contribute to reduction in the concentration of H_2O_2, while Bcp also reduces organic peroxides, such as cumene hydroperoxide and tert-butyl hydroperoxide (Atack *et al*., 2008). *ahpC* expression is controlled by iron concentrations at the transcriptional level (Baillon *et al.*, 1999). Superoxide dismutase (Sod) is considered the primary enzyme responsible for the detoxification of superoxide (Winterbourn *et al.*, 1975).

There are three *sod* genes in *E. coli*: *sodA*, *sodB,* and *sodC,* encoding superoxide dismutases. *C. jejuni* possesses one *sod* gene, *sodB.* Superoxide *dismutase* dismutates superoxide ($O_2^{\cdot-}$) to hydrogen peroxide (H_2O_2).

Deletion of *sodB* in *C. jejuni* leads to an increase in susceptibility to both superoxide and peroxide stress (Flint *et al.*, 2014) fig 1.4. Other *C. jejuni* gene products that have been identified in the reduction of the effects of high oxygen concentration are listed in Table 1.2.

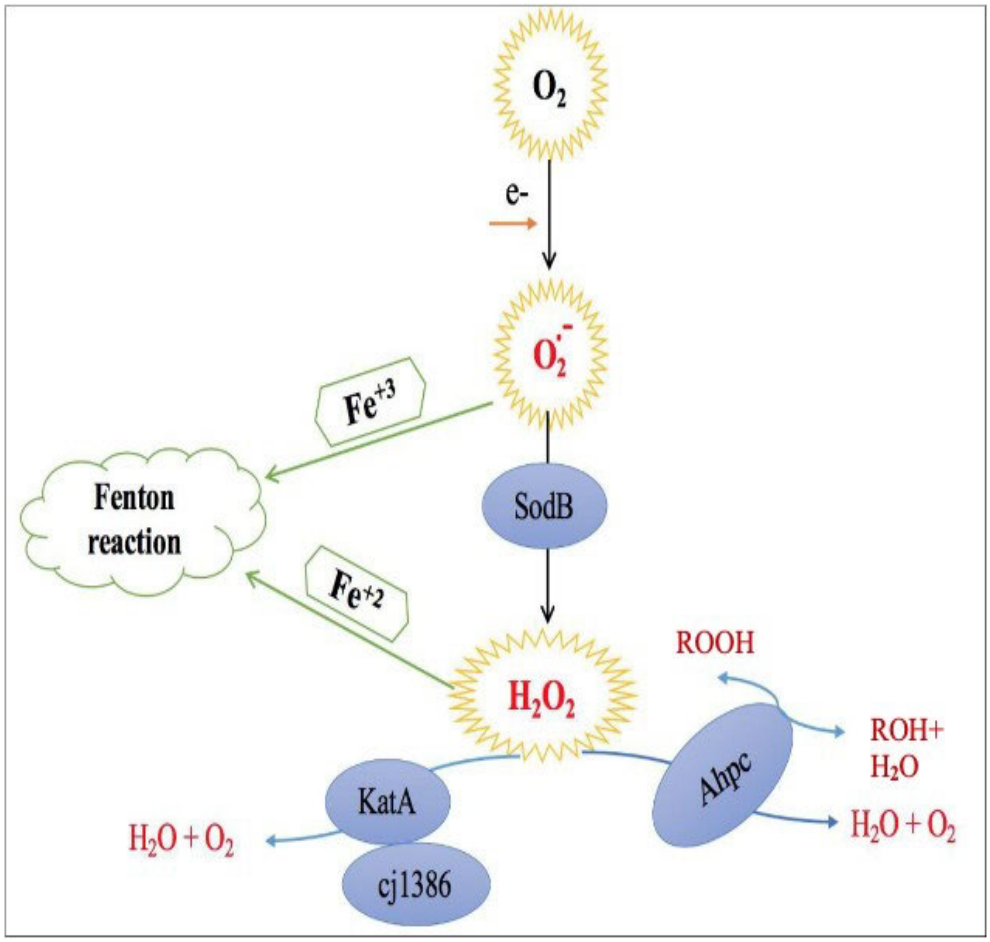


Figure 1.4 Schematic representation of oxidative stress defense by KatA, AhpC, SodB in *C. jejuni*. The antioxidant proteins are highlighted in blue; the oxidants are highlighted in red; green indicates Fenton reaction, see figure 1.4B. KatA catalyses conversion of H_2O_2 to water and oxygen, AhpC catalyses the reduction of organic hydroperoxide (ROOH) and H_2O_2 to their corresponding alcohols, and SodB catalyses the dismutation of superoxide anion ($O^{\cdot-2}$) to hydrogen peroxide H_2O_2 and oxygen. A dapted from (Palyada *et al*., 2009).

Gene ID(NCBI)	Gene Symbol (NCTC1168)	Protein	Function	Susceptibility increases by mutation/ Reference
905678	*katA*	Catalase	Decomposition of H_2O_2	Increased sensitivity toward hydrogen peroxide/ Flint *et al.*,2012.
904658	*ahpC*	Alkyl hydroperoxide reductase	Reduction of alkyl peroxides. Scavenger of endogenous H_2O_2	*ahpC* knockout mutant is highly sensitive to cumene hydroperoxide (an organic peroxide), but not to H_2O_2/ Baillon *et al.*,1999.
665448	*sodB*	Superoxide dismutase	Dismutation of superoxide expresses the iron-co factored	Increases *C. jejuni* susceptibility to both superoxide and peroxide stress/ Palyada *et al.* 2009; Flint *et al.*,2014.
905679	*cj1386*	heme -trafficking protein	*cj1386* is located downstream of *katA*, is involved in the full catalase activity, and contributes to *C. jejuni* colonisation of chicken intestines	*cj1386* mutant has an increased sensitivity towards hydrogen peroxide(Flint *et al.*,2012)
905488	*cj1198*	S-ribosylhomocysteine lyase	Produces functional AI-2 activity.	A L*uxS* mutation in *C. jejuni*81–176 increases the sensitivity to peroxides (H_2O_2 and cumene hydroperoxide)/ Elvers and Park ,2002.
905834	*cj1556*	Transcriptional regulator	Involved in oxidative and aerobic stress responses.	*rrpA* mutant led to increased sensitivity to oxidative and aerobic stress, reduced ability to form biofilms, and negative effect on downregulation of genes associated with oxidative and aerobic stress responses, such as *katA*, *perR*, and *hspR* /Gundogdu *et al.*, 2011.

905827	*cj1546*	Transcriptional regulator	Has role in regulating the response to oxidative and aerobic stress. RrpB is a DNA binding protein with auto-regulatory activity	– /Gundogdu *et al.,* 2016.
905664/ *cj1371*	*cj1371*	Periplasmic protein	Its molecular function has not been defined in *C. jejuni*.	A mutation of *cj1371* increased *C. jejuni* susceptibility to paraquat./Garénaux *et al.*, 2008a.
905088 904596	*tpx(cj0779)* *bcp(cj0271*	2-Cys peroxiredoxin Bacterioferritin comigratory protein	tpx and bcp product together play important roles in resistance to a number of oxidative and nitrosative stress agents, as well as to molecular oxygen.	Single mutant strains were no more sensitive than the wild-type strains, but the double mutant was hypersensitive./Atack and Kelly,2008
904360 904682	*docA* *cj0358*	Cytochrome C551 peroxidase	Both are periplasmic proteins with apparent haem-dependent peroxidase activity. The actual role of *ccps* in resistance to ROS during *C. jejuni* colonisation remains unclear.	Deletion of *docA* or *cj0358* did not significantly impair the resistance of the strain to H_2O_2./ Hendrixson and DiRita, 2004;Bingham-Ramos and Hendrixson, 2008.
904967 905403	*msrA(cj0637)* *msrB(cj1112)*	peptide-methionine (S)-S-oxide reductase peptide-methionine (S)-S-oxide reductase	Both reduced oxidised methionine to methionine and restored methionine function in protein synthesis and protection of *C. jejuni* against oxidative and nitrosative stress.	*C. jejuni* became more sensitised to peroxide and superoxide stress and nitrosative stress when there was a double mutation in *msrA/B* ./ Atack and Kelly,2008).

Table 1.2 Summary of *C. jejuni* systems against oxidative stress. Genes, proteins name, function and effect of mutations are listed.

1.6.2.2 Regulation of response to oxidative stress in *C. jejuni*

In many gram-negative food-borne pathogens such as *E. coli* and *Salmonella enterica*, oxidative stress defence mechanisms are regulated by the peroxidesensing and superoxide regulators OxyR and SoxRS. Both are absent in the *C. jejuni* genome.

However, *C. jejuni* possesses the peroxide resistance regulator (PerR), which replaces OxyR (Parkhill et al., 2000), PerR regulates the genes encoding enzymes dealing with peroxide stress genes, including *katA* and *ahpC*. The autoregulation of PerR in *C. jejuni* is mediated by iron (Kim *et al.*, 2011). The Fur homolog, which directly or indirectly regulates at least 104 genes in *C. jejuni*, is also involved in the regulation of oxidative stress defence and peroxide stress resistance (Palyada *et al*., 2009).

Mutation in p*erR* significantly increases the expression of the antioxidant proteins KatA and AhpC. In addition, the perR *fur* double mutant leads to the abolishment of all iron regulation. PerR also controls defence against suoeroxide. According to van Vliet *et al., C. jejuni* PerR regulates the transcription of both peroxide and superoxide defence genes (van Vliet *et al.,* 2002). PerR has also been shown to bind to the *sodB* promoter and negatively regulate transcription of *sodB* gene, the most representative gene in superoxide defence (Kim *et al.*, 2015).

C. jejuni also contains another regulator, CosR*,* which plays an important role in the control of oxidative stress resistance by regulating the oxidative stress defence genes *ahpC, dps*, *katA*, and *sodB* (Hwang *et al.*, 2011).

Results of Western blotting and reverse transcription-quantitative PCR revealed increased expression of KatA upon acid stress. Electrophoretic mobility shift assays (EMSA) showed that the binding affinity between Fur and the *katA* promoter was reduced at low pH, rationalising the higher levels of expression of *katA* under acidic conditions (Askoura *et al.*, 2016).

1.7 Carbon metabolism and energy sources

The study of metabolic processes of *C. jejuni* can help to understand its pathology and colonisation. *Campylobacter* is generally defined as asaccharolytic; that is, it cannot utilise carbohydrates as a substrate for growth (Hooper *et al.*, 1999; Coyne *et al.*, 2005). It relies on amino acids and tricarboxylic acid (TCA) cycle intermediates as energy and carbon sources. This has been supported in genomic sequencing studies that failed to identify any complete carbohydrate metabolic pathways in the *C. jejuni* NCTC11168 genome (Parkhill *et al.*, 2000). The TCA cycle is an important source of energy for *C. jejuni* and many organic acids, such as pyruvate, oxaloacetate, and 2-oxoglutarate, which are products of catabolism and feed into the cycle (Velayudhan and Kelly, 2002). Some TCA intermediates can also be transported into the bacterial cell and used as a nutritional source. The KgtP permease transports 2-oxoglutarate and DcuA, and DcuB (C4dicarboxylate transporters) mediates the transport of fumarate, succinate, and malate (Guccione *et al.*, 2008). Citrate transporters have been identified in numerous *C. jejuni* strains (Guccione *et al.*, 2008). However, in 2011, both Muraoka and Zhang, and Stahl demonstrated the ability of certain strains of *C. jejuni*, including NCTC11168 to utilise fucose (Muraoka and Zhang, 2011; Stahl *et al.,* 2011).

1.7.1 Catabolism of amino acids

Within the gut, *C. jejuni* utilises serine, aspartate, asparagine, and glutamate, which are readily acquired as a primary nutrient source, acting as a source of intermediates in the TCA cycle and as a carbon source (Hofreuter *et al.*, 2008). *C. jejun*i depends on the two proteins, SdaC (Cj1624c) and SdaA (Cj1625) (Velayudhan *et al*., 2004) for serine metabolism. SdaA acts as an L-serine dehydratase and SdaC is a transporter of serine into the cell. Mutation of *sdaC* leads to loss of serine uptake into the cell. Hence, SdaC is the sole transporter capable of transporting significant amounts of L-serine into the cell (Velayudhan *et al.*, 2004).

Although L-serine is not essential for the growth of *C. jejuni in vitro*, L-serine utilisation was shown to be important for the efficient colonisation of chicks (Velayudhan *et al.*, 2004). Glutamate and aspartate are both taken in and catabolised using the Peb1 system, which comprises the proteins Cj0919c, Cj0920c, Cj0921c (Peb1A) and Cj0922c (PebC) (Del Rocio Leon-Kempis *et al.*, 2006). Peb1ABC protein is a transporter for both glutamate and aspartate. Mutation of *cj0921c* in *C. jejuni* led to complete loss of transport of glutamate and impaired uptake of aspartate. AspB converts glutamate to aspartate, which is then converted into the TCA cycle intermediate fumarate by AspA (Leon-Kempis *et al.*, 2006; Guccione *et al.*, 2008). Proline is a less preferred substrate and proline catabolism occurs only following the exhaustion of serine, and glutamate (Wright *et al.*, 2009). The uptake of proline significantly increases during the stationary phase (Wright *et al.*, 2009; Mohammed, 2018). The PutP protein (Cj1502c) transports proline into the cell, and PutA acts to convert proline to glutamate. The ability of *C. jejuni* to use asparagine and glutamine depends on the presence of the secreted enzymes asparaginase and gamma-glutamyl transpeptidase (*ggt*). GGT is an enzyme pertinence to the family of N-terminal nucleophile hydrolases, acts the degradation of glutathione (Rossi *et al*.,2012).

Possession of the *ggt* gene enables the utilisation of both glutamine and glutathione, while asparagine is converted to aspartate by the product of the *ansA* gene (Hofreuter *et al.*, 2008). Among strains of *C. jejuni,* there is a diversity in their ability to use these amino acids, which is identifiable by the presence or absence of *ggt* and *ansA* this may affect their ability to colonise different tissues or animals (Hofreuter *et al.*, 2008) fig 1.5.

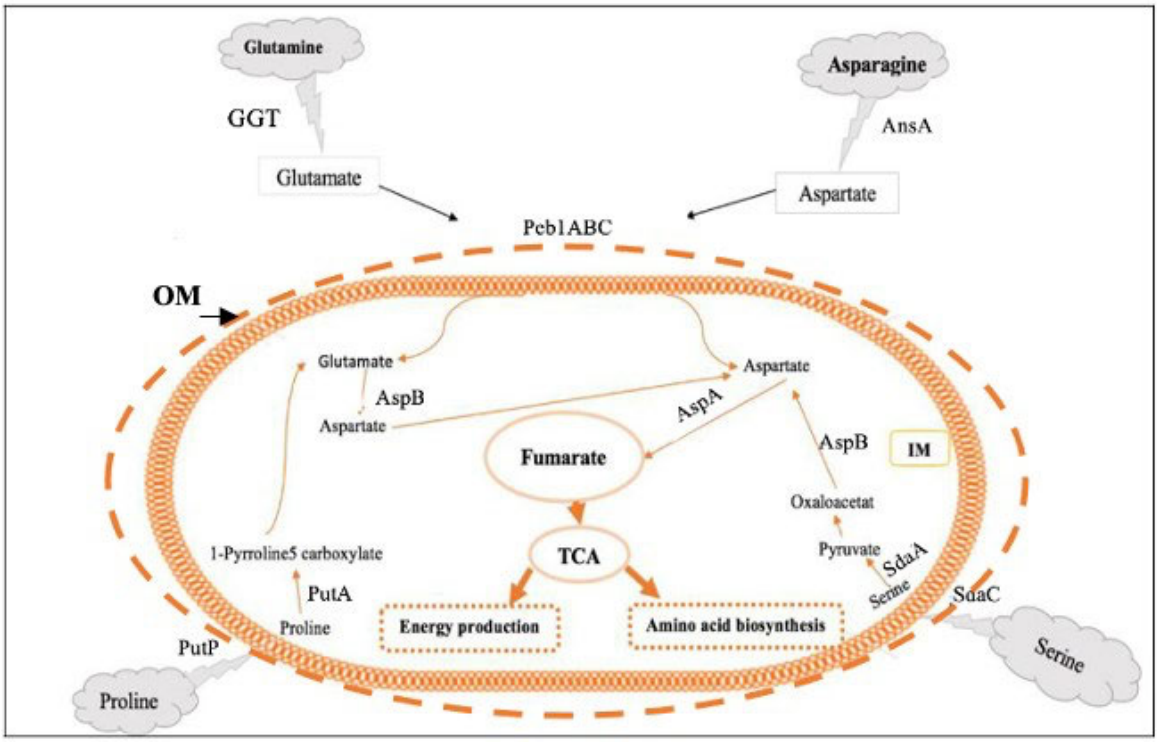


Figure 1.5 Central metabolism in *C. jejuni.* Schematic of transporters and amino acid metabolism, which are considered to fuel the growth of *C. jejuni*: IM, inner membrane; orange circle; OM outer membrane; orange dash line; grey cloud, main amino acids, gene products involve in uptake and metabolism of serine, proline, glutamate and aspartate are labelled.. Outputs of amino acid metabolism are indicated in orange circles; dashed rectangles indicate the process of metabolism. A dapted from (Stahl *et al*., 2012).

1.7.2. Carbohydrate utilisation

As established, *C. jejuni/coli* depend on amino acids as primary energy and carbon sources, some organic acids, and TCA intermediary compounds. The core genome lacks several genes required for the catabolism of glucose and other sugars. It was not until 2011 that evidence was provided of the ability of some strains of *C. jejuni subsp. jejuni* to metabolise any carbohydrate, such as fucose (Muraoka and Zhang, 2011; Stahl *et al.*, 2011). Subsequently, in 2015, a locus encoding the Entner-Doudoroff (ED) pathway permitting the utilisation of glucose was identified (Vorwerk *et al.*, 2015).

1.7.2.1 Fucose utilisation

Some strains of *C. jejuni* possess a genomic island (*cj0480c–cj0490*), including the gene (c*j0486/fucP)* that encodes an L-fucose permease. Possession of this locus, encoding 11 genes, allows the utilisation of L-fucose as a substrate for growth (Dwivedi *et al*., 2016). Over 50% of *C. jejuni* strains harbour the 9kb operon (*cj0480c-cj0490*) (Muraoka and Zhang 2011).

Mutations in *cj0481, cj0486*, or *cj0487* resulted in loss of the ability to grow on fucose. Strains mutated in *cj0486* exhibited a competitive disadvantage in a piglet colonisation model of human disease (Stahl *et al.*, 2011; Martiny *et al.*, 2011).

The conclusion was that when *C. jejuni* colonises the mucus layer of the intestinal epithelium in humans, chickens, and other animals, soluble L-fucose released from mucin glycoproteins is available. However, *C. jejuni* lacks fucosidases that act to release fucose residues from oligosaccharides, thus permitting transport into the cell for metabolism (Hooper and Gordon, 2001). A recent study showed that *C. jejuni* can grow on fucosylated mucin glycoproteins when the commensal, *Bacteroides vulgatus* is included. *B. vulgatus* acts to release L-fucose from mucin which is then available for metabolism by *C. jejuni* (Garber *et al*., 2020).

1.7.2.2 Glucose metabolism and the Entner-Doudoroff pathway

Until several years ago, there was general agreement that *C. jejuni* lacks the ability to catabolise glucose (Parkhill *et al.*, 2000; Velayudhan and Kelly, 2002). The core metabolism in the glucose assimilation of *C. jejuni* by the classical Embden-MeyerhofParnas (EMP) glycolysis pathway does not work because it lacks phosphofructokinase (PfkA), and therefore cannot convert fructose-6 phosphate to fructose 1,6 phosphate and thus cannot metabolise glucose. However, it can undergo the reverse reaction using the enzyme fructose-1,6-bisphosphatase (Fbp); hence, it can synthesise glucose as required for polymer and polysaccharide synthesis via gluconeogenesis (Guccione *et al.*, 2008; Hofreuter *et al.*, 2008; Parkhill *et al.*, 2000; Pendleton *et al.*, 2013; Velayudhan and Kelly, 2002). The absence of an identifiable glucose transporter gene in the genome of reference C. *jejuni* strain NCTC11168 was also consistent with the inability to utilise glucose.

In 1952, Michael Doudoroff and Nathan Entner discovered the Entner-Doudoroff (ED) pathway in *Pseudomanas saccharophila,* and following numerous biochemical studies, the ED pathway was shown to be an alternate pathway of glucose metabolism used in a wide variety of organisms, including archaea and plants (Conway, 1992), such as mosses and ferns (Chen *et al.*, 2016).

In *Campylobacter*, the ED pathway was initially identified in the genome sequence of *C. jejuni* subsp. *doylei* 269.97 (Miller, 2008). Sequence analyses identified seven genes on an 8669bp region of DNA, the *glc* locus, encoding enzymes for catabolising glucose plus a transport permease. The gene encoding the permease was subsequently annotated as *glcP,* encoding a glucose permease. The other genes encode enzymes required for the catabolism of glucose via the ED pathway, are shown in fig1.6.

The seven identified genes including a putative glucose transporter are located within an *rrn* locus between 16SrRNA, isoleucyl-tRNA and 23SrRNA. fig1.7

Recently, a similar *glc* locus was identified in the genome sequences of a subset of *C. coli* strains and the functionality of this ED pathway was demonstrated (Vorwerk *et al.*, 2015; Vegge *et al.*, 2016). The *C. coli* isolates were divided into clades 1, 2, and 3 based on rMLST analysis (Sheppard *et al.*, 2008). Clade 1 contained all the ED-positive *C. coli* strains except one. These isolates were drawn from a range of sources included animals, food and the environment. WGS has also identified a *glc* locus in a small percentage of *C. jejuni* isolates. Interestingly, these could be defined as primarily from environmental isolates: 51.4% of identified ED-positive strains were from Norway rats and 42.9% from wild birds (Vegge *et al.*, 2016). It has been shown that this *glc* locus can be transferred between *C. coli* strains by horizontal gene transfer (HGT) (Vorwerk *et al.*, 2015). Possession of the *glc* locus permits the growth of both *C. coli* (Vegge *et al.*, 2016) and *C. jejuni* on glucose (Mohammed, 2018). Following uptake via the glucose permease, the enzymes of this pathway convert glucose to glyceraldehyde- 3-phosphate and pyruvate via 2-keto-3-deoxy-6-phosphogluconate (KDPG) fig1.6. Glucose is initially phosphorylated by glucokinase (Glk). The enzyme glucose-6-phosphate dehydrogenase (Zwf) converts glucose-6phosphate to Glucono1,5 lactone -6P. The phosphogluconolactonase (Pgl) enzyme then converts glucono1, 5 lactone-6P to Gluconate-6P, while the dehydratase (Edd) enzyme converts gluconate 6P to 2-keto-3-deoxy-6-phosphogluconate (KDPG). The final, specific step in the ED pathway is the hydrolysis of the 6-carbon KDPG intermediate to pyruvate and glyceraldehyde 3-phosphate by aldolase (Eda) (Conway, 1992; Vorwerk *et al.*, 2015). *Pseudomonas* spp. grow aerobically and use glucose via the ED pathway (Conway, 1992).

They have been described as having an EDEMP pathway, which is a cyclic fusion of the ED and EMP reactions, where a portion of the triose-phosphate formed by the (Eda) aldolase reaction is converted to hexose phosphate via the gluconeogenic reactions of the EMP pathway. *Helicobacter pylori* also metabolises glucose via an ED pathway comprising three key gene products, Edd, Eda, and Zwf (Mendz *et al.*, 1994). A study conducted *in vivo* with mutated *H. pylori* showed that glucose utilisation by this pathway was not essential for the colonisation of mice (Wanken *et al.*, 2003). The benefit of the ED pathway for certain strains of *C. jejuni* is unknown. Studies on *C. coli* have indicated enhanced biofilm formation in some ED-positive strains and extended survival in the stationary phase (Vegge *et al.*, 2016). The prevalence of possession of the ED pathway by strains associated with environmental rather than farmed animals and humans could potentially indicate a role in enhanced survival in the environment.

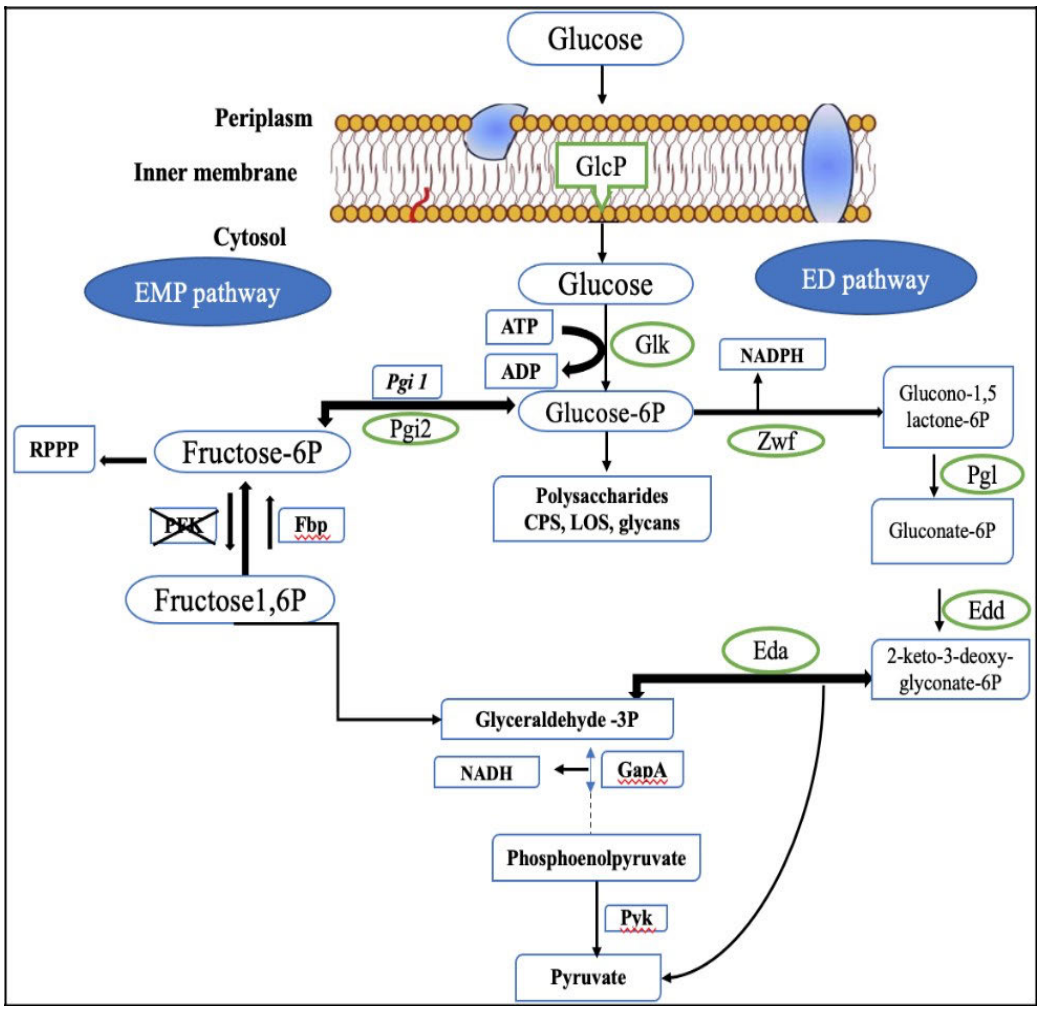


Figure 1.6 Entner-Doudoroff pathway of glucose metabolism in *Campylobacter.* Gene products encoded on the *glc* locus that participate in glucose metabolism via the ED pathway are labelled with a green circle (metabolic enzymes) or rectangle (uptake); Glk (glucosekinase) phosphorylates glucose forming glucose-6P, Zwf (glucose-6-phosphate dehydrogenase) converts glucose-6phosphate to Gluconol1,5 lactone-6P, which is converted to Gluconate-6P by Pgl (6- phosphogluconolactonase); Edd (6phosphogluconate dehydratase) and Eda (2-keto-3-deoxy-6- phosphogluconate aldolase), the main enzymes in the ED pathway, catalyse the conversion of the hexose gluconate-6P to 2- keto-3-deoxy-gluconate-6P and then to glyceraldehyde-3P. Pgi1and Pgi2(*glc* locus) are two phosphoglucose isomerases. Fbp is an enzyme of the EMP pathway and converts fructose-1,6-bisphosphatase to fructose in the EMP pathway. The conversion of glycerate -3P to pyruvate and the entry of pyruvate into the TCA cycle occurs via standard reactions. RPPP is the reductive pentose phosphate pathway (Adapted from Vegge *et al.*, 2016).

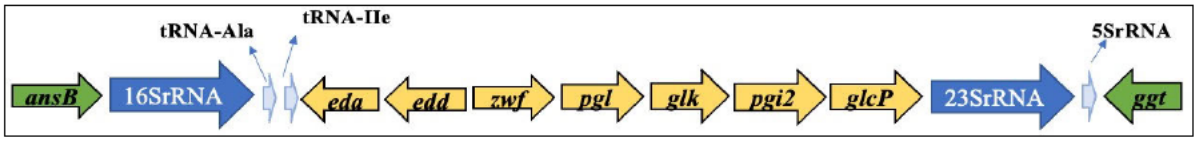


Figure 1.7 The *glc* locus within a ribosomal RNA operon in C. *jejuni* Dg275.The yellow arrows indicate genes encoding proteins required for glucose uptake and metabolism via the ED pathway; blue colour-coded arrows, the *rrn* locus; green arrows are flanking genes (Adapted from Mohammed, 2018).

1.8 Genetic diversity in *Campylobacter*

1.8.1 *Campylobacter* genome

In 2000, the first reported genome sequence and annotation for *C. jejuni* NCTC11168 was published, (Parkhill *et al*., 2000). and the re-annotated sequence followed in 2007 (Gundogdu *et al*., 2007). *C. jejuni* NCTC11168 has a circular chromosome of 1,641,481 base pairs.

The genome has a low GC ratio of around 30% (Taylor *et al.* 1992). The asaccharolytic nature and low GC content of *Campylobacter* were reasons for its separation from the genus *Vibrio* (Moore *et al.*, 2005; On, 2001). Genomes of the majority of *Campylobacter* species have a low GC content of 30–36%. However, in the entire genus the range is 29–46% (Vandamme and De Ley, 1991).

The NCTC11168 genome contains several repetitive sequences including three copies of the ribosomal rRNA operon *(rrn* A, B and C) each of which encodes, 16S, 23S *rRNA* and 5S *rRNA*) (Vorwerk *et al.,*2015). In addition, there are a number of repetitive genes, *maf* genes, associated with a flagellar gene cluster (Karlyshev *et al.*, 2002). A close phylogenetic relationship was found between *C. jejuni* and *H. pylori.* More than 50% of *C. jejuni* genes have orthologues in *H. pylori* most of which are related to housekeeping function, resistance, transmission, and pathogenesis (Parkhill *et al.*, 2000).

Genome sequences are available for two other well characterised strains of *C. jejuni,* strains 81116 and 81176. NCTC 81176 differs from NCTC11168 and 81116 by the possession of two plasmids pVir (37468 bp), which contains several genes that encode for a Type 4 secretion systems (T4SS) (Bacon *et al*., 2002), and pTet plasmid (45205bp), which carries a *tetO* gene encoding tetracycline resistance. (Batchelor, *et al*., 2004; Hofreuter *et al*., 2006; Pearson *et al*., 2007). Neither *C. jejuni* 81116 nor 11168 contains plasmids (Bacon *et al*., 2000; Manning, *et al*., 2001).

Recently, MLST and WGS have helped to show the wide genetic diversity in strains of *C. jejuni* and *C. coli* and that certain *C. jejuni* genotypes are host-specific (Sheppard *et al.*, 2011; Sheppard *et al.*, 2014).

1.8.2 Genetic diversity

Genetic diversity is considered a major trait of many pathogen populations. Variation in genetic sequence provides many advantages such as adaptation to new environments, varied metabolism and survival of pathogens in a specific niche and hostile host environments. In *Campylobacter* genetic variation occurs by intracellular genome rearrangement and slipped strand mispairing at runs of short sequences leading to hypervariable sequences (Karlyshev *et al*., 2005).

DNA is subject to various kinds of damage caused by a range of endogenous and environmental factor, which cause strand breaks, nucleotide modifications, mismatches (Eisen and Hanawalt, 1999). In general, bacteria have DNA repair mechanisms to protect DNA against damage (Friedberg, 2008), however DNA repair systems for survival of *C. jejuni* is stillpoorly understood. One recent study investigating repair associated with oxidative DNA damage studied the role of *cj0595c* (named *nth* gene) in *C. jejuni* NCTC11168. Inactivation of *cj0595c*,

led to an increase in numbers of oxidative stress resistant (OX^R) mutants, compared with the wild-type strain.

Resistance correlated with transitions in the *perR* gene. Inaddition, recombinant Nth protein showed endonuclease III activity and ability to excise the thymine glycol (Tg) base from a double stranded DNA. These results are consistent with the hypothesis that Nth functions as a BER (base-excision repair) enzyme in *C. jejuni* (Dai *et al*., 2019).

Based on previous research, the genome of *C. jejuni* is characterised by rapid microevolutionary changes during the passage through the gastrointestinal tracts of rats and chickens (Kim, *et al.*, 2012), as well as humans. A laboratory worker became infected with the *Campylobacter* strain with which they were working, NCTC11168-GSv. WGS analysis of an isolate from stool samples identified that a number of genetic changes occured after passage through this human host. Indel mutations within 12 contingency loci conferring phase variations were identified in homopolymeric tracts, as well as SNPs in 2 genes (Dallas *et al.*, 2014).

Contingency genes are found in many bacteria and *C. jejuni* NCTC11168, contains more than 20 contingency genes (Parkhill *et al.*, 2000). These act primarily to synthesise or modify surface structures, such as lipooligosaccharide (LOS), pilus, flagella and capsule, and have a role in host interaction and virulence (Moxon *et al.*, 2006; Parkhill *et al.*, 2000).

Hypervariability commonly occurs in genes, that influences the makeup of cell surface structures, such as the flagellum, lipooligosaccharide, and capsular polysaccharide. (Gundogdu *et al.*, 2007). The flagellar biosynthesis and modification area contains seven hypothetical paralogous genes: *maf1 (cj1318), maf2 (cj1333), maf3(cj1334), maf4 (cj1335*6), *maf5 (cj1337), maf6 (cj1341), and maf7 (cj1342),* which belong to the motility accessory factor (*maf*) family (Karlyshev *et al.*, 2002). The genes *maf1* and *maf4* were found to be identical, and both contained homopolymeric G tracts. The majority of *maf* gene products are predicted to be cytoplasmic, but *maf3* has a potential transmembrane region.

The ability to rapidly vary the cell surface is considered a major contribution to immune avoidance and the colonisation of dynamic host environments (Parkhill *et al.*, 2000).

1.8.3 Horizontal gene transfer (HGT)

Gene transfers profoundly shapes a primary mechanism in the evolution of bacteria, and it is a driver of the diversification among bacterial species and the acquisition of new and varied functionality in pathogens (Schjørring and Krogfelt, 2011). Three mechanisms contribute to gene transfer: conjugation, transduction, and transformation. The conjugation mechanism requires plasmids to mediate their own transfer from donor to recipient cells through direct contact. Transduction is a process that requires a virus to transfer DNA from one bacterium to another. Transformation is a mechanism that allows the uptake of DNA directly from the environment (Dubnau, 1999). *Campylobacter* has been shown to be naturally transformable both *in vitro* and in the natural environment, which potentially leads to greater genetic diversity between strains, as large genomic changes can take place rapidly (Taylor *et al*., 1992). Many previous studies have proposed that the horizontal transfer of DNA contributes to phenotypic changes in *Campylobacter*, including the acquisition of antibiotic resistance (Wittwer *et al.*, 2005), changes in capsule (Karlyshev *et al.*, 2005), and lipooligosaccharide composition (Gilbert *et al.*, 2004), as well as flagellar synthesis (Harrington *et al.*, 1997). HGT contributing to generating diversity in MLST data was identified by comparing the number of different alleles at each locus for a given number of STs (Sheppard and Maiden, 2015). Each new allele was generated by the progressive accumulation of mutations. Each new ST contained a new allele in at least one locus. *C. jejuni* is considered naturally competent in transformation, and it represents one of more than 40 naturally competent bacterial species that are able to import

DNA from the environment and incorporate it into their genomes (Wang and Taylor, 1990). Many reports have been published about R plasmids that are

transmissible among *Campylobacter* spp. (Sagara, *et al*., 1987). The plasmid pVir(37kb) was identified in more than 15% of 104 *C. jejuni* clinical isolates and it contains components of a type IV secretion system (T4SS) (Bacon *et al*., 2002), that has a role in the virulence of some major bacterial pathogens (Christie 2001; Bacon *et al*., 2000) *In vitro*, studies have identified importance of the ,pVir plasmid in promoting adherence and invasion of intestinal epithelial cells in culture. The *tetO* gene located on pTet (45-kb) confers resistance to tetracycline. (Friis *et al.*, 2007).

In the related species *H. pylori*, two pVir-encoded genes required for natural transformation *virB11* and *comB3*, were tested to determine their role in natural transformation in *C. jejuni* (Hofreuter *et al.,*2001; Bacon *et al.,*2000). Mutants of *virB11* and *comB3* showed no difference in transformation compared with the wild type; however, a defect in transformation efficiency was observed in a *comB3* mutant (Bacon *et al*., 2000).

1.9 Project aims

A bank of *C. jejuni* isolates had been collected from faecal samples of farm associated Norway rats in the SE of England between 2011 and 2013. MLST and phylogenetic analysis of WGS (Fig1.8) assigned approximately two thirds of the strains to clonal complexes, commonly associated with human disease, chicken and farm animal colonization, for example, ST45CC-45, ST21CC-21, ST22CC-22 and ST42CC-42 (de Haan *et al*., 2012; Harvala *et al*., 2016). However, many isolates that were classified as unassigned clonal complexes, fell into two major groups – a distinct clade, RG1 (red in fig 1.8). and a broad group of strains more closely related to ST45CC45 (blue and black in fig1.8).

This latter group contained 37 strains of *C. jejuni*, possessing genes encoding enzymes of the ED pathway and glucose utilisation. These isolates were of interest for two reasons, firstly as *C. jejuni* has an incomplete EMP pathway, strains of this species are generally unable to grow on glucose as a carbon source and have been defined as asaccharolytic.

Secondly, the unusual distribution of these strains suggested a possible preference for host or environment.

The *glc* locus appears to be a mobile element located within an *rrn* operon, but almost exclusively within wild bird and Norway rat isolates (Vorwerk *et al*., 2015, Vegge *et al*., 2016, Mohammed, 2018). Glc positive Norway rat associated *C. jejuni* strains fell into two broad groups, one group (highlighted in blue and green, fig1.8) is closely related to ED negative CC45 strains and all have a complete *glc* locus, while sequencing data for strains in a second broad group (black in fig 1.8) suggested that some of these strains possess a complete *glc* locus but in others the *glc* locus was incomplete (Mohammed, 2018). The ultimate aim of this study was to contribute to the understanding of the benefit of glucose utilisation via the ED pathway to these strains of *C. jejuni* and any contribution this may have on their ecological niche.

Specific objectives addressed in this Thesis:

1. This study began by assessing glucose utilisation of these strains and revisiting sequence data, where the presence of incomplete genes of the *glc* locus in draft WGS data of several strains indicated instability of the *glc* locus.

2. Identify the location and number of copies of *glc* locus inserted in selected strains. Confirm this by PCR combined with hybrid Illumina/ nanopore sequencing

3. Study the effect of glucose and disaccharides such as maltose on growth properties of several Glc positive strains under standard microaerobic condition and high and low concentrations of oxygen.

4. Attempt to create a complete *glc* (*rrnA*) knockout in Dg275 strain to assess impact of possession of *glc* locus. Analyse and assess naturally evolved mutants, unable to utilise glucose recovered from a chicken colonisation trial.

5.Study the influence of possession of the ED pathway on aerobic and oxidative stress, specifically assessing sensitivity to H_20_2

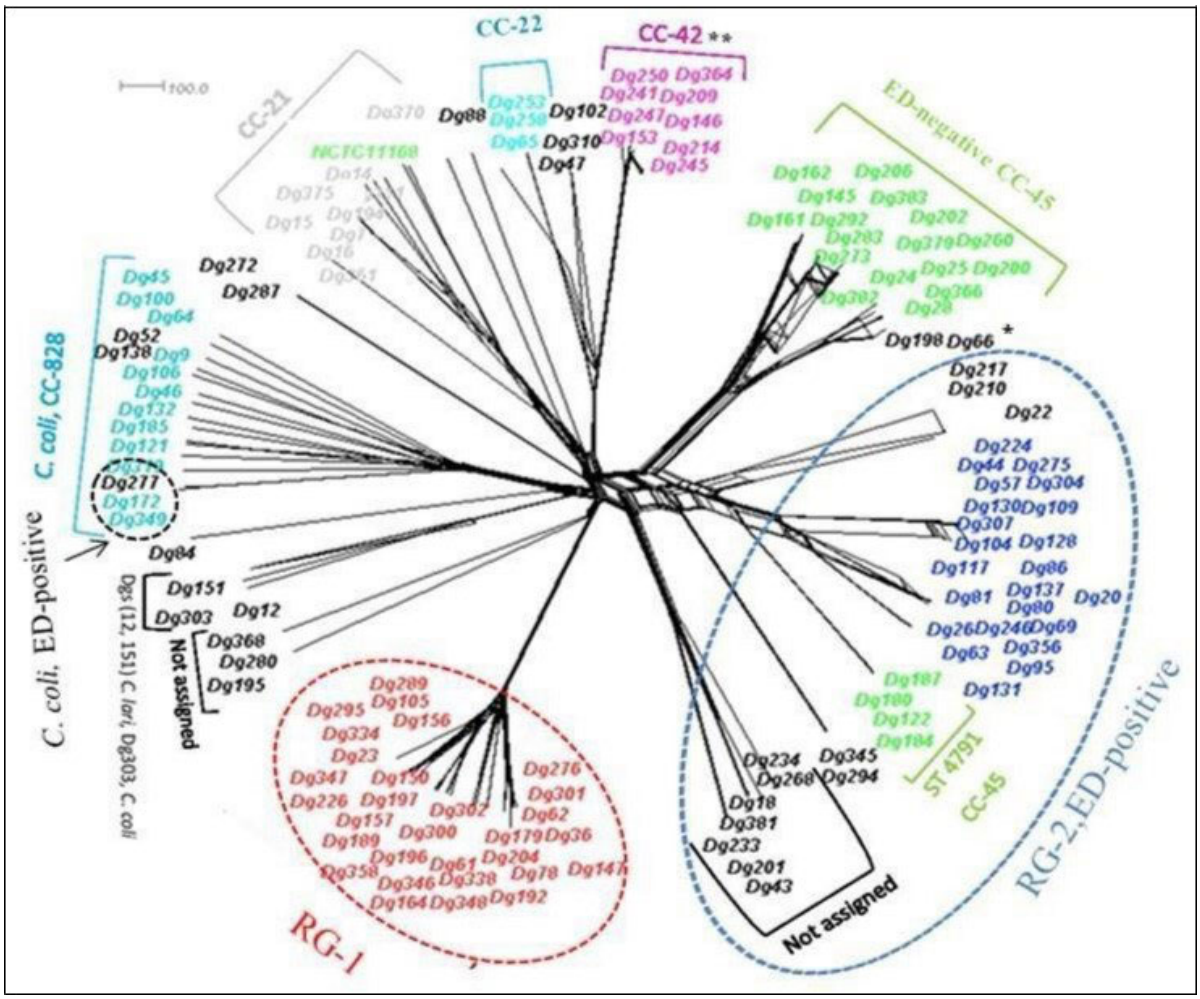


Figure 1.8 WGS phylogenetic network of 143 Campylobacter isolates from farm associated Norway rat. *Campylobacter* strains isolated from fresh Norway rat faecal samples from eight different farms within the South-West of England (Sites C, E, H, G, F, I, J, K) between 2010 and 2012. Genomic DNA was isolated and draft genome sequences obtained by IIumina sequencing and all data deposited on *Campylobacter jejuni/coli* PubMLST database, 2014a) (MacIntyre and co-workers, unpublished data). Gene-by gene comparison of WGS data was performed using genome comparator on PubMLST/ campylobacter. CC- clonal complex and ST-sequence typing. 137 isolates are from farm associated Norway rats, 6 are from farm animals (goose Dg333, Dg326, Dg328 and Dg327; chicken Dg311; pig Dg319). Red colour, novel clade, Rat group 1; Green bracket, classic STs of the CC-45 complex; blue colour, RG2 new ST (7259,7258 & 5130)- shares at least 4 loci with ST45CC45. Phylogenetic network taken from Mohammed,2018).

Chapter2

Material and Method

2.1 Chemicals, media, buffers, and consumables

Chemicals and media were purchased from Fisher Scientific, unless otherwise indicated and are listed in Appendix 1. Recipes for all media are also listed in Appendix 1. Media routinely used for growth were Blood Agar (BA) prepared with Blood Agar base No. 2 and 7% defibrinated horse blood or Columbia Blood Agar base with 5% horse blood. BA-2% was blood agar with additional agar to reduce motility and facilitate colony isolation and routine colony forming unit (cfu) quantitation. Liquid media were used for metabolic studies and hydrogen peroxide (H_2O_2) sensitivity assays, such as MHB, Modified Eagle Medium, (DMEMf).

Glucose, maltose, and lactose were added from a 400 mM filter, sterilised stock to a final concentration of 20 mM in the culture medium. CampyGen Atmosphere generation system gas packs for 2.5 L and 3.5 L (jars were used for the growth of *C. jejuni.* Alternately, a Don Whitley workstation M35 was used to create the required microaerophilic conditions. Routinely, 5% O_2, 10% CO_2, 2% H_2 and 83% N_2, 2%O_2 ,10%CO_2,2%H_2 ,86%N_2 ;15.9%O_2 ,4.5%CO_2 ,0%H_2**,**7 9.6%N_2. Phosphate-Buffered Saline (PBS) or Maximal Recovery Diluent (MRD) were used for serial dilution preparation. Where indicated, Bolton selective supplement, which contains Vancomycin, Cefoperazone, Trimethoprim, and Cycloheximide, was used to prevent contamination. *Campylobacter* isolates were stored at −80°C in Brain Heart Infusion (BHI) containing 15% v/v glycerol.

2.2 Bacterial strains and routine culture conditions

Table 2.1 lists the bacterial strains used in this study. Including source, date of isolation, sequence type (ST), and/or clonal complex (CC) number. All Dg strains were originally isolated from Norway rat faecal pellets. For strains indicated as a, b and c, the original Dg stocks of each strain were subculture onBA2%, and three individual colonies (a, b, and c) were picked, subcultured individually on BA, and restocked as glycerol stocks. All solid and liquid cultures were incubated at 37°C under microaerophilic conditions.

Lack of contamination was routinely checked using gram staining to confirm the spiral shape for *C. jejuni* and absence of growth on BA plates at 37°C under aerobic conditions, as *C. jejuni* cannot grow under atmospheric oxygen concentrations.

2.3 Harvesting and stocking of *C. jejuni* strains

A loopful inoculum was used as the source of the bacterium in many procedures. Routinely, a subculture from a −80°C stock was prepared on BA2% and incubated at 37°C under typical microaerobic conditions for 48h. One or more isolated colonies were subcultured on BA plates for approximately 24–36h. Depending on the application, a half to a loopful of bacterial growth was recovered from a plate and suspended in a liquid medium as required.

Stock cultures were prepared by suspending a loopful of culture in Brain Heart Infusion (BHI) containing 15% v/v glycerol and storing immediately at −80°C. All stock cultures were prepared in a Biological Safety Cabinet.

[1] Strains	[2] ID	[3] CC(ST)	[4] ED type	[5] Source-, Date/ Ref
Dg 16b (a, b ,c)	25973	CC-21	I	E -23/06/2011
Dg 18a (a, b, c)	25977	Not assigned	I	G -14/7/2011
Dg 161(a, b, c)	26016	CC- 45	I	C- 22/05/2012
Dg 43a (a, b, c)	25978	Not assigned	21/23	X- 22/8/2011
Dg 268(a, b, c)	25948	Not assigned	21/23	E - 03/07/2012
Dg 234(a, b, c)	26049	Not assigned	8	E - 26/6/2012
Dg 345(a, b, c)	26009	CC-45 (7256)	10	C - 26/4/2012
Dg 381(a, b, c)	26002	Not assigned	I	I - 3/8/2012
Dg 201(a, b, c)	25993	Not assigned	I	I - 30/5/2012
Dg 275	26058	CC- 45(7259)	2	X- 03/07/2012
Dg 200	25985	CC- 45	-	I- 30/05/2012
Dg 95	26020	CC- 45	3	G-05/12/2011
Dg 57a	25947	CC- 45	2	C-22/08/2011
Ch95-95 (1,2,3,4,5)	-	CC- 45	(3*)	Recovered isolates of Ch95 from Chicken trial (Mohammed, 2016)
NCTC- 11168	48	CC-21	-	Human stool,1977/ (Parkhill *et al*., 2000)

Table 2.1 *Campylobacter jejuni* strains.[1]Dg indicates the Reading strain stock number assigned to stocks prepared during gDNA preparation for sequence data available on PUBMLST/*C. jejuni.* All bacteria were restocked as RM-1 stocks, following one round of culture on MHA motility agar, to enhance growth in culture media (Mohammed, 2016). A, b and c are the three individual colonies selected, subcultured, and restocked for ED locus confirmation. [2]ID, Identification Number on the *Campylobacter jejuni/coli* PubMLST database. [3]CC(ST), MLST Clonal Complex number and (Sequence Type), not assigned—strains without *gltA* number and hence no ST or CC was assigned.[4]ED type, based on the allelicprofile of all seven *glc* genes; (-) no *glc* locus (ED negative) (3*), *glcK* is a pseudogene in Ch95-95 (1); I, one or more genes within the *glc* locus indicted as incomplete on PUBMLST/ *campylobacter/jejuni.* [5] The letter indicates farm site code from which Norway rat faecal pellets used for *Campylobacter* isolation were collected/source for reference strain (Primary farm animals: E, cattle; G, turkey, cattle; C, pigs, sheep; X, pigs, sheep, I, cattle, poultry).

2.4 *C. jejuni* growth assays

2.4.1 Enhancing growth on MHA motility agar

Growth on MHA motility agar and stocking of *C. jejuni* cells from the perimeter of the motility ring enhanced the growth of recent isolates on MHB (Mohammed, 2018). Therefore, all strains listed in table 2.1 with subcultures a, b and c were subject to one round of motility on MHA motility agar, and the restocked isolates from these plates were used in subsequent growth assays. To do this 5µl standardised cell (OD_{600} 0.02) (see below, 2.4.2) was delivered into the center of a 0.4% MHA plate (MH medium containing 0.4% agar) using a sterile pipette tip. Plates were incubated and 48h at 37°C. Glycerol stocks were prepared directly from a stab of agar plus bacteria taken from close to the outer ring of motility using a sterile 3 ml plastic dropper. Bacteria and agar were resuspended in BHI, 50% glycerol, to give an approximately 12–15% final volume of glycerol and stocked at –80°C. From these RM-1 stocks, subcultures were prepared on BA–2%, followed by subculture and glycerol stocking of three separate colonies on BA, as described above 2.3. The three stocks were labelled a, b, and c as in table 2.1 and used as bacterial stocks for all studies described here.

2.4.2 Preparation of standardised bacterial cell suspension

A cell suspension standardised to 0.02 or 0.2OD_{600} was routinely used for inoculation in growth studies. Strains were subcultured on blood agar (BA–2%) incubated at 37°C for 36– 42h, followed by subculture of individual colonies on MHA for 36-42h (microtitre plate assay), or BA for 24-36h (shaking culture). Using a biological safety cabinet, one loopful (5µl blue loop) of culture, or about five colonies were evenly suspended in 1 ml of fresh media and centrifuged at 8000rpm for 4min in a Mini spin, Eppendorf centrifuge at RT to pellet the cells. The supernatant was removed and cells were carefully resuspended in 1ml of media MHB or DMEM as required.

The OD_{600} was monitored using 800µl suspended cells in a 1-cm path length cuvette and a WPA CO8000 cell density meter. The remaining sample was used to standardise the samples to 0.02 or 0.2 OD_{600} in the same media. An OD_{600} of 0.02 and 0.2 from BA corresponded to an average of ~7 × 10^7 and ~7 × 10^8 cfu/ml of Dg95 at 36h of growth, respectively. A loopful of standardised cells was routinely plated on BA and incubated aerobically for 48 h at 37°C to confirm the absence of contamination. An OD_{600} of 0.2 using the WPA CO8000 was equivalent to a reading of 0.078 at OD_{600} using the Spectra MAX 340 pc spectrometer with 200 µl culture in a 96-well plate.

2.4.3 Microtitre plate growth assay

Microtitre plates were used to screen multiple strains of *C. jejuni* and, in initial studies, to monitor growth under a range of conditions at 37°C. Standardised cells, 20µl prepared from MHA plates as a standardised culture of 0.02 OD_{600}, as described above 2.4.2, were added to 180µl sterile media to give a starting concentration ~ 7 × 10^7 cfu/ml. Triple wells were inoculated per strain. See (Appendix 2 for preparation of media). Where indicated, microtitre plates were incubated without shaking at 37°C in a 3.5L anaerobic jar with a Campy gas pack. For incubation periods exceeding 48h, the gas-generating pack was replaced. In some experiments, where indicated, Bolton's broth selective antibiotic was added to ensure the absence of any contamination. When the Bolton broth supplement antibiotic was added to the growth culture, there was no significant effect on campylobacter growth.

Following incubation, the OD_{600} of the 24h and 48h samples was read directly in a Spectra MAX 340 plate reader. Due to aggregation of cells at 72–96h, these later samples were first resuspended by carefully pipetting up and down four times with a 200µl multichannel pipettor. A 100µl aliquot of suspended cells was then transferred to 100µl PBS in a new plate before reading at OD_{600} to calculate cell density using a Spectra MAX 340 plate reader.

In later studies, to screen multiple colonies of Ch95–95–isolates (1-2-3-4 and-5) and Dg95 for glucose utilisation microtitre plates were incubated in a Don Whitley M35 workstation cabinet, with shaking was done at 600rpm under standard microaerobic conditions at 37°C and with 70% humidity.

2.4.4 Growth in flasks with shaking and sampling carbohydrate utilisation

Conical flasks with cotton wool plugs were used for growth with shaking. For early experiments, testing growth on different carbohydrates, 25ml conical flasks with 10 ml DMEMf–FBS plus antibiotics were incubated in 2.5L anaerobic jars with a Campy gas pack and shaking at 150rpm in a New Brunswick orbital shaker at 37°C. DMEMf containing 20 mM glucose, 20 mM maltose, or 20 mM lactose, prepared as described in Appendix 2, was inoculated with 1 ml bacterial cells, standardised to an OD_{600} of 0.02. Later experiments were performed in a Whitley M35 Workstation using 15ml media, such as MHB, MHB-FBS, or DMEMf, in 50ml conical flasks with shaking at 150rpm on an orbital shaker **(**Heidolph Unimax 1010) at 37°C. In some experiments, where indicated, antibiotic was added to prevent contamination. Flasks were routinely inoculated with 150µl bacterial cells, standardised to an OD_{600} of 0.2, to give a zero-time OD_{600} of 0.002 and approximately 5×10^{6} cfu/ml. Growth was monitored roughly every 12 h (time points as indicated) by removing 550–800µl of culture to measuree bacterial density OD_{600} using a WPA CO8000 cell density metre and to perform cfu calculation as in 2.4.5.

2.4.5 Calculation of colony-forming units (cfu/ml)

The Miles–Misra method (Miles *et al.,* 1938) was used to estimate viable bacteria by calculating the number of colony-forming units (cfu) in standardised suspensions and growth studies. For each sample, 10-fold serial dilutions from 10^{-2} to 10^{-7} were prepared in duplicate or triplicate in a 96-well plate by adding 20µl sample to 180µl of cold PBS diluent or MRD. Plates were predried for 30min at 37C with the lid slightly opened. Five replicate 10 µl drops from dilutions 10^{-2} to 10^{-7} were then dropped onto the surface of the dried BA–2% plate using a multichannel pipettor After the drops had dried, the plates were incubated for 48 h at 37°C. A magnifying lens was used to count cfu, ideally between 3–30 cfu/drop.

2.4.6 pH measurement

Cultures were grown as described in 2.4.4 in 15 ml DMEMf with and without glucose in 50 ml conical flasks with shaking at 150rpm. The broth was inoculated with relevant *C. jejuni* strains to give a starting OD_{600} of 0.002 and incubated at 37°C for three days in standard microaerophilic conditions. The pH was measured using a HI-98100 Checker Plus pH Tester, Hanna Instruments at zero time and then at 24h, 48h, and 72h without removing cultures samples from the cabinet.

2.4.7 Gram staining

Gram staining was performed to confirm *Campylobacter* morphology using a 20µl heat-fixed culture. Cells were stained with crystal violet 0.4% w/v for 1min, grams iodine 1% w/v for 1 min, destained with ethyl alcohol 96% for 20 s, and followed by a water wash and counter stain with dilute carbol fuschin for 1min. Slides were examined using light microscopy for spiral to curved or coccoid cell morphology.

2.5 Maltose and glucose quantification

Glucose and maltose levels were monitored during growth in DMEMf and/or MHB (with or without 20 mM glucose) using a glucose oxidase based assay and for future NMR spectroscopy. Spent growth media (1ml) was recovered at different times of growth by centrifugation at 13,000rpm for 10min. Recovered supernatant fractions (1ml) were sterilised by filtration using a 0.22 µm MillexGV filter tip and stored at −20°C for subsequent analysis. A maltose and glucose assay kit was used for the rapid, sensitive, and accurate measurement of maltose and glucose levels in spent growth media using a colorimetric. In this assay, maltose was converted to glucose by glucosidase and glucose quantitated via glucose oxidase activity. Hence, the assay measured maltose plus free glucose. Free glucose alone was quantitated by measurement in the absence of glucosidase, and maltose concentration was determined by subtracting free glucose level from the quantity of maltose plus free glucose. The assay was performed in a final volume of 100µl in sterile 96-well clear F-bottom, polystyrene plates with lid. A standard curve using the kit-supplied maltose was performed following the instructions of the maltose + glucose assay kit. This demonstrated that the amount of maltose was proportional to the colours density between maltose concentrations of 1 and 5nmol, equivalent to 2 and 10nmol glucose, fig 2.1. Growth studies were performed with media containing 20 mM maltose. Before performing the assay, the glucose assay buffer was thawed and brought to room temperature (RT). All other reagents were then brought to RT. Glucose reaction mix (GRM) was prepared freshly before each assay: 50µl of GRM containing 2µl glucose probe, 2µl glucose oxidase, and 46µl glucose assay buffer.

Reactions contained sample (approximately 1–10nmol glucose), plus/minus 2µl glucosidase, glucose assay buffer to a final volume of 50µl, followed by the addition of 50µl GRM. Samples were mixed and plates incubated for 60min at 37°C, protected from light.

Absorbance was read at 570 nm, without lid using a Spectra MAX 190 Microplate Reader at endpoint.

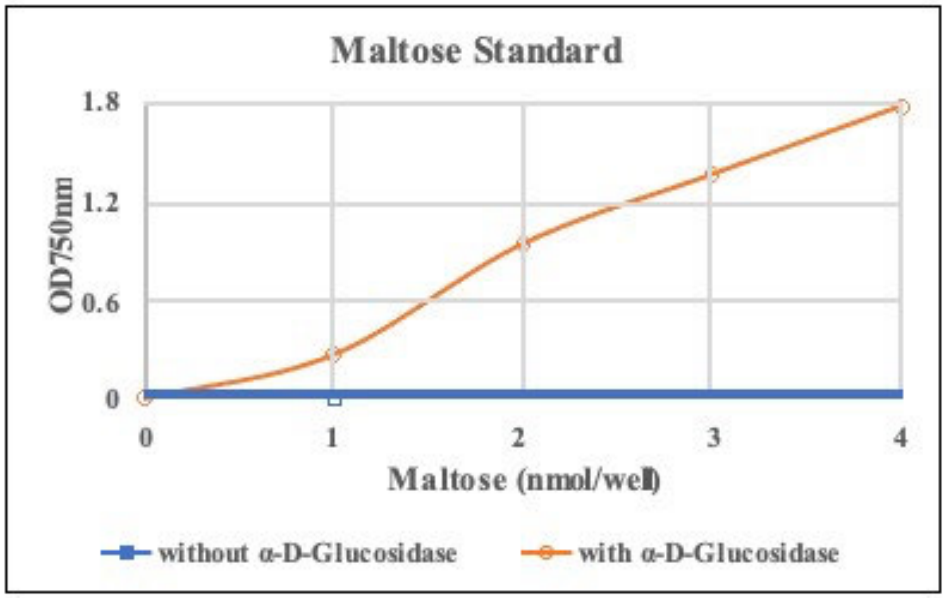


Figure 2.1 Standard curve for monitoring maltose in biological culture media using ab65335 Maltose and Glucose Assay Kit. The 0, 1, 2, 3, and 4µl of the maltose standard (10nmol/well) were added into each well individually. The wells were adjusted to 50µl by adding the glucose assay buffer to generate 0, 2, 4, 6, 8, and 10nmol/well of the maltose standard. Also, 50µl of a glucose reaction mix was added into each well plus 2µl glucosidase enzyme to evaluate maltose. The open orange circle is maltose with α-D-Glucosidase. The solid square is maltose without α-D-Glucosidase.

2.6 Fluorescence microscopy for cell shape and viability

The Bacterial viability assay kit was used to monitor coccoid formation and cell viability. First, bacterial cells were recovered from stationary or death phase culture media and extensively washed as follows: From a 5ml culture, bacterial cells were harvested by centrifugation at 5000 rpm for 20min and resuspended in 2ml of 1x wash buffer. Then 1ml of this bacterial suspension was diluted to 5ml with 1x wash buffer. After 1h incubation at RT, centrifugation was repeated, and the pellet was resuspended in 5ml of 1x wash buffer and centrifuged again. The final pellet was resuspended in 1ml of 1x wash buffer. To prepare samples for Fluorescence Microscopy, 1µl of live cell stain and 1µl of dead cell stain was added to the 1ml sample of washed cells and incubated at RT in the dark for 15min. Two to three drops of sample was then spotted on a microscope slide covered with a coverslip. Zeiss–zen software was used for sample viewing and photography by selecting the dye for samples from the databases. Using excitation/ emission wavelengths of 490nm/525nm and 536nm/617nm (FITC/TAMRA filter) with oil lens, as set on a fluorescence microscope. Images of the selected areas recorded viable (green fluorescence) and dead (red fluorescence) cells.

2.7 Growth Assay with variable oxygen levels

Experiments comparing growth with different oxygen levels used the Whitley M35work station at 37°C and 15ml MHB-FBS in 50ml conical flasks with shaking at 150rpm (section 2.4). Cultures were inoculated to give a starting OD_{600} of 0.002 (approximately 3-5 $\times 10^6$ cfu/ml, as described above in section 2.4.4 and incubated under each of the following atmospheric conditions: (a) standard conditions 5% O_2,10% CO_2, 2% H_2, 83% N_2; (b) low oxygen 2%O_2, 10%CO_2, 2%H_2, 86%N_2, (c) high oxygen 13%O_2,5%CO_2,1.8H_2, 80.2%N_2 and very high oxygen 15.9%O_2, 4%CO_2,0%H_2, 80.1% N_2.

Bacterial density OD_{600} and cell viability cfu/ml were monitored at intervals of up to 72h, as described in sections 2.4.4 and 2.4.5. All samples were removed from the flask in the cabinet. To monitor the impact of aerobic incubation, i.e. in 21% oxygen on cell viability, 15ml cultures were grown with shaking as described above for 24h at 37°C. Then at 24h, cultures were removed from the workstation and transferred to an aerobic incubator for further incubation aerobically with or without shaking. Both OD_{600} and cell viability were monitored.

2.8 Hydrogen peroxide sensitivity

Cultures(15ml) were inoculated and grown at 37°C understandard microaerophilic conditions, as described above. Samples were taken at times during growth, as indicated, and both OD_{600} and cfu were determined. At each time point, bacteria were recovered from an equivalent of 0.9 OD_{600} units by centrifugation at 5000rpm for 10min. After discarding all supernatants, the pellet was resuspended in 4.5ml of PBS. To monitor sensitivity, a 30% solution of H_2O_2 (9.8 M) was used to prepare a working 1M H_2O_2 stock (102µl of H_2O_2 plus 898µl of water). In 1.5ml Eppendorf tubes, 1µl, 5µl, 10µl, and 15µl of 1M H_2O_2 were then added to 900µl of cell suspension in PBS, plus 99µl, 95 µl, 90µl, and 85µl nanopure water, to give test concentrations of 1mM, 5mM, 10mM, and 15mM peroxide, respectively, in a final volume of 1ml. The 0mM peroxide control contained 900µl of cells plus 100µl water. All tubes were incubated at 37°C under microaerobic conditions with gentle shaking at 150rpm on a Grantbio PMS-1000i Microplate Shaker. Samples (20µl) were taken after 15min, 30 min, and 1h and added to 180µl cold PBS buffer without removing tubes from the cabinet.

Serial dilutions were prepared for cfu calculations, section 2.4.5, 10µl of the 10^{-1} to 10^{-6} dilutions were then spotted onto BA2%plates, incubated for 48h, and colonies counted. For these assays, each day, a solution of 1M H_2O_2 was freshly prepared from the stock of 30% (w/v)

H_2O_2 which had been stored at 4°C and was no more than 1year old. Triplicate biological repeats for growth curves and assays were performed on different days, with the average and SD cfu/ml calculated.

2.9 Preparation of genomic DNA (gDNA)

2.9.1 GeneJET Genomic DNA Purification kit

GeneJET genomic purification kit was used routinely for isolation of genomic DNA (gDNA) for PCR analysis and NGS/Illumina sequencing of some samples.

Single colonies were subcultured on BA for 24-30h, and half of a 5µl loopful of bacterial growth was suspended by mixing with the loop in 1ml MHB. Washed cells were recovered by centrifugation for 5min at 5000rpm and resuspended in 180µl of digestion solution, thoroughly mixed with a loop. To digest and degraded all proteins particularly nuclease that breakdown isolated DNA, 20µl ProteinaseK was added, then the sample was heated for 60min at 65°C in a heat block with vortexing every 15min. A 20µl RNAase A, was then added and incubated 10 min at RT to degrade RNA, followed by 200µl lysis solution and vortexed for one minute. Then 400µl of 50% ethanol was added, and the sample was mixed and transferred to a gDNA purification column. This was centrifuged for 3min at 13,000rpm to bind gDNA, washed with 500µl of wash buffer I, centrifuged for 3min at 8000Xg 11000rpm, then washed with 500µl of wash buffer II, followed by further centrifugation for 3min at 13000rpm. In the final step, the purified DNA was eluted in 50µl of nH_2O and centrifuged at 12000rpm for 3min. Samples were stored at −20°C.

2.9.2 Monarch genomic DNA purification

The monarch genomic purification method resulted in longer gDNA fragments and was used for nanopore sequencing and Illumina sequencing with Novogene. Single colonies were subcultured on BA for 24h under standard conditions (5% O_2,10% CO_2,2% H_2, 83%N_2). Half of a 5µl loopful of growth was used to isolate gDNA using a Monarch genomic DNA purification kit Cells from growth on the BA plate were suspended in 1 ml-MHB and mixed well. Cells were recovered by centrifugation for 3min at 13000rpm, resuspended well in 90µl of 10mM Tris-Cl with gentle mixing. Also, 10µl lysozyme solution (25mg/ml) was added together with 100µl tissue lysis buffer and mixed. The sample was heated at 37°C for 10–20min or until clear (not exceeding 20 min). ProteinaseK (10µl) was added, and the sample was incubated at 65°C for 30–45 min in the heating block with shaking by hand or vortex briefly. After adding 3µl RNAase A, the sample was incubated for 5min at 56°C. Following a brief vortex 400µl gDNA binding buffer was added to the sample and mixed thoroughly by vortexing for 5-10s or shaking by hand. The lysate/binding buffer mix was then transferred to a gDNA purification column, which was then centrifuged for 3min at 1,000rpm to bind gDNA, followed by centrifugation for 1min at 12000rpm. The column was then transferred to a new collection tube, and the genomic DNA washed twice, with 500µl gDNA wash buffer, by closing the cap and inverting a few times so that the wash buffer reaches the cap, followed immediate centrifugation for 1min at 12,000rpm and discard the flow through. The gDNA Purification Column was then placed in 1.5ml microfuge tube and 50µl preheated (60°C) water added. With, cap closed, this was incubated at room temperature for 1min. The column was then centrifuged for 1min at 12,000rpm to elute DNA. Genomic DNA samples were stored at −20°C.

To determine the DNA concentration, nanodrop and agarose gel electrophoresis (AGE) with gel red staining was used. While extraction of gDNA for Illumina sequencing does not require long fragments of DNA and vortexing of samples can be used during preparation preparation of gDNA for nanopore sequencing analysis requires a gentle mixing step to avoid shearing DNA and facilitate long reads from a single molecule.

2.9.3 DNA quantification and quality

2.9.3.1 Nanodrop spectrophotometer DNA quantification

To routinely measure the DNA concentration of isolated genomic DNA for PCR, PCR products, and plasmid, 2µl of a DNA sample was measured for quantification (A260) and purity (A260:A280) using a Nanodrop spectrophotometer (ND1000, Thermo Scientificor DNA Quantification Spectrophotometer from DeNovix. Water was used as a blank.

2.9.3.2 Fluorescence quantification assay

The DeNovix® dsDNA Broad Range Assay (Cambridge bioscience, DSDNA-BROAD-1) was used to quantitate and reveal the purity of the double-stranded DNA (dsDNA) samples. The standard detection range was from 2 to 2000ng in 200µl final volumes. All solutions were equilibrated to room temperature before use reagents were vortexed and then centrifuged briefly to reduce loss on the vial cap. Working solutions were prepared by mixing 10mL of the assay buffer with 100µl of the dye and 100µl of the enhancer.

Both standards (0 or 200 ng/µl) were prepared in different two tubes, 190µl working solutions plus 1µl of the both standards+ 9µl of water), also, DNA sample was prepared by 190µl working solution plus 1µl of DNA + 9µl of water. All tubes mixed well and incubate standards and samples at room temperature for 5mins in a dark place. The final step generates the standard curve and then measures the samples using a DS-11 DNA Quantification Spectrophotometer from DeNovix.

2.9.3.3 Testing gDNA quality

DNA quality was estimated using the two ratios 260/280 and 260/230. 260/280 ratios are routinely used to determine DNA purity, ideally with a ratio of 1.85-1.87 and with a range of 1.80-1.90 considered acceptable. Values less than 1.80 indicate potential proteincontamination, and values in the range of 1.90 to > 2.0 may indicate potential RNA contamination (Novogene instruction). The 260/230 values were used as secondary measure of nucleic acid purity. Very high purity is considered when A260/A230 ratios are in the range of 2.20-2.50, while a range between 1.80-2.50 is generally considered acceptable and clean. Contamination of the sample by organic compounds such as Trizol, phenol, or by salts such as the chaotropic salt guanidine thiocyanate (GTC), any form of aromatic molecules, and substances like silica fibers all induce a lower A260/A230 ratio. To check the purity of DNA preparations by gel electrophoresis 1.0% agarose gels with 1.0% TAE (0.5g of agarose+50ml of the 0.5ml of 50xTAE buffer) were used and run at 100V for 40min. The absence of gDNA degradation, protein contamination (indicated by DNA in wells), and RNA contamination were all monitored by gel electrophoresis, see fig 2.2.

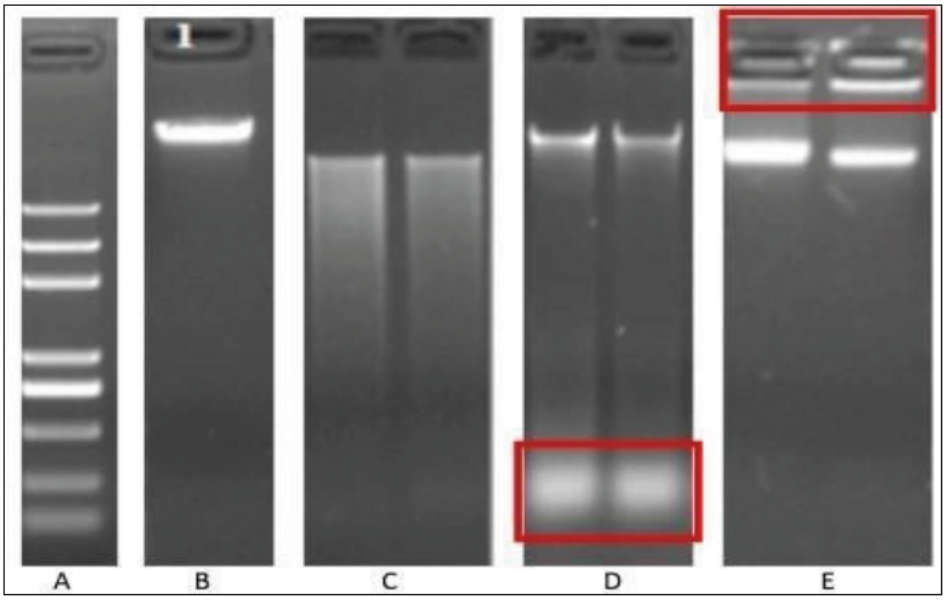


Figure 2.2 Gel electrophoreses showing examples of gDNA quality. (A) DNA marker, (B) qualified sample, (C) degraded sample, (D) sample contaminated with RNA, (E) sample contaminated with a protein and /or DNA aggregates. Red boxes refer to areas of contamination (taken from Novogene).

2.9.3.4 Agarose gel electrophoresis

A horizontal agarose gel electrophoresis (75000500) system (Bio-Rad) was used to analyse DNA samples. Gels typically contained 0.6 to 0.7% (w/v) agarose in TAE buffer (40mM TrisHCl, 1mM EDTA, pH 8.0), prepared from 50xTAE (242gTris base, 57.1ml Glacial acetic acid, 100ml 5M EDTA pH 8.0 per liter). The nucleic acid stain, Gel Red, or safe DNA gel stain (1µl) was mixed with 50ml of melted agarose cooled to 45–50°C. For PCR products, routinely 2µl DNA loading buffer (x2) was added to a 2µl DNA sample and analysed using 1 µl DNA Hyper Ladder 1 with 2µl DNA loading buffer (fig2.3) as a length standard. Gels were run at 80V for 70 min and imaged using a G-Box (SynGene).

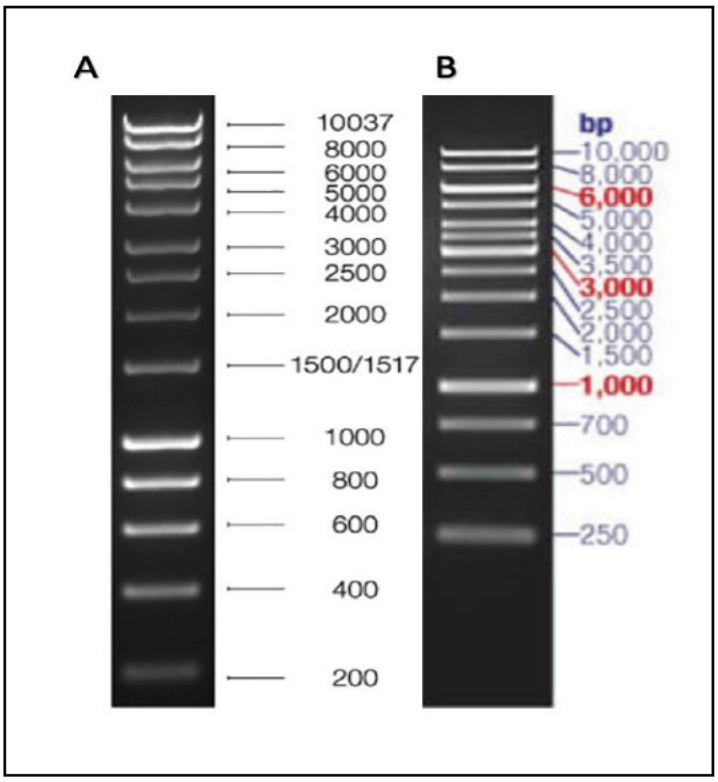


Figure 2.3 DNA standards for AGE. A–Hyper DNA ladder, B DNA ladder 10KB [Fermentas, Lithuania). The molecular size (bp) and the respective molecular mass (ng) of each band are shown in the above figure (taken from Bioline).

2.10 Oligonucleotide primers

Oligonucleotide primers listed in table 2.2 were used for PCR or sequencing were synthesised by MWG Eurofins, Germany, and stored as stock primers in nuclease-free H_2O at a concentration of 200pmol/µl, diluted to a working concentration of 10pmol/µl, and stored at -20°C. Primer sequences for the target genes were designed using DNA Dynamo and Artemis software. Also, TM Calculator of Oligonucleotide Properties Calculator and MWG were used to design the primers. Based on MWG criteria, typical properties for optimal primer design were 18–22 bp, TM (54-62), GC% (40-60), TM as close as possible and no more than 3°C apart.

A. Standard PCR primers (short length)				
Primer	**Sequence 5'-3'**	**TM (°C)**	**Size(bp)**	**amplified genes**
hipO F	GAAGAGGGTTTGGGTGGTG	53.2	19	*hipO*
hipO R	AGCTAGCTTCGCATAATAACTTG	51.7	23	
aspK	AGCTTGCGGTAAGAGCCTTGC	60	21	*aspK*
aspK R	ATAAAAGACTATCGTCGCGTG	60	21	
glk 18F	TACGGGCATGTATCAGCAGAG	58	21	*pgi*
glcP18R	ATAGCAACCTTTCCGCCAAC	57	20	
glk 381F	CTTATTAGCGAGCAAGCTTTAAGTGG	60	26	*pgi*
glcP 381R	CCTTGAACTGGAGGTATTAGAGC	61	23	
ansA 381F	CAAATGATGGTAGTGGTGTCG	60.1	21	*edd, eda*
zwf 381R	CCCCAACTACCTGCAAGATA	59.9	21	
glcP 201R	CCTTGAACTGGAGGTATTAGAGC	60.6	23	*pgi*
glk 201 F	TACGGGCATGTATCAGCA GAG	57.6	21	
ansA 201F	AGTTGTTGCAGGTAGTGGTGCAG	55	23	*edd, eda*
zwf 201 R	GGATCAATCCAAGTCCAA GCTGC	55.8	23	
pglF	AAGCTGAAGAGTTCGAGTGG	57.5	20	*glk*
pgiR	AACGCCCACCTACCCAATTC	60	20	
eddF	ACACCCGCTATGTGTGATGG	58	20	*edd, eda*
edaR	TAGCAAACGAAGTTCCGCAAG	57	21	

B. Primers for amplification of long products spanning entire *rrn* loci, plus *glc* locus if present. Distance between F and R to *glc* loci					
***rrn/glc* locus**	**Target Primers**	**Sequence 5′–3′**	**TM°C**	**Size (bp)**	**Distance between F and R to *glc* loci**
Dg18 *rrnA*	Dg18F	GTTCAAGGGTTGTGGCAGGGCGT	60	23	264
	Dg18R	GAAAACGAACGGTTTTTGCATGACGTG	58.2	27	824
Dg18 *rrnB*	Dg43F	AGGTAAATCAAGAGATAGCCGCCCAAG	59.5	27	512
	Dg43R	CATCAATATCCATAAAATCAAATAGATCAA ACACT	58.4	26	1469
Dg18 *rrnC*	Dg43F	TTGGGCATAGAAAGAACTTATAAAGG	58.6	26	554
	Dg43R	TGCGTATGAGTGGGGAGG TTCTTGTAG	61.3	27	952
Dg43 *rrnA*	Dg43F *ansA*	AGCAGGTTGTGTTGCTGTGAGTGATTC	57.9	27	211
	Dg43R	GAAAACGAACGGTTTTTGCATGACGTG	58.2	27	770
Dg43 *rrnB*	Dg43F *murD*	TGAAAGCGCGGTAATGGCTGCACTTAA	59.7	27	350
	Dg43R	AGGTAAATCAAGAGATAGCCGCCCAAG	59.5	27	469
Dg43 *rrnC*	Dg43F	TTGGGCATAGAAAGAACTTATAAAGG	58.6	26	535
	Dg43R *tonB3*	TGCGTATGAGTGGGGAGG TTCTTGTAG	61.3	27	461
Dg268 *rrnA*	Dg18F *ansA*	GTTCAAGGGTTGTGGCAGGGCGT	60	23	226
	Dg43R	GAAAACGAACGGTTTTTGCATGACGTG	58.2	27	754
Dg268 *rrnB*	Dg43F *murD*	TGAAAGCGCGGTAATGGCTGCACTTAA	59.7	27	325
	Dg18R	AGGTAAATCAAGAGATAGCCGCCCAAG	59.5	27	509
Dg268 *rrnC*	Dg43 F	TTGGGCATAGAAAGAACTTATAAAGG	58.6	26	584
	Dg43R *tonB3*	TGCGTATGAGTGGGGAGG TTCTTGTAG	61.3	27	573
Dg201 *rrnA*	Dg18F *ansA*	GTTCAAGGGTTGTGGCAGGGCGT	60	23	246
	Dg43R	GAAAACGAACGGTTTTTGCATGACGTG	58	27	840
Dg201 *rrnB*	Dg43F	AGGTAAATCAAGAGATAGCCGCCCAAG	59.5	27	975
	Dg234R	CAAATAGATCAAACACTCTATTTTTACCCAT AGAA	58.1	35	1399
Dg201 *rrnC*	Dg43F	TTGGGCATAGAAAGAACTTATAAAGG	58.6	26	575
	Dg43R *tonB3*	TGCGTATGAGTGGGGAGG TTCTTGTAG	61.3	27	567
Dg381 *rrnA*	Dg18F *ansA*	GTTCAAGGGTTGTGGCAGGGCGT	60	23	262
	Dg43R	GAAAACGAACGGTTTTTGCATGACGTG	58	27	824
Dg381 *rrnB*	Dg43F	AGGTAAATCAAGAGATAGCCGCCCAAG	59.5	27	988
	Dg234R	CAAATAGATCAAACACTCTATTTTTACCCAT AGAA	58.1	35	1021
Dg381 *rrnC*	Dg43F	TTGGGCATAGAAAGAACTTATAAAGG	61.3	26	517
	Dg43R *tonB3*	TGCGTATGAGTGGGGAGG TTCTTGTAG	58.6	27	625
Dg234 *rrnA*	Dg18F *ansA*	GTTCAAGGGTTGTGGCAGGGCGT	60	23	228
	Dg43R	GAAAACGAACGGTTTTTGCATGACGTG	58.2	27	862
Dg234 *rrnB*	Dg43F*murD*	AGGTAAATCAAGAGATAGCCGCCCAAG	59.5	27	482
	Dg234R	CAAATAGATCAAACACTCTATTTTTACCCAT AGAA	58.1	35	1065
Dg234 *rrnC*	Dg234F	CCTGAGACTTTAGGTTTATCCTTAGAAGAA	58.3	30	315
	Dg43R *tonB3*	TGCGTATGAGTGGGGAGG TTCTTGTAG	58.6	27	308
Dg345 *rrnA*	Dg345F *ansA*	CGTGTTGCTGTGAGTGATTC	58.6	20	300
	Dg345R *cj0031*	CGATGATGACTTGGGTAAGGCC	62.5	22	760
Dg345 *rrnB*	Dg 345R *cj0431*	TTGATGAAATGGGCAGAGTG	57.6	20	366
	Dg 345F *murD*	GCGGTAATGGCTGCACTTAATC	61.5	22	323
Dg345 *rrnC*	Dg345F *cj0742*	ATGGAGAACATCGAGCAAGAG	59.3	21	120
	Dg 345R *hrcA*	CTGAGTGATCAAACCCTCATCGC	62.7	23	563
Dg16 *rrnA*	Dg16F *recJ*	CACTTAAAAGGCAGCGC	52.8	17	1568
	Dg16R *cj0034c*	GATCAACTACAACTCGCC	53.7	18	446
Dg16 *rrnB*	Dg 16 F *murD*	AGTTCAACTCTTACGCAGAGC	56	21	473
	Dg 16 R *cj0431*	TTGATGAAATGGGCAGAGTG	54	20	124

Table 2. 2 Primers used for PCR. A. Primers amplifying short products used in standard PCR confirmation of a*spK* (*C. coli* specific), *hipO* (*C. jejuni* specific) and analysis of target segments of the *glc* locus. B. Primers used to amplify long PCR products > 6 kbp for amplification of entire *rrn/glc* loci. *Distance from for word primers to 16SrRNA and from Reverse primers to 23SrRNA.

2.11 PCR amplification

2.11.1 Colony PCR

For lysed colony PCR, bacteria were cultured on BA–2% plates at 37°C for 30-36h. One or two (if pure culture) colonies were resuspended in 100µl of Tris-EDTA buffer (TE) (10mM Tris and 1mM disodium EDTA, pH 8.0) and heated for 5min at 95–100°C in a heating block. Unlysed cells and debris were removed by centrifugation for 3min at 13,000 rpm, and the supernatant was stored at -20°C. Dilutions of 1/50 in nH_2O were used as templates in the PCR reactions. Dream Taq polymerase was used to amplify short PCR products to confirm presence/absence or product length < 5-6 kb. PCR reactions were performed using 0.75µl of forward and reverse primers (10pmol/µl), 2.5µl of Template DNA (lysed cells, 1/50), 12.5µl of Dream Taq PCR Master Mix (2x) and a final volume made up to 25µl with nH_2O. Reaction cycles are shown in table 2.3A with annealing temperatures between 55-65°C. Examples included identification of *C. jejuni* and *C. coli*, respectively, using forward and reverse hipO and aspK primers, and confirmation of *glc* locus using primers targeting different *glc* locus genes table 2.2.

2.11.2 Long range RCR

To amplify *rrn* loci with and without *glc* loci, PCR products > 6 kb, PCR reactions were performed using CloneAmp HiFi and isolated gDNA, following the manufacturer protocol. Briefly, the reaction mixture contained 12.5µl of CloneAmp HiFi, 1µl each of forward and reverse primers (10pmol/ µl), 50ng-100ng of template DNA, and a final volume made up with water to 25µl. The amplification cycle is shown in table 2.3B.

A- Steps		**Temperature (°C)**	**Time**
1 cycle	Initiation	95.0°C	2 min
30 PCR Cycles	Denaturation	95.0°C	30 s
	Annealing	60°C	30 s
	Extend	72°C	1 min/kb
1 cycle	Final extension	72°C	10 min
		4°C	Hold
B- Steps		**Temperature (°C)**	**Time**
1 cycle	Initiation	98.0°C	2 min
30 PCR Cycles	Denaturation	98.0°C	10 s
	Annealing	55°C	15 s
	Extend	72°C	5min/kb
		4°C	Hold

Table 2.3 A& B Reactions for PCR. A-The reaction used in primer walking of the small region with Dream Taq PCR Master MixB. The reaction used in primer walking of big gaps, with CloneAmp HiFi.

2.11.3 Purification of PCR products

A GeneJET PCR Purification Kit was used to purify PCR products before DNA sequencing. Binding buffer (1volume) was added to 1volume of the PCR product mix. Samples were added to a GeneJET PCR column and centrifuged for 30–60s at 13,000rpm to bind DNA. DNA bound on the column was washed with 700µl wash buffer, and the centrifugation step repeated. An additional 1min of centrifugation removed any residual wash buffer. Finally, 20µl sterile nH_2O was used to elute DNA into a new sterile Eppendorf tube with centrifugation at 13000rpm for 1min. The DNA concentration was estimated by Nanodrop and quality confirmed by AGE.

2.12 DNA sequencing

2.12.1 PCR products

PCR products were sent to MWG Eurofins (Germany) for the Sanger sequencing tube service. A total of 15µl PCR product (between 100–3000bp) at a concentration of 10ng/µl was sent with 15µl of a 10pmol/µl sample of appropriate sequencing primer(s). PCR products were generally sequenced using the same primers used for PCR amplification.

2.12.2Whole -genome sequencing

2.12.2.1 IIumina whole- genome sequencing

gDNA extracted using Monarch genomic DNA purification 2.9.2 was quality checked as above 2.9.3. All samples met the conditions in terms of purity, where A260/A280 was 1.8– 1.92 and A260/A230 ratios 1.99–2.0. DNA concentration was estimated using the fluorescence assay 2.9.3.2. The integrity of the genomic DNA was assessed by gel electrophoresis 2.9.3.4. gDNA (30µl at a concentration of 20ng/µl) was sent to NOVOGENE-HK (Hong Kong), in 1.5ml Eppendorf Safe-lock with Parafilm to seal each tube. Samples were kept in cool pack during shipment.

2.12.2.2 Nanopore sequencing

The same gDNA sent for Illumina genome sequencing was used for nanopore sequencing using Oxford nanopore technologies. The nanopore sequencing was performed by Dr. Soon Gweon (Harbone building, lab 118, School of Biological Sciences, University of Reading).

2.13 Bioinformatics analysis

2.13.1 PubMLST

The PubMLST.org website contains files of open-access curated databases that integrate population sequence data with provenance and phenotype information for over 100 different microbial species and genera. High molecular diversity of *C. jejuni* makes it difficult to compare and identify the source of infection and transmission routes. MLST is considered one of the genotyping methods that started in 1998. It helps in the molecular differentiation of *Campylobacter* isolates and is the most widely applied due to its high discriminatory power and reproducibility (Colles and Maiden, 2012). The *Campylobacter jejuni/coli* PubMLST database (http://pubmlst.org/campylobacter/) contains sequence information for each of the MLST alleles of all *C. jejuni* and *C. coli* recorded in the database, plus epidemiological data. Since approximately 2012, WGS data is now routinely recorded. Besides, ED alleles, ST and CC types. WGS data can be retrieved and analysed on a gene-by-gene basis via PUBMLST. The draft genome sequences of all Dg strains analysed in this study are stored in the BIGSdb genomics database and are accessible via PUBMLST/campylobacter. They are defined as from an 'environmental' source. Data on these isolates accessed using the ID number, as listed in table 2.1. Genome comparator was used to compare alleles of the *glc* locus and to identify incomplete or missing genes. Individual genes were downloaded to compare the sizes of the complete and incomplete genes. Downloading genes of the *glc* locus with surrounding bases was used to further compare the integrity of the *glc* locus and related contig sizes.

2.13.2 DNA dynamo

DNA dynamo is DNA sequencing and Analysis programme provides several services that helps to Import or retrieve annotated sequence files from a variety of formats and online databases. It was used to analyse and assemble sequencing data, analyse mutations, design, primers,

design cloning and create plasmid maps. It was also used for DNA and protein multiple sequence alignments via Clustal(http://www.bluetractorsoftware.co.uk).

2.13.3 Identifying the genomic location of *rrn* loci using CONTIGuator and Artemis

The Artemis Comparison Tool (ACT) is a free genome browser and annotation tool that aids interactive visualization of annotated genomes and comparisons between genome sequences (Cole et al., 2001). In this study, all draft genome contigs of RG2 strains were downloaded from the BIGSdb database as an archive file containing FASTA files of each contig. The NCTC11168 reference genome sequence was downloaded from the NCBI database (https://www.ncbi.nlm.nih.gov/genome/149). CONTIGuator web server (http: combo.dbe.unifi.it/contiguator) was used to align the draft genome of each test strain with the FASTA file NCTC11168 reference genome. FASTA files of contigs assembled with the NCTC11168 sequence were extracted from the CONTIGuator program and aligned with the gb file of NCTC11168 reference strain using ACT (Carver *et al*., 2008). This alignment displayed mapped contigs and identified gaps. Gaps in test genomes corresponding to location of the 3 *rrn* loci (A, B, and C) of NCTC11168 were identified. Primers were designed from the end of both contigs and, where possible, within an ORF, typically with a TM of 55– 63°C and less than 3°C between pairs of forward and reverse primers, using DNA Dynamo. CloneAmp HiFi was used to amplify the DNA within the gaps, as described above.

2.13.4 Data analysis

Simple calculations and graph development were carried Microsoft Excel. Statistical comparisons were performed by SPSS software for statistical analysis with two to three biological replicates and to calculate the standard deviation, the standard errors of means, and check significance differences.

Chapter 3

Revisiting incomplete *glc* loci

3.1 Introduction

As defined by the core genome, *C. jejuni* has an asaccharolytic metabolism, meaning that it cannot use any kind of carbohydrate, such as glucose, and relies on certain amino acids, short chain fatty acids and citric acid cycle intermediates, as its main carbon and energy sources (Parkhill *et al*., 2000; Velayudhan and Kelly, 2002; Cody *et al*., 2017). This has been confirmed by many *in vitro* and *in vivo* studies (Velayudhan and Kelly, 2002; Stahl *et al*., 2012; Hofreuter, 2014 and Hofreuter *et al*., 2012). However, in 2008, a locus encoding enzymes of the ED pathway was identified in the genome sequence of *C. jejuni,* subsp. *doylei* 269.97 (Miller, 2008). Sequence analysis identified seven genes on this 8669bp region of DNA, between the 16S and 23S *rRNA* genes of the *rrn* locus (Miller, 2008). Encoded products were predicted to confer the ability to transport and phosphorylate glucose and then metabolise glucose via the ED pathway (see chapter 1.7.2.2 for details). Following WGS of large numbers of *C. jejuni* and *C. coli* isolates, presence of the same locus, named *glc* locus, was identified in the genomes of many additional strains, primarily from wild birds and Norway rats (Vorwerk *et al*., 2015; Vegge *et al*., 2016). Vorwerk *et al* demonstrated functionality of this *Campylobacter glc* locus and catabolism of glucose via the ED pathway in a clinical isolate of C. *coli,* strain CHW470. Interestingly, possession of the *glc* locus has also been linked to biosynthesis of amino acid and surface carbohydrates, with evidence of strain preference for catabolism (energy production) or biosynthesis and biofilm formation (Vorwerk *et al*., 2015; Vegge *et al*., 2016). Subsequently, Mohammed demonstrated glucose utilisation in *C. jejuni* for a group of farmyard isolates, with focus on strains Dg275 and Dg95 (Mohammed, 2018). Available *Campylobacter* strains of the *C. jejuni* group (350 strains) had been isolated from Norway rats associated with eight farms (C, E, H, G, F, I, J and K) in South East England over a period of three years (Mohammed, 2018).

Draft WGS data were available for 137 of these strains. Of these, 23 *C. jejuni* isolates closely linked to Clonal complex ST45CC-45, possess the *glc* locus and have been shown to utilise glucose, thus form closely related phylogenetic clades of ED-positive CC-45 strains (see Phylogenetic tree below, fig3.1 strains coloured blue and green) (Mohammed, 2018).

A number of other ED positive strains are more distantly related (strains coloured black in fig 3.1) have not been assigned a CC group. The whole group of ED positive strains ie strains possessing the *glc* locus, are assigned as group RG2 (encompassed by dashed circle, fig3.1) (Mohammed, 2018). In addition, a couple of other strains annotated as having genes of the *glc* locus belonged to ST21CC21 (strain Dg16) and ST45CC45 (strain Dg161). Both of these clonal complexes are considered as generalist and associated with clinical disease (Vegge *et al*., 2016; Kwan *et al*., 2008).

The *glc* locus is located on a genomic island and intraspecies transfer between *C.coli* strains by natural transformation has been demonstrated at low frequency (Vorwerk *et al*., 2015).Transfer to or within *C. jejuni* has never been demonstrated *in vitro*. In light of the limited correlation of association of the *glc* locus in *C. jejuni* with clinically isolated with strains (Vegge *et al*., 2016), and annotation of incomplete loci within draft genome sequences, the possibility of gene transfer and gene decay was considered. Hence, this part of the study began by confirming functionality of the *glc* locus in these diverse farmyard *C. jejuni* strains and revisiting sequence data and genome organisation of the *glc* locus in these strains.

3.2 Comparison of the *glc* locus in *C. jejuni*

3.2.1 Allele assignment of *glc* loci in farmyard *C. jejuni* isolates

Genes of the *glc* locus are defined as ED genes in PUBMLST/campylobacter. As with MLST for EDMLST, each gene sequence not identical to one in the database is defined as a new allele and given a new allele number. The ED type is then defined by the combination of each set of all 7 allele numbers for example ED type 2 has the profile 3-4-4-5-4-4-5 corresponding to the genes *glcP, pgi, glk, pgl, zwf, edd* and *eda.* Currently, 36 ED types have been identified in *C. jejuni* and 10 in *C. coli* (Vegge *et al*., 2016). Genome comparator was used to identify the ED type, the comparison of different sequence types (alleles) for each gene of the *glc* locus and to identify any missing and incomplete genes. Eighteen of the *C. jejuni* farm-associated rat isolates belong to ED type 2 with the allelic profile 3-4-4-5-4-5-5 (Table 3.1). Dg 95 represents a closely related group of *glc* loci from five strains belonging to ED type 3, varying only in the *pgl* locus. Four additional strains (Dg 180 and Dg 184; Dg 122 and Dg 187) belonging to ST-4791CC45 were assigned as ED types 9 and 11, respectively, and differed from each other only in the *pgl* locus. Dg 180 and Dg 184 (ED type 9) differed from ED Type 2 only in the *pgi* locus, while ED type 11, differed from ED type 2 in both *pgi* and *pgl* locus. All of these strains were classified as belonging to the broad clonal complex ST45CC-45 and each different ED type belonged to a specific phylogenetic branch when analysed by wgMLST (fig 3.1). This relationship held when analysed by cgMLST (not shown). For example, all strains with ED type 2 *glc* locus belonged to the 2 wgMLST clades indicated by Dg275 and Dg81, while ED type 3 correlated with the phylogenetic branch assigned to Dg95 and the four other ED type 3 strains.

Strains carrying ED types 9 and 11, also belonged to this broad phylogenetic clade, but to an earlier divergent branch. Notably, all strains (within the RG2 group) possess the *glc* locus. The closest strains isolated that were negative for the *glc* locus, were those assigned ST45-CC45, ED negative (fig3.1). Strains harboring ED type 2 *glc* locus were isolated over two years from four farm sites C, E, L and X, while those harbouring ED type 3 were isolated from sites G and H (Table 2.1). The ED-negative ST45CC-45 strains were isolated from some of the same farm sites (G, C, I, X and J). Thus, the close correlation of core genome phylogenetic group with ED type for these strains is consistent with acquisition of this *glc* locus by a shared ancestor of these strains and vertical transmission with gradual mutation in a specific niche, rather than extensive HGT between phylogenetically distinct strains. The *glc* locus, in ED types 10 and 22, which includes strains Dg 345, Dg 294 and Dg22, is interesting. Half of the locus, *pgl*-e*da* ie the segment encoding enzymes of the ED pathway is identical to ED type 2, while the other half of the locus *glcP-glk* is assigned completely different alleles (Table 3.1). This could reflect HGT and recombination between two different *glc* loci and deserves further investigation. The final group of 7 strains carrying the ED locus (ED types 8, 21, 23) possess a different pattern of alleles over the entire *glc* locus when compared to ED types of CC 45 strains (coloured black in table 3.1 and fig 3.1). They all shared the same allele for *glcP* (allele 4) and for *zwf* (allele 7), indicating a much closer relationship of these ED types to each other than to those of ED type 2. These strains are also phylogenetically distinct based on wgMLST and may represent a different origin of *glc* locus. These strains had been isolated from Farms G, E, I and X over two years (Table2.1). Within this final more distantly related group, Dg 43, Dg 268 and Dg233 were all annotated with an intact *glc* locus.

However, based on the WGS data available via PUBMLST, Dg18, Dg201 and Dg381 all had a truncated CAMP2018 (*pgi*), while Dg201 and Dg 381 also had a truncated CAMP2022 (*edd*) and CAMP2023 (*eda*) (Table 3.1).

Two additional strains, Dg16 and Dg161, annotated with parts of the *glc* locus were from very different phylogenetic groups and were potentially of particular interest. Dg16 belongs to the clonal complex ST21CC21 (commonly associated with human disease), and Dg161 belongs to the classic ST45CC45 group of *glc* negative strains. The draft genome sequence indicated that Dg16 contained two complete alleles of the *glc* locus, CAMP2019 (*glk*) and CAMP2020 (*pgl*), which correspond to the alleles in Dg275, four incomplete loci, CAMP2017 (*glcP*), CAMP2021 (*zwf*), CAMP2022 (*edd*) and CAMP2023 (*eda*), and one missing gene, CAMP2018 (*pgi*). The Dg161 draft sequence data indicated an even more incomplete *glc* locus with only one complete gene, CAMP 2023 (*eda*), four incomplete loci, CAMP2018 (*pgi*), CAMP2019 (*glk*), CAMP2020 (*pgl*), *CAMP2020(edd)* and two missing genes, CAMP2017 (*glcP*) and CAMP2021 (*zwf*). Contig numbers for the assembled sequence data were essentially unacceptably high, with 162 for Dg16 and 186 for Dg161, compared to 32-41 contigs for the CC45 strains analysed. However, because of the significance of these two isolates and possibility of instability of the *glc* locus in other strains, both were included in further analysis.

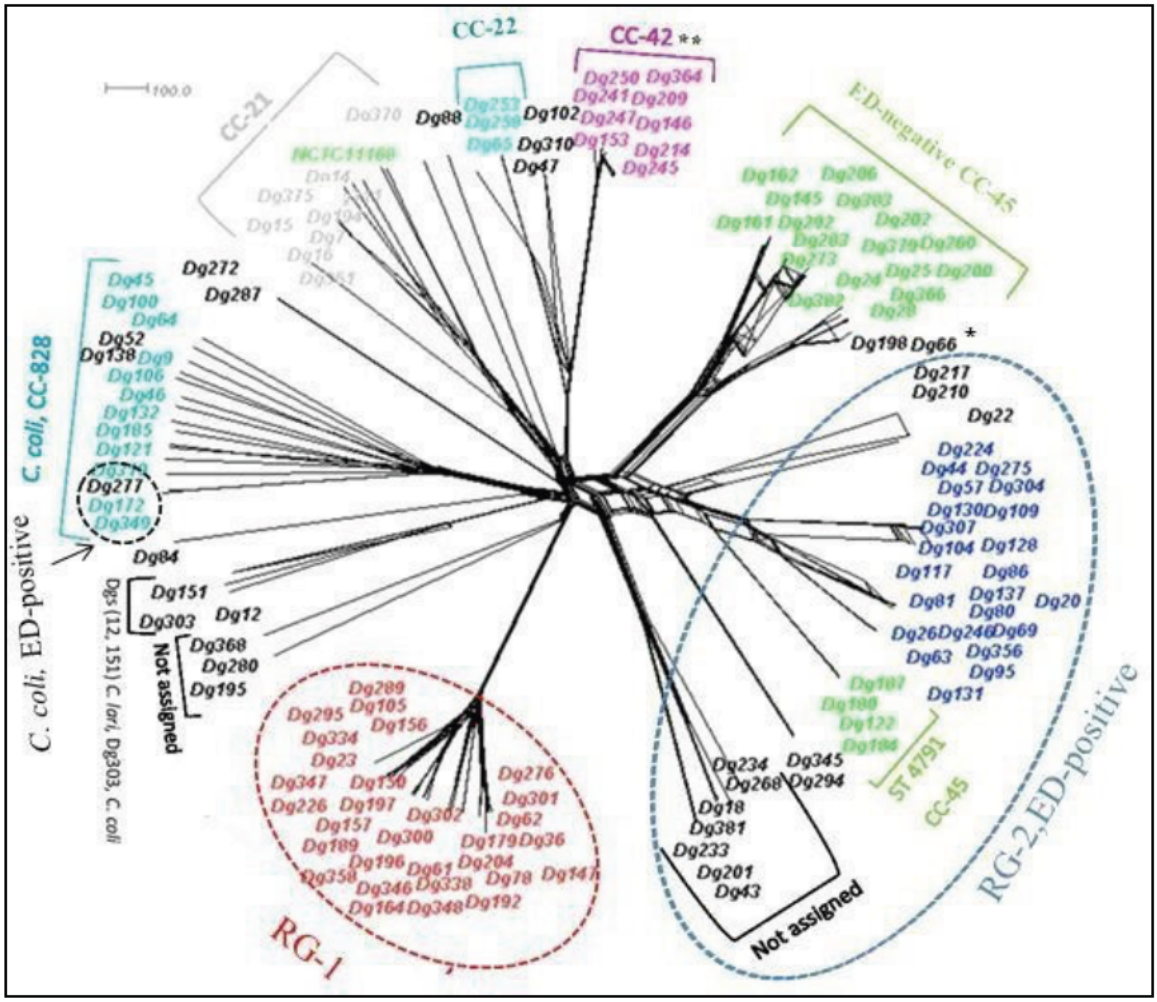


Figure 3.1 The WGS phylogenetic network of 143 Campylobacter isolates from farm-Norway rats. Campylobacter strains isolated from fresh Norway rat faecal samples from eight different farms within South West England (Sites C, E, H, G, F, I, J and K) between 2010 and 2012. *C.jejuni* / *coli* strains were isolated the same day by mashing and transferring the samples to Bolton broth with selective antibiotic supplement and incubation for 4h at37°C, followed by 20h at either 37°C or 42°C to enrich for campylobacter. Genomic DNA was isolated and draft genome sequences were obtained by Illumina sequencing, and all data on *Campylobacter jejuni/coli* were deposited on the PubMLST database (MacIntyre and co-workers, unpublished data). Gene-by-gene comparison of the WGS data was performed using a genome comparator on PubMLST/campylobacter. There were 137 isolates from the farm-associated Norway rats and six from farm animals (goose: Dg333, Dg326, Dg328 and Dg327; chicken: Dg311; pig: Dg319). Red text: novel clade, Rat group 1; Green bracket: classic STs of the CC-45 complex; blue text: RG2 new ST (7259, 7258 and 5130) shares at least four loci with ST45CC45. The phylogenetic network was taken from (Mohammed, 2018).

Locus											
ID	**Dg**	**CC/ST**	**Contigs**	**ED type**	**CAMP 2017 *(glcP)***	**CAMP 2018 *(pgi)***	**CAMP 2019 *(glk)***	**CAMP 2020 *(pgl)***	**CAMP 2021 *(zwf)***	**CAMP 2022 *(edd)***	**CAMP 2023 *(eda)***
25973	Dg16b	Classic 21 / 21	162	(-)	I	X	4	5	I	I	I
26016	Dg 161	Classic 45 -45	186	(-)	X	I	I	I	X	I	5
25977	Dg 18a	Not assigned	65	(I)	4	I	8	8	7	7	8
25993 26002	Dg201 Dg381	Not assigned	68 77	(I)	4	I	8	10	7	I	I
25978	Dg43	Not assigned	58	21/23	4	10	8	8	7	8	7
25997 26049	Dg233 Dg234	Not assigned	61 64	8	4	8	6	11	7	10	7
25948	Dg268	Not assigned	71	21/23	4	8	6	7	7	6	7
Other isolated RG2 (CC-45)											
26009 26022	Dg345 Dg294	ST- 7256/45	33 36	10	6	13	13	5	4	5	5
25974	Dg22	ST -3471	32	22	8	9	9	5	4	5	5
26027 25945	Dg210 Dg217	ST-7276	41 33	7	3	7	12	5	4	5	6
26040 26034	Dg184 Dg180	ST-4791/45	40 32	9	3	12	4	5	4	5	5
25999 26054	Dg122 Dg187	ST-4791/45	40 35	11	3	12	4	12	4	5	5
26058	Dg 275	CC-45	34	2	3	4	4	5	4	5	5
26020	Dg95	CC-45	35	3	3	4	4	6	4	5	5
Ch95-95 (1)		CC- 45	35	-	3	4	I	6	4	5	5

Table 3.1 Comparison of genes of the *glc* locus in rat isolates of *C. jejuni.* Dg275 (turquoise/ blue) is a representative of the large RG2 group (ED type 2) with 17 other strains, and Dg95 (green) of ED type 3, with four other isolates. These were isolated from farm sites C, E, L, X, and G, H, respectively. ED type (I) (incomplete glc locus), including Dg18, Dg 381 and Dg201- assigned with one or more truncated genes. ED type (-) many genes missing or incomplete, poor sequence data. Black numbers, strains with unassigned MLST status; all other isolates are CC45, except strains of ST- 3471 and ST7276 which are not assigned a CC group and Dg16 which belongs to ST21CC21. Ch95-95(1) is a naturally recovered mutant of Dg95 (described in chapter 5). Genes, X: missing gene; I: incomplete gene; numbers, different alleles of each individual gene, and together a code for ED type assignment. Contigs, number of contigs assembled in the draft genome accessible via PUBMLST/campylobacter. Genome comparator on PUBMLST/campylobacter (Jolley and Maiden, 2010) was used to compare alleles. (Camp name on PUBMLST website).

3.2.2 Comparison of sequences of incomplete genes in draft WGS data

To localise the predicted truncation of genes within the *glc* locus of Dg16, Dg18, Dg201, Dg161 and Dg381, the length of each annotated gene, as in PUBMLST/ campylobacter was compared to that of Dg275 and Dg43, both of which have complete *glc* loci (Table 3.2A). Shorter genes were identified primarily for *pgi* and also *edd* and *eda.* To correlate truncations with breaks in DNA sequence assembly (ends of contigs), each gene was once again downloaded with selection of the option to include 50kbp flanking nucleotides on either side. This ensured download of the complete contig in each case.

(Table 3.2B). The length of the complete *glc* locus is 8358bp in Dg275 and this lies within a contig of 9662bp in the WGS draft. In the Dg43 draft sequence, the *glc* locus lies within a contig of 14750bp. When this was repeated for Dg18, the *glc* locus was split over two separate contigs, with a break between *pgi* and *glk.* With Dg201 and Dg381, the *glc* locus was split over three separate contigs, with an additional break between *eda* and *edd*. Evidence of any *glc* locus in Dg16 and Dg161 was fragmented over four or five contigs. Each break correlated with truncation of a gene *pgi* in Dg18 and additionally *eda* and *edd* in Dg201 and Dg381 (Table 3.2 A, B).

A-Strains	***glcP***	***pgi***	***glk***	***pgl***	***zwf***	***edd***	***eda***
Dg275	1200	1644	1005	681	1401	1803	624
Dg43a	1215	1644	999	681	1401	1803	624
Dg18a	1215	(1119)	999	681	1401	1803	624
Dg201a	1215	(1090)	999	681	1401	(1050)	(594)
Dg381	1215	(1090)	999	681	1401	(1050)	(594)
Dg16	(754)	X	1005	681	(739)	(1026)	(543)
Dg161	X	(1326)	(558)	(607)	X	(932)	624

B-Strains	***glcP***	***pgi***	***glk***	***pgl***	***zwf***	***edd***	***eda***
Dg275	9662	9662	9662	9662	9662	9662	9662
Dg43a	14750	14750	14750	14750	14750	14750	14750
Dg18a	4042	4042	8050	8050	8050	8050	8050
Dg201a	3948	3948	4777	4777	4777	4777	2214
Dg381	3948	3948	4777	4777	4777	4777	2214
Dg16	754	X	2897	2897	739	1225	902
Dg161	X	1887	1887	798	X	1667	1238

Table 3. 2A&B Comparison of each individual gene of the *glc* locus in the draft genome sequences. Sequences of the *glc* locus were downloaded from PUBMLST by downloading the sequence, as follows A: the complete nucleotide sequence for each gene of the *glc* locus, individually. Number in brackets, truncated genes. X: missing gene; B: assesses breaks in the assembled DNA sequence, as each gene was again downloaded from PUBMLST but with the 50kb parameter on either side selected**.**

The downloaded DNA sequences for complete and incomplete genes were then aligned using DNA dynamo programme to identify the location of truncations (fig 3.2-3.4). Dg43 was used as reference, as it has the closest *glc* locus to Dg18, Dg201 and Dg381, as shown in Table 3.2. Dg18, CAMP2018 (*pgi*), had a truncation of 525bp, which corresponds to loss of the N terminus and approximately a third of the protein (fig 3.2). Dg201 and Dg381 *pgi* were missing the same region, with an additional 29 bp (554 bp total missing). Alignments for *edd* indicated that both Dg201 and Dg381 were missing 753 bp from the end of the *edd* gene with the initial 1050 bp of this 1803 bp gene identical to Dg43 *edd* (fig 3.3). Similarly, there were 28 bp missing from the start of the *eda* gene with the remainder of the gene identical to *eda* in Dg43 (fig3.4). The sequence truncations correlated with contig ends and break in DNA sequence assembly.

Figure 3.2 Alignment of the Dg43 *pgi* and incomplete *pgi* genes in Dg18, Dg201 and Dg381 from the WGS draft data. The *pgi* gene was downloaded from the genome sequence data of each strain on the PUBMLST site and aligned using DNA dynamo. Top image, complete alignment indicating no differences post truncation.

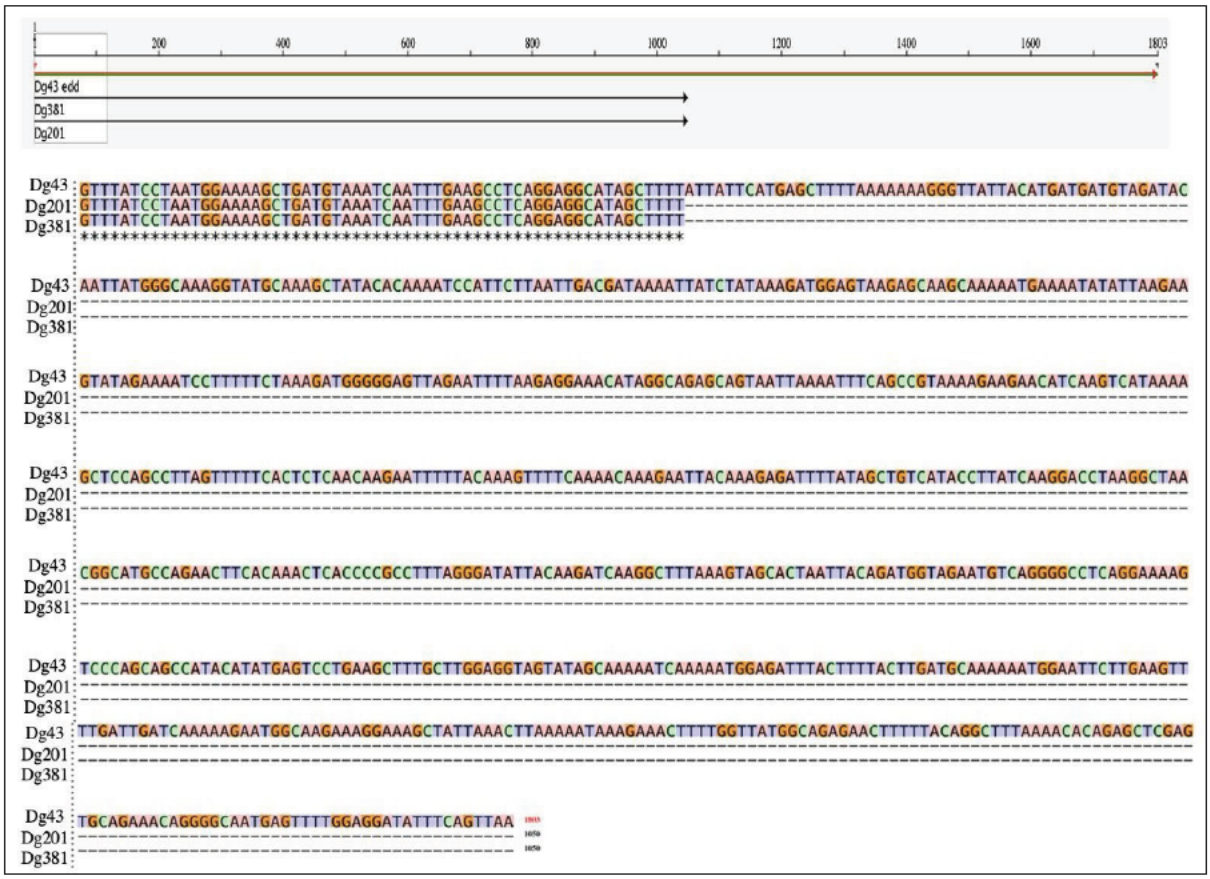


Figure 3.3 Alignment of complete Dg43 *edd* with incomplete *edd* genes Dg201 and Dg381 from the draft WGS data. The *edd* sequence for each strain was downloaded from the PUBMLST website. Then the DNA dynamo programme was used to align sequences. Top image, complete alignment indicating no differences up to the truncation.

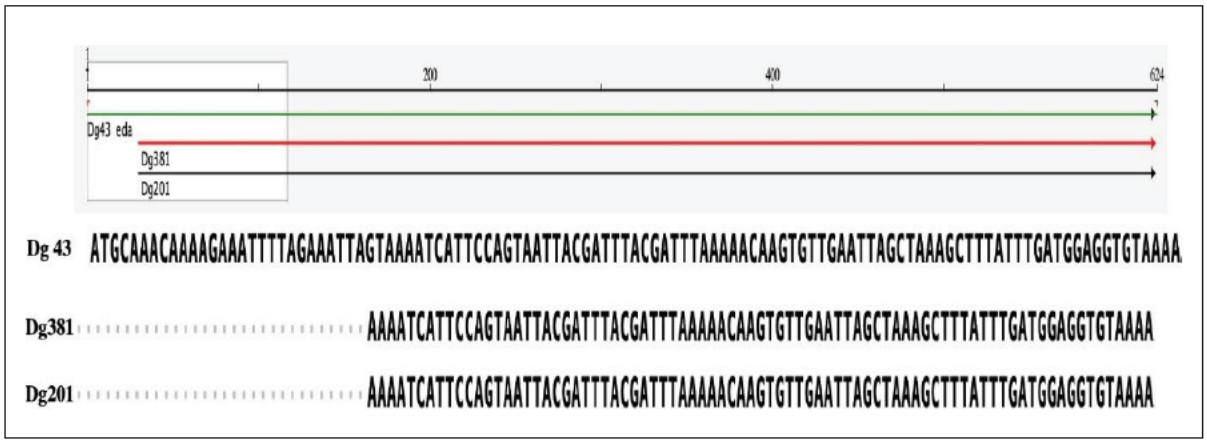


Figure 3.4 Alignment of the complete Dg43 *eda* with incomplete *eda* gene in Dg381 and Dg201 from the WGS draft data. The *eda* sequence for each strain was downloaded from the PUBMLST website, and DNA dynamo was used for comparison. Top image, depicts alignment showing truncation and identical residues in sequenced region after that.

3.3 PCR amplification and confirmation of truncated sequences

To confirm sequence data of the *glc* locus of Dg18, Dg 201, Dg381, Dg16 and Dg161, 3 subcultures of each strain were analysed by lysed colony PCR (2.11.1).

Cells from subculture stocks a, b and c of each strain were tested for aspartokinaseK (*aspk*), which is specific for *C. coli* (Banowary *et al*., 2015) and hippuricase (*hipO*), which is specific for *C. jejuni* (Banowary *et al*., 2015) The expected PCR product for *C. coli aspK* was 400 bp, as obtained with the *C. coli* control, Dg349, and for *C. jejuni hipO* the product was 735 bp, as seen with Dg275 (fig 3.5). None of the test samples produced a band of around 400 bp with the aspK primers and all produced the expected product of close to 800 bp with the HipO primers. This simply confirms that all 3 subcultures (a-c) for each strain are *C. jejuni* and there is no contamination with *C. coli*.

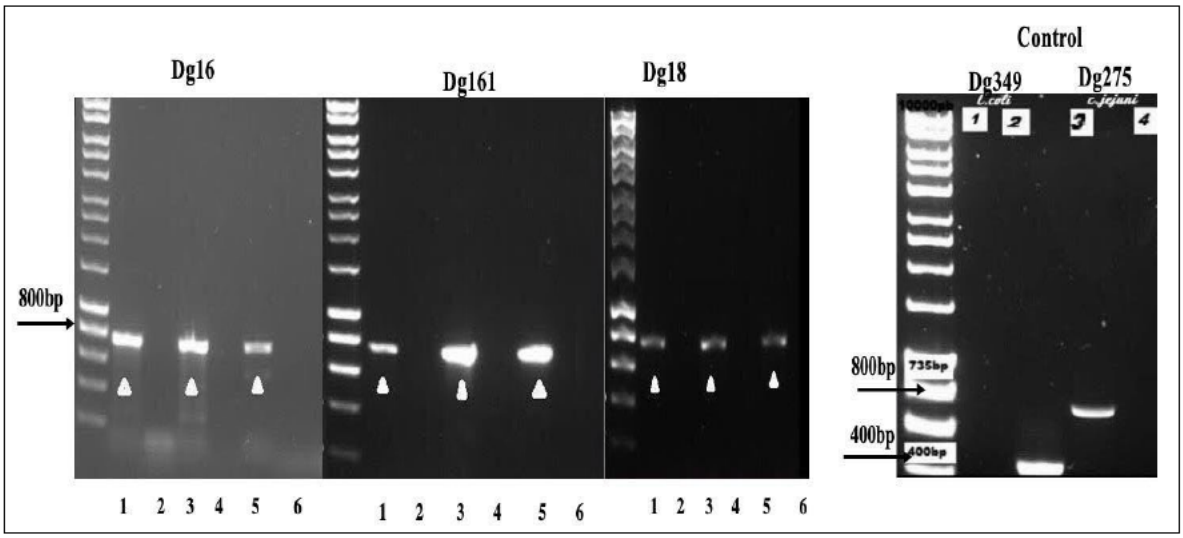


Figure 3. 5 Detection *C. coli* (aspK) and *C. jejuni* (hipO) by colony PCR.PCR was performed using lysed colonies of strains as shown and the *C. coli* specific primer pairs (aspK forward and aspK reverse) lanes 2, 4 and 6 or the *C jejuni* specific primers (hipO forward and hipO reverse, lanes 1, 3 and 5). a, b and c are subcultures of each test strains, Dg18, Dg16 and Dg161. Dg275 *C. jejuni,* positive control, lanes 3 and 4, Dg 349, *C. coli*, positive control, lanes 1 and 2.

Confirmation of, *pgi*, *edd* and *eda* in subcultures a, b and c of each strain was completed by PCR amplification and Sanger sequencing.

Location of primers used to amplify each gene is shown in fig 3.6, and the sequence of primers is listed in table 2.2. Amplification of the *pgi* gene from Dg18a, b and c, Dg201a, b and c and Dg381a, b and c are shown in fig 3.8.

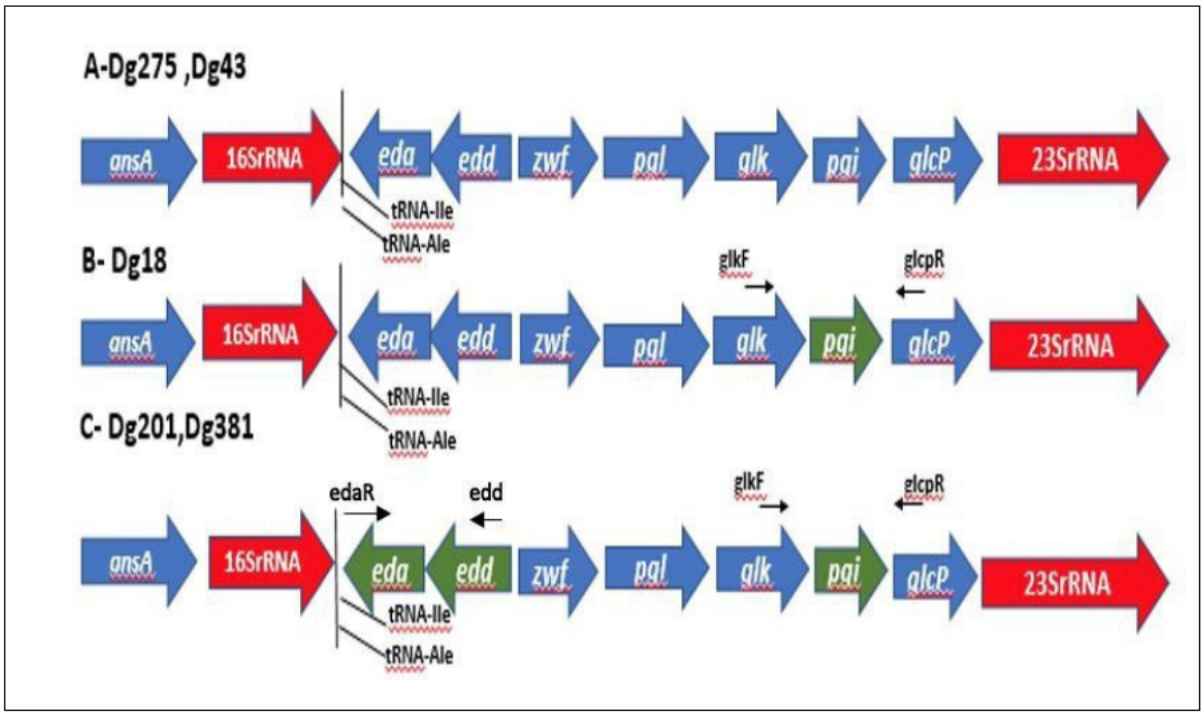


Figure 3.6 Structure of the *glc* locus for RG2 strains. A: complete locus, as presented in Dg275 and Dg43; B: ED pathway in Dg18 sequence data only (*pgi*) is incomplete C: Dg381 and Dg201 sequence data *pgi*, *edd* and *eda* are all incomplete. Arrows indicate primers used for amplification and sequencing. Green, genes annotated as incomplete in draft genome sequence, blue complete genes.

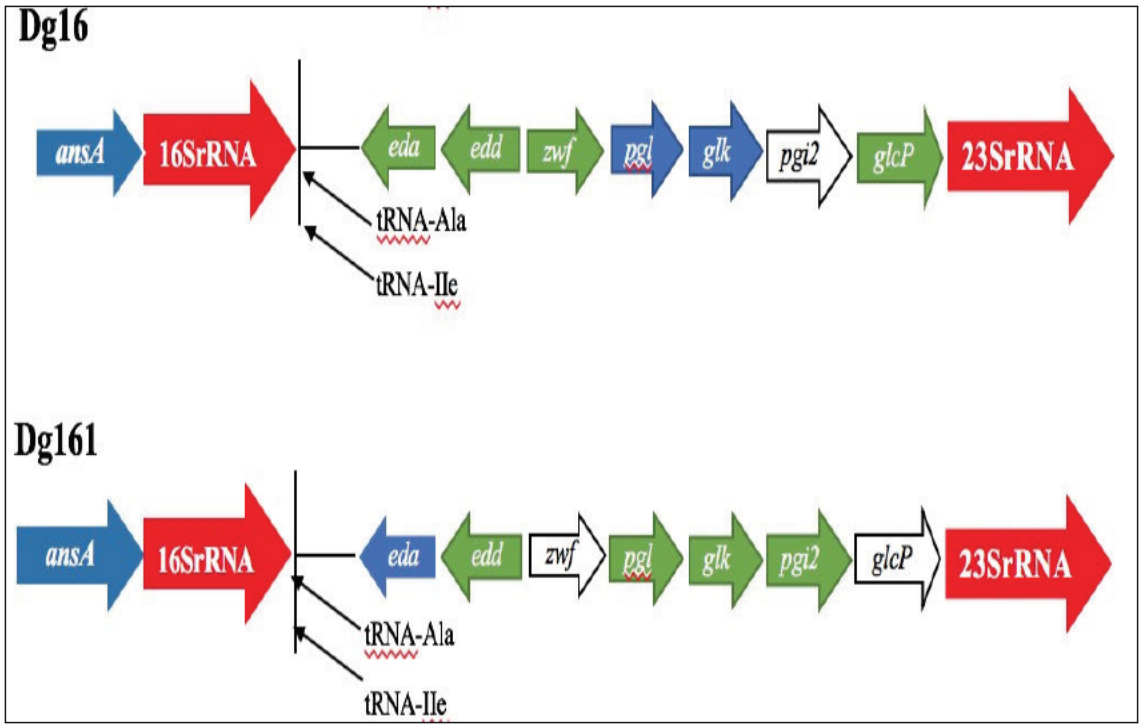


Figure 3.7 Corresponding location of *glc* genes annotated in Dg16 and Dg161 WGS data. *glc* genes predicted in the WGS data of Dg 16 and Dg 161 are mapped onto the *glc* locus of Dg275. In the putative incomplete ED pathway: Dg16 has missing *pgi2* while *edd, eda* and *zwf* are incomplete only *pgl* and *glk* are complete. Dg161 has two genes missing *zwf* and *glcP*, and the rest of the genes are incomplete except for *eda*. Genes assigned in the draft genome sequence are as follows: green (incomplete), blue (complete) and white (missing gene). See section 3.4 below for analysis of any inserts within the *rrn* loci of these strains.

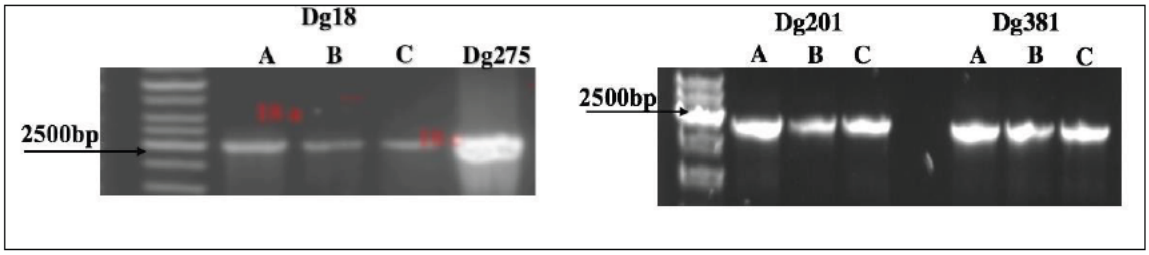


Figure 3. 8 PCR amplification of *pgi* gene from Dg18, Dg381 and Dg201 with Dg275 as control. Lysed colony PCR was used for amplification (as described in section 2.11.1) with primers glk F and glcPR for Dg18 and primers with glkF and glcP18R with Dg201 and Dg381. A, B and C are stocked subcultures of each strain. Dg275, control for *pgi* gene using isolated genomic DNA as template.

Product size of the amplified *pgi* gene in each case was the same for each of the subcultures and the amplified product from Dg275 (1644 bp + flanking region to the primers), fig 3.8. Amplification of the same size product was reassuring as *pgi* in the draft WGS data for Dg18 was 400bp shorter (1256 bp) than that of Dg275 (Table 3.2A). The additional genes missing in Dg201 and Dg381 were *edd* and *eda*. Primers used to amplify this region were eddF and eda R, table 2.2. Product size (expected size 1698 bp) of the amplified *edd/eda* fragment for both Dg201 and Dg381 was the same for each of the subcultures and also the same as the amplified product from Dg43 which has an intact *glc* locus fig 3.9.

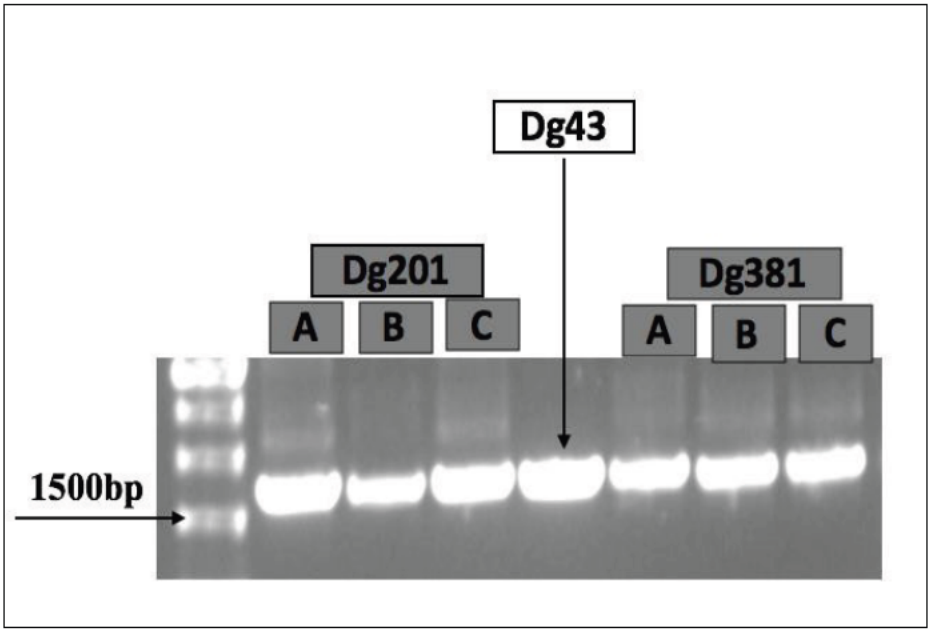


Figure 3.9 PCR products of *eda* and *edd* genes from strains Dg201, Dg381 and Dg43. Primers eddF and edaF were used in a PCR reaction with isolated genomic DNA from subcultures a, b and c of strains Dg201 and Dg381 and genomic DNA of strain Dg43 as control DNA ladder (the molecular marker).

All PCR products were purified and sent with the amplification primers for Sanger sequencing by MWG Eurofins, Germany. DNA Dynamo and Clustal omega were used to analyse the sequences for *pgi* of Dg201 a, b, c and Dg381 a, b and c, the *edd* gene of Dg201 a, b, c and Dg381 a, b, c and the *eda* gene of Dg201 a, b and c, as well as Dg381 a, b and c (See Appendix 3 for more detail). Dg43 and Dg18 *pgi*, *edd* and *eda* sequences were used a reference. Based on these Sanger sequence results of the problem areas, both strains Dg201 and Dg381 possess a complete *glc* loci. In each case, the 'missing' sequence was clearly there and correct. The fact that the PCR product of Dg18 *pgi* was the correct size suggests that this strain also has a complete *glc* locus, however, sequence data from the PCR product was inconclusive. Genomic DNA of subculture (a) for each of these strains has been reisolated and sent for Illumina sequencing to Novogene. Assuming improved sequencing, the new assembled sequence will replace the original WGS sequence data. This will also confirm if the *pgi* gene of Dg18 is complete. Assignment of these areas as falsely truncated in the WGS data can most easily be explained by the fact that these sequences lie at the end of a contig within the *glc* locus and reinforces the need to confirm any unusual WGS sequence data. Fig 3.7 depicts the predicted annotated of *glc* genes in Dg16. Isolated genomic DNA from these strains was tested for presence of *pgl* and *glk* by amplification with primers targeting both genes. No product was obtained. This is consistent with absence of these genes from Dg16 Combined with the high contig number it was most likely that presence of *glc* genes in this strain and Dg161 was due to poor sequencing results, possibly contamination at some point along the process. However, because of the potential interest of both presence of *glc* locus in these generalist strains and also the possibility of evidence of instability of the *glc* locus in some strains, genomic DNA from Dg16 and Dg161 (subclones a in both cases) was submitted for Illumina WGS.

3.4 Confirmation of glucose utilisation by RG2 strains annotated as 'truncated' *glc.*

After confirmation that there was no evidence of truncated *glc* genes in Dg18, Dg201 and Dg381, the likelihood that the 'truncated genes' were simply incomplete sequence data was considered.

Subcultures (a, b and c) were tested for the ability to grow on glucose, using DMEMf- FBS minimal growth media with and without 20 mM glucose in a microtitre plate growth assay. Cultures were grown at 37°C, with a microaerobic gas pack as described in 2.4.3 for 72h. Growth was monitored by OD_{600} and cfu. Subcultures a, b and c for each strain revealed beyond any doubt the ability of each of these strains to grow and utilise glucose. As results were comparable for all 3 subcultures, a, b and c for each strain were treated as repeats to produce the data shown in fig 3.10. Enhanced growth and survival in the presence of glucose was evident from 48h onwards. OD_{600}, at 48h was in the range of 0.8- 0.7 with and 0.4 - 0.65 without glucose for all 3 strains. At 60h, for Dg18 the cfu/ml was approximately 2.5 $\log_{10}$ higher with glucose (1.13×10^9/ml), compared to without glucose (6.89×10^6/ml)/ (P=0.003). Dg381 showed approximately 1.5$\log_{10}$ higher cfu count in glucose (1.17×10^9/ml) compared to (5.67×10^7/ml) in the absence of glucose (P=0.02, 60h) and Dg201 showed a six-fold higher cfu count with glucose (6.83×10^8/ml) compared to without glucose 1.1×10^8/ml) (P=0.004). See fig 3.11 for absence of enhanced growth of the negative control Dg200 (no *glc* locus) in glucose, and increased growth and survival with Dg275 (positive control for the *glc* locus). These growth results are in accord with previous studies that indicated the use of glucose by Dg275 only after the depletion of amino acids (Mohammed, 2018). Dg16 (ST21CC21) and Dg161 (ST45 CC45), the two strains possessing genomes annotated with fragments of the *glc* locus in the draft WGS, were also tested for growth on glucose (fig 3.11). Measurement of growth was by OD_{600} only in a microtitre plate assay.

Dg275 (RG2) the positive control for *glc* locus showed 4-fold higher OD_{600} in glucose after 72h growth compared to growth in the absence of glucose. Dg200 the negative control (no *glc* locus) actually showed decreased growth in the presence of glucose. A similar decrease in OD_{600} in the presence of glucose was seen with both Dg16 and Dg161 (fig 3.11), as might be expected confirming absence of a functional *glc* locus. Growth results being consistent with PCR results, when PCR showed no product for Dg16 and Dg161.

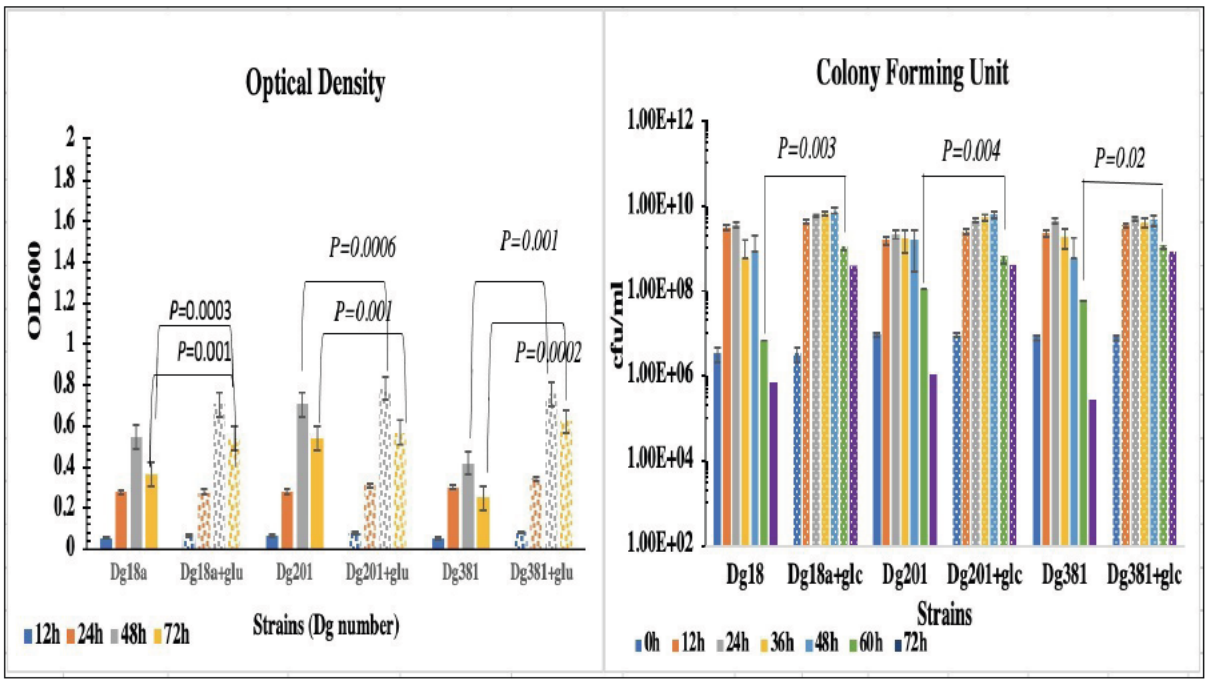


Figure 3.10 Impact of glucose on growth of Dg 18, Dg201 and Dg381. Twenty µl of a standardised sample of 0.02 OD_{600} was prepared from bacteria grown on MHA, and used to inoculate in DMEMf- FBS in the presence (hatched bars) or the absence (solid bars) of 20 mM glucose. Subcultured stocks (a, b and c) for each strain were grown separately. As results were comparable for a, b and c in each case, they were used as triplicate repeats to provide the average growth shown with standard deviation. Samples were monitored for OD_{600} and cfu at the time indicated, as described 2.4.3.

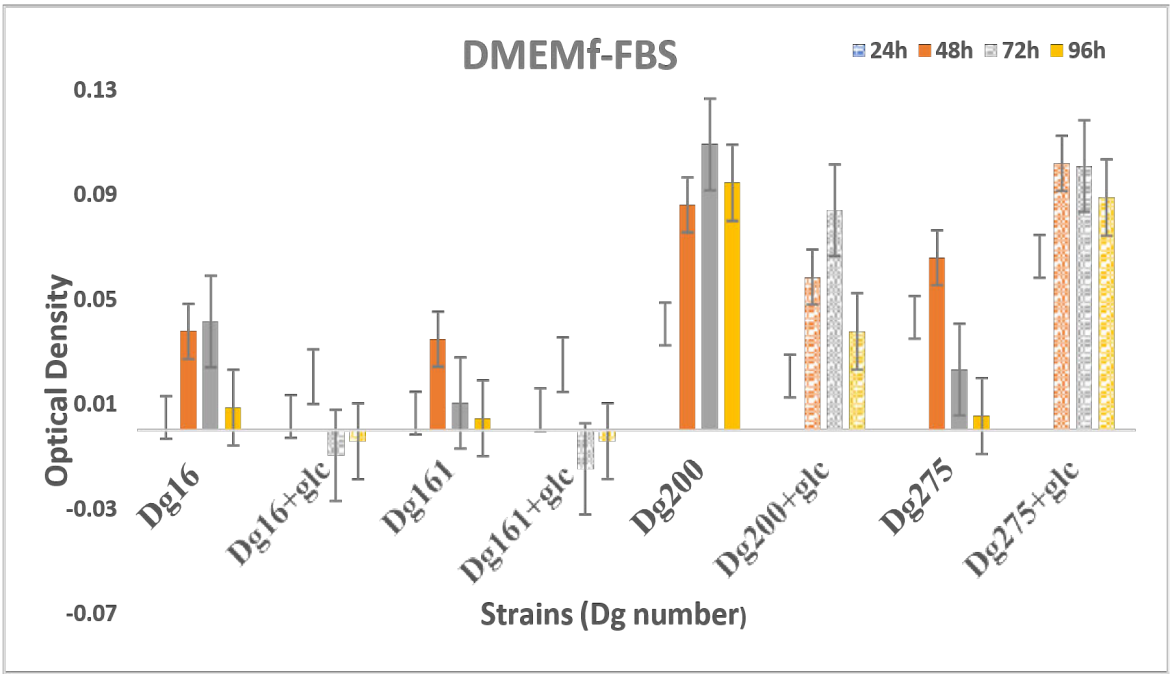


Figure 3.11 The impact of glucose on growth of Dg16 and Dg161. Twenty µl of a standardised sample of 0.02OD_{600} was prepared from bacteria grown on MHA, and used to inoculate in DMEMf- FBS in the presence (hatched bars) or the absence (solid bars) of 20 mM glucose, in a microtitre plate assay. Subcultured stocks (a, b and c) for each strain were grown separately. As results were comparable for a, b and c in each case, they were used as triplicate repeats to provide the average growth shown with standard deviation. Samples were monitored for OD_{600} at the time indicated.

3.5 Association of *glc* locus with *rrn* loci

Published data shows that in *C. jejuni* subsp. doylei 269.97 the *glc* locus is located within an *rrn* locus between the 16S *rRNA*, alanyl-tRNA, isoleucyl-tRNA and 23SrRNA,5S rRNA (Miller, 2008; Vorwerk *et al*.,2015). There are three copies of *rrn* loci, 4676 bp in length, in *C. jejuni* and *C. coli* (Taylor *et al*., 1992; Parkhill *et al*., 2000). Importantly, evidence has been reported that in Dg275 the *glc* locus is inserted within two different *rrn* loci: *rrn*A and *rrn*B (Mohammed, 2018).. In this section the genome location of the *glc* locus in Dg43 and related strains was investigated. Genetic arrangement of the *rrn* loci of Dg16 and Dg161 was also analysed.

3.5.1 Genomic organisation of *rrn* and *glc* locus in Dg43 and related strains

For each strain (Dg18, Dg 43a, Dg234, Dg345, Dg 201, Dg268, Dg381 and Dg275), the programme CONTIGuator (was used to map all contigs using NCTC11168 as a reference. Because the *rrn* sequence is a repeat sequence, it was never assembled and was always on a contig by itself. The Artemis programme (Carver *et al*., 2011) was used to locate *rrn*A, *rrn*B and *rrn*C in NCTC11168 and identify the encompassing genes. Correlation of *rrn* flanking genes identified in the NCTC11168 genome sequence with the partially assembled test sequence for each strain, permitted mapping of the 3 copies of *rrn* in all test strains. The assumption was that the *glc* locus would be within one or more of the three *rrn* loci. Fig 3.12 is a schematic diagram of the predicted genetic organisation of all 3 *rrn* loci, for each strain, with the *glc* locus inserted where identified by PCR.

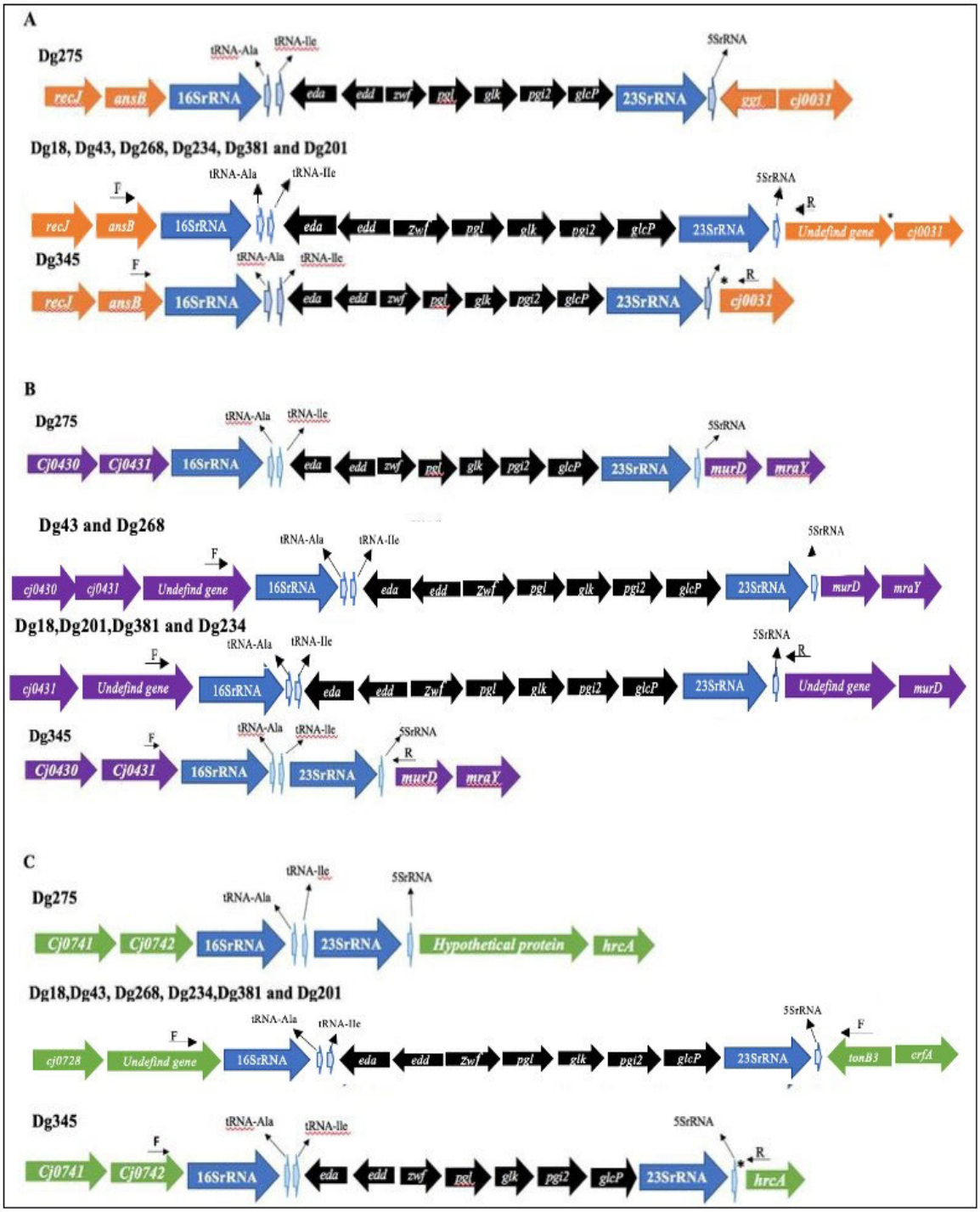


Figure 3.12 Diagram of the genetic arrangement of rRNA loci A, B and C with *glc* insert. Predicted genetic arrangement of *C. jejuni* strains as indicated. Organisation based on mapped contigs and NCTC11168 *rrn* flanking genes as a guide for genetic organisation around *rrn*A (A)*rrn*B (B) and *rrn*C (C). Insertion of *glc* locus was based on results of PCR amplification (Figure3.18 and Table 3.3). Blue arrows, genes of *rrn* locus and light blue, tRNA Ala, tRNA IIe and 5S rRNA black arrows genes of *glc* locus. Flanking genes of *rrn*A (orange arrows) *rrn*B (purple arrows) of *rrn*C (green arrows) *Indicates predicted existence of additional genes. Amplification primers indicated by arrows. *cj0741* is a hypothetical protein, and *cj0742* is a pseudogene (putative outer membrane protein). *cj0728* is a putative periplasmic protein. *cj0430* is a putative integral membrane protein. *cj0431* is a putative periplasmic ATP/GTP- binding protein. *cj0030* is a hypothetical protein. *cj0031* is a putative type IIS restriction/modification enzyme.

To identify which *rrn* loci carried an inserted *glc* locus, primers were designed for amplification of each of the three *rrn* sites, using the mapped flanking genes as primer targets. PCR amplification of the *rrn* A, B and C loci DNA of each strain Dg43, Dg18, Dg201, Dg381, Dg268, Dg234 and Dg345- used the flanking primers as follows: Gap A was between *ansB* and hypothetical gene for all strains except for Dg345, which was between *ansB* and *cj0031*. Gap B was between *murD* and a hypothetical gene for Dg43 and Dg268; however, Dg345 was between *murD* and *cj0431.* For the rest of the strains Gap B was between a hypothetical gene on both sides. Gap C was between *tonB3* and a hypothetical gene for all strains except for Dg345, which was between *hrcA* and *cj0742* (fig3.12). To interpret the outcome, the expected size of each product with the *glc* locus was calculated. This included 4674bp for *rrn* locus, 8358bp (or shorter length if truncated) for the *glc* locus and variable size of flanking DNA as summarised in table 3.3. Including a complete *glc* locus, the minimum size of the PCR product would be 13 kbp. The PCR product profile for Dg275 was consistent with previously identified genome arrangement, that is insertion of the *glc* locus into *rrn* A and *rrn* B but not *rrn* C, fig 3.12 (Mohammed, 2018). Surprisingly, the products for amplification of all 3 *rrn* sites (A, B and C) for Dg43 were well over the 10 kbp marker and consistent with the predicted sizes of about 14 kbp for each site. The same profile was seen for each strain (Dg18, Dg201, Dg381, Dg234, Dg268) that was closely related phylogenetically to Dg 43 (fig3.1).

Each of these strains had a large insert of substantially greater than 10kb within *rrn* A, B and C. As each strain had been shown to grow on glucose, the conclusion was that the genomes of all six of these strains carry a *glc* locus inserted within all three copies of *rrn*. This was confirmed for Dg268 and Dg43. The PCR products for *rrn* A, *rrn* B and *rrn* C gaps were sent for Sanger sequencing with primers glcp381R and glk18F.

This confirmed presence of *glk*, *pgi* and *glcP* hence confirming the existence of *glc* loci in all three *rrn* loci of these two strains. The only other strain tested to carry only two copies of the *glc* locus was Dg345. Based on wgMLST see fig3.1, Dg345 is more distantly related to the Dg43 group of strains and closer to the CC 45 related group. However, in contrast to Dg275, with Dg345 the *glc* locus appeared to be inserted in *rrn* A and *rrn* C, but not in *rrn* B.

Strains	Expected size of amplified product including *glc* loci (bp)[1]		
	A	B	C
Dg43	14015	13853	14030
Dg18	14077	15015	14540
Dg268	14014	13868	14191
Dg201	14120	15408	14176
Dg381	14120	15408	14176
Dg234	14124	13781	14581
Dg345	13088	8578	12707

Table 3.3 Expected sizes of the amplified *glc* loci for all three *rrn* loci.[1]Expected product size on amplification with primers (Table 2.2) targeting genes flanking 16S *rRNA* and 23S *rRNA* genes (see fig 3.12). Predicted size shown is that including the *glc* locus, either complete or truncated as predicted in WGS data, with the exception of Dg345 for which the PCR product was far below the minimum size of 13000 bp for *rrn* + *glc* genes.

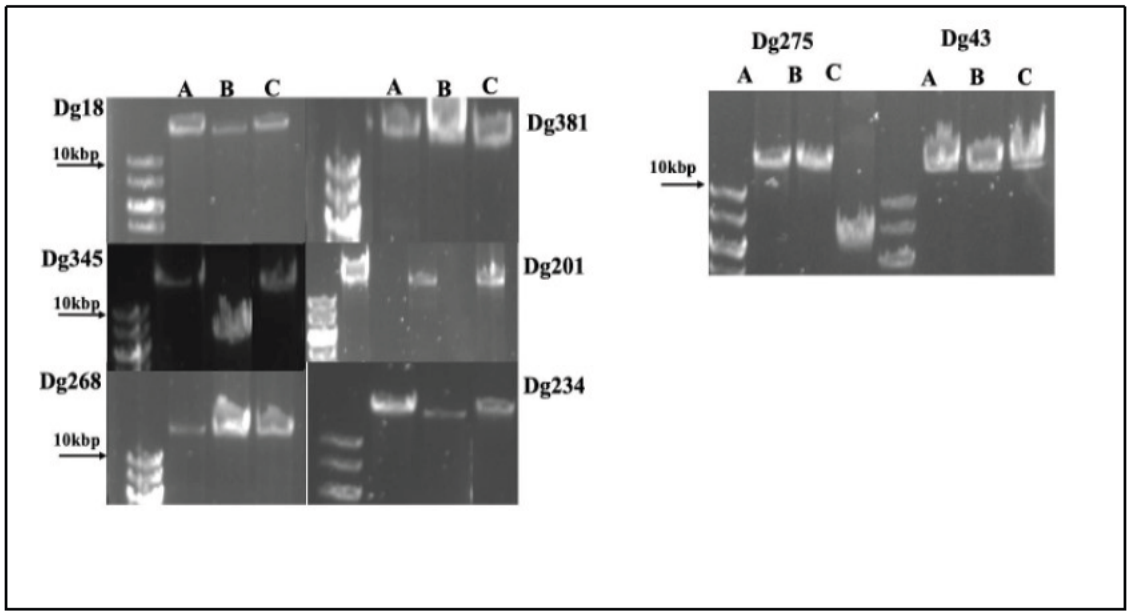


Figure 3.13 PCR products of amplified *rrn* loci A, B and C. *rrn* loci of Dg43, Dg18, Dg268, Dg201, Dg381, Dg234, Dg345 were amplified, using primers depicted in figure 3.17. Dg275 had previously been shown to have *glc* locus inserted into *rrn*A and *rrn*B but not *rrnC* (Mohammed, 2018). PCR products were obtained using CloneAmp HiFi and isolated genomic DNA as template. Primers and primer characteristics are listed in table2.2B and table 3.3 summarises expected product sizes.

Finally, to conclusively establish insertion of the *glc* locus in each of the three *rrn* loci of these strains, Dg43 was chosen as a representative of this group of strains for Nanopore sequencing. The long reads achieved with Nanopore sequencing would confirm genetic organisation around each of the three *rrn* loci and permit closure of the draft genome by hybrid assembly. Genomic DNA was reisolated using a Monarch genomic DNA purification kit, as described 2.9.2. Illumina sequencing was performed commercially by Novogene and Nanopore sequencing and associated bioinformatics analysis was kindly performed by Dr. Soon Gweon (University of Reading). The assembled annotated genome of Dg43 was 1777980 bp around 100000 bp larger than Dg275 and Dg95 (see Chapter 5 below). Each of the three copies of the *glc* loci were confirmed inserted into *rrnA* position (42860-51533) *rrnB* (428529-437202) and *rrnC* (733771-742444).

All copies of the *glc* loci have the same size (8673bp) and identical sequence. This also provided the closed annotated sequence of Dg43 for future analysis.

3.6 Conclusion

Catabolism of carbohydrates is not associated with central metabolism of *C. jejuni* in part due to a lack of phosphofructokinase in the classical Embden-Meyerhof-Parnas glycolysis pathway and relevant transport proteins (Parkhill *et al*., 2000; Velayudhan and Kelly, 2002). The mobile genetic locus, termed *glc* locus, and associated ability of *C. coli* and *C. jejuni* strains possessing this locus to metabolise glucose via the ED pathway, has more recently been identified (Vorwerk *et al*., 2015; Vegge *et al*., 2016; Mohammed, 2018). Approximately 25 % of 138 sequenced isolates of *C. jejuni*/ *coli* from farmyard Norway rats carry the *glc* locus and have been studied here. The isolates could be broadly divided into two groups based on ED type and wgMLST phylogenetic grouping. The largest group was most closely related to the ED negative CC45 generalist isolates and formed ED types 2, 3, 9 and 11. These are characterised by strains Dg275 and Dg95. Despite isolation of strains of other clonal complexes from the same source, no strains belonging to classic CC groups were identified as possessing this ED locus. This would be consistent with more vertical transmission and gradual drift than frequent horizontal transfer of the *glc* locus to more distantly related strains. A smaller distinct phylogroup, carrying ED types 8, 21 and 23 also included 3 strains annotated with incomplete *glc* loci. This study initially focused on this group of strains. Strain Dg43 has a complete *glc* locus (8367bp) and showed enhanced growth on minimal media supplemented with glucose. The three strains with an incomplete *glc* locus (Dg18 *pgi*; Dg201 *pgi, edd and eda;* and Dg381 *pgi, edd and eda*) also showed enhanced growth with glucose.

This was potentially interesting for two reasons. First, it raised the possibility that the ED pathway might still function despite the truncation of *pgi* (phosphoglucose isomerases), or less likely, in the absence of *edd* (6phosphogluconate dehydratase) and *eda* (2-keto3-deoxy-6-phosphogluconate aldolase).

Second, the possession of the *glc* locus is rare in *C. jejuni* and is essentially absent from clinical isolates (Vegge *et al*., 2016). The occurrence of a fragmented *glc* locus with incomplete genes might indicate instability of this locus. In accordance with this, the two isolates (Dg16a and Dg161) belonging to ST21CC21 and ST45CC45, respectively, were classified as having an extensively fragmented *glc* locus. However, annotation of truncated *glc* loci in Dg18, Dg201 and Dg381 were shown to be errors of draft WGS data. Full sequences of each assigned annotated gene were identified by Sanger sequencing. The sequence data of Dg16a and Dg161, while interesting coming from generalist strains, was unreliable. Hence, genomic DNA was reisolated from fresh subcultures of each of these 5 isolates for repeat Illumina WGS to confirm the complete *glc* locus sequence, and absence of any *glc* genes, as relevant.

During this analysis, the genome organisation of the *glc* locus in relation to the three *rrn* copies (A, B and C) of *C. jejuni* was established for Dg43 and related strains. In 2015, presence of the *glc* locus within *rrn*A in *C. coli* was reported (Vorwerk *et al*., 2015). Mohammed demonstrated that in *C. jejuni* Dg275 and Dg95, the *glc* locus was inserted within two different *rrn* loci: *rrnA* and *rrnB*, between isoleucyltRNA gene and 23SrRNA gene (Mohammed, 2018). By targeting *rrn* flanking genes, PCR amplification and sequencing of *rrn* A, B and C, surprisingly, a *glc* locus was identified within each copy of the *rrn* locus (*rrn*A, *rrn*B and *rrn*C) in *C. jejuni* Dg43 and five other related strains. Existence of the *glc* locus within Dg43 was confirmed by hybrid assembly of the genome based on both Nanopore and Illumina sequencing. This has also provided a closed genome sequence of strain Dg43, which representsa more divergent group of the Glc positive strains of *C. jejuni*.

Chapter 4

Impact of glucose on growth and survival

4.1 Introduction

Campylobacter jejuni depends on amino acids for growth and energy production both *in vitro* and *in vivo* (Hofreuter *et al.,* 2012). In rich media, the reference strain NCTC11168 uses the amino acids Ser, Asp, Glu and Pro generally in decreasing order of preference (Velayudhan *et al.,* 2004; Guccione *et al.,* 2008; Leach *et al*., 1997), while C. *jejuni* 81-176 prefer to grow in media containing only Asn and Gln as carbon sources (Hofreuter *et al*., 2008).

In 2016, genes encoding enzymes of the Entner–Doudoroff (ED) pathway were identified in the genome of *C. jejuni* subsp. *doylei* 269.97 (Miller, 2008). The possession of the ED pathway by *Campylobacter* has been shown to be more widespread and has been studied in *C. coli* (Vegge *et al*., 2016; Vorwerk *et al*., 2015) and more recently in *C. jejuni* (Mohammed, 2018). *In vitro* growth conditions, the two *C. jejuni* RG2 strains Dg275 (ED 2) and Dg95 (ED 3) were shown to use Ser and Asp in preference over glucose in both the rich media, MHB, and in DMEM (Mohammed, 2018).

This part of the thesis focuses on the specific strain Dg275 to provide an example of a strain that shows optimal growth in the presence of glucose. The growth of Dg275 was tested on media containing pyruvate with and without glucose. Furthermore, the ability of some strains of RG2 to utilise other sources of carbohydrates, such as maltose and lactose was evaluated.

4.2 Dg275 growth dynamics and effect of glucose with rich and minimal media

As with other bacteria, *C. jejuni* undergoes distinctive dynamic stages during growth. The lag phase, or time post inoculation can be quite variable and depends on the state of the *Campylobacter* used as inoculum. During this period the *Campylobacter* adapt to the growth conditions and show little if any increase in cell number. The phase of rapid cell division and exponential growth then follows and this is followed by the stationary phase (Wright *et al*., 2009). In this part of the study, *C. jejuni* Dg275 growth dynamics was initially studied. This C. *jejuni* strain typically reached early stationary phase sometime between 12h and 24h depending on the length of lag phase and growth conditions (see fig4.1 below). During stationary phase bacterial division continues, but this is balanced by bacterial death and in this stage, nutrients are often limiting. By late stationary phase, around 48h, spiral cells begin to go through a morphological transition to non-viable coccoid cells (Ikeda and Karlyshev, 2012) and the culture enters the final decline stage, where the number of cells decreases often rapidly to low numbers by 60–72h (Frirdich, *et al*., 2019). For example, see fig 4.2 below. The optical density (OD_{600}) of *Campylobacter cultures* increases during exponential growth and continues increasing during stationary phase. Colony forming unit (cfu) monitors viable bacterial numbers, this was considered a more reliable measure of bacterial growth and survival from stationary phase. The growth dynamics of *C. jejuni* Dg275 strains, ED type2 and influence of 20mM glucose was established in Mueller Hinton broth (MHB) and Dulbecco's modified Eagle medium with iron ascorbate (DMEMf).

Growth was in 50ml conical flasks agitated at 150rpm for 72h at 37°C under microaerobic conditions (5% O_2, 10% CO_2 , 2% H_2, 83% N_2) in the M35 cabinet.

All media were inoculated to a starting OD_{600} of 0.002 (cfu, ~$3.4X10^6$ /ml), with a standard inoculum prepared as described in chapter 2.4.2 and fig 4.1. Hydrogen (H_2) gas was included as it has been shown to act as a major electron donor during strict anaerobic growth and to aid growth of *C. jejuni* strains at the lower limits of oxygen during microaerophilic growth (Borden, 2004). Although in contrast to the enhanced growth for *C. concisus* with hydrogen as electron donor in microaerobic conditions (Yeow *et al*., 2020 ; Benoit *et al*., 2018; Lee *et al*., 2014), there is no proof of a beneficial effect of hydrogen gas on *C.jejuni* growth under optimal conditions.

In MHB, after 6h growth, the culture was in mid-exponential phase (OD_{600}, 0.015 and cfu, 3.5×10^8/ml) and by 18h had reached early stationary phase (OD_{600}, 0.32 and cfu, 4.3×10^9/ml). Between 48h and 72h the culture was in exponential decline, with a decrease in OD_{600} 0.4-0.27, and cfu decrease from 3.8×10^6-6.7×10^4 cfu/ml, between 60-72h. There was no significant impact of glucose during exponential or stationary phase of growth. But at 60h, there was close to a 1 $\log_{10}$ difference in viable count with $4.8X10^7$cfu/ml surviving in the presence of glucose compared to $3.88X10^6$cfu/ml in the absence of glucose (P=0.05). At 72h, the enhanced survival in the presence of glucose was clearer. Without glucose, the cfu was 6.70×10^4cfu/ml but with glucose, the cfu was almost 2logs higher at $4.43X10^6$cfu/ml (P=0.01, 72h).

Growth of Dg275 in DMEMf in the absence of glucose followed a similar pattern but was slightly lower than in MHB, reaching a maximum cfu of (7.12×10^8 /ml) at 24h with a sharp decline phase between 48-60h that decreased to 6.5×10^5/ml at 72h. The presence of 20 mM glucose in DMEMf had a more pronounced effect than in MHB. This can be explained by the limited availability of preferred nutrients in DMEMf and hence earlier use of glucose.

A significant difference in viable count was already detectable at early stationary phase. At 24 h, the cfu was 4.56×10^9/ml with glucose and 8.7 $X10^8$cfu/ml without glucose (P=0.05, 24 h). As with MHB, the benefit of glucose in the decline phase was also substantial in DMEMf, at 60 h, there was a 2 $\log_{10}$ difference between growth with glucose 2.70×10^8cfu/ml and without glucose $3.06X10^6$cfu/ml. The most striking difference in growth of Dg275 in DMEMf compared to MHB was the marked increase in OD_{600} of the culture when glucose was included in DMEMf, but not seen with MHB. Significant differences between growth (OD_{600}) with and without glucose when grown in DMEMf were seen at 24, 36 and 60h (P = 0.01, P = 0.005 and P = 0.0001, respectively).

In summary, addition of glucose had a similar effect in both media, conferring enhanced survival in the decline phase of growth. Dg275 grew better in the rich media MHB but addition of glucose to DMEMf had a bigger effect with significant impact and enhanced growth earlier in the stationary phase.

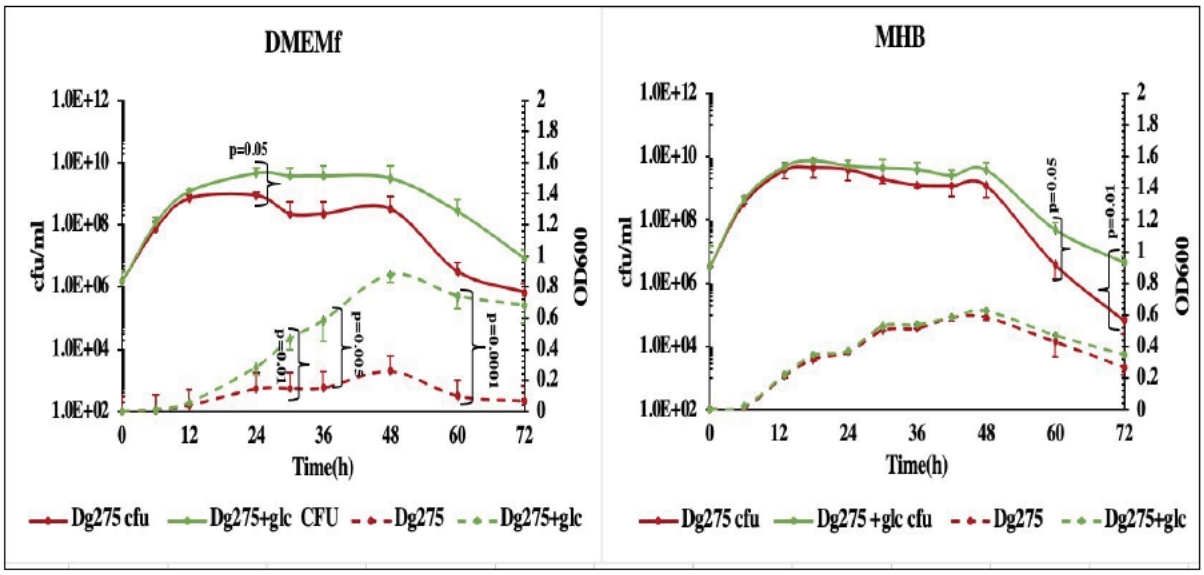


Figure 4. 1 Growth enhancement of Dg275 in the presence of glucose in shaking cultures. A standardised inoculum was prepared in MHB or DMEMf from bacteria grown on MHA for 38 h, as described in (chapter 2.4.2).150µl of the standardised sample (OD_{600} = 0.2) was used to inoculate 15 ml of respective media with (green) and without (red) 20 mM glucose in a 50 ml conical flask. The isolates were grown in an atmosphere of 5% O_2, 10% CO_2, 2% H_2 and 83% N_2 in an M35 cabinet with shaking at 150rpm at 37°C. Growth was monitored based on OD_{600} (dashed line) and cfu (solid line) at the intervals shown. The plotted points indicate the mean measurements of triplicate flasks; error bars indicate data from three independent experiments.

Transformation of *C. jejuni* from spiral into a coccoid form correlates with entrance into a nonculturable state. Hence, these cells have also been defined as being in a viable but nonculturable state, although whether they remain viable remains controversial (Ayrapetyan *et al*., 2015; Ikeda and Karlyshev, 2012). The effect of glucose on the shape and viability of Dg275 at late stationery and decline phase of growth in DMEMf was monitored by gram staining and light microscopy, as described in section 2.4.7 and with a bacterial viability assay kit and fluorescence microscopy as described in section 2.6.

Gram stained samples clearly showed spiral cells in both samples at 48h, see fig 4.2, but in the absence of glucose some cells remained spiral and others transitioned to the coccoid form. Notably, the lower density of cells in the absence of glucose was marked and this correlated with the very low OD_{600}, fig 4.1.

By 60h without glucose few cells remained and all cells were coccoid, while when grown with glucose, the image showed a dense mix of both spiral and coccoid cells.

The Bacterial viability assay kit permitted determination of viability and shape at the same time. Dg275 was inoculated and grown, as described in fig 4.3 for 48 and 72h. At each time point, cells were recovered from a 5ml culture, fixed, stained and visualised by fluorescence microscopy, as described section 2.6. Red colour of cells indicates that the dye can permeate the membrane and the cells are dead. With the 48h samples, live (green) spiral cells were present in both samples, although without glucose viable (green) coccoid cells dominated and some non-viable (red) coccoid cells were present, fig 4.3. In the last stage of growth at 72h, the difference was pronounced. All cells grown without glucose were coccoid and red (dead). In contrast, with glucose while half of cells were also red and coccoid (dead), while around half of cells remained green (viable). Of the green cells many were coccoid, but spiral cells were still visible. These microscopy results show the impact of glucose to aid survival of *C. jejuni* Dg275 in unfavourable conditions in the late stationary and decline phase of growth. However, if the viable (green) coccoid cells are culturable and can revert to the spiral form is not known.

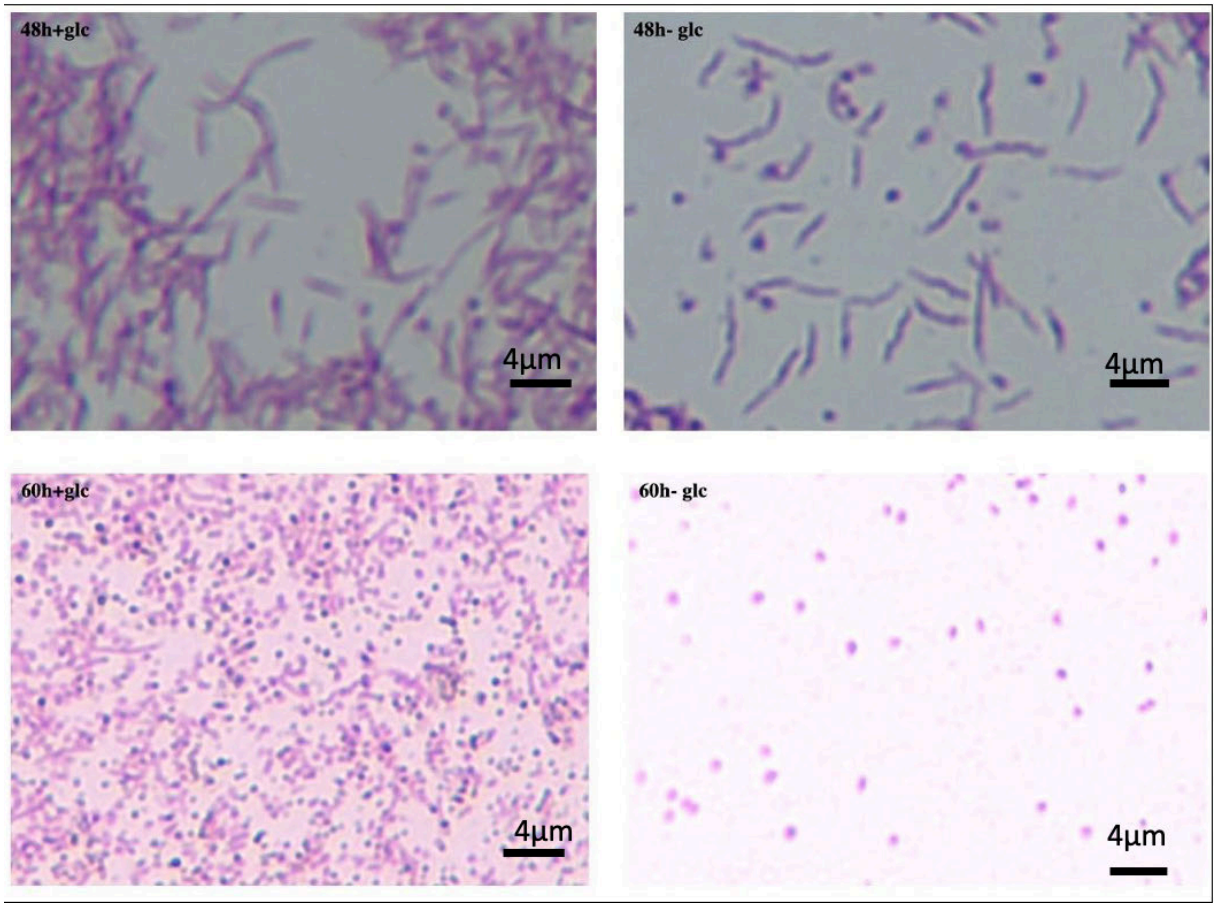


Figure 4.2 Spiral and coccoid morphology of *C. jejuni*. The Gram-stained isolates were observed with a high-powered light microscope at 1000×magnification. The bar represents 4 µm. The isolates were cultured in DMEMf with and without glucose under microaerophilic conditions at 37°C.

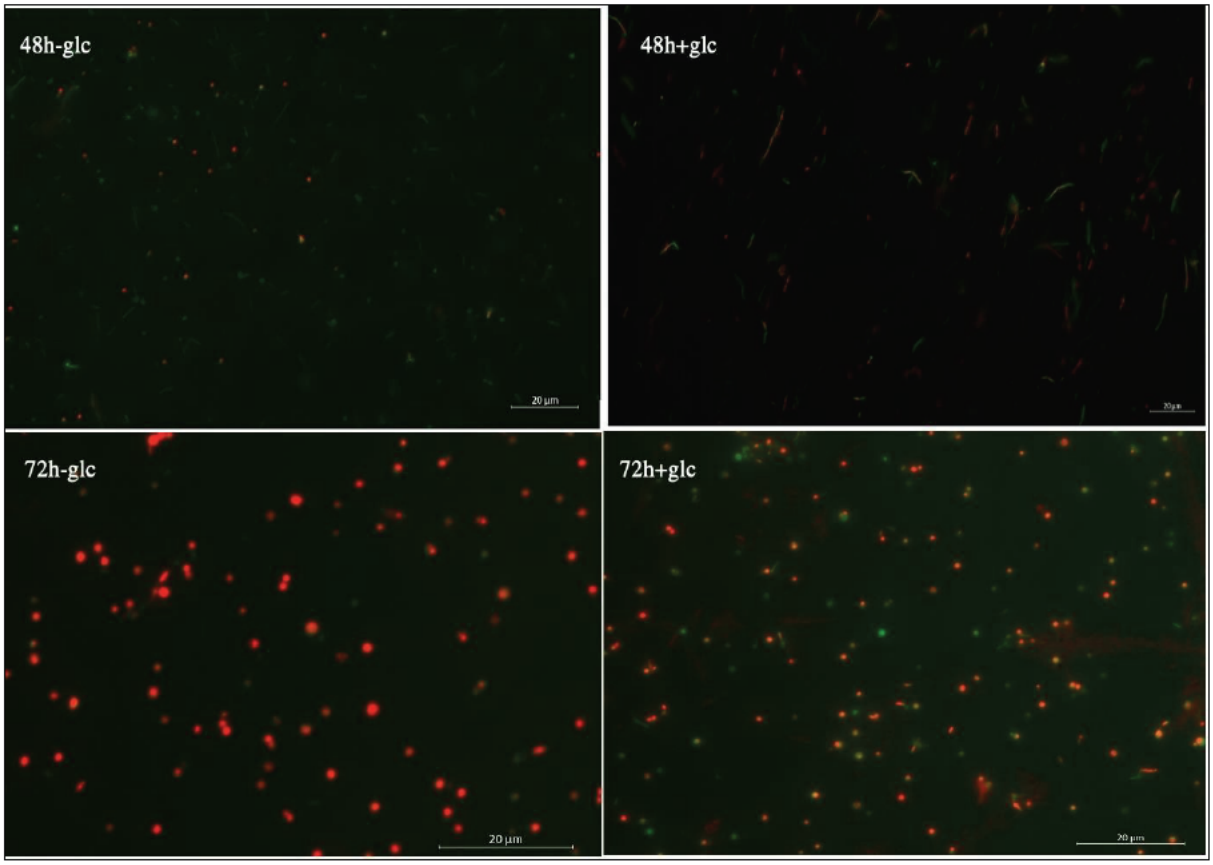


Figure 4.3 Viability and morphology of live and dead Dg275 (ED+) cells with and without glucose in DMEM media. Dg275 was grown in 15ml DMEMf under microaerophilic conditions at 37°C with shaking at 150rpm for 48 and 72h with and without glucose. Cells were then recovered, stained and fixed, as described in section 2.6. Samples were visualised using Ex/Em 490 nm/525 nm and 536 nm/617 nm FITC/TAMRA filter sets in a fluorescence microscope, at 600 × magnification.

4.3 Effect of glucose on growth dynamics of other ED positive strains in DMEMf

Dg43ED type 21/23 and 5 related strains were shown to possess a complete *glc* locus and showed enhanced growth when glucose was included in the medium. These strains represent a more phylogenetically distinct group of *C. jejuni* and of the *glc* locus. The dynamics of growth of three of these strains Dg18, Dg201 and Dg381 in DMEMf with and without glucose is shown in fig 4.4. These are compared to growth of Dg275, ED type2 and also to Dg200 a *C. jejuni* strain belonging to clonal complex CC-45, but with no *glc* locus. This reaffirmed the impact of the *glc* locus on *C. jejuni*. All strains exhibited very similar profiles with enhanced survival in the decline phase. All also exhibited the same 2-3fold increase in OD_{600} from stationary phase onwards, presumably correlating with increased growth in addition to survival in DMEMf in the presence of glucose. Notably, Dg200, *glc* negative, exhibited a small decrease in growth in the presence of 20 mM glucose, a result frequently noted.

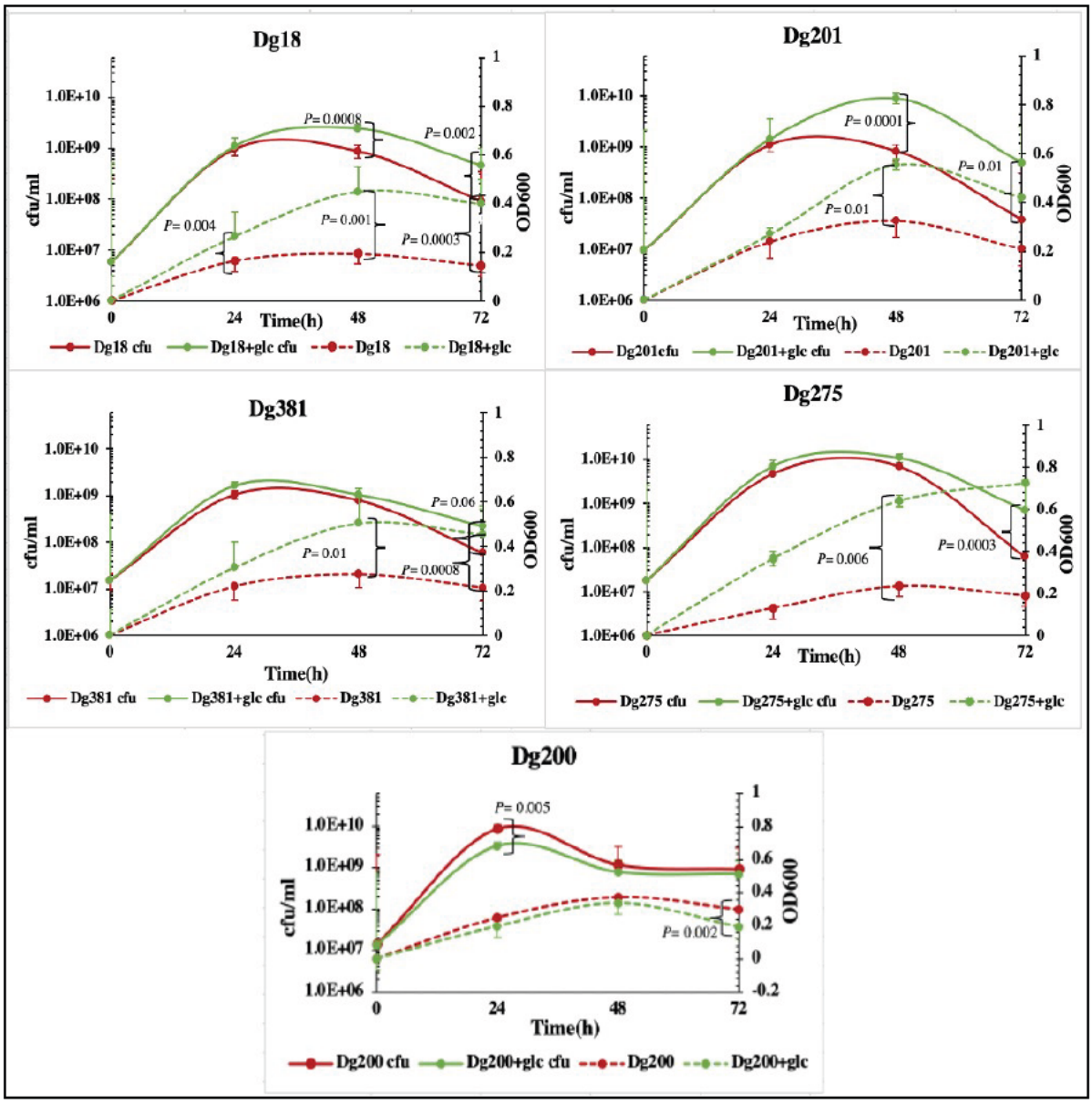


Figure 4.4 Growth enhancement of the *glc* loci Dg18, Dg201 and Dg381 in the presence of glucose in shaking cultures. A standardised inoculum was prepared in DMEM from bacteria grown on MHA for 38h, as described in chapter 2.4.4. 150µl of the standardised sample OD_{600} = 0.2 was used to inoculate 15ml of respective media with (green) and without (red) 20mM glucose in a 50ml conical flask. The isolates were grown in an atmosphere of 5% O_2, 10% CO_2, 2% H_2 and 83% N_2 in an M35 cabinet with shaking at 150rpm at 37°C. Growth was monitored based on OD_{600} (dashed line) and cfu (solid line) at the intervals shown. The plotted points indicate the meanmeasurements of triplicate flasks; error bars indicate data from three independent experiments.

4.4 Enhanced survival during growth in DMEMf with glucose is not related to protection from increased pH

C. jejuni grows optimally within a pH range of 6.5–7.5 and can grow in a more acidic pH but is inhibited at levels below pH 5.0. In the alkaline range, growth is inhibited above pH8.0 (Doyle and Roman 1981; Garénaux *et al*., 2008).

Similar to other *C. jejuni* strains, Dg275 preferentially utilises the amino acids Ser, Asp, Pro, Gln and Asn as carbon and energy sources, and its metabolism of Ser and other amino acids would result in an increased pH as ammonia is released. Hence, glucose metabolism could in theory protect ED positive strains of *C. jejuni*, such as Dg275 by decreasing the pH because of the production of acidic compounds following glucose metabolism. Dg275 was grown micro aerobically in DMEMf with or without 20mM glucose for 72h, and the samples were monitored for pH every 24h. pH was measured inside the M35 cabinet by using a hand-held pH monitor (Hanna Instruments). As shown in table4.1, during growth for 72h, the pH slightly increased to pH7.3 in the absence of glucose but was 0.2pH units lower pH7.1 in the presence of glucose. Although this small difference may be attributable to metabolic products from glucose utilisation, no difference in pH was found at 48h, and all pH levels monitored were within the optimal growth pH for *C. jejuni.* Hence, the maintenance of an optimum pH is unlikely to explain the marked protection from the decline in cfu during growth in the presence of glucose. In addition to monitoring pH, samples were collected during growth for NMR analysis to compare metabolic products during growth with and without glucose. Analysis of these samples is currently under investigation.

pH	DMEMf	24 h	48 h	72 h
Dg275(1)	7.5	7.03	7.19	7.33
Dg275(2)		7.03	7.15	7.3
Dg275(1)+glc		7.02	7.18	7. 01
Dg275(2)+glc		7.02	7.18	7.08
Blank-glc /Blank + glc		7.05/7.03	7.05/7.08	7.03/7.05

Table 4.1 pH of DMEMf during growth of Dg275 with and without glucose. Dg 275 was cultured on BA-2% plates for 42-48h. Approximately 1ml of a standardised sample (OD_{600}=0.002) prepared from bacteria grown on MHA was used to inoculate 15 ml of DMEMf with and without 20mM glucose. Cultures were incubated with shaking at 150rpm under microaerophilic conditions at 37°C. Samples of 1ml were taken and measured inside an M35 cabinet to check for pH at each time point.

4.5 Growth of Dg275 on pyruvate

Pyruvate is a main metabolic junction linking carbohydrate or amino acid utilisation to energy generation and biosynthetic pathways. In the ED pathway, pyruvate is considered a final product of glucose utilisation. Pyruvate plays an important role in decrease in the concentration of hydrogen peroxide, and helps *C. jejuni* to grow aerobically (Verhoeff-Bakkenes *et al*., 2008). *C. jejuni* uses pyruvate as an electron acceptor (Mend *et al*., 1997). Furthermore, pyruvate works as an alternative electron acceptor for fermentation (Hodge and Krieg, 1994). The effects of pyruvate on cell growth in the presence of glucose were tested by growing the ED-positive *C. jejuni* strain, Dg275, in 50ml conical flasks containing 15ml of DMEMf plus 20mM glucose or DMEMf plus 20mM pyruvate -/+ 20mM glucose in 5% O_2, 10% CO_2,2% H_2 and 83 % N_2 at 37°C with shaking at 150rpm. As previously described, growth in DMEDf+glucose was monitored based on OD_{600} and cfu/ml values.

The rapid metabolism of pyruvate was evident from the marked increase in growth of Dg275 during the exponential phase in DMEMf with addition of 20mM pyruvate, fig 4.5.

This was evident in both OD_{600} and cfu values. By 24h (early stationary phase) there was approximately 6-fold increase in OD_{600} and 10-fold difference in cfu/ml comparing growth in DMEMf plus 20mM pyruvate and DMEMf plus 20mM glucose.

There was no difference in either OD_{600} or cfu counts at 24h comparing growth in DMEMf with pyruvate alone and growth in DMEMf with pyruvate plus glucose. Thus, pyruvate was rapidly utilised and stimulated growth from early exponential phase and there was no evidence of inhibition or stimulation of growth by the presence of glucose at this stage. From 24h onwards (stationary phase and decline phase) the benefit of glucose was evident. At 36h and 60h growth cfu/ml values were significantly higher for growth in DMEMf plus glucose and pyruvate compared to growth in DMEMf with pyruvate alone (P=0.04, 36h and 0.006, 60 h). In the absence of glucose, cells grown in DMEMf plus pyruvate also died more rapidly than cells grown in DMEMf plus only glucose, fig 4.5.

These results suggest that pyruvate was rapidly depleted at 24h and then in DMEMf+pyruvate with glucose, the cells used glucose for growth and survival. Once again, the key factor is better survival of ED positive *C. jejuni* when glucose is available.

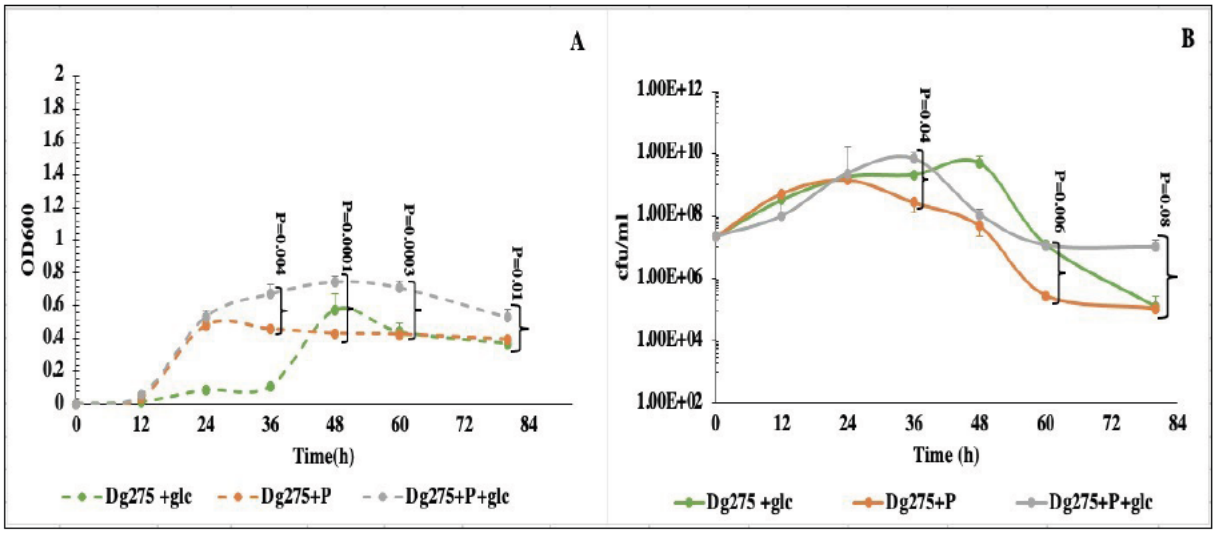


Figure 4.5 Growth of *C. jejuni* strain Dg275 in DMEMf supplemented with pyruvate. Cells were cultured on BA2% plates, and a single colony was subcultured in MHA for 36h and grown in DMEMf+g or DMEMf+ 20mM pyruvate -/+ glucose in 50ml conical flasks. Samples were incubated in an optimal condition (5% O_2, 10% CO_2 and 2% H_2) with shaking at 150rpm and with 70% relative humidity at 37°C for 80h. Samples were monitored for OD_{600} (dashed line) and cfu (solid line) at the time points indicated. Green = with DMEMf+ 20mM glucose, orange = DMEMf+20mM pyruvate only, grey = DMEMf+20 mMpyruvate+20mM glucose. The data presented are from triplicate flasks for each strain, which were prepared from the same standard inoculum and performed at the same time.

4.6 Testing of the ability of *C. jejuni* to utilise other sources of carbohydrates

Given that ED-positive strains of *C. jejuni* could clearly use glucose, the ability of several strains to grow on disaccharides containing glucose was tested. The two common disaccharides, lactose (galactose and glucose) and maltose (two units of glucose linked by an α (1-4) bond were tested for their ability to enhance the growth of several strains in DMEMf. The metabolism of either disaccharide would require either the secretion of an enzyme capable of cleaving the disaccharide or an uptake system, followed by specific cleavage. The first step was performed to test whether either could support growth *in vitro*.

4.6.1 Screening of several strains for enhanced growth on lactose or maltose

The ED-positive strains Dg18a, Dg43a, Dg268, Dg345 and Dg 275 were selected to test for maltose or lactose utilisation, whereas the ED-negative strain Dg200 was used as a negative control. These EDpositive strains are closely related phylogenetically, with slightly different ED profiles, see chapter 3. DMEMf supplemented with FBS was used, as this was the best media for growth with glucose. Either maltose or lactose was added to a final concentration of 20 mM, and the media were inoculated and incubated at 37°C with 150rpm shaking, as described in fig4.4 &fig 4.5 respectively. In initial studies, the OD_{600} of the cultures of all the ED-positive strains Dg18, Dg43, Dg268, Dg345 and Dg275 was enhanced in the presence of maltose, fig4.6A. These cultures typically grew to a maximum OD_{600} of approximately 0.7-0.8, but the maximum OD_{600} was typically 0.4- 0.55 in the absence of maltose. In each case, the difference was significant, with *P* values of (0.0008, 0.001 and 0.002) at 36h of growth of Dg18, Dg43 and Dg268, respectively.

At the later phase of growth 48–72h, differences in OD_{600} remained significant, with *P* values in the range of $P = 0.001–0.008$ for all the ED-positive strains. Cfu/ml were determined for the ED-positive strains Dg18, Dg43 and Dg275 and the ED-negative strain Dg200, fig 4.6 B. For Dg18 and Dg43, the samples at 24 and 36h also reflected enhanced growth in maltose, with a six-fold increase in cfu/ml at 24h for both strains and three- to five-fold increase at 36 h at Dg18 and Dg43, respectively ($P < 0.001$ at 24 and 36h for Dg18, < 0.01 for 24h and < 0.001 for 36h for Dg43). By 48 and 72h, the cfu/ml for these strains was similar both in the presence and absence of maltose. For Dg275 the only significant difference was found in the 48h sample. In contrast to the cultures of the ED-positive strains, those of the EDnegative strain Dg200 showed no enhanced growth in the presence of maltose based on either their OD_{600} or cfu values.

Maltose appeared to marginally suppress the growth of Dg200. This result is expected, as Dg200 does not contain an ED pathway, which is required for the downstream catabolism of glucose.

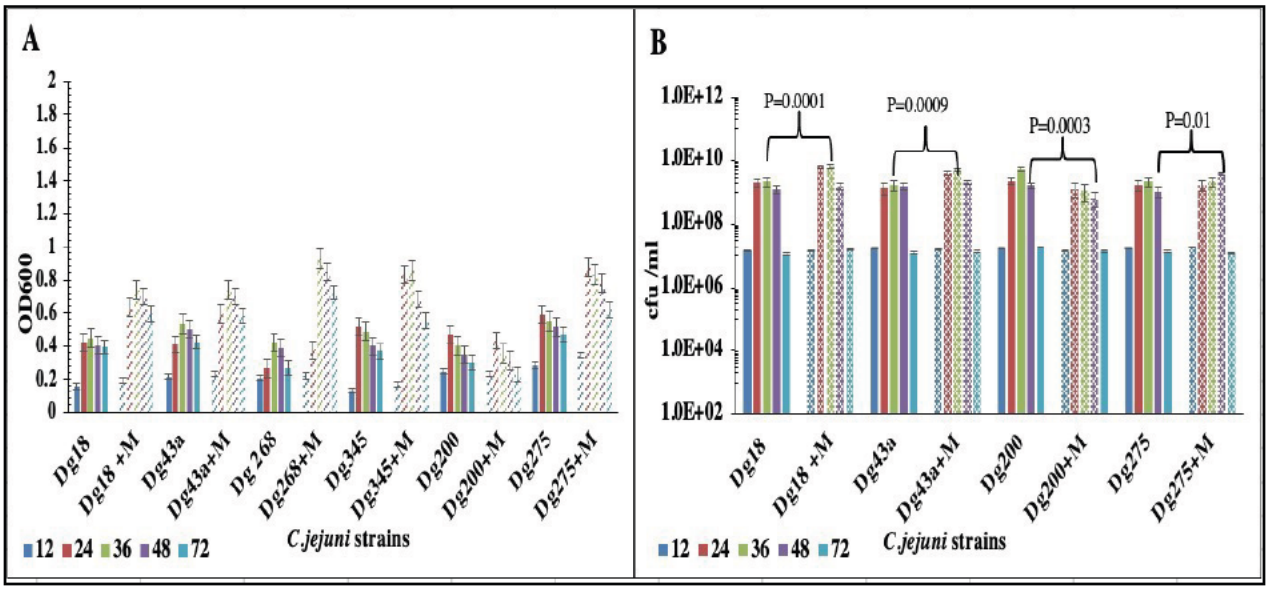


Figure 4.6 Screening for the ability of maltose to support the growth of ED-positive *C. jejuni* strains. Approximately 1ml of a standardised sample (0.02 OD_{600}) prepared from bacteria grown on MHA was used to inoculate 10ml of DMEMf + FBS in the presence (hatched bars) or absence (solid bars) of 20mM maltose. *C. jejuni* strains Dg18, Dg43a, Dg268, Dg345, Dg275 and Dg200 are as indicated. Cultures were grown with shaking at 150rpm in a jar with a microaerophilic gasbag. Growth was monitored based on OD_{600} (A) and cfu/ml (B). Average and SE were calculated from triplicate experiments performed on separate occasions. *P*= statistical differences between growth in DMEMf-FBS with and without 20mM maltose at the same timepoint of growth.

The five *C. jejuni* strains Dg18a, Dg43a, Dg268, Dg345 and Dg 275, +glc with Dg200-glc were screened for their ability to grow on lactose, along with the same negative control Dg200, ED-. Strains were tested in 25ml conical flasks with DMEMf-FBS with or without 20 mM lactose for 3 days. These initial trials indicated no evidence of enhanced growth of any strains in the presence of lactose when monitored based by cfu.

The cfu count was tested for the five ED-positive strains Dg18, Dg 43, Dg268, Dg345 and Dg275, with Dg200 as the negative control, fig4.7B. For all the ED-positive strains and the negative strain, no significant difference in cfu was found between the cultures with and without lactose. Furthermore, all the strains showed no growth at 72h, fig 4.7B. Hence, in contrast to the preliminary evidence that the ED- positive strains may be able to use maltose, no evidence showing that they could use lactose for growth was found.

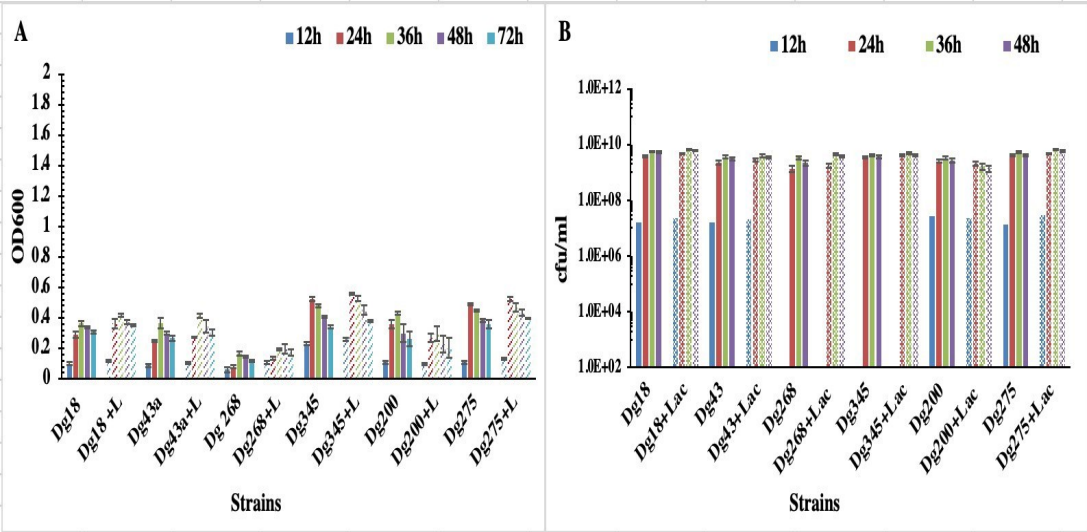


Figure 4.7 Screening for the ability of lactose to support the growth of ED-positive *C. jejuni* strains. Approximately 1ml of a standardised sample (0.02 OD_{600}) prepared from bacteria grown on MHA was used to inoculate 10ml of DMEMf + FBS in the presence (hatched bars) or absence (solid bars) of 20mM lactose. *C. jejuni* strains Dg18, Dg43, Dg268, Dg345, Dg275 and Dg 200 are as indicated. Cultures were grown with shaking at 150rpm in a jar with a microaerophilic gasbag. Growth was monitored based on OD_{600} (A) and cfu/ml (B). Average and SE were calculated from triplicate experiments performed on separate occasions. Data were collected from three biological replicates.

4.6.2 Measurement of maltose utilisation

The ED positive strains Dg95 and Dg43 and the mutant Ch95-95(1) *glk* (see chapter 5 for detail) were tested for their ability to utilise maltose in DMEMf medium. Both RG2 strains Dg95 and Dg43 grew well in the presence of glucose. These strains contain a complete *glc* locus and Ch95-95(1) carries a *glk* pseudogene (Mohammed, 2018) and hence cannot utilise glucose.

Strains were subcultured from a 36h MHA culture into fresh DMEMf with and without maltose in the M35 cabinet with optimal microaerobic conditions (5% O_2,10% CO_2,2% H_2, **and** 83%N_2). In contrast to the earlier experiment, Dg43 grew similarly in DMEMf with and without maltose, the improvement in growth may be due to the addition of FBS. Dg 95 showed only a small but significant enhanced survival at the 72h time point. As would be expected Ch95-95*glk* (1) showed no difference in growth whether glucose was present or not.

The ability of the *C. jejuni* strains to utilise maltose and the maltose concentration and glucose amount in culture were measured using the Maltose and Glucose Assay Kit (Abcam, ab65335) as described 2.5. Maltose concentration was calculated at zero time and at the end of culture. No evidence of decrease in maltose (as measured by glucose concentration) was evident in the 72h sample.

In fact, there appeared to be a slight increase in free glucose, possibly related to period of incubation of maltose till 72h. These results correlate with the very low level of enhance growth in the presence of maltose alone fig4.8. From these results the conclusion is that Dg95 does not possess the ability to degrade and utilise maltose.

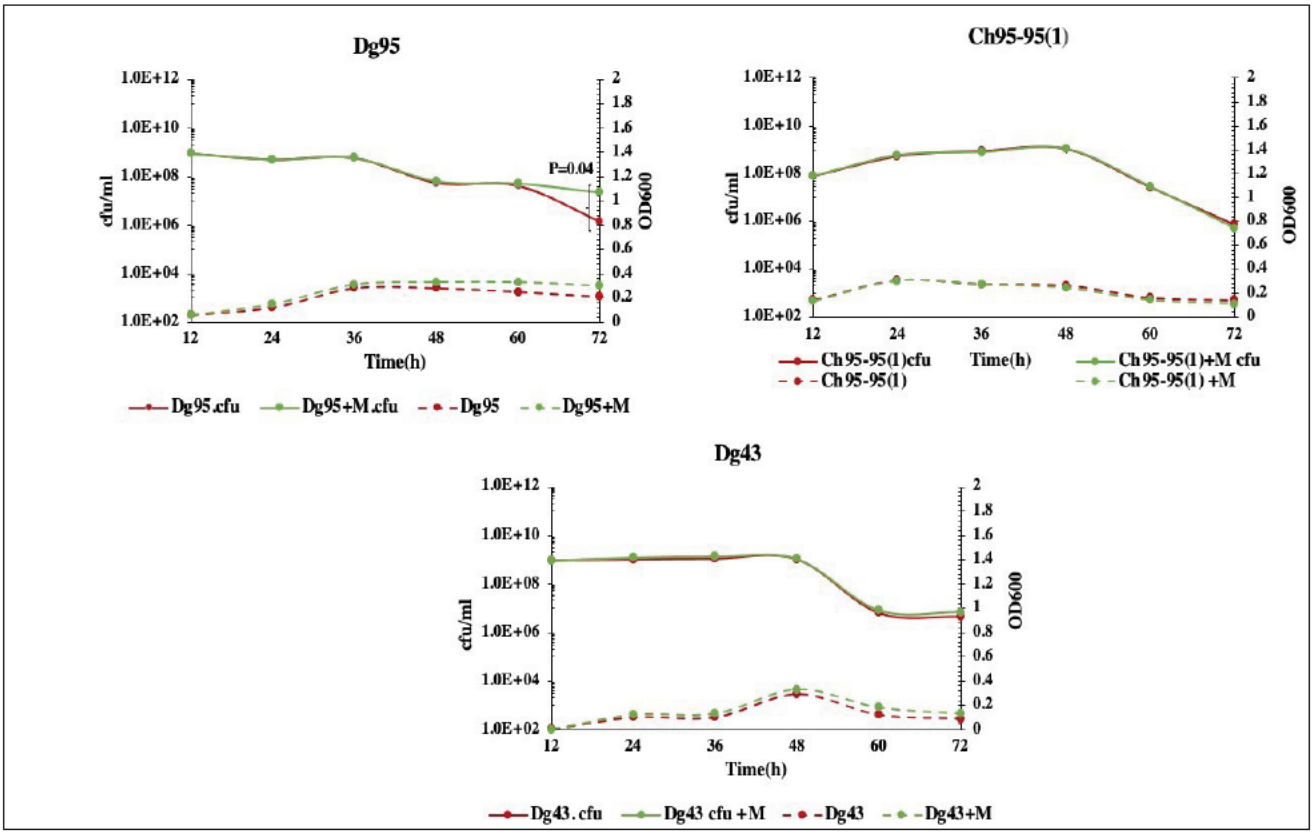


Figure 4.8 Growth of RG2 in DMEMf minimal media supplemented with maltose. Stock cultures of strains were subcultured on BA2% plates, incubated for 30–36h and then subcultured on MHA for 36h. Standardised cells from the MHA plate were diluted in DMEM medium to an OD_{600} of 0.2, and 150µl was used as the inoculum. The strains were grown in 50ml conical flasks containing DMEM with 0.2mM iron and 20mM maltose with shaking at 150rpm. Growth was monitored based on OD_{600} (A) and cfu (B). The dotted line refers to the OD_{600} of the control. The solid line refers to the cfu results. The red and green colours refer to DMEMf medium without and with maltose, respectively. The error bars of all the strains are data from three biological replicates.

4.7 Discussion

The observed growth dynamics of *C. jejuni* showed that its growth depends on its nutritional environment. The growth stages were similar for both media based on cfu and OD_{600}. However, the growth in MHB was better than that in DMEMf. As shown in the results presented in section 4.2, glucose has a significant effect on the survival of the cells in DMEMf media (minimal media). Catabolism of glucose, via the ED pathway has been demonstrated for *C. coli* strains carrying the *glc* locus (Vorwerk *et al*., 2015; Vegge *et al*., 2016). Mohammed demonstrated the consumption of glucose by *C. jejuni* Dg275, correlating with enhanced survival at later stages of growth, and studies presented here confirm a similar glucose enhanced growth for all *glc* positive *C. jejuni* strains tested (Mohammed, 2018). Hence, the assumption is that all of these *C. jejuni* strains carrying the *glc* locus, are metabolising glucose via the ED pathway, contributing to energy provision and potentially also biosynthesis.

During growth of Dg275 in DMEMf with glucose the benefit of glucose availability was seen earlier than when grown in the rich medium, MHB. Thus, the ED pathway may be useful during colonization of certain hosts or in an environment with readily available glucose, in the absence of other preferred nutrients. During *in vitro* growth of Dg275, glucose is utilised only following depletion of Ser and Asp in both MHB and DMEMf (Mohammed, 2018). It would be interesting to further explore benefit of possession of the *glc* locus in different *in vivo* environments. A bacterial viability assay confirmed extended survival of Dg275 cells in the spiral form if grown in glucose and also demonstrated that a substantial proportion of coccoid cells in the late decline phase were identified as viable (green) as opposed to those grown without glucose which were all defined as non-viable (red). This result correlates with the higher cfu counts in these samples, although whether the higher count is solely due to the presence of spiral cells or whether the viable coccoid cells contribute to the cfu would require more detailed investigation.

pH plays a role in the growth of *C. jejuni*. Reid *et al.*, indicated that the regulation of genes, such as *hrcA* and *hspR*, plays a role in reducing the effect of pH changes; *hrcA* or *hspR* deletion caused a slight but statistically significant increase in the sensitivity of *C. jejuni* to acid killing (Reid *et al*., 2008). The results in the current study indicated that the *in vitro* growth of Dg275 in DMEMf did not significantly affect pH. MLST data showed that Dg275 contains two genes, which encode the putative heat shock regulator (*hrcA*, K03705) and heat shock transcriptional regulator (*hspR,* K13640) (Reid *et al*., 2008). After the ability of *C. jejuni* to use glucose was evaluated, six strains of RG2 were tested for their ability to use other types of sugars, such as maltose and lactose. The strains included Dg 18, Dg43a, Dg268, Dg345, Dg95 and Dg275 as positive strains and Dg200 and Ch95-95(1) as negative control strains. The initial screen indicated enhanced growth of Dg275 in DMEMf with 20mM maltose to the minimal media, but repeat assays and maltose quantitation were inconsistent with maltose utilisation. FBS was omitted from the repeat assays. Contaminating glucose, either in the maltose or FBS, might explain the initial enhanced growth on addition of maltose. Growth on maltose would require either a transport system to bring maltose into the cell and then metabolise it as described for *E. coli* (Raibaud and Schwartz, 1980; Freundlieb and Boos, 1986). Or a secreted enzyme such as amylase to degrade the maltose externally. The conversion of maltose to glucose would be followed by the phosphorylation of glucose by *glcK* to produce glucose-6P and entry into the ED pathway. No annotated genes for maltose uptake or degradation in *C. jejuni* Dg275 were readily identifiable. The results showed also that strains could not utilise lactose. Hence, it was concluded that Dg275 can transport and metabolise glucose but not disaccharides or polymers containing glucose.

Chapter 5

Characterisation of naturally evolved mutants unable to utilise glucose

5.1 Introduction

Chickens are considered a natural host for *C. jejuni* (Yogasundram *et al*., 1989), which is a commensal of the intestinal flora of chickens (Hermans *et al.,* 2012). However, the *glc* locus had not been found in any of the chicken isolate genome sequences in the PUBMLST/campylobacter database (Vegge *et al*.,2016). In a previous chicken colonisation trial (Mohammed, 2018), Ross chickens were infected with ~10^7 cfu of *C. jejuni* strains isolated from Norway rats, using the oral gavage method. The two *glc* positive strains tested were Dg275 and Dg95. Both of these RG2 strains colonised the caecum of the chickens with a recovery of ~2.5 $\log_{10}$ cfu/g at 7dpi (days post infection). Although Dg275 and Dg95 were less effective colonisers than several 'classic' *glc* negative Norway rat isolates of *C. jejuni*, such as Dg200 (ST45CC45), Dg153 (ST42CC42) and Dg194 (ST21CC21), the study demonstrated efficient colonisation of Ross chickens at both 3 dpi and 7 dpi by Dg275 and Dg95. Strains recovered from chicken caceal contents were confirmed by PCR as the correct *C. jejuni* strain and monitored for glucose utilisation (Mohammed, 2018). Interestingly, the five separate colonies (designated Ch95-95 (1-5) analysed from Chicken number 95, which had been colonised with *C. jejuni* Dg95, were unable to utilise glucose despite possessing the *glc* locus. This was in contrast to the 20 colonies analysed from the other 4 chickens infected with Dg95 and the 25 colonies analysed from chickens infected with Dg275. All retained the ability to utilise glucose. In this study, attempts to delete the entire *glc* locus (~6 kbp) from *C. jejuni* strain Dg275 were unsuccessful. Therefore, as a naturally derived mutant was already available, mutations in Ch95-95 isolates were further analysed to enable use of this strain as a *glc* negative derivative of Dg95.

5.2 Loss of the ability to utilise glucose following passage of Dg95 in chickens

Five single colonies had been stocked from the day seven caecal samples from each of the six chickens colonised with Dg95 (Mohammed, 2018). To test ability to use glucose, strains were tested in a microtiter plate growth assay for enhanced growth in DMEMf in the presence of glucose, fig5.1. The results demonstrated typical enhanced growth of the challenge input strain Dg95 in the presence of glucose. For Dg95, the OD_{600} reached a maximum of 0.3±0.060 without glucose compared to 0.5 ±0.0017 with glucose at 48 h (P<0.004). Also, the Dg95 strain continued to grow better with glucose at 72h, when OD_{600} was 0.32±0.024 without glucose compared to 0.46±0.028 with glucose (P<0.001). At the last point of growth, at 96h, the difference was still evident. The OD_{600} with DMEMf +glc was 0.45±0.188, compared to DMEMf –glc, which was 0.31±0.052, fig 5.1. In contrast, all five recovered colonies from Ch95-95(1-5) showed no enhanced growth with glucose.

Indeed, the addition of 20 mM of glucose to DMEMf led to a small decrease in the optical density of these cultures. For example, for Ch95-95(1), grown in the presence of glucose, the OD_{600} at 48h was 0.35± 0.054, compared to 0.43±0.021 with cultures grown in the absence of glucose (P<0.08), fig 5.1. Continued growth without glucose was slightly better than cultures with glucose. At a later stage of growth, the OD_{600} was 0.3±0.13 and 0.25±0.141 without glucose, compared to 0.26±0.072 and 0.23 ±0.163 with glucose. P values were not significant at the last two-time points72h and 96h. Ch95-95(2-5) also grew in the same way as Ch95-95(1). In conclusion, the presence of glucose had no apparent beneficial effect on the growth of recovered strains from chicken Ch95. These strains behaved in the same way as other *glc* negative strains that have been tested previously, such as Dg200, fig4.4, chapter 4.

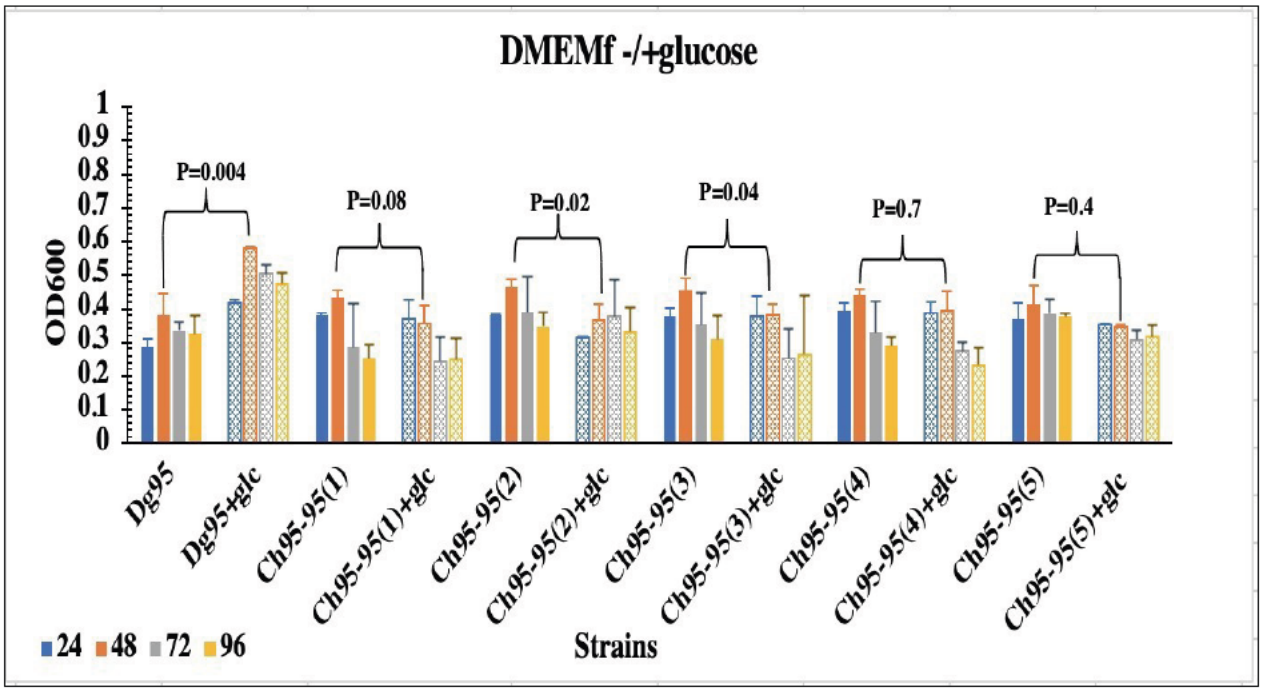


Figure 5.1 Testing ED-positive isolates recovered from chicken trials for glucose utilisation. DMEMf medium was inoculated, starting OD_{600} 0.002, with Dg95 (chicken inoculum) and 5 isolates Ch95-95(1-5) from Chicken number 95 from previous chicken trials (Mohammed, 2018). Strains were incubated for 96 h at 37°C in microtiter plates with and without 20mM glucose, shaking at 600rpm. Aliquots were taken at indicated time points and monitored by OD_{600}. In the presence (hatched bars) or absence (solid bars) of 20mM glucose, SE was determined from three replicate wells for each strain.

After the inability of Ch95-95 strains to use glucose in DMEMF media was confirmed, Ch95-95(1) was tested in MHB-FBS in a 50 ml conical flask growth assay for enhanced growth in the presence of glucose, fig 5.2. The results showed the enhanced growth of the input strain Dg95 in the presence of glucose. The OD_{600} reached a maximum of 0.52±0.002 without glucose compared to 0.76±0.007 with glucose at 48h (P<0.005). At the same time, the OD_{600} actually decreased from 0.7± 0.007 without glucose, to 0.66±0.0141 with glucose (P<0.01) for Ch95-95(1). At the last time point of growth (72h), the Dg95 strain survived better with glucose than

without; OD_{600} was 0.27±0.014 without glucose compared to 0.5±0.007 with glucose (P<0.001). In contrast, growth and survival of Ch95-95(1) was not significantly different with or without glucose, where OD_{600} with glucose was 0.4±0.006 and 0.46± 0.03 without glucose. Cfu results also indicated that there was no significant difference in the rate of growth and survival of Ch95-95(1) during culture in the presence or absence of glucose, at any of the time points that were tested, fig 5.2. This confirmed the failure of Ch95-95 (1) to use glucose. In contrast, for Dg95, in the presence of glucose viable count was two times higher, 4.28E+09 cfu/ml compared to $2.19X10^9$ cfu/ml following 48h growth in the absence of glucose, while at 60h, cfu was $1.57X10^8$cfu/ ml without glucose, compared to $4.75X10^8$cfu / ml with glucose, fig 5.2.

In conclusion, the results of this experiment confirmed the inability to use glucose and that it had no apparent effect on growth in the Ch95-95 strains, with there being no significant difference between growth with and without glucose. On the contrary, for Dg95, the glucose aided growth.

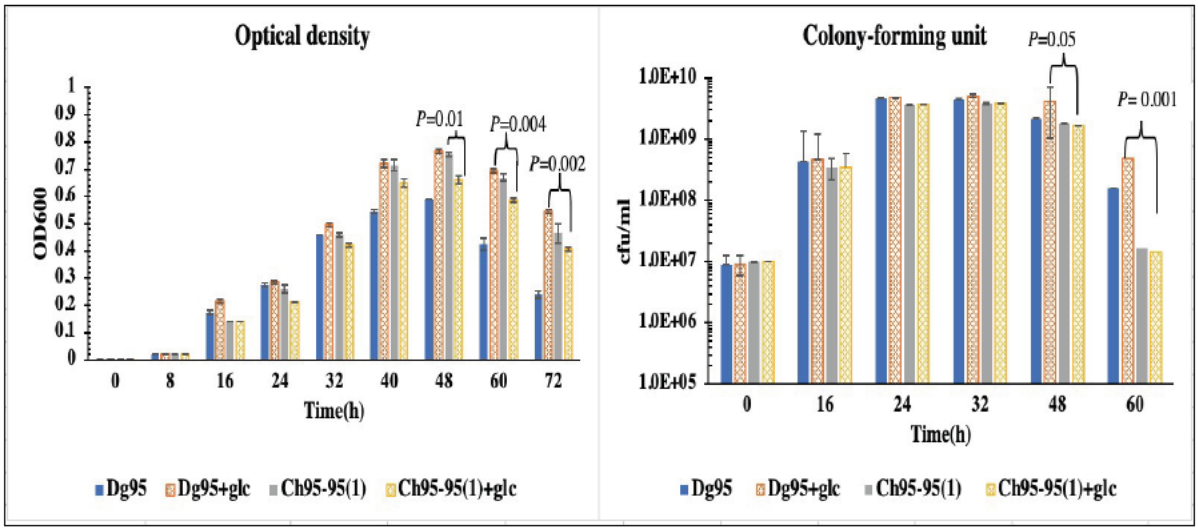


Figure 5.2 Growth of wild type Dg95 and natural mutation Ch95-95(1) in MHBFBS supplemented with glucose. Strains were subcultured from BA2% plates, onto MHA medium and grown for approximately 36h at 37°C. Standardised cells, from the MHA plate, were diluted in MHB medium to 0.2 OD_{600} and 150µl used as inoculum in 15ml MHB- FBS ± 20 mM of glucose. 50ml conical flasks incubated at 37°C, 150rpm with 60% humidity. Solid bars represent growth without glucose, while dotted bars represent growth with glucose. Error bars were derived from duplicate technical repeats. *P* values, compare significant differences between Dg95 and Ch95.

5.3 Identification of mutations affecting the utilisation of glucose by Sanger and Nanopore sequence

The presence of selected genes of the *glc* locus in each of the Ch95-95 isolates had been confirmed by PCR analysis using primers eda-zwf, zwf-pgi, and pgi-glcP and WGS of Ch95-95(1) had identified the mutation Gly167 to a TGA stop codon in glucokinase (*glk*) of Ch95-95(1) (Mohammed, 2018). In this study, to establish if all five Ch95-95 isolates carried the same mutation in the *glk* gene, *glk* was amplified from isolated gDNA of each strain, fig5.3 and sent for Sanger sequencing. The same size PCR fragment was amplified from all strains, confirming no major deletion within the *glk* gene.

5.3.1 Sanger sequence

Three of the five strains Ch95-95(1,2,3,4,5) were tested using Sanger sequence analysis. DNA sequence from these three strains, Ch95-95-1,2,3, each showed differences in the section of the *glk* gene that was sequenced fig5.4. In Ch95-95(1), there is a single nucleotide polymorphism (SNP) GGA to TGA at 490bp in the *glk* gene, corresponding to codon Gly167. TGA acts as a stop codon and results in a truncated glucose kinase which would be consistent with non-functionality of the *glc* locus. Surprisingly, the *glk* gene in Ch95-95(2) did not have a stop codon at 490bp as in Ch95-95(1), but possessed a different mutation within *glk*. In Ch95-95(2) *glk* there is a SNP at 499 bp leading to the codon change GGG to TGG. This results in mutation of the consensus carbohydrate binding site, EXGH (Coombes *et al*., 2020), where Gly(G) encoded by GGG is replaced by Trp (W) (TGG) resulting in the amino acid sequence EGWH fig5.4.

For Ch95-95(3), no defect appeared at 490bp and499bp as in Ch95-95(1), Ch95-95(2), respectively. As this strain was incapable of using glucose, this strain needs more sequencing and analysis.

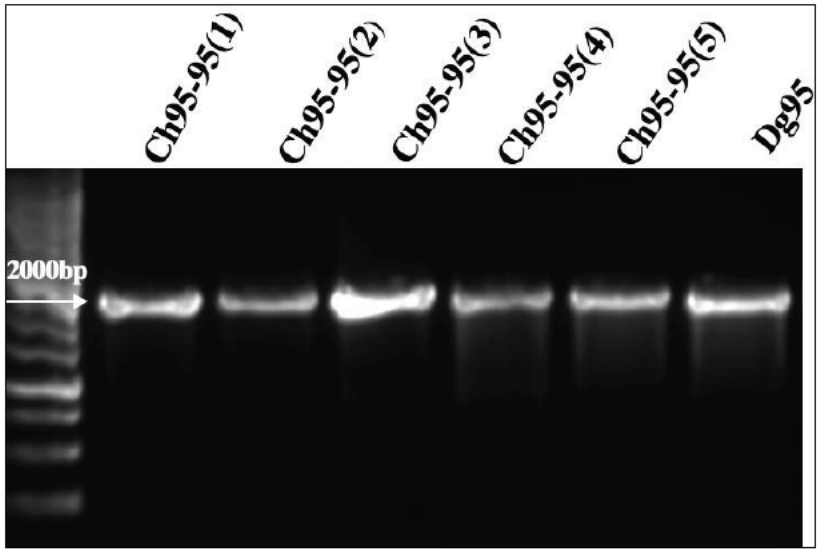


Figure 5.3 Agarose gel of amplified *glk* from Dg95 and recovered Ch95-95 colonies. Primers and primer characteristics are listed in the Methods section of Chapter 2. Primers pglF and pgiR were used to amplify a 2.09 kbp fragment, including the complete *glk* gene, using purified gDNA from Dg95 and isolates Ch95-95(1-5) as template.

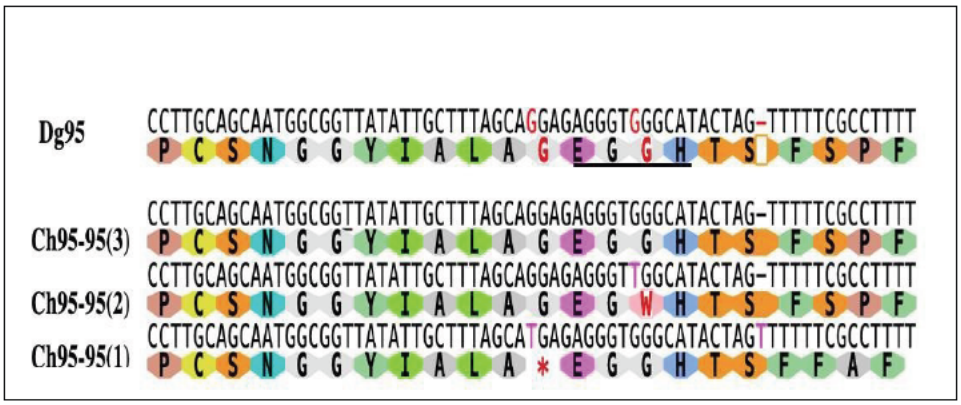


Figure 5.4 Multiple alignment *glk* nucleotide and amino acid sequences for Dg95 and Ch95-95 strains. PCR products (Figure 5.3) were sequenced with primers pglF and pgiR, and sequences were aligned using DNA Dynamo. Shown are nucleotide residues 457 to 522 of the *glk* gene. The black uppercase lettering refers to DNA, while aa residues are bolded. The asterisk (*) represents the stop codon in Ch95-95(1) Gly 167, W in Ch95-95(2). The bold black line refers to the EGGH consensus sequence of *glk*.

5.3.2 Whole genome sequencing for some chicken strains and Dg95

Draft Illumina genome sequences of Dg95 and Ch95-95(1) were already available (Mohammed, 2018). Mutation within *glk* was the sole mutation within the *glc* locus of this strain. To search for the presence of other common mutations outwith the *glc* locus that might affect expression or function of the *glc* locus, gDNA from Ch95-95(2) and Ch95-95(3) was also subjected to Illumina whole genome sequencing.

There are two copies of the *glc* locus, and hence *glk*, within Dg95 (see Chapter 3). There was no evidence of heterogeneity within the site of mutation in either Ch95-95(1) or Ch95-95(2), on analysis of PCR amplified *glk* by Sanger seqeuncing. While this indicates the same mutation within both sites, it was important to confirm this and this was done using Nanopore sequencing. Nanopore sequencing was also used to confirm draft assembly and establish a closed genome sequence, gDNA was prepared using a Monarch genomic DNA purification kit and the quality of DNA used for sequencing is shown in fig 5.5 and Table 5.1. Illumina sequencing was performed commercially by Novogene. Nanopore sequencing and associated bioinformatics analysis was kindly performed by Dr. Soon Gweon (University of Reading). The assembled annotated genomes of Dg95, Ch95-95(1) and Ch95-95(2) were 1.667.271 Mbp, 1.666.696 Mbp and 1.667.268 Mbp, respectively. Dg95 is a wild type glucose utilising strain of *C. jejuni* isolated from farm associated Norway rats, while Ch95-95(1) and Ch95-95(2) represent non-glucose utilising mutated strains of Dg95, which evolved during colonisation of chicken 95.

Strains	Nano drop ng/ µl	260/280	260/230	Conc. (ng/ µl (Fluorescence)
Ch95-95(1)	61.8	1.77	1.96	51.1
Ch95-95(2)	45.65	1.76	2.5	39.3

Table 5.1An example of DNA purification, estimated from the two ratios 260/280 and 260/230. DNA purity is routinely determined using 260/280 ratios. Table showed 260/280 ratios within an acceptable range. A260/A230 within a range of 1.80 to 2.50 is generally considered acceptable and clean.

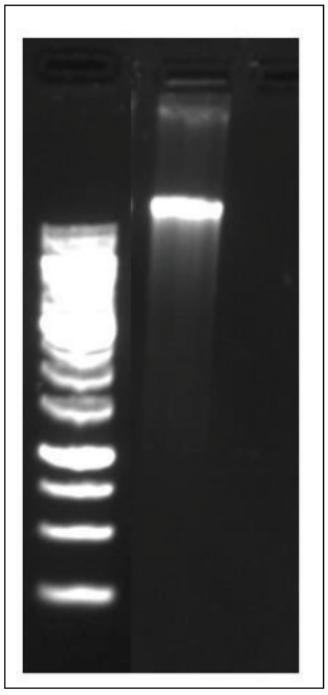


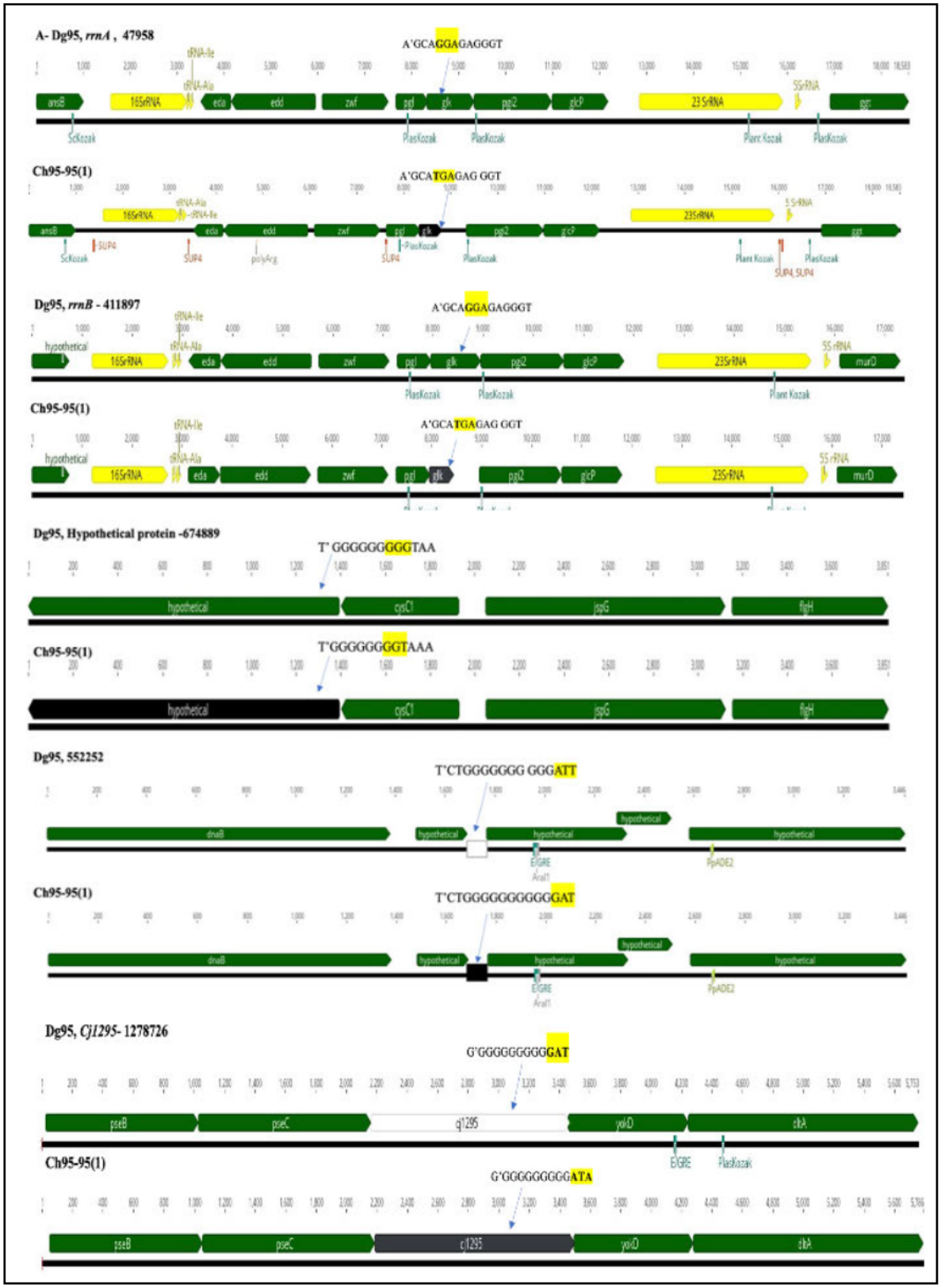
Figure 5.5 Gel electrophoresis for quality of DNA for sequencing. The Ch95-95(2) strain was used for extraction. DNA using a Monarch genomic DNA purification kit. 1 µl of DNA ladder and 2 µl of the genomic DNA plus 2 µl of blue dye for both. It ran for 40 min at 100 V in 1.0% = 0.5 g of Agarose + 50 ml of 1XTAE buffer.

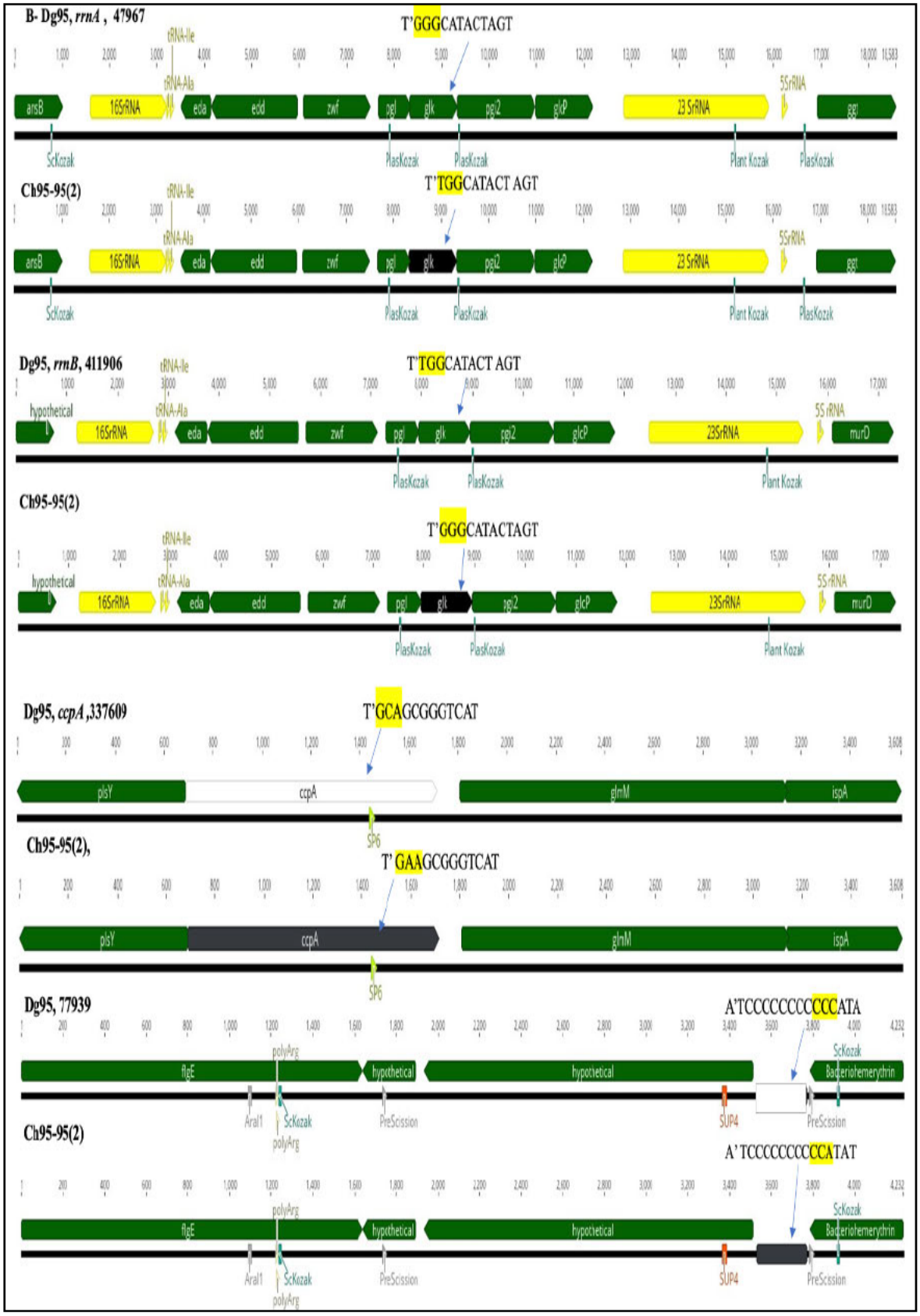
A comparison of the closed genome sequences of Ch95-95(1) and Dg95 confirmed the single mutation within the *glc* locus, a point mutation in *glk* encoding a stop codon in place of Gly167, and hence, a truncated glucokinase. The presence of the same single *glc* mutation in both copies of *glk* positions 47958 within *rrnA* and 411895 within *rrnB* was confirmed for Ch95-95(1). In addition, a comparison of the closed genome sequences helped to detect other changes outside of the *glc* loci. These are listed in table 5.2. At position 552252, there was an additional G in Ch95- 95(1), not in Dg95. Also, there were two sites where a single G was deleted, at positions 674889 and 1278726.

Interestingly, a comparison of Ch95-95(2) with Dg95 confirmed that the G to T substitution in Ch95-95(2), as highlighted in fig5.4 and 5.6, is the only mutation within the *glc* locus. This strain did not possess a GGA to TGA SNP, as was seen in Ch95-95(1). Again, the same substitution was present in both copies of the *glc* locus, at positions 47967 and 411906 on the closed genome. In this case, as highlghted by Sanger sequencing, this substitution leads to a significant active site amino acid substitution Gly170Trp. Also, the comparison identified eight sites with changes outside of the *glc* loci. In five positions a single G or C had been deleted (77939, 485112, 564023, 674889 and 1278726), one position involved a substitution (337609), and in two sites 668755 and 138379 an additional C had been inserted, fig5.6, table5.2. Significantly, Gly170 was within the consensus sugar binding site, EXGH, of glucokinase (Coombes *et al*., 2020). Hence, both the substitution to a stop codon and the mutation of the sugar binding site can explain the loss of a functional glucokinase, and hence, glucose metabolism via the ED pathway. Of the 14 chickens that were inoculated with Dg95 or other *glc* positive strains, Ch95-95 was the only chicken in which a non-functional *glc* locus was recovered (Mohammed, 2018).

Strains	Gene/ gene product	Position (Dg95)	Ref	M	Sequence		Position	Types of mutation
Ch95-95 (1)	***glk* (CAMP2019)**	47958	G	T	W	A'GCAGGAGAGGGT	Gly164	
					M	A'GCATGAGAG GGT	Stop, pseudogene	Nonsense
	***glk* (CAMP2019)**	411897	G	T	W	A'GCAGGAGAGGGT	Gly164	
					M	A'GCATGA GAG GGT	Stop codon (pseudogene)	Nonsense
	Intergenic (Hypothetical protein)	552252	G	GG	W	T'CTGGGGGGG GGGATT	-	SSM / Insertion
					M	T'CTGGGGGGGGGGGAT		
	Hypotheticalprotein (C)	**674889**	**GG**	**-G**	W	TTT ATGGGGGGGGGT AAA	Phe182, Met183, nine amino acid to Lys192	SSM, frameshift
					M	CTT TATGGGGGGGGT AAA	Leu182, Tyr183, nine amino acid to , Stop (pseudogene)	
	***cj1295*(CAMP1213)**	**1278726**	**GG**	**-G**	W	G'GGGGGGGGGGAT.....TCT	Gly32, Gly, Gly, Asp35, six amino acids, Ser41	
					M	G'GGGGGGGGGATA ....TAA	Gly32, Gly, Gly, Ile35, six amino acid, Stop (pseudogene)	SSM, frameshift
Ch95-95 (2)	***glk* (CAMP2019)**	47967	G	T	W	T'GGGCATACT AGT	Gly167, His168, Thr169, Ser170	
					M	T'TGGCATACT AGT	Trp167, His168, Thr169, Ser170	Missense
	***glk* (CAMP2019)**	411906	G	T	W	T'GGGCATACTAGT	Gly167, His168, Thr169, Ser170	
					M	T'TGG CAT ACT AGT	Trp167, His168, Thr169, S er170	Missense
	Intergenic (hypotheticalprotein)	77939	CC	-C	W	A'TCCCCCCCCCCCATA	-	
					M	A' TCCCCCCCCCCATAT		SSM / Deletion
	Hypothetical protein (C)	564023	CC	-C	W	A'TCCCCCCCCCCCCCAT	Gly5, Gly6, Gly7, Gly8, Asn1, three amino acids, Ala12	SSM/frameshift
					M	A'TCCCCCCCCCCCCATT	Gly5, Gly6, Gly7, Gly8, Asn1, three amino acids, Stop (pseudogene)	
	Hypothetical protein (C)	**674889**	**GG**	**-G**	W	TTT ATGGGGGGGGGT AAA	Phe182, Met183, nine amino acid to Lys192	Missense
					M	CTT TATGGGGGGGGT AAA	Leu182, Tyr183, nine amino acid Stop (pseudogene)	/frameshift
	ccpA (Cj0358)	337609	C	A	W	T'GCAGCGGGTCAT	Ala97, Ala98, Gly99, His100	
					M	T' GAAGCGGGTCAT	Gly97, Ala98, Gly99, His100	Missense
	Intergenic (hypothetical protein and *selU* gene	485112	GG	-G	W	CGGGGGGGGGGT ATG	Arg94, Gly95, Gly96, Gly97, Met98	
					M	CGGGGGGGGGTATAG	Arg94, Gly95, Gly96,Val97 ,**Stop codon**	SSM/ Deeletion
	Putative methyltransferase (C)	668755	C	CC	W	A'CCCCCCCCCCGTAAC	Pro125, Pro126, Arg 127, Asn128.	
					M	A'CCCCCCCCCCCGTAA	Pro125, Pro126, Pro 127, Stop (pseudogene)	SSM , Frameshift
	***cj1295*(CAMP1213)**	**1278726**	**GG**	**-G**	W	G'GGGGGGGGGGAT.....TCT	Gly32, Gly, Gly, Asp35 six amino acid, Ser41	
					M	G'GGGGGGGGGATA .....**TAA**	Gly32, Gly, Gly, Ile35, six amino acid Stop(pseudogene)	SSM, frameshift
	Putative methyltransferase(C)	1380379	C	CC	W	A'CCCCCCCCCCGTAAC	Pro125, Pro126, Arg 127, Asn128.	
					M	A'CCCCCCCCCCCGTAA	Pro125, Pro126, Pro 127, Stop (pseudogene)	SSM , Frameshift

Table 5.2 SNPs, deletions and insertions in Ch95-95(1) and Ch95-95(2). This table explains the sites for five changes in the Ch95-95(1) genome and 10 changes in the Ch95-95(2) genome, compared with Dg95. Both strains had substitution in the *glk* gene in different positions (Ch95- 95(1) in *rrnA* (position 47958) and *rrnB* (position 411897), Ch95- 95(2) in *rrnA* (position 47967) and *rrnB* (position411906). Both had deletion in PseC (UDP-4- amino-4, 6dideoxy-N- acetyl-beta-L-altrosamine transaminase (position 1278726). Five sites (positions 485112, 564023, 668755, 674889 and 1278726) had deletion in Ch95-95(2). There were three insertions at positions (668755) and (1380379) for Ch95-95(2), and one position insert interageneic hypothetical protein (position 552252) in Ch95-95(2).*ccpA* 'Cytochrome c551 peroxidase, Putative polysaccharide. (**C bold**) refers to complementarity strand, (SSM); slipped-strand mispairing.


A- Dg95, rrnA , 47958
A'GCAGGAGAGGGT
Ch95-95(1)
A'GCATGAGAG GGT
Dg95, rrnB - 411897
A'GCAGGAGAGGGT
Ch95-95(1)
A'GCATGAGAG GGT
Dg95, Hypothetical protein -674889
T' GGGGGGGGGTAA
Ch95-95(1)
T'GGGGGGGGTAAA
Dg95, 552252
T'CTGGGGGGG GGGATT
Ch95-95(1)
T'CTGGGGGGGGGGGAT
Dg95, Cj1295- 1278726
G'GGGGGGGGGGAT
Ch95-95(1)
G'GGGGGGGGGATA


B- Dg95, rrnA , 47967
T'GGGCATACTAGT
tRNA-Ile
tRNA-Ala
5SrRNA
ansB
16SrRNA
eda
edd
zwf
pgl
glk
pgi2
glcP
23 SrRNA
ggt
ScKozak
PlasKozak
PlasKozak
Plant Kozak
PlasKozak
Ch95-95(2)
T'TGGCATACT AGT
Dg95, rrnB, 411906
T'TGGCATACT AGT
hypothetical
murD
Ch95-95(2)
T'GGGCATACTAGT
Dg95, ccpA ,337609
T'GCAGCGGGTCAT
plsY
ccpA
glmM
ispA
SP6
Ch95-95(2),
T' GAAGCGGGTCAT
Dg95, 77939
A'TCCCCCCCCCCCATA
polyArg
flgE
hypothetical
hypothetical
Bacteriohemerythrin
AraI1
PreScission
SUP4
Ch95-95(2)
A' TCCCCCCCCCCATAT

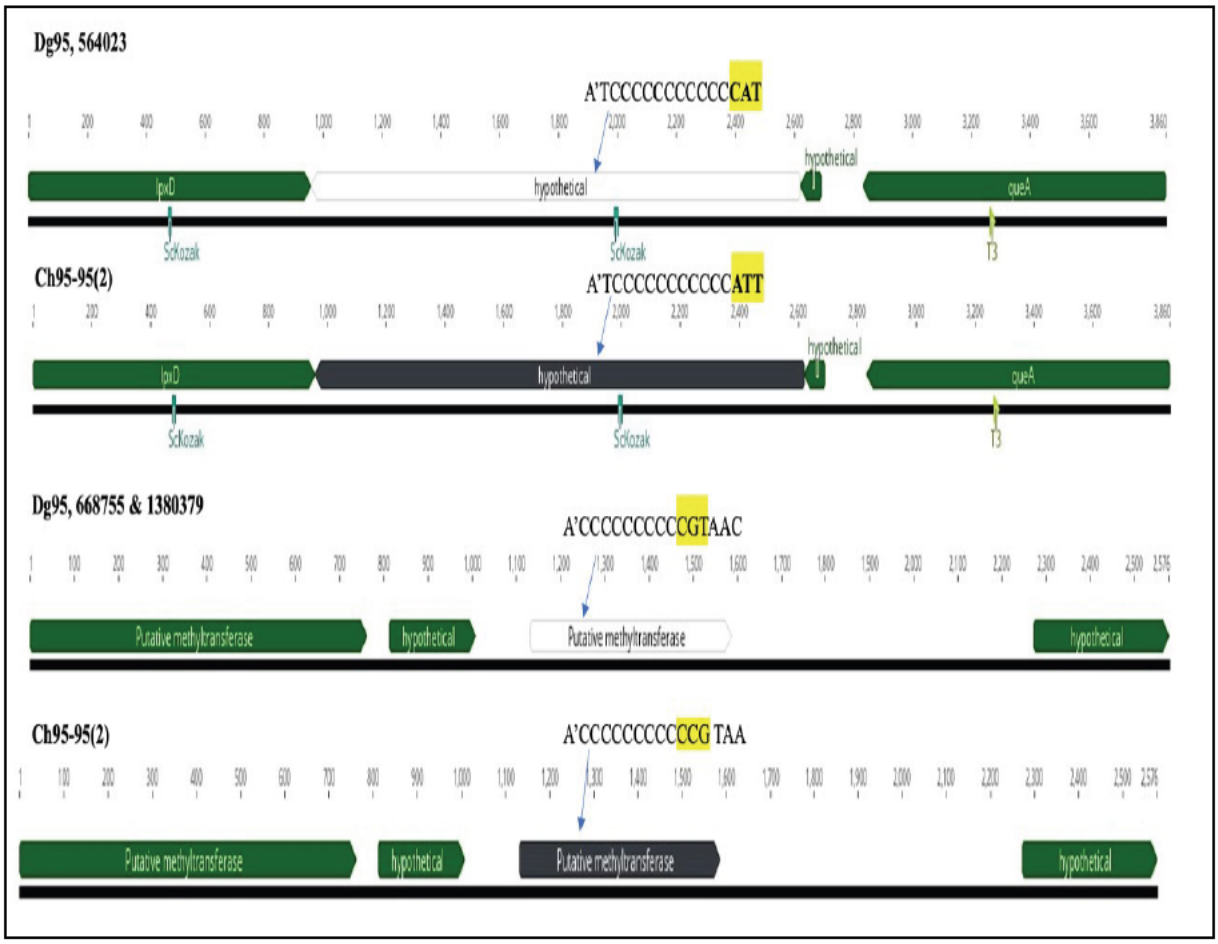


Figure 5.6 Genetic context of mutations in Ch95-95(1&2). (A) Schematic presentation of the location of mutations in Ch95-95(1) *glk* gene in *rrn*A and *rrn*B and additional mutations. (B) Schematic presentation of location of mutations in Ch95-95(2). Black color indicates genes/ intergenic regions which contain a mutation and white color indicates location sequence in wild type strain.

5.4 Comparing the *glk* gene in RG2 strains with the chicken trial strains

To compare the *glk* gene in 39 *C. jejuni* strains, a *glk* sequence was downloaded for all strains from the PubMLST website, and the Clustal Omega programme was used for alignments. Based on the alignment results in table 5.3 and Table 3.1, there are seven different *glk* alleles among RG2 strains. Both ED type 2 Dg275 and type 3 Dg95 have the common *glk* allele number 4.

In the Dg22 strain, the *glk* gene (allele number 9) showed the next highest identity with the *glk* gene in Dg95, with only one mismatch at 970bp (T instead of C in *glk* Dg95), fig5.7. Additionally, strains Dg217 and Dg210, which have *glk* allele number 12 and belong to ED type7, showed 99.8%, identity with the *glk* gene in Dg95 with two mismatches at 142bp (G instead of A) and 281bp (G instead of A). The strains also showed high identity in Dg294 and Dg345 (ED type 10) strains, with 99.05% identity and 10 mismatches, table 5.3. However, the more distantly related strains Dg43 and Dg234 possessed a *glk* gene with only 999bp encoding 2 fewer C-terminal amino acids, fig5.8. These were the least similar to Dg95 *glk*. With 25 mismatches, Dg234, Dg233, and Dg268, shared only 87.3% identity with Dg95 *glk* table5.3 Dg234, Dg233 had been isolated from farm E on 26/06/2012, while Dg268 was isolated from farm C on 03/07/2012, see table 3.1. Dg43, Dg18, Dg381, and Dg201 had 116 mismatches and showed 88.2% identity compared to Dg95 *glk.* As highlighted above the strains recovered from the chicken trial, Ch95-95(1) and Ch95-95(2) had only a single mutation at 490 bp (TGA instead of GGA), and TGG instead of GGG, respectively.

Notably, sequences around the region of *glk* mutation in Ch95-95(1) and Ch95-95(2) were conserved. While there was evidence of third position wobble (position 492bp) in Gly167 codon and in Glu168 codon, the amino acid sequence GEGGH was conserved. This emphasises that the nucleotide changes observed in Ch95-95 chicken isolates is not common and that these mutations leading to loss of glucose kinase function are a valid explanation for loss of glucose utilisation by these 2 strains.

Strains	Identity %	Nucleotide length bp	Mismatches	Contigs	Gaps
[1]Dg95, Dg275	100	1005	-	35/34	-
Dg22	99.9	1005	1	32	0
Dg217 Dg210	99.8	1005	2	33 41	0
Dg294 Dg345	99.05	1005	10	36 33	0
Dg43 Dg18 Dg381 Dg201	88.2	999	116	58 65 77 68	2
Dg234 Dg268 Dg233	87.3	999	125	64 71 61	2
Ch95-95(1)	99.9	1005	1	-	0
Ch95-95(2)	99.9	1005	1	-	0

Table 5.3 Percent identity and mismatches in the *glk* gene among RG2 strains and chicken trial strains. The *glk* sequence was download from the PubMLST website, and DNA Dynamo and Clustal Omega were used for alignment.[1]Dg95 and Dg275 are representative of the following large group with an identical *glk* nucleotide sequence: Dg20, Dg26, Dg44, Dg57, Dg63, Dg68, Dg69, Dg80, Dg81, Dg104, Dg109, Dg117, Dg122, Dg130, Dg131, Dg137, Dg180, Dg184, Dg187, Dg224, Dg246, Dg304, Dg307and Dg356.

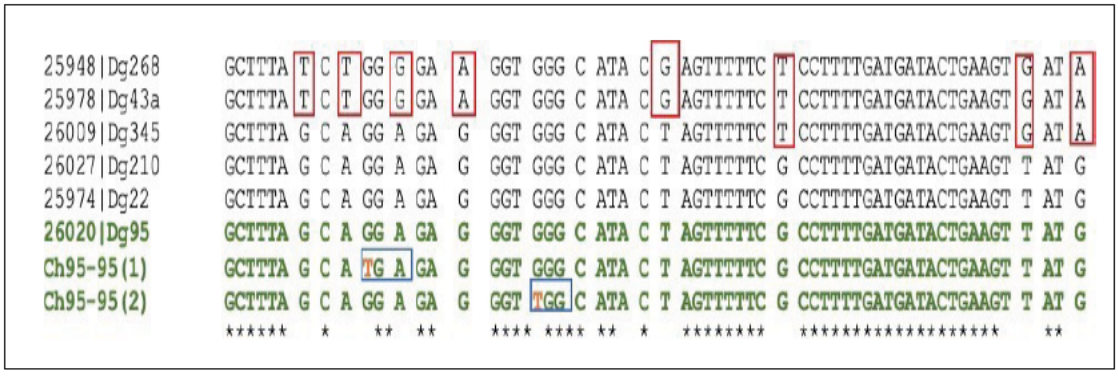


Figure 5.7 Alignment of the *glk* gene for the RG2 group compared to Ch95-95(1) and Ch95-95(2). The PubMLST website was used to download the *glk* gene for all RG2 strains, and alignment was done using the DNA Dynamo programme and Clustal Omega. One representative of each *glk* ST (Table 5.3) is included. The red squares refer to the differences among the RG2 strains, while the blue squares (540 bp) show the differences between the RG2 (rat) strains and Ch95-95(1) and Ch95-95(2).

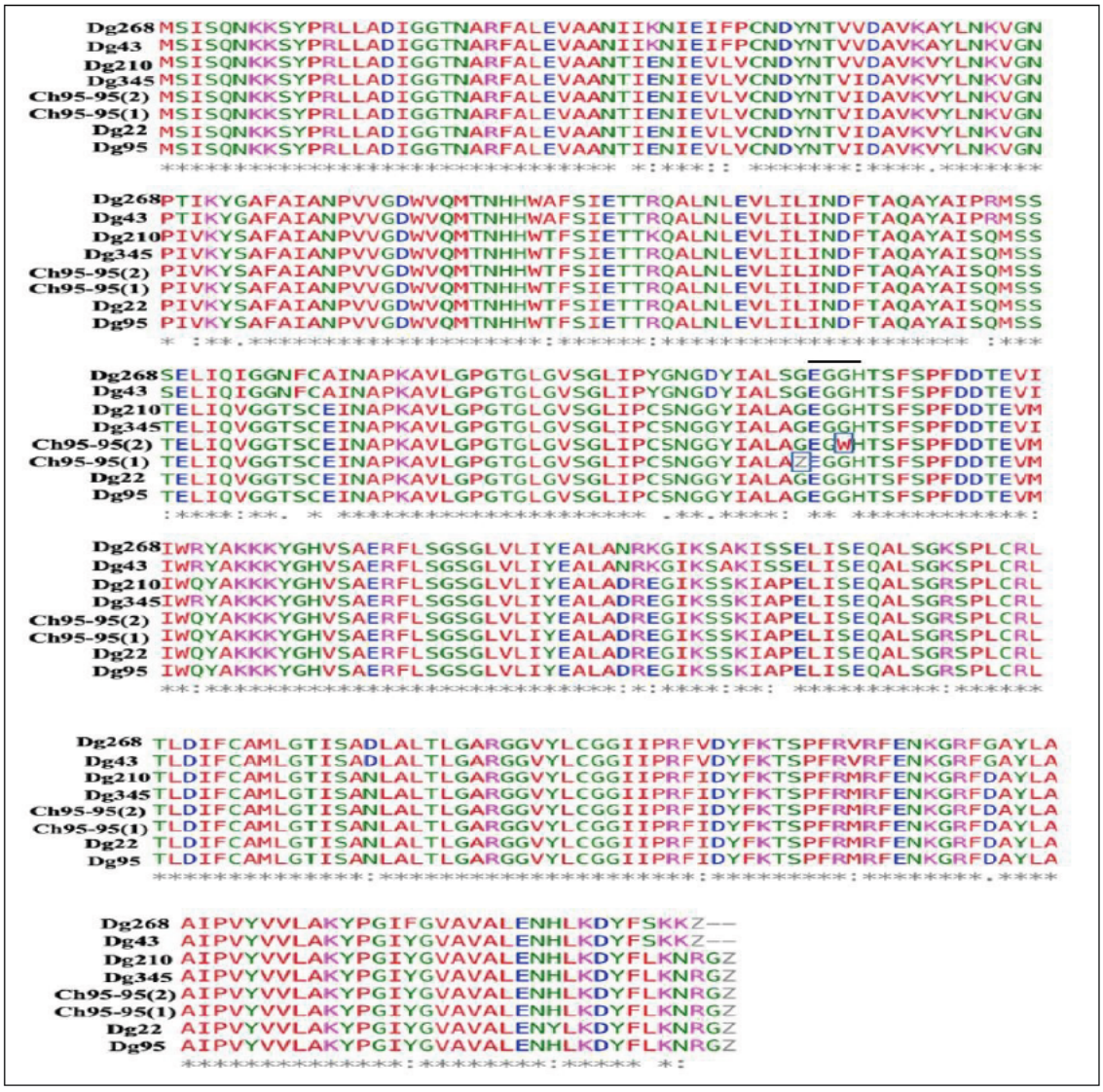


Figure 5.8 Protein sequences of the *glk* gene of strains. that are listed in Table 5.3 were aligned using Clustal Omega (Sievers *et al.*, 2011). The whole *glk* gene was translated to create the amino acids sequences. Blue squares represent the mutations TGA-Z stop codon and TGG-W in the strains recovred from the chicken trial, Ch95-95(1) and Ch95-95(2), respectively. The bold black line refers to EGGH consensus sequence. The rest of strains were farm-associated *C. jejuni* strains. Dg95 and Dg201 were isolated from farm G, Dg268 and Dg345 from farm C, while Dg22 from H and Dg43 from farm X.

5.5 Conclusion

Based on systematic genomic analyses, *glc* loci are present in only 1.7% of >6,000 genomes of *C. jejuni* and *C. coli* from different sources, clinical and environmental animals (Jolley *et al*., 2012). Of the *C. jejuni* strains that were positive, 51.4% were isolated from rats, 42.9% were isolated from wild birds, 2.9% were isolates from farm environments, and two isolates were from an unknown source (Vegge *et al.,* 2016). Further, 43.2% of *C. coli* strains were isolated from human disease and 8.1% were isolated from rats and soil (Vegge *et al.,* 2016). The RG2 strains that were tested were highly adapted to the colonisation of chickens (Mohammed, 2018). The data presented in this chapter confirm the inability of recovered strains in chickens to use glucose, by growth assay *in vitro*. In addition, genomic studies confirmed that Ch95-95(1) contained five mutations in different positions and that two of them were in the *glc* loci at the *glk* gene, one in the *glc* locus within *rrnA*, position 47958 and the same mutation in the *glc* locus within *rrnB*, position 411897. The other three were outside of the *glc* loci in different positions 552252, 674889, and 1278726. On the other hand, Ch95-95(2) had 10 mutations. This included the mutation in *glk* within the *glc* loci in *rrnA,* position 47967, and in *rrnB* position 411906, and eight mutations outside of the *glc* loci in positions 77939, 674889, 337609, 485112, 668755, 1278726, and 1380379. These mutations at positions 674889 and 1278726 were common to both Ch95-95 (1) and (2) and hence may potentially have an additional common negative impact on glucose utilisation.

Chapter6

Oxidative stress

6.1 Introduction

C. jejuni is oxygen sensitive and grows optimally in the presence of low oxygen concentrations. For the best growth, it requires the following concentration of gases: approximately 5-6% oxygen and 10% carbon dioxide with the complement as nitrogen (Bolton and Coates, 1983; Garénaux *et al*., 2008). *C. jejuni* does not grow under strict anaerobic conditions. In *C. jejuni* respiration is preferentially aerobic with O_2 as terminal electron acceptor, hence a requirement for oxygen for good growth (Garénaux *et al*., 2008; Pittman and Kelly, 2005). However, a number of the key enzymes of central metabolism of *C. jejuni* have oxygen-labile iron-sulphur clusters and are more closely related to corresponding enzymes of anaerobic bacteria than those of aerobic bacteria. Examples include, serine dehydratase (SdaA) that deaminates serine to produce pyruvate and ammonia, pyruvate: acceptor oxidoreductase (POR) that catalyses decarboxylation of pyruvate to acetyl-CoA for entry into the TCA cycle and 2-oxoglutarate: acceptor oxidoreductase (OOR) (Kelly, 2001). *C. jejuni* posseses several alternate electron acceptors such as fumarate, nitrite and nitrate that can be used for anaerobic respiration and to support growth in the lower gut (Sellars *et al*., 2002; Garénaux *et al*., 2008). Under such conditions, a small amount of oxygen is also required for functional ribonucleotide reductase and nucleic acid synthesis which provides an explanation for the inability to grow under strict anaerobic conditions (Garénaux *et al*., 2008; Pittman and Kelly, 2005).

The natural environment of *C. jejuni* is the intestinal tract of chickens and other animals (Mughini, *et al*., 2012; Thakur and Gebreyes 2005). Survival in the environment and during food processing are important steps in transmission between animals and to humans. During these stages, *C. jejuni* will be exposed to much higher than the preferred levels of oxygen, up to atmospheric oxygen levels (21%), and hence the bacteria are exposed to exogenous aerobic as well as endogenous oxidative stress as a result of aerobic metabolism.

Oxidative stress results in extensive damage to DNA, lipid and protein, due to the production of toxic reactive oxygen species (ROS). *C.jejuni* employs many defence mechanisms to deal with oxidative stress. ROS-detoxification enzymes of *C. jejuni* 11168 include one superoxide dismutase (SodB) one catalase (KatA) and three different enzymes of the peroxyredoxin family, including alkylhydroperoxide reductase (AhpC), Tpx and Bcp. *sodB*, *katA* and *ahpC* have been shown by mutagenesis to have a primary role in reducing damage caused by high oxygen (Imlay, 2008; Oh *et al.,* 2015). SodB catalyses the breakdown of the superoxide anion $O_2^{\bullet-}$ to hydrogen peroxide (H_2O_2), catalase breaks down H_2O_2 producing water and oxygen while alkyl hydroperoxide reductase detoxifies organic peroxides (Seaver and Imlay, 2001; Parsonage *et al*., 2008). Cells mutated in *ahpC* and *katA*, resulted in a coccoid morphology, while the *sodB* mutant had an elongated cellular morphology under aerobic condition (Oh *et al*., 2015). PerR and CosR are the two primary regulators controlling expression of genes involved in the oxidative stress response. (See chapter 1.6 for detail on the genes oxidative stress response).

C. jejuni strains carrying the *glc* locus encode products for uptake and phosphorylation of glucose as well as key enzymes of the ED pathway. These strains have been identified primarily from wild birds and Norway rats (Vegge *et al*., 2016). It is likely that transmission of these strains includes passage through water, mud and the environment and substantial exposure to high levels of oxygen. *Pseudomonas* spp. like *Campylobacter* have an incomplete EMP pathway and metabolise glucose via the ED pathway. The importance of the ED pathway and production of the reducing factor NADPH to survival against oxidative stress has been highlighted for the strict aerobe *P. putida* (Chavarria *et al*., 2013). In addition, pyruvate, the product of catabolism of glucose via the ED pathway, is known to act as an anti-oxidant when added to *C. jejuni* growth media by decreasing the level of H_2O_2 thus permitting growth in higher levels of oxygen. (Verhoeff-Bakkenes *et al*., 2008).

In this chapter, the impact of a functional ED pathway on growth and survival of these *Campylobacter* strains at different oxygen concentrations and protection against hydrogen peroxide is investigated.

6.2 Confirmation of presence of key genes related to oxidative stress in Dg275 and Dg95

Before studying the effect of glucose on growth and survival under conditions with non-ideal levels of oxygen, a survey of presence of genes encoding enzymes and regulators related to oxidative stress was performed. Draft genomes of the two Glc positive strains, Dg275 and Dg95, as well as the relatively closely related Glc negative strain, Dg200 were analysed using genes of the well characterised strain NCTC11168 as reference. The PUBMLST/ campylobacter website was used as a source of the sequence of each gene from all strains for subsequent alignment. As shown in table 6.1, all three strains possessed the complete sequence for all genes analysed and in most cases the genes were greater than 97 % identical to the corresponding gene/ gene product in NCTC11168. Notably, both *rrpA* and *rrpB* encoding regulators are present in all strains. These MarR- type regulators have been linked to control of stress under aerobic conditions and oxidative stress but possession of these genes by *C. jejuni* strains was shown to be variable (Gundogdu *et al*, 2016). Of all comparisons, *rrpA* shared the lowest identity (89.2 %) with the corresponding gene in NCTC11168; while the *rrpB* gene in both Dg275 and Dg95 shared 97.9% identity with NCTC11168 *rrpB*.

Although potential impact of point mutation(s) or differences in regulation of any of these genes cannot be excluded, it can be concluded that both Glc positive strains, Dg275 and Dg95, and the Glc negative strain, Dg200, to be studied possess the main genes linked to response to aerobic and oxidative stress.

Genes	**Dg275**	**Dg95**	**Dg200**
catalase *(katA- cj1385)*	98	98	97
Alkyl hydroperoxide reductase *ahpC- cj0334*	98.6	98.6	98
Superoxide dismutase (*sodB-cj0169)*	99	99	99
heme -trafficking protein *(cj1386)*	96.9	96.9	99.3
rrpA (cj1556)	89.2	89.2	89.2
rrp B (cj1546)	97.9	97.9	97.4
a putative periplasmic protein *(cj1371)*	97.2	97.2	97.9
tpx (cj0779)	99.2	99.2	99.2
bcp (cj0271)	99.1	96.4	96.2
fdxA (cj0333c)	97.8	97.8	97.1
s-ribosylhomocysteinase -*LuxS (cj1198)*	97.5	97.5	97.9
fur(cj0400)	98.9	98.9	98.9
perR(cj0322)	99.2	99.2	99.0
cosR (cj0355c)	99.7	99.7	99.1

Table 6.1 Survey for presence of genes encoding enzyme and regulator related to ROS resistance. Genes were identified in PUBMLST/campylobacter, download and aligned using Clustal Omega with NCTC11168 as reference strain. Percent nucleotide identity over the entire gene in each case is shown. Dg275 and Dg95 possess the *glc* locus, Dg200 does not. See chapter 1.6.2 for detail of gene product function.

6.3 Effect ED pathway on growth in different concentrations of Oxygen

6.3.1 Comparison of growth of ED positive and negative strains in 5%O_2

C.jejuni is routinely grown in an atmosphere of 5%O_2, 10%CO_2, 2%H_2 and 83%N_2. Growth of the two *C.jejuni* ED positive strains, Dg95 and Dg275, in addition to two ED negative strains, Dg200 and NCTC11168, was monitored, using these standard gaseous conditions, in the rich growth medium MHB-FBS. All strains were cultured in 50ml conical flasks with 15ml media, including Bolton broth supplement antibiotic at 37°C, with shaking at 130rpm.

ED positive strains were tested for growth in the presence and absence of 20mM glucose, while growth of the ED negative strains was tested only in the absence of additional glucose. Both NCTC11168 and Dg200 grew much faster then the two *glc* positive strains, Dg275 and Dg95. By 12h both NCTC11168 and Dg200 had reached a viable count of $2x10^8$ cfu/ml and early stationary phase, as reflected by cfu. In contrast, both Dg275 and Dg95 had a much longer lag phase, evident in both the cfu count and OD_{600}. From the 13h to 24h time points, for Dg 275, growth appeared to be exponential while for Dg95 increase in cfu continued up to 38h. This was the same whether these latter strains were grown in the presence of glucose or not. As previously reported, chapters 4 and 5 the impact of glucose correlated with enhanced survival in the very late stages of growth where both OD_{600} and viable count decreased more slowly in the presence of glucose, fig 6.1

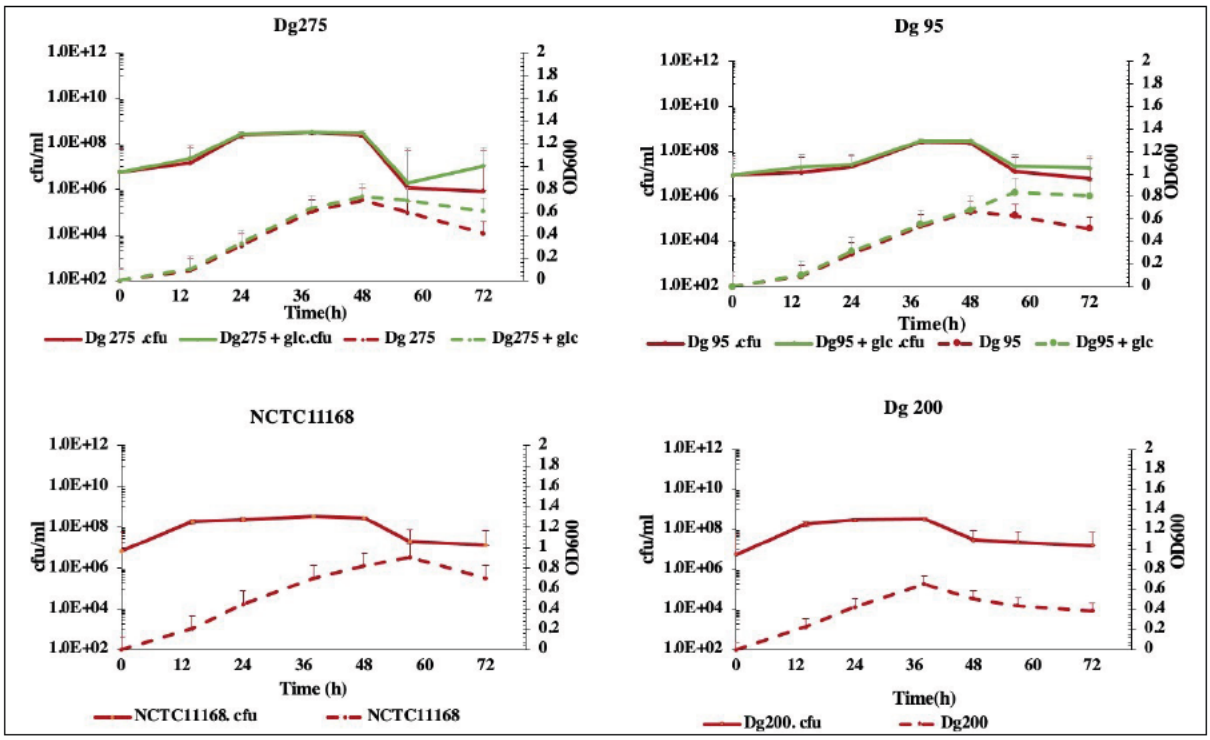


Figure 6.1 Growth at 5%O2, 10%CO2, 2%H2 in rich media with and without glucose. Strains tested were: RG2 Dg275 and Dg95 as (+ED), one of ST21CC-21, NCTC11168 and one of ST45CC-45, Dg200 as (ED).MHB-FBS (15ml) in a 50ml conical flasks was inoculated with 150µl standardized cells, prepared from BA plates as described in chapter 2(2.4.4) with shaking at 130rpm in M35 cabinet. Samples were monitored for OD_{600} (dash line) and cfu (solid line) at time indicated. Green, with 20mM glucose; red no glucose.

6.3.2 Comparison of growth of ED positive and ED negative strains in 2%O_2

Growth of the same strains was tested in MHB-FBS with 2%O_2, 10%CO_2, 2%H_2 and 86 % N_2, see fig6.2. The pattern of growth of all 4 strains was similar and much more limited, as judged by OD_{600} under these more oxygen limited conditions. Interestingly, Dg275 and Dg95 (both ED+) no longer showed the extended lag seen when grown in 5% O_2 fig6.1 Maximum OD_{600} reached for all strains was 0.3-0.4, and cfu around $3x10^8$/ml. For all strains decline in cfu began between 48 and 60h. Impact of glucose under these growth conditions was minimal marginal, if any, of the characteristic increased survival of Dg275 in the presence of glucose at 72h.

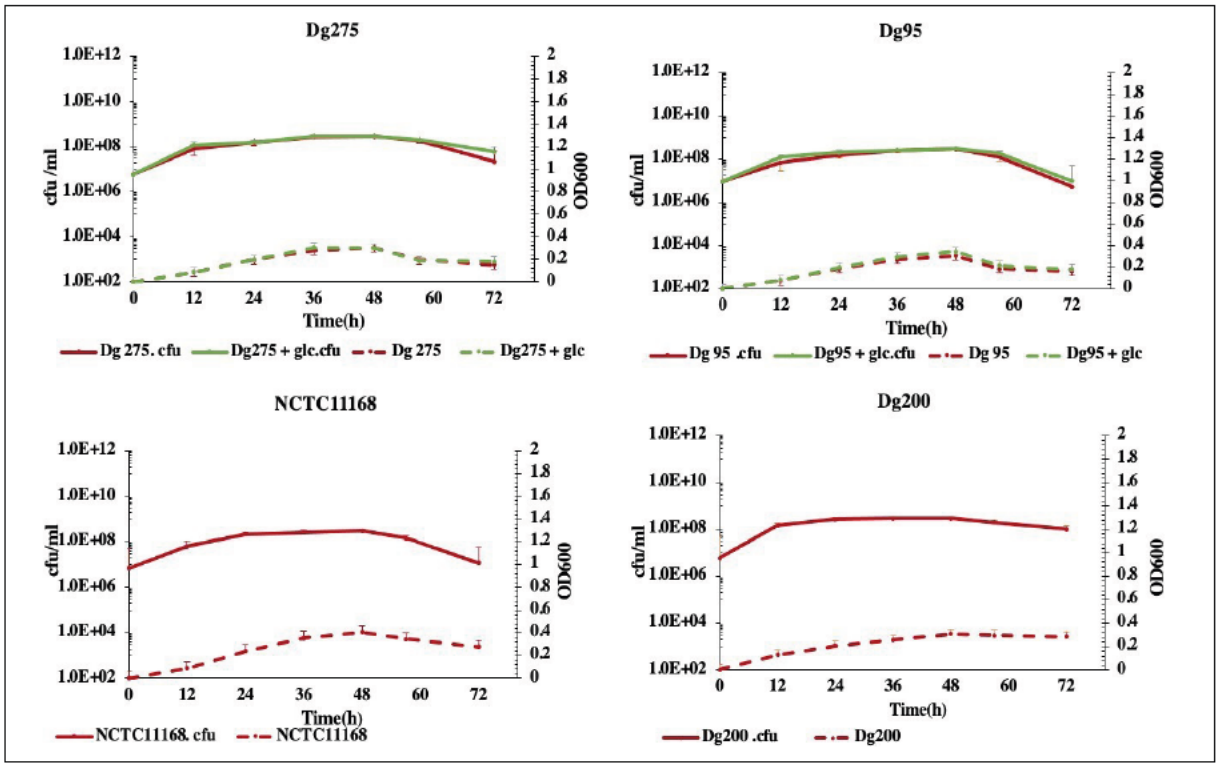


Figure 6.2 Effect of 2%O_2,10%CO_2,2% H_2 on growth with and without glucose. MHB-FBS (15ml) in a 50ml conical flasks was inoculated with 150µl standardized cells, prepared from BA plates as described in chapter 2(2.4.4) with shaking at 130rpm in M35 cabinet. Samples were monitored for OD_{600}(Dash line) and cfu (Solid line) at time indicated. Green, with 20mM glucose; red no glucose. Means and error bars show from two biological cultures.

6.3.3 Comparison of growth of ED positive and ED negative strains in 15.9%O_2

Strains Dg 275 and Dg 95 (both ED+), Dg200 and NCTC11168 (both ED-), were then tested for growth properties in high oxygen 15.9%O_2, 4%CO_2, 0% H_2 with 79.9 % N_2 in MHB-FBS media with and without glucose, fig6.3. Dg200 and NCTC11168 survived in the presence of 15.9 %oxygen, but showed a striking delay in growth. NCTC11168 started growing only after 48 h incubation, as monitored by both OD_{600} and cfu. Dg200 showed evidence of an increase in cfu between 36 and 48h but no increase in OD_{600} until 48-60h. Both strains grew to a maximum of $1.8x10^8$ cfu/ml.

In contrast, the growth curve of both Dg275 and Dg95 in 15.9%oxygen mirrored the characteristic lag and slow start to growth up to around 12h, as seen with growth in 5% oxygen, fig6.1. Both Dg275 and Dg95 showed exponential growth by 24h (monitored by cfu and OD_{600}) and reached a maximum at 48h. At 48h for both strains the OD_{600} was 1.3 and cfu approximately $3.4X10^8$/ml. Growth then decreased by 1 $\log_{10}$ by 60h.

Interestingly, cultures of Dg275 and Dg95 both showed the same enhanced growth (cfu/ml) early in exponential phase, at 24h and enhanced OD_{600} between 48 and 72h when grown in the presence of glucose. Irrespective of the impact of glucose, both Dg275 and Dg95 were evidently more acclimatized for growth in high levels of oxygen.

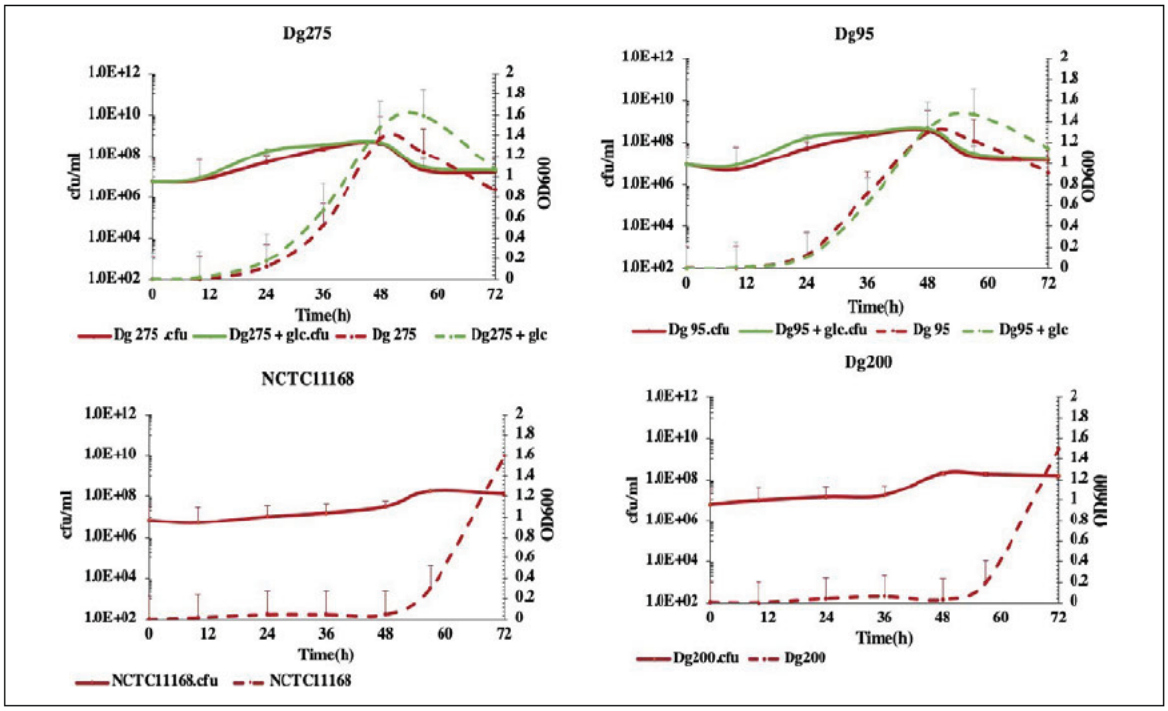


Figure 6.3 Effect of 15.9%O_2,4.5%CO_2,0% H_2 on growth with and without glucose. MHB-FBS (15ml) in a 50ml conical flasks was inoculated with 150µl standardized cells, prepared from BA plates as descried in chapter 2(2.4.4) with shaking at 130rpm in M35 cabinet. Samples were monited for OD_{600}(Dash line) and cfu (Solid line) at time indicated. Green with 20mM glucose, red no glucose. SE is from duplicates for Dg275 and Dg95 (+ED), Dg200 and NCTC11168 (-ED). Based on OD_{600}, P value gave results 0.01 for Dg95 at 57h growth with glucose compare without glucose. Cultures at initial cells densities at OD_{600} =0.002.

It is well documented that some strains of *C. jejuni* show better growth than others in high oxygen, and also that strains, including NCTC11168, can adapt to growth at high oxygen concentrations (Boysen *et al*., 2007). For this reason, subcultures taken from 60h growth of both Dg200 and NCTC11168 were glycerol stocked and retested along with the original stocks for growth in MHB-FBS with 15.9 % oxygen, fig6.4. Purity of all cultures was confirmed by gram stain, and comparison of aerobic and microaerobic subculture on BA at 37°C.

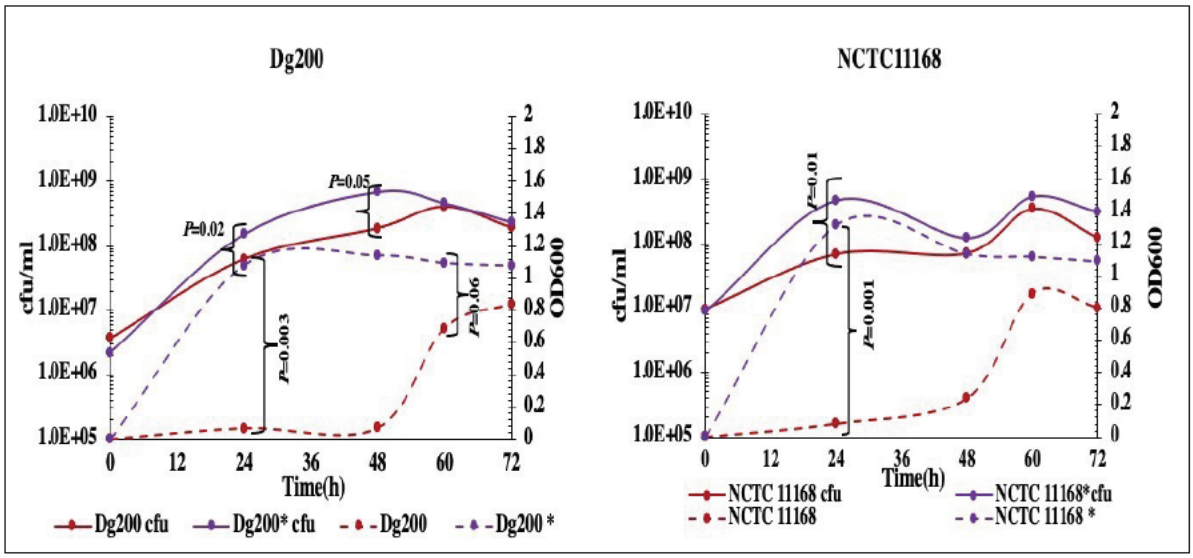


Figure 6.4 Adaptation of Dg200 and NCTC11168 to growth in 15.9%O_2. *C. jejuni* NCTC 11168*, and Dg200* are the adapted strains which had been isolated and glycerol stocked from samples of 60 h culture previously grown in 15.9 % oxygen, fig6.3. Inoculation and culture of both adapted* and original strain (no asterisk) in MHB-FBS with 15.9 % O_2, 4.1 % CO_2, 79 % N_2 and sampling were as described in fig6.3. OD_{600}, dash line; viable cells (cfu/ml), solid line. The errors in bacterial growth were calculated by determining the standard deviation from the mean of triplicate experiments.

As might be predicted, NCTC 11168* (previously exposed to high oxygen) showed good evidence of adaptation to growth in 15.9% oxygen, while the original stocked NCTC11168 again only grew following a long delay, see fig6.4). After 24h, the OD_{600} of the NCTC 11168* culture was approximately 10-fold higher than that of NCTC 11168, which had an OD_{600} <0.1 (*P*=0.001,24 h NCTC11168* vs NCTC11168).

Similarly, with a cfu/ml of $4.58X10^8$ at 24 h for NCTC 11168* compared to one of $6.83X10^7$ for NCTC 11168 (*P* = 0.01) the difference in colony forming units also confirmed good early growth of the adapted strain. By 48-60h the original stock was beginning to grow while the adapted culture entered the decline phase. A similar pattern was evident with Dg200 with delayed growth of the original stock to 48-60h and early growth of the previously exposed strain Dg200*. At 24h, Dg200* reached an OD_{600} of 1.08 while Dg200 was 0.07 (*P*= 0.003). The difference in OD_{600} between the two cultures continued till 60h when a significant difference remained (*P*= 0.06). Quantitation of cfu followed a similar pattern with a ten-fold higher count for Dg200* and significant difference at 24h (*P*= 0.02) increasing to *P* = 0.05 at 48h. These results indicate that adaptation of Dg200 like NCTC11168 to high oxygen can be readily acquired.

6.3.4 Comparison of growth and survival of ED positive strains in DMEMf with 15.9 % O_2

Growth of ED positive *C. jejuni* strains in DMEMf provided a suitable screen for identification of strains capable of metabolising glucose, see chapter3. As with growth in MHB, supplementation of DMEMf with 20mM glucose enhanced survival of ED positive cultures in the decline phase of growth. The benefit of glucose metabolism was more marked with DMEMf, fig6.5, due to the more restricted availability of suitable nutrients.

Therefore, the impact of high oxygen on growth of ED positive strains in DMEMf with and without 20mM glucose was investigated. The enhanced growth and survival in the presence of glucose was even more marked when cultures were incubated with 15.9 % oxygen. With Dg275 a difference in cfu/ml was already evident at 24h and by 48h there was a 2 $\log_{10}$ higher number of viable cells in the media with added glucose. This difference continued up to 72h, when no viable cells were recovered from the media without glucose.

While the difference was not as great with Dg95, the pattern was the same, at 48h the difference was significant (P=0.01) and by 60h no viable cells were recovered from the media without glucose. Both bacteria were clearly much more susceptible when grown in DMEMf in high oxygen, fig6.5 than under standard microaerobic conditions, fig6.1, in the absence of glucose. This suggests that availability of glucose as a nutrient cannot be the only explanation for enhanced growth in the presence of glucose and that glucose catabolism via the ED pathway may contribute to protection from stationary phase and oxidative stress.

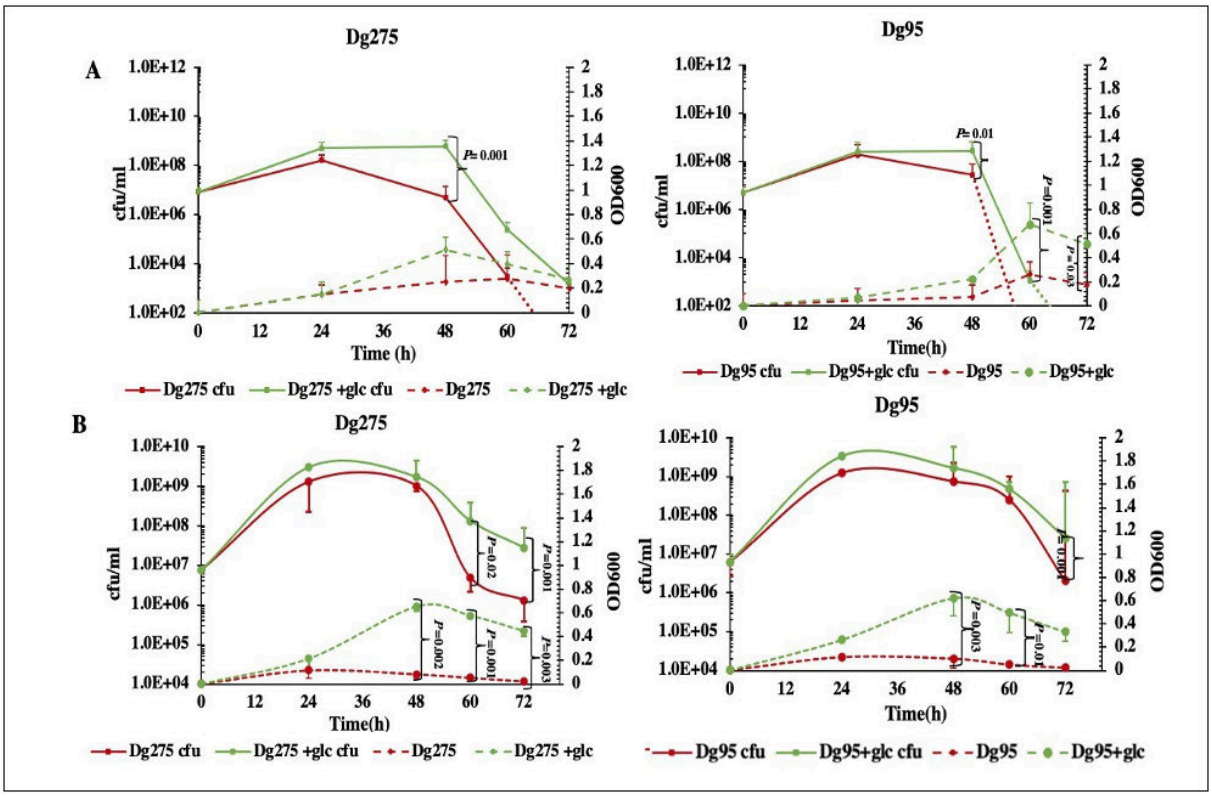


Figure 6.5 Impact of glucose of growth of ED positive strains in DMEMf with 15.9%(A) or 5%(B) oxygen. 15ml in a 50ml conical flasks was inoculated with 150µl standardized cells, prepared from MHA plates and grown at 150rpm in M35 cabinet. Sample were monitored for OD_{600} (Dash line) and cfu (sold line) at times indicated. Green, with 20mM glucose, red no added glucose, Means and error bars are from three independent biological cultures.

6.3.5 Ch95-95*glk* is susceptible to15.9%O_2

Ch95-95*glk* (1) is a non-glucose metabolising mutant derived from Dg95 in a chicken colonisation trial (Mohammed, 2018). The gene encoding glucokinase, *glk*, is a pseudogene due to a stop codon immediately upstream of the predicted glucose binding site, chapter5. In the absence of glucose phosphorylation, Zwf (G6P dehydrogenase) cannot function to produce 6P gluconate, the substrate for the ED pathway, see fig 1.6A&B. As discussed in chapter 5, under standard microaerobic conditions with 5%O_2, presence of the *glk* pseudogene in Ch95-95 (1) abolishes the benefit of glucose metabolism to enhanced survival at 72h. Otherwise the mutation had little impact on growth and survival of the strain. The impact of this mutation was also tested on growth and survival at high oxygen. Fig6.6A&B show growth of both the wild type, Dg95, and Ch95-95 *glk* (1) during growth in MHB with and without 20mM glucose in the presence of 15.9 % O_2. Fig6.6 C&D show growth of both strains when cultured under standard microaerobic conditions for comparison. There was a small difference, 1.5 fold higher OD_{600} value for Dg95 compared to Ch95-95 *glk* at 36h, corresponding to a 1.6 fold increase in cfu at the same time point with 5 %O_2, fig6.6 C&D (P =0.04), grown without glucose. There was difference between growth of parent and mutant strain, without glucose, up until 36h in 15.9 %O_2. But by 48h, recovery of Ch95-95 *glk* had dropped significantly, by 2 $\log_{10}$ compared to Dg95 ($2.6x10^6$ cfu/ml compared to $2.4x10^8$ cfu/ml, respectively, fig 6.6B), even in the absence of glucose. The *glk* mutation had no readily apparent effect on growth in MHB no glucose under standard culture conditions (5%O_2) but with 15.9% oxygen Ch95-95 *glk* was much more susceptible to aerobic stress in the late stationary phase of growth irrespective of presence or absence of glucose.

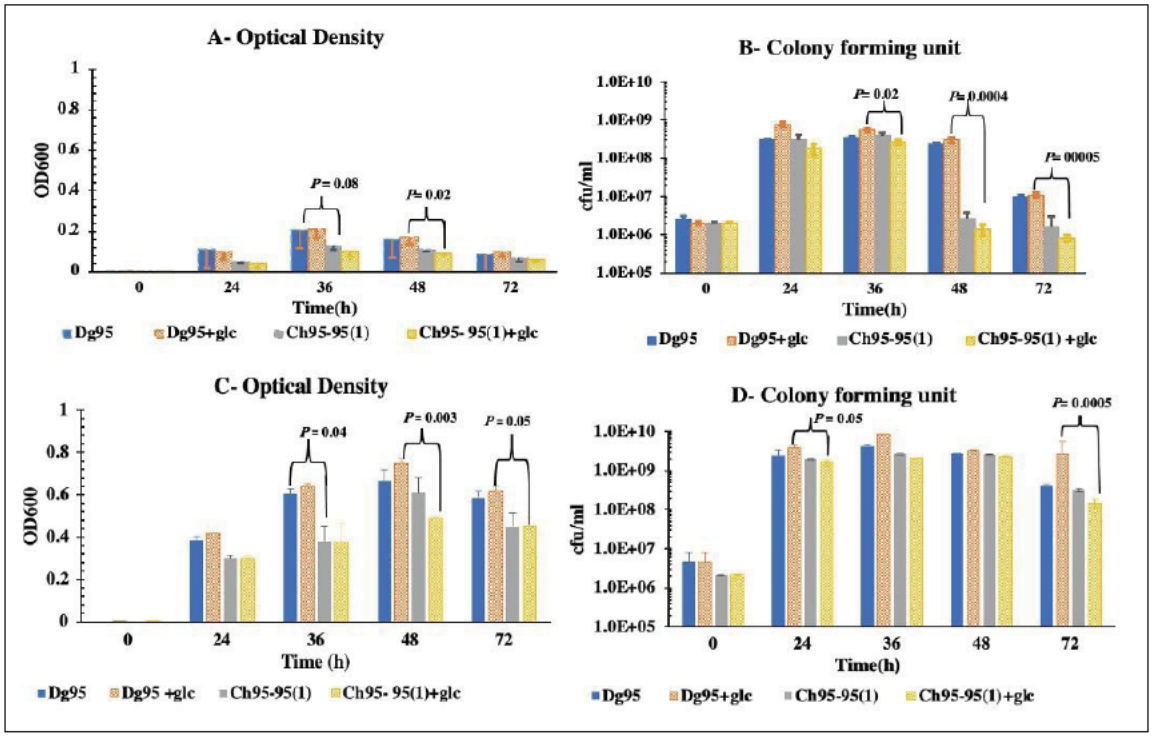


Figure 6.6 Growth of Dg95 and Ch95-95*glk* in MHB with glucose in 15.9%O2(A, B).and 5% (C-D). Strains were grown on a BA-2% plate for 48h, asingle colony was re -subculture on MHA for 36h and used for inoculum. Strains were incubated in 15ml MHB medium, 20mM glucose, and incubated for 72h in 50ml conical flasks with shaking at 150 rpm, inoculated with 150µl standard of 0.2 OD_{600} suspension of culture. Cell density and cfu at 24h till 72h are shown. Aliquots were taken during growth, as indicated, and monitored for OD_{600} and viable cells (cfu). Solid bars, growth without glucose, while Dotted bars growth with glucose. Means and error bars from three independent biological cultures.

6.3.6 Effect of atmospheric conditions on survival

During transmission to humans and in the environment, *C. jejuni* is exposed to atmospheric oxygen tension of 21% O_2 with 0.04 % CO_2 and 78 % N_2. The ability of *C. jejuni* to survive under atmospheric conditions are likely to substantially impact successful transmission from the environment to other animals and humans. Here, the impact of glucose on survival of *C. jejuni* Dg275 (RG2, ED positive) under aerobic conditions was compared to that of NCTC11168 ST21CC-21, ED negative. Both strains were initially grown, with shaking at 37^{O}C, under standard microaerophilic conditions (5% O_2, 10%CO_2, 2%H_2) in MHB or MHB+FBS and for Dg275 with or without glucose. After 24h growth, the flasks with culture were transferred to a shaking incubator in the air and cultured for a further 12h at 37^{O}C. Fig 6.7 shows bacterial density OD_{600} and viable count cfu up to 12h. The curves for Dg275 and NCTC11168 in MHB were similar, fig 6.7. In both cases the cells survived for 8-10h under the atmospheric conditions and then suddenly entered a rapid decline phase. The drop in survival was more pronounced for Dg275 with a decrease of 5 $\log_{10}$ cfu/ml between 8 and 10 h for Dg275 grown in MHB plus 20mM glucose. Again, provision of glucose appeared to protect cells. In the absence of glucose in MHB, fig 6.7B, there was no difference in survival up to 8h, but between 8 and10 h the number of culturable cells plummetted to <100 cfu/ml (none were recovered). The decrease in cfu of NCTC11168 was slower with a decrease of 2 $\log_{10}$ cfu/ml between 10 and 12h. The presence of FBS in the media did not affect the survival curve of NCTC11168. For Dg275, addition of FBS to the media completely changed the shape of the curve to a gradual decrease in cfu/ml of about 1 $\log_{10}$/ 2h over the entire curve, with approximately 1 x10^{6}cfu/ml still surviving at12h.

In this case, there was no difference between presence/ absence of glucose, suggesting that something else in FBS was protecting Dg275 cells. The results are consistent with reports of the high resistance of NCTC11168 to atmospheric O_2.

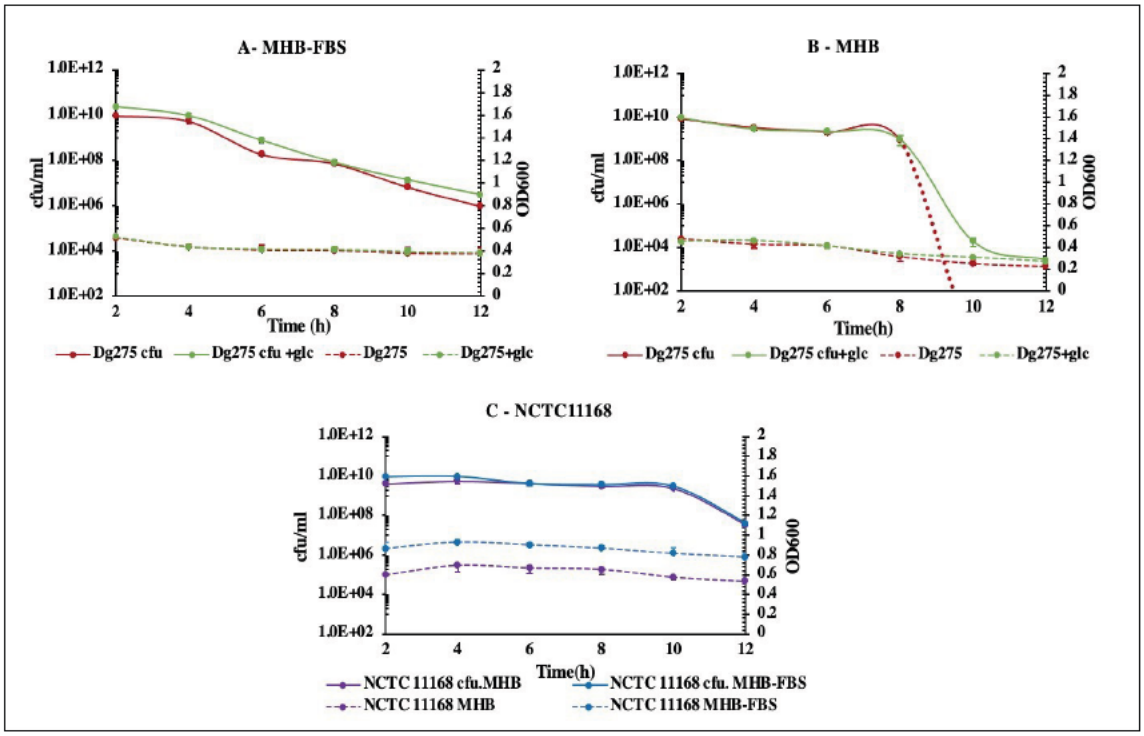


Figure 6.7 Survival of Dg275 and NCTC11168 in atmospheric oxygen. (A) Dg275 in MHB-FBS plus or minus glucose (B) Dg275 in MHB plus or minus glucose (C) NCTC11168 in MHB or MHB-FBS. Cultures were inoculated to give a final OD of 0.002 and grown for 24h in 50ml conical flasks in the M35 cabinet(5%O_2,10%CO_2,2%H_2) at 37°C, 0 time indicates point of transfer of culture to atmospheric conditions at 37°C, where shaking continued at 150rpm. Atmospheric cultures were monitored for OD_{600} (dash line) and cfu (solid line) at times indicated. Green with 20mM glucose, red no added glucose. Error bars derived from three technical repeats.

Glucose was earlier shown to substantially enhance survival of Dg275 during growth in DMEMf, see fig6.5. Extension of this to monitor survival of Dg275, Dg95 (ED + strains), Dg 200 and NCTC11168 (ST45CC-45 and ST21CC21, respectively), in atmospheric conditions following growth in DMEMf was also tested.

As above, cultures were grown at 37˚C, in 5%O_2, 10%CO_2, 2%H_2 for 24h and then transferred to air where shaking continued at 37˚C. The results reinforce the extent to which glucose protects Dg275 from aerobic stress in a minimal medium. At 24h, Dg275 cultures are in early stationary phase and grow well for a further 24h under microaerobic conditions 5%O_2 even in the absence of glucose, see fig 6.8. However, when switched to atmospheric oxygen, 4h later no cells were recovered from Dg275 cultures without glucose, whereas Dg275 cultures grown in DMEMf with 20mM glucose could still be recovered at 10^6 cfu/ml after 8h incubation with shaking in air, fig 6.8. Dg95 showed the same trend although cfu were only detected at 4h post aerobic incubation.

Both Dg200 and NCTC 11168 grow faster than Dg275. Hence the poor recovery of these strains could be related to the state of cells at 24h microaerobic incubation, glucose would not be expected to enhance survival of either of these Glc negative strains. In DMEMf media, it has been demonstrated that in the absence of glucose cultures of Dg275 and other ED positive strains become nutrient limited and die faster (Mohammed, 2018).

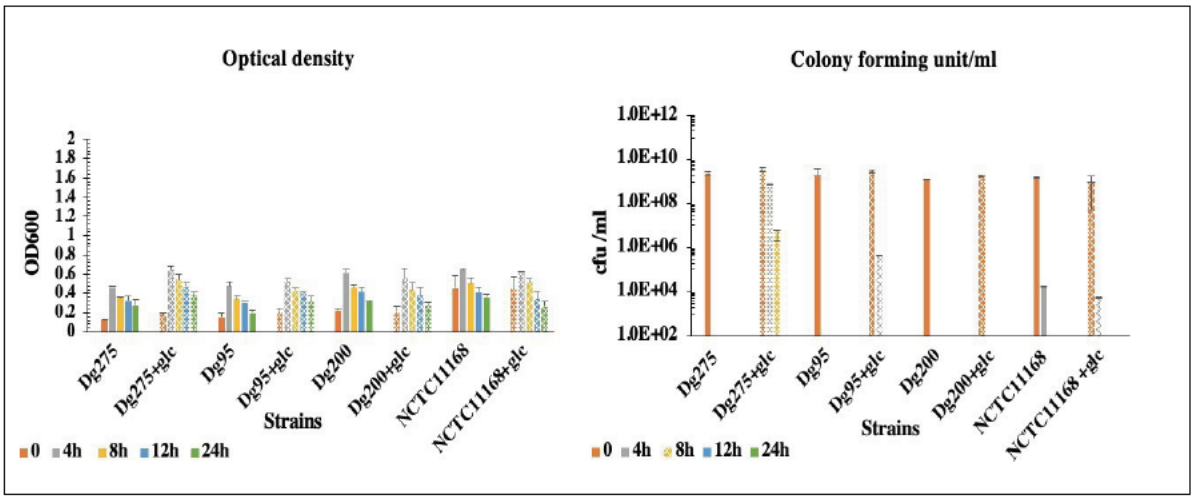


Figure 6.8 Impact of glucose on growth and survival of Dg275 and Dg95 under atmospheric condition in DMEMf with and without glucose. 15ml DMEMf in a 50ml conical flask was inoculated with 150µl standardized cells (0.2 OD_{600}), prepared from MHA plates and grown at 150rpm in M35 cabinet. Samples were monitored for OD_{600} and cfu at time indicated. After 24h the culture was transferred to air with shaking at 150rpm, at 37°C. Cfu was determined following 10-fold serial dilutions, spotted on BA-2% incubated at 37°C for 48h in 5%O_2, 10%CO_2, 2%H_2 . Pattern fill, with 20mM glucose, solid fill no added glucose. SE is from triplicate repeats, strains Dg275 and Dg95(ED pathway), Dg200 and NCTC11168(-ED). Results shown are the means and SD of triplicate samples. The experiment was repeated three times and all results produced similar results. Statistical analysis was conductedusing the t-test in excel.

6.4 Effect of H_2O_2 on survival of NCTC11168 and Dg275 during growth

Exposure to hydrogen peroxide (H_2O_2) was used as one indicator of the resistance of Dg275 and Dg95 to oxidative stress. The impact of growth in the presence of glucose on sensitivity of cells H_2O_2 was monitored.To establish optimal assay conditions, initial studies assessed sensitivity of the well- studied strain NCTC11168 during growth and then compared this to sensitivity of Dg275.

Bacteria were cultured in 15ml MHB- FBS broth in 50ml conical flasks, at 37°C, with shaking at 150rpm under standard conditions, 5% O_2, 10% CO_2, 2% H_2 and 83%N_2 with 70% humidity, as described in fig6.9 and chapter2, 2.1.4. Bacterial cells were recovered at times during exponential (4 and 6h), stationary (12 and 24h), late stationary (48h) and death phase (72h) of growth and resuspended in PBS at a final OD_{600} of 0.18 to monitor for sensitivity to hydrogen peroxide, as described in chapter 2.2.8.

Initial tests monitored sensitivity to increasing concentrations of H_2O_2 1-15mM for different time periods 15, 30 and 60 min, at 37°C under microaerobic conditions. Survival of bacterial cells was monitored by calculation of cfu, chapter2, 2.4.5, following serial dilution of samples, spotting and incubation on BA-2% plates at 37°C for 48h under microaerophilic conditions. As previously documented (Rodrigues *et al*., 2015), NCTC11168 was much more resistant to hydrogen peroxide during the stationary phase of growth, showing high resistance to 10mM hydrogen peroxide following 30min exposure with samples from 12h, 24h and 48h growth, fig6.9 C-E. There was no significant difference in cfu recovered from these growth samples treated with 1 mM H_2O_2 for up to 30min. With 5mM H_2O_2 or 10mM H_2O_2 and 30 min exposure of these stationary phase NCTC11168 cells, recovery of colony forming units was 45-56 % and 29–34 % respectively.

Only with increased exposure 60min and/or increased concentration of H_2O_2 15mM did recovery of NCTC11168 cells decrease by more than 1 log_{10}.

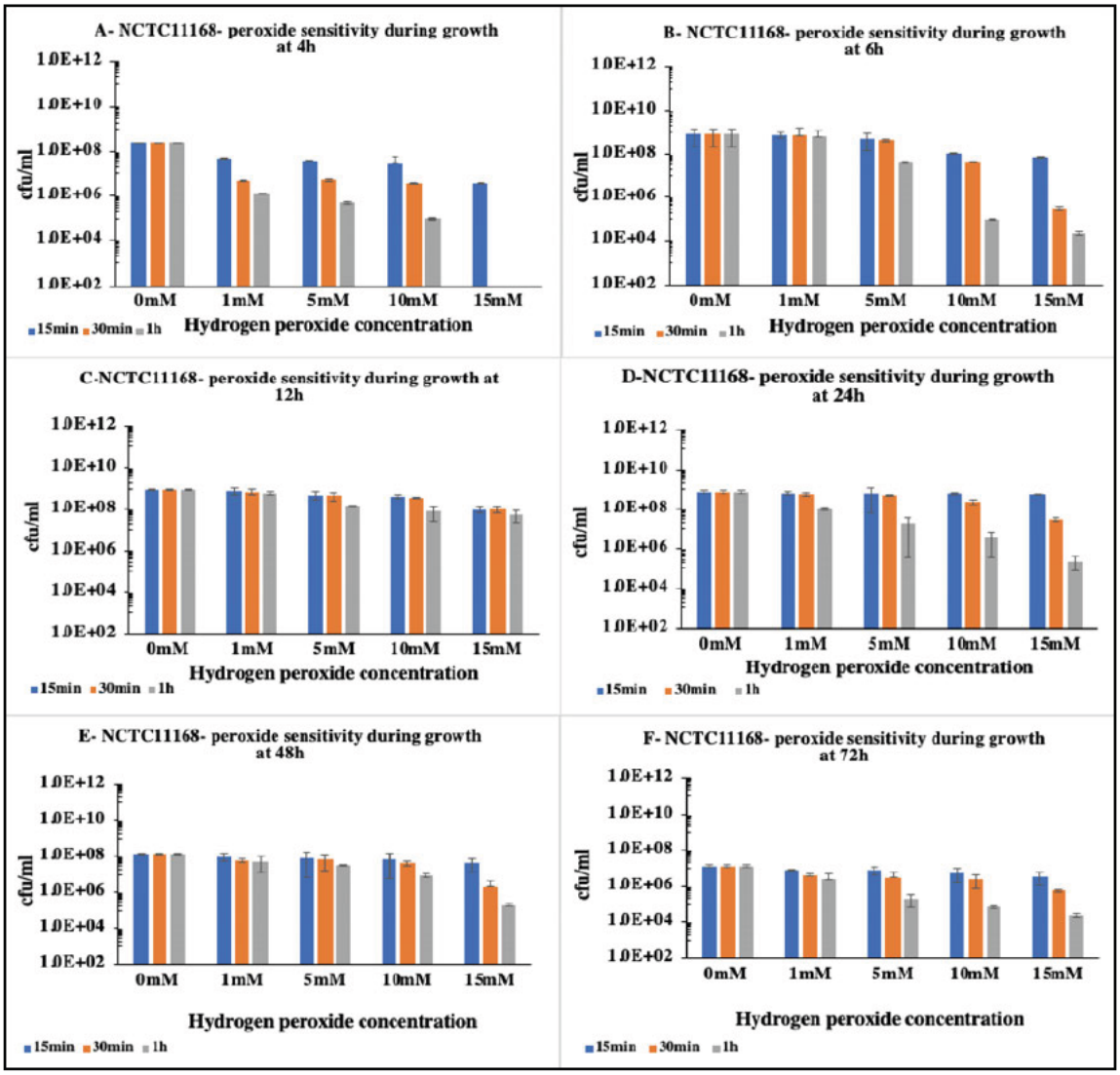


Figure 6.9 Sensitivity of *C. jejuni* NCTC11168 to H_2O_2 at different stages of growth. NCTC11168 was cultured in 15 ml MHB-FBS in 50 ml conical flasks, at 37°C, with shaking at 150 rpm under standard microaerophilic conditions, in the M35 cabinet with 70% humidity, as described in chapter2, 2.1.4. At indicated times, samples of culture equivalent to 0.9 OD_{600} units were taken and processed for the peroxide sensitivity assay 2.8. The final concentration of cells in each assay (1ml) corresponded to an OD_{600} of 0.18, variation of cfu with time is indicated by 0mM samples, monitored at 60 min. For 4 h sample, OD_{600} was not assessed. Samples were exposed to increasing concentrations of H_2O_2, as indicated for 15,30 and 60 minutes, under standard microaerobic conditions in the M35 cabinet at 37°C section 2.8. Susceptibility to H_2O_2 was assessed by cfu of serial dilutions as described, 2.4.5. Averages and SD shown are from triplicate flasks inoculated with the same starting culture 5.0X 10^6 /ml.

Strains	Growth time/h	Survival (%) of cfu								
		15 minutes			30 minutes			60 minutes		
		1mM	5mM	10mM	1mM	5mM	10mM	1mM	5mM	10mM
NCTC11168	4	19.7	14.10	10.40	1.93	1.97	1.4	0.48	0.19	0.04
	6	80	55	12	74	46.1	5	66.4	4.6	0.01
	12	75	58	41	70	44.4	33.6	59.6	14.2	7.5
	72	56	43	27	32	22.6	18.7	17	1.37	0.5
Dg275	4	39.8	3.93	0.02	3.8	0.44	0.02	0.31	0	0
	6	12.51	7.71	0.01	2.08	1.58	0.09	0.21	0.10	0.02
	12	67.7	43.4	0.04	70.3	57.2	0.55	54.6	42.5	0.03
	72	4.39	4.31	0.01	7.7	6.09	5.41	0.77	0.49	0.33

Table 6.2 Survival of exponential and death phase cells of NCTC11168 and Dg275 to H_2O_2 exposure.

In the early phases of growth, 4h-6h, when the bacteria are rapidly multiplying, NCTC11168 cells were much more sensitive to H_2O_2, fig 6.9 A,B. Table 6.2 highlights survival values of mid-exponential phase cells, following different treatments. After 4h growth and exposure to 1 mM H_2O_2 for 15 min, 30 min or 60 min only 20%, 1.9% or 0.5% of cells survived. A similar profile of 14%, 1.9% and 0.2% survival, respectively, was seen with 5 mM H_2O_2, and with 10 mM H_2O_2, 10.4%, 1.4% and 0.04% survival was observed. Exposure of 4h growth to 15mM H_2O_2 was very detrimental, only 1.4% of cells survived 15 min exposure, with no colonies recovered following longer exposure times. Following 6h growth, NCTC11168 cells were already resistant to 1 mM H_2O_2 even following 60min exposure, although at 10mM H_2O_2

sensitivity followed a similar profile to the 4h sample with approximately 10%, 5% and 0.01% recovery following 15, 30 and 60 min exposure, respectively.

In the later (death) phase of growth, 72h, the decrease in viability of cells during growth was evident. Colony forming units of the control samples 0 mM peroxide, 0.18 OD_{600} had already dropped from ~$1x10^9$/ml at peak stationary phase, to ~$1x10^7$/ml. These bacteria were comparatively resistant to H_2O_2, although more sensitive than 48h growth samples at each concentration of H_2O_2 tested fig6.9 E and F. Comparison of percent survival of 72h samples to 4h growth samples is summarised in table 6.2. A 10-35 fold higher survival was recorded for the 72h samples compared to 4h samples at each H_2O_2 concentration tested following 30 or 60 min exposure. A concentration of 1 mM H_2O_2, only had a pronounced effect on the 4 h sample, where cfu values dropped from $2.3x10^8$/ml in untreated sample to $5x10^6$/ml and $1.2x10^6$/ml following 30 min and 60 min incubation, respectively.

The 72h sample was also affected by 1 mM H_2O_2 but only marginally with the cfu decreasing from $1.3x10^7$/ml in untreated sample to $2.4x10^6$/ml following 60 min exposure. In contrast, 15 mM H_2O_2 impacted survival of bacteria at all growth stages and led to at least a 4-5 log_{10} drop in colony forming units of exponential cells, following exposure for 30 min or more. Notably, sensitivity of cells was generally affected more by increase in exposure time than by increase in H_2O_2 concentration, for example see fig 6.9 A and F 1,5 and10mM.

An exception was 60 min exposure where increase in H_2O_2 concentration correlated with increased sensitivity. Thus while 1 mM H_2O_2 with 30 and 60min exposure is a useful concentration to test the most sensitive cells, inclusion of concentrations of 5 and 10mM is appropriate to monitor less sensitive samples and to reinforce results. Having established the peroxide sensitivity assay using NCTC11168, the assay was repeated for cultures of Dg275.

Results are shown below in fig6.10. Data for survival of Dg 275 grown in MHB-FBS (no glucose) has been extracted and added to table 6.2.

As with NCTC11168, samples from early cultures are particularly sensitive to H_2O_2. Dg 275 has a much longer lag phase during growth than NCTC11168, with cultures in the exponential phase between 6 and 12h, see fig6.9. Hence, the 4 and 6h cells of Dg275 were highly sensitive. For example, with the 4h sample, only 3.4%, 0.39% and 0.02% survived exposure for 30 min to 1mM, 5mM and 10mM H_2O_2, respectively and the 12h sample also showed a 2 $\log_{10}$ decrease in survival on exposure to 10mM H_2O_2 for 30 min. Stationary phase samples of Dg275 exhibited resistance to hydrogen peroxide at the lower concentrations of 1mM and 5mM, but showed some sensitivity at concentrations of 10mM and 15mM. Cultures of 60 and 72h growth already had a low cfu count of $1.2x10^7$/ml and $7.0x10^6$/ml for the test suspension OD_{600} 0.18 and cells from72 growth were ~4 fold more sensitive to H_2O_2 than cells grown for 60h. Although these late-stage cells exhibited increasing sensitivity to H_2O_2, they remained more resistant than Dg275 cells from early exponential phase, table 6.2. Overall, the pattern of H_2O_2 sensitivity was similar for both strains.

6.5 Impact of glucose metabolism on resistance of Dg275 and Dg95 to H_2O_2

To study the effect of growth on glucose on survival in the presence of hydrogen peroxide, focus was on the later stage of growth of the ED positive strain Dg275, at a time when this strain has been shown to metabolise glucose (Mohammed, 2018). Both Dg275(EDtype2) and Dg95(ED type 3) have been shown to utilise glucose. In addition the inability of the mutant, Ch95-95*glk*(1) to grow on glucose has been demonstrated, fig 5.2 ,fig 5.4 and hence this mutant was included for comparison to investigate the impact of the ability to utilise glucose on these *C. jejuni* strains.

Fig6.10 shows the survival of Dg275 cells, harvested at different times during growth with or without glucose and exposed to H_2O_2. As previously established, growth of Dg275 in MHB-FBS with and without 20mM glucose produced similar viable counts up to 36-48h, with a notable decrease in cfu in the absence of glucose at 60h and 72h (not shown). The enhanced survival of Dg275 in the presence of glucose is evident in the untreated, 0mM H_2O_2 control samples, fig6.10.

Following growth for 60h in the absence of glucose, the untreated control had a cfu count of $1.2x10^7$/ml and for 72h of $7.0x10^6$/ml ; in contrast to growth in the presence of glucose where the cfu counts for untreated control were $5.8x10^8$/ml at 60 h and $4.3x10^8$/ml at 72h.

The classic profile of hydrogen peroxide sensitivity of the strain Dg275 grown in the absence of glucose is discussed above, section 6.4. With respect to the impact of growth on glucose, following 30 min exposure to H_2O_2, there was no difference in sensitivity of cells grown in the presence or absence of glucose up to 60h, while at 72h there was approximately a 3-5-fold increase in percent recovery of cells that had been grown on glucose, fig6.10 and table 6.3.

Following 60min exposure to H_2O_2 the difference was greater, samples taken at 48,60 and 72h growth in glucose all exhibited ~4-8 fold higher recovery over cells grown without glucose, table 6.3. In contrast to sensitivity of exponential phase cells, sensitivity of death phase cells was independent of H_2O_2 concentration and more dependent on time of incubation, possibly correlating with H_2O_2 enhanced rate of death of a sensitive subset of cells.

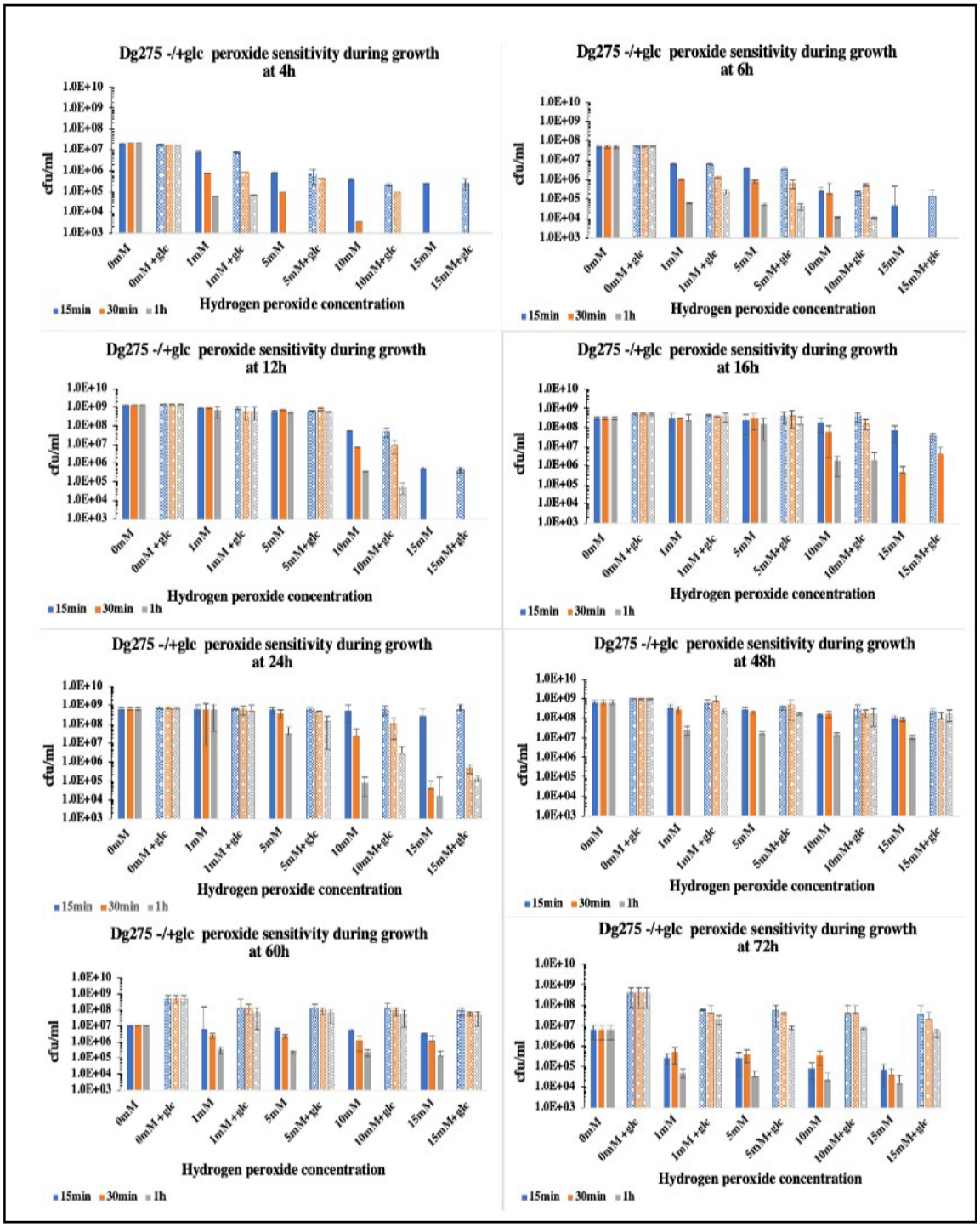


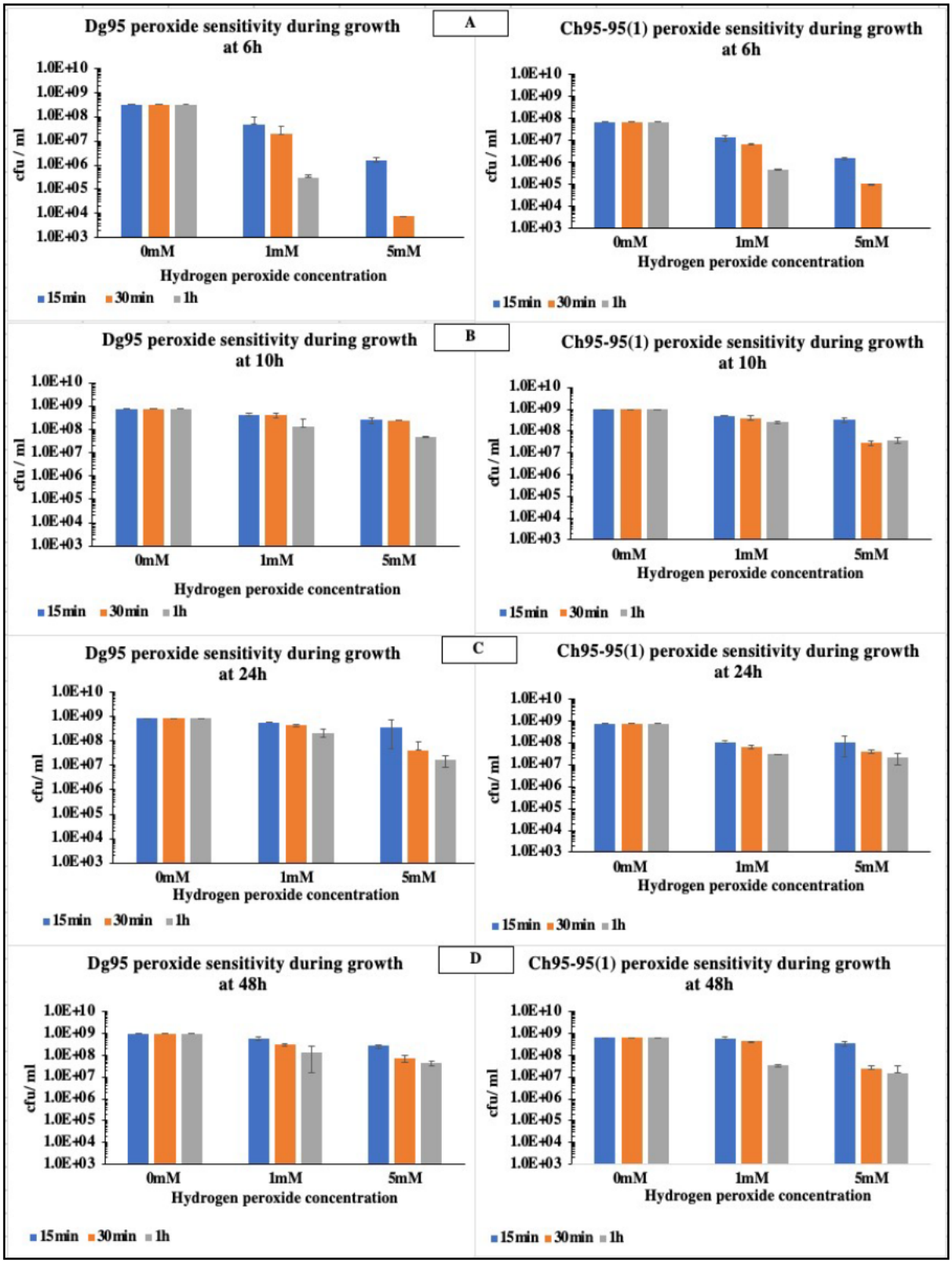
Figure 6.10 Sensitivity of Dg275 to H_2O_2 grown in the presence or absence of glucose. Dg275 was cultured in MHB- FBS with or without 20mM glucose and assayed for sensitivity to peroxide, as described in fig6.9. The final concentration of cells in each hydrogen peroxide assay (1ml) corresponded to an OD_{600} of 0.18, variation of cfu with time is indicated by 0 mM (H_2O_2- control) samples, monitored at 60 min. For 4h sample, OD_{600} was not assessed. Averages and SD shown are from triplicate flasks inoculated with the same starting culture 6 x 10^6/ml.

Strains	**Growth /hour**	**Survival rate (%) at hour exposure with different concentration of H_2O_2**			
		1mM	**5mM**	**10mM**	**15mM**
Dg275	60	3.20	2.27	2.14	1.16
Dg275+glc		14.17	11.79	10.5	9.1
Dg275	72	0.77	0.49	0.33	0.23
Dg275+glc		5.29	2.05	1.80	1.19

Table 6. 3 Impact of glucose in growth media on survival of Dg275 following 60 min exposure to H_2O_2.

Dg95 (+ED) and the mutant Ch95-95*glk* (1) (-ED), were grown in MHB-FBS plus 20mM glucose, sampled throughout the growth phase and tested for susceptibility to H_2O_2 at concentrations of 1mM and 5mM for 15, 30 and 60 min exposure, fig6.11. Growth of both strains in this medium was comparable up to approximately 48h, see fig5.2. After this time the impact of glucose utilisation was evident as recovery of cfu decreased more rapidly with continued growth of Ch95-95*glk* than with the parent strain Dg95. This was also evident in cfu/ml values from the '0 mM' control samples shown in fig6.11. Control samples from the parent strain had cfu counts of $9.8x10^8$/ml (48h), $8.3x10^7$/ml (60h) and $7.2x10^7$/ml (70h) whereas the mutant strain had values of $6.5x10^8$/ml (48h), $3.3\ x10^7$/ml (60h) and $5.7x10^6$/ml (70h). On treatment of exponentially growing cells (6h) with H_2O_2, both strains exhibited a high sensitivity. Following 30min exposure to 5mM H_2O_2, survival was <1.58% (no colonies recovered) for both strains. With 1mM H_2O_2 and 60min incubation, recovery was 0.1% for Dg95 and 0.67% for Ch9595*glk*, fig6.11.

Late logarithmic stationary phase cells (10h-48h) exhibited the characteristic more resistant profile fig6.11B&C. Following 60h and 70h growth, fig6.11 E&F, the peroxide sensitivity pattern was similar to that of Dg275 with and without glucose. In addition to decreased survival of Ch95-95*glk* during growth, reflecting the inability to metabolise glucose, the percent survival of viable cells on exposure to H_2O_2 was reduced compared to Dg95, table 6.4. This was particularly evident following 60 min exposure to either 1mM or 5mM H_2O_2 where recovery of the parent strain Dg95 was 15-fold and 10-fold higher than that of Ch95-95*glk*, respectively.


A
Dg95 peroxide sensitivity during growth at 6h
Ch95-95(1) peroxide sensitivity during growth at 6h
B
Dg95 peroxide sensitivity during growth at 10h
Ch95-95(1) peroxide sensitivity during growth at 10h
C
Dg95 peroxide sensitivity during growth at 24h
Ch95-95(1) peroxide sensitivity during growth at 24h
D
Dg95 peroxide sensitivity during growth at 48h
Ch95-95(1) peroxide sensitivity during growth at 48h
cfu / ml
cfu/ ml
1.0E+10
1.0E+09
1.0E+08
1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
0mM
1mM
5mM
Hydrogen peroxide concentration
15min
30min
1h

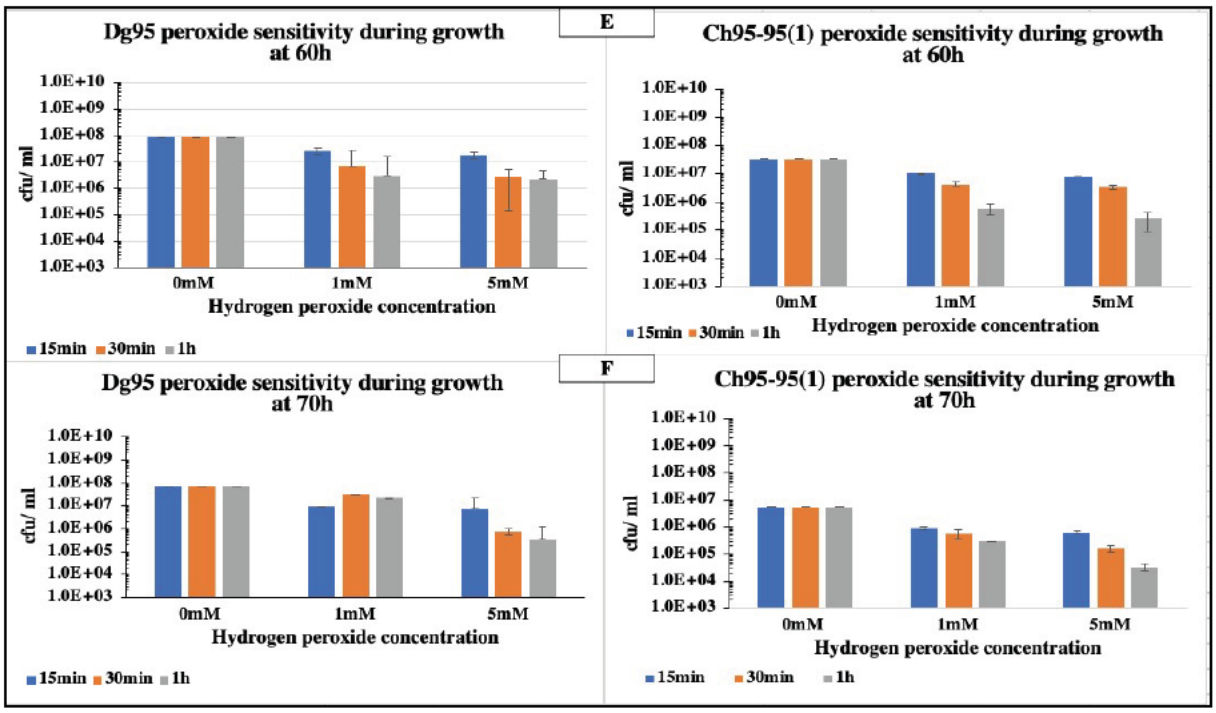


Figure 6.11 Sensitivity of Dg95 and Ch95-95*glk* to H_2O_2. Dg95 and Ch95-95*glk*(1)were cultured in MHB- FBS with 20mM glucose and assayed for sensitivity to peroxide, as described in fig 6.9. The final concentration of cells in each hydrogen peroxide assay (1ml) corresponded to an OD_{600} of 0.18, variation of cfu with time is indicated by 0mM samples, monitored at 60 min. For 6h sample, OD_{600} was not assessed. Averages and SD shown are from duplicate flasks inoculated with the same starting culture 9 x 10^6/ml for Dg95 and 7x10^6/ml for Ch95- 95*glk* (1).

Strains	**Growth (h)**	**Survival rate (%) at 60-70h exposure with different concentration of H_2O_2**					
		15 minutes		**30 minutes**		**60 minutes**	
		1mM	**5mM**	**1mM**	**5mM**	**1mM**	**5mM**
Dg95	60	30	21	8.01	3.0	34	7.6
Ch95-95(1)		30.6	23	13	10	1.7	0.75
Dg95	70	13	10	42	11	30.3	4.6
Ch95-95(1)		16	11	10	3	5.3	0.6

Table 6.4 Relative survival of Dg95 and Ch95-95*glk* to H_2O_2 exposure following growth in MHB-FBS with glucose.

6.6 Conclusion

Campylobacters require a low concentration of oxygen, and cannot grow in atmospheric oxygen (Kelly, 2008), however there are some studies demonstrating that *C. jejuni* can grow *in vitro* in atmospheres with an oxygen tension of 21%O_2 (Kaakoush, *et al*., 2007). The ED pathway has been associated with aerobic growth and it was considered that possession of the ED pathway may enhance survival of these glucose utilising bacteria on exposure to oxygen levels above 10 % and even up to atmospheric condition (21%O_2).

Under growth with suboptimal concentrations of oxygen (2%), the ED positive strains Dg275 and Dg95 both grew as well as the ED negative strains tested. However, during growth with 2% Oxygen there was no evidence of benefit of addition of glucose to the growth media. Viable cell count of cells grown with and without glucose all decreased at the same rate. This was in contrast to growth under standard conditions of 5%O_2, where glucose consistently enhanced survival during the decline phase of growth whether in minimal or rich media.

In high oxygen, adaptation of the ED negative strains, Dg200 and NCTC11168, was required to overcome aerobic stress. In contrast, the ED positive strains Dg275 and Dg95, were shown to have the ability to grow immediately in high oxygen and there was some evidence of enhanced growth in the exponential phase which should be confirmed. This may indicate that these strains carrying the *glc* locus are already primed to deal with aerobic stress.

At 15.9%O_2, the impact of the presence of glucose with cultures grown in DMEMf was particularly marked. The difference in stationary phase growth and survival with and without glucose was much greater than seen with the same strains grown under standard microaerobic conditions in DMEMf. A strain carrying a mutation within the *glc* locus, abolishing glucose utilisation, was required to confirm the significance of the glucose metabolism, rather than another property of these strains, to survive oxygen stress.

The Ch95-95*glk*(1) strain in which the gene encoding glucokinase (*glk*) is a pseuodogene was used. It was demonstrated that inclusion of glucose in growth media with Ch95-95*glk*(1) had no benefit to survival of this strain under standard microaerobic conditions (See Chapter 5 for details). Under 15.9% O_2, Ch95-95*glk*(1) also showed no growth or survival benefit with inclusion of glucose in the media. However, interestingly the survival of Ch95-95*glk*(1) both with and without glucose was greatly decreased in the decline phase, although like the parent strain, Dg95, it required no pre-adaptation for growth under these conditions. These data all support the hypothesis that glucose utilisation via the ED pathway is aiding survival of cells from aerobic and oxidative stress in the decline phase of growth.

Hydrogen peroxide is considered a major ROS and, in bacterial cells, is generated during the process of aerobic metabolism (Day *et al*., 2000). In this study, susceptibility to H_2O_2 was studied as an example of the response of *C. jejuni* to oxidative stresses by either exogenous or endogenous H_2O_2. . Initial studies confirmed the high susceptibility of Dg275 and Dg95 to H_2O_2 in early exponential phase, at 4h and 6h growth, when cells are actively dividing. At this time cells were sensitive to 30 min exposure 1mM H_2O_2 by stationary phase. Cells were highly resistant to H_2O_2 and only sensitive to a high concentration of 15mM H_2O_2 and/or longer exposure times of 1h. All strains were again more susceptible to H_2O_2 during the decline phase of growth at 60h and 72h, but still not as sensitive as in early exponential phase of growth. Addition of glucose to culture media had a clear effect on protecting Dg275 and Dg95 cells (both ED positive) from the effects of H_2O_2. This correlates with enhanced survival of these cells during the decline phase. In general, the results indicate that glucose has a positive effect on the survival of *C. jejuni* in the decline phase of growth and that this in turn also enhances survival on exposure H_2O_2.

Metabolism via the ED pathway produces 2 products known to have a role in production against oxidative stress. NADPH is an important reductant in protection against oxidative stress and is a product of the conversion of glucose phosphate to Glucono1,5 lactone -6P by glucose-6-phosphate dehydrogenase, the gene product of *zwf*.

In addition, one molecule of glucose metabolised via the ED pathway, in a few steps produces one molecule of pyruvate and in a few additional steps produces a second molecule of pyruvate, see chapter1, fig1.7A. While pyruvate is the entry level to the TCA cycle and energy production via aerobic respiration, it can also be fermented to acetate reducing production of ROS products. Additionally, pyruvate is a recognised anti-oxidant and can chemically convert H_2O_2 via an enzyme independent chemical reaction to produce acetate and release water and carbon dioxide, although enzymatic removal by catalase is likely more effective (Guarino *et al*., 2019).

Chapter 7

General discussion and future work

7.1 Introduction

C. jejuni is one of major causes of human bacterial gastroenteritis worldwide, causing campylobacteriosis by both foodborne and waterborne infections (Liu *et al*., 2017). In addition, *C. jejuni* infections can occur by direct contact with farm animals including sheep, pig, cattle and chickens, the latter of which are considered to be the main vector for human campylobacteriosis via contaminated food (Hartley-Tassell *et al*., 2018; Domingues *et al*., 2012). The focus of this study was on some unusual *C. jejuni* strains frequently associated with Norway rats, a potential source for *C. jejuni* in the food chain. The presence of rats on farms was shown to be associated with an increase in *Campylobacter* introduction into chicken houses (Kapperud *et al*., 1993). Also, another study confirmed that 87% of rat faecal samples tested were positive for *C. jejuni* (Kasrazadeh *et al*., 1987). Thus, contamination of animal feed and water as well as environmental soil is likely to contribute to transmission of *C. jejuni* in the farmyard with subsequent colonisation of farm animals (Nkogwe *et al*., 2011). The *C. jejuni* strains used in this study were isolated from farmyard associated Norway rats. These strains included generalist *C. jejuni* strains belonging to ST21CC and ST45CC clonal complexes commonly associated with human disease, as well as the newly identified RG2 group of ED positive *C. jejuni* carrying the *glc* locus (Mohammed, 2018). This study primarily focused on the glucose utilising strains of *C. jejuni*.

7.2 Distribution of the *glc* locus among strains of *C. jejuni*

The first indication that any strains of *C. jejuni* might have the ability to utilise glucose was in 2008, when a locus encoding enzymes of the ED pathway was identified in the genome sequence of *C. jejuni* subsp. *doylei* 269.97 (Miller, 2008). This sequence analysis identified the seven genes constituting the *glc* locus as a gene cluster on a 8669bp region of DNA. In a survey of available sequences, Vegge and workers identified the *glc* locus in only 1.7% of over 6,000 genomes, indicating that glucose utilisation was a property of a small subset of *C. jejuni* strains, primarily from wild birds and rats, that is from environmental rather than clinical sources (Vegge *et al*., 2016). The *glc* loci were found in Norway rats (39 strains), wild birds (30 strains), farm environments (2 strains), and environmental waters (1 strain) (Jolley and Maiden. 2010; Vegge *et al*., 2016). Functional utilisation of glucose in *Campylobacter* was first demonstrated in *C. coli* (Vegge *et al*., 2016; Vorwerk *et al*., 2015) and only recently confirmed in strains of *C. jejuni* (Mohammed, 2018), thus putting to rest the established criterion that *C jejuni* never utilises glucose. The glucose utilising strains isolated from Norway rats (termed RG2 strains) mostly fall into two to phylogenetic groups. The largest group containing Dg275 (ST7259CC45) and Dg95 share 4 of the 7 MLST alleles with the centrally defined clonal complex ST45CC45. Based on wgMLST and cgMLST, these CC45 strains possessing the *glc* locus represent a phylogenetic group clearly distinct from the ED-negative CC45 rat-associated strains, represented by Dg200, ST45CC45 (Mohammed, 2018). Based on allele analysis of each gene within the *glc* locus, the *glc* loci identified thus far in different *Campylobacter* strains have been classified into different EDMLST types. Thirty-six different ED types have been identified in *C. jejuni* and ten in *C. coli* (Vegge *et al*., 2016).

The majority of glucose utilising *C. jejuni* strains isolated from Norway rats were shown to belong to ED type 2 (3-4-4-5-4-5-5), including Dg275 and ED Type 3 (3-4-4-6-4-5-5), varying only in the *pgl* locus and includingDg95. Several other strains, Dg 180, Dg 184, Dg 122 and Dg 187 also belong to clonal complex CC45 (ST4791CC45) are assigned ED types 9 and 11 and differ from ED Type 2 only in *pgi* or in *pgl* + *pgi*. For all of these strains, the EDMLST correlates well with wgMLST and notably none belong to a large distinct phylogenetic group of strains.

Some other of the glucose utilising *C. jejuni* strains isolated associated with Norway rats are more distantly related to the above group, based on wgMLST or rMLST. These include strains Dg 43, Dg 268, Dg233, fig3.1. These strains were shown to have the same set of 7 genes within the *glc* locus as Dg275 and Dg95 (ED Types 2 and 3) but the *glc* locus from these strains were also defined as phylogenetically distinct (ED type 21, 23). Several additional strains, Dg18, Dg201and Dg381, from this group were initially annotated as having an incomplete *glc* locus, but PCR amplification and Sanger sequencing confirmed that each strain possesses a complete *glc* locus and functional studies using DMEMf confirmed the characterised benefit of extended growth and survival with inclusion of glucose in the media. Indeed, comparison of the impact of glucose on growth in minimal and rich media, demonstrated that all strains, irrespective of ED type and wgMLST grouping, exhibit the same growth profile with no benefit of glucose during exponential growth and similar growth profiles reflecting benefit of glucose only in late stationary and the decline phase of growth.

The *rrn* operon copy number in bacteria varies from 1 to 15 per genome (Rainey *et al*., 1996). For example, *Clostridium paradoxum* and *Bacillus subtilis* (Loughney *et al*., 1983), contain between 10 to 15 rRNA operons (Rainey *et al*., 1996) while the enteric bacterium *E.coli* has seven copies (Ellwood and Nomura, 1980), and both *Rickettsia prowazekii* (Andersson *et al*.,

1995) and *Mycoplasma pneumonia* possess one copy of the RNA operon (Bercovier *et al*., 1986). It has been reported that deletion of one of the *rrn* operons in *E. coli* did not have a significant negative effect on its growth (Condon *et al*., 1995).

C.jejuni possesses three copies of the *rrn* locus, each containing 16S rRNA, 23S rRNA, 5S rRNA genes and several tRNAs (Taylor *et al*.,1992; Parkhill *et al*., 2000). The *glc* locus was located within one copy, *rrn*A in *C. coli* (Vorwerk *et al*., 2015) and in Dg275 it was identified in both *rrn*A and *rrn*B but not in *rrn*C (Mohammed, 2018). In this study, a combination of Illumina and Nanopore sequencing with hybrid assembly has confirmed insertion of the *glc* locus in *rrn*A and *rrn*B of Dg95 but not in *rrn*C and surprisingly, combined with PCR analysis has identified 3 copies of the *glc* locus in Dg43, one associated with each of the 3 *rrn* loci. No marked correlation of growth properties with location or number of *glc* locus insertions within the genome was seen in this study. The sequencing results from both Ch95-95 (1) *glk* and Ch95-95 (2) *glk* where *glk* is a pseudogene or has a missense mutation, respectively identified the same *glk* mutation in the complete *glc* locus within both *rrn*A and *rrn*B. This would be consistent with rapid recombination of the *glc* locus within the genome of one bacterial cell (Karlyshev and Wren, 2005). The extent of recombination and transfer of the *glc* locus between different strains of *C. jejuni* remains to be established. Transfer by natural transformation between two strains of *C. coli* has been demonstrated in the laboratory, with a low level of efficiency (Vorwerk *et al*., 2015) and this has yet to be demonstrated between strains of *C. jejuni*. The general correlation of ED type with whole genome and core genome phylogenetic grouping of glucose utilising strains together with the small percent of *C. jejuni* isolates having a *glc* locus, is consistent with only a very low frequency of transfer of the *glc* locus between *C. jejuni* strains. However, it may be that in some natural environments there is strong selection pressure for acquisition of the *glc* locus. This requires further study.

7.3 Metabolism of sugars by *C. jejuni*

A variety of different bacteria have been shown to possess the ED pathway including *Pseudomonas* spp and *Escherichia coli.* In *E. coli* the ED pathway enzymes are inducible and serve primarily in gluconate catabolism, while glucose itself is catabolized preferentially by EMP-mediated glycolysis (Conway, 1992). In *Pseudomonas* spp. as with *Campylobacter* the EMP pathway is incomplete. In *C. jejuni* strains carrying the *glc* locus, the ED pathway would be beneficial in hosts with readily available glucose, and in particular in hosts when the preferred amino acids are limiting. In addition to free glucose, *C. jejuni* in the digestive gut of animals, will be exposed to macromolecules containing glucose, for example amylose, starch, cellulose. For this reason, the ability of several of the glucose utilising strains to metabolise lactose and maltose was tested. It was shown that neither were metabolised by the strains tested and this correlates with the absence of any readily identifiable maltose or lactose degradation enzymes or uptake system in these *C. jejuni* genomes. However, despite being unable to directly metabolise glucose containing disaccharides and polysaccharides such as cellulose, starch, maltose and lactose these ED positive strains of *C.jejuni* are very likely to have access to these molecules through co-association with other microbes of the animal gut microbiota. For example, this has recently been shown for fucose metabolism by *C. jejuni*. Fucose is a common component of mucins, but *C. jejuni* strains carrying the *fuc* locus do not possess a gene encoding a fucosidase enzyme and hence are unable to cleave the fucose moieties from glycosylated host mucins. However, a recent study has shown an inter- dependence of *C.jejuni* with *Bacteroides vulgatus* to acquire free fucose, as *B. vulgatus* was shown to cleave 4-nitrophenyl-α-Lfucopyranoside (Garber *et al*., 2020).

C. jejuni strains possessing the *glc* locus have been primarily identified from Norway rats, wild birds and environment sources (Vegge *et al*., 2016). This is despite the very large numbers

Of complete human and chicken genome sequences available (PUBMLST/campylobacter). Presence of the ED pathway in *C. jejuni subsp jejuni* strains from humans or chickens is extremely rare. It may be that there is some particular benefit to glucose metabolism in these environmental strains. Glucose metabolism via the ED pathway supported stationary and decline phase survival of all *C. jejuni* strains carrying the *glc* locus.

This is consistent with published studies demonstrating glucose catabolism via the ED pathway in *C. coli* (Vorwerk *et al*., 2015). The ED pathway typically produces 1 mol each of ATP, NADH, and NADPH per mol glucose (Flamholz *et al.*, 2013), providing energy for continued growth. It produces pyruvate which can be fed into the TCA cycle and aerobic respiration. NADPH produced via the ED pathway is considered to be important in anabolic processes.

Glucose metabolism in *C coli* has been shown to contribute to anabolic processes including surface polysaccharide production (Vorwerk, *et al.,* 2015) and biofilm formation where with one strain of *C. coli* a large increase in a pellicle or floating biofilm was observed, while in another strain of *C. coli* glucose metabolism was diverted to catabolic processes (Vegge *et al.,* 2015). *C. jejuni* consistently transitions from a spiral morphology to a coccoid morphology during nutrient starvation and the late stationary and decline phases of growth, as well as under other conditions of stress (Reezal and Anderson, 1998). Glucose utilisation of Dg275 protected this strain from coccoid formation in the late stationary/ decline phase and maintained viability of the cultures.

It is not known what control mechanism is involved ensuring that glucose was only used once preferred amino acids are deplete and if this may also be linked to stationary phase growth, but availability of glucose provides an additional carbon and energy source to prevent cell death.

It has also been suggested that 1mol of glucose produce 1mol of NADPH via the ED pathway (Flamholz *et al*., 2013), and its reductive characteristics may be utilised for oxidative stress protection (Stanton, 2012).

7.4 Impact of different concentrations of oxygen on the survival of ED-positive *C.jejuni* strains

C. jejuni is defined as a microaerophilic bacterium which means that it requires sub-atmospheric concentrations of oxygen. It requires a low oxygen tension for growth (Kelly, 2008) and will not grow in air alone. *C.jejuni* growth in the presence of oxygen inevitably produces reactive oxygen species (ROS), such as H_2O_2, these products lead to damage of intracellular macromolecules, such as DNA, proteins, and lipids (Imlay, 2008). *C. jejuni*, including the glucose-utilising strains studied here, possesses genes for each of the key enzymes typically involved in dealing with oxidative stress resistance. These include *sodB, katA*, and *ahpC* (Parkhill *et al*., 2000). Mutations within *ahpC*, *katA*, and *sodB* result in *C. jejuni* strains that are defective in production of the corresponding ROS-detoxification enzymes, peroxidase, catalase, and superoxide dismutase and in each case exhibit growth reduction under aerobic conditions compared to the wild-type (Euna *et al*., 2015). It is proposed here that the products of glucose utilization by the *glc* locus have a role alongside the products of *sodB, katA*, and *ahpC* in protecting ED positive RG2 strains against aerobic and oxidative stress. The ED pathway has been associated with aerobic growth and it was considered that possession of the ED pathway may enhance survival of these glucose utilising bacteria on exposure to oxygen levels above 10 % and even up to atmospheric condition (21%O_2). Glucose metabolism enhanced survival of both Dg275 and Dg95 following growth at 5%O_2 and 15.9%O_2, but there was no difference in growth with or without glucose with 2% O_2.

Moreover, the increase in glucose dependent survival in limited media (DMEMf) was significantly higher when grown in 15.9 % O_2 than with 5.0 % O_2. In addition, a contribution of glucose utilisation for protection was confirmed by comparing the growth with and without glucose of Dg95(ED+) and Ch95-95*glk*(1)(ED-).

Addition of anti-oxidants during culture has been shown to protect *C. jejuni* from high oxygen for short times, examples include ferrous sulfate, sodium metabisulfite and sodium pyruvate, and been shown to help survival of *C. jejuni* exposed to high oxygen (Verhoeff- Bakkenes *et al*., 2008; Chou *et al.,*1983). Addition of catalase, sodium dithionite or histidine to the growth medium have also been shown to enhance the survival of *C. jejuni* at 17- 20% O_2 (Hoffman *et al.,* 1979; Juven and Rosenthal, 1985). Pyruvate is a product of glucose catabolism. Verhoeff-Bakkenes et al reported that pyruvate helps to decrease the concentration of hydrogen peroxide and thus enables *C. jejuni* to grow aerobically (Verhoeff-Bakkenes *et al.,* 2008). The final step in catabolism of glucose via the ED pathway is hydrolysis of the 6 carbon KDPG intermediate to pyruvate and glyceraldehyde 3-phosphate by Eda, phosphogluconate aldolase. Thus, during growth on glucose, ED positive strains of *C. jejuni* are likely to have an increased level of pyruvate. Hence pyruvate accumulation could in theory contribute directly to combatting oxidative stress. The impact of growth on glucose on protection against hydrogen peroxide was demonstrated for both Dg275 and Dg95.

Dg275 and Dg95 exhibited a typical profile of sensitivity to hydrogen peroxide, with early exponential phase being the most sensitive and a later sensitive period in decline phase (Hwang *et al*., 2011). Growth in glucose led to a significantly better difference in survival in the decline phase, but no difference in exponential phase of growth. This would correlate with the fact that in exponential phase cells are primarily in spiral morphology and 'healthy' cells. Dense cultures are better able to survive high oxygen,although this was unlikely to contribute here in a shaking liquid culture. Other ways in which *Campylobacter* has been shown to survive a high oxygen tension *in vivo* include metabolic commensalism with aerobic

microorganisms found on foods. *Pseudomonas* spp. is one where *C. jejuni* is able to withstand conditions of atmospheric oxygen tension till 48h, when co-cultured with *Pseudomonas* species (Hilbert *et al*., 2010).

When *C. jejuni* strains were exposed to atmospheric oxygen, NCTC11168 survival had decreased more than 1,000-fold after 12h at 37°C and by 24h no detectable viable cells remained. This is in accord with published studies (Kaakoush *et al.,* 2007) suggested that low density cultures may be particularly susceptible to oxygen toxicity.

7.5 Mutations in the Ch95-95 mutants

In light of the very low presence of ED positive strains recovered from chickens, a chicken colonisation trial had been run with Dg275 and Dg95 (Mohammed, 2018). While both strains successfully colonised chickens, colonisation was approximately at 2 $\log_{10}$ cfu/g lower than with the best colonising strains. From one chicken, chicken 95, that had been colonised with *C. jejuni* Dg95, only non-glucose metabolising colonies Ch95-95 (1-5) were recovered. This study confirmed the inability of all 5 recovered colonies to use glucose despite still possessing the *glc* locus and investigated the sequence of two strains Ch95-95 (1 and 2) by both Illumina and nanopore sequencing. Both strains had a mutation in *glk*, glucose kinase, but surprisingly in different positions.In Ch95-95(1) *glk* was now a pseudogene, following a single point mutation changing Gly167 to a TGA stop codon, while in Ch95-95(2) the mutation resulted in a significant amino acid substitution in the consensus sugar binding site, GluXGlyHis. In Ch95-95 (2) Gly170 was mutated to Trp, changing this sequence to GluGly TrpHis and thus effectively abolishing the predicted binding site. Neither of these mutations would be expected to be functional. Interestingly, in both cases the sequencing results identified the mutation in *glk* in both copies of *glc* locus. In both Ch95-95(1) and Ch95-95(2) strains, there were several additional differences between the genome sequence of the parent Dg95 and the recovered Ch95-95 strains. Two of these differences were common to both Ch95-95 strains and in both cases involved deletion of a single G in a poly G series, typical of slipped strand mispairing.

One of these changes was a deletion of G at position-1278726 in *cj1295* which is located beside PseC (UDP-4-amino- 4,6dideoxy-N-acetyl-beta-L-altrosamine transaminase).

The other was within a Hypothetical protein (position- 674889). It is interesting that the inoculated Glc^+ *C. jejuni* strains in only one single chicken of around 10 chickens tested had mutated to a nonfunctional *glc* locus and that, in this case, all 5 recovered colonies had lost the ability to utilise glucose but possessed different mutations within *glk*. It would be interest to understand this in more depth and investigate mutations in the 3 other Ch95-95 isolates as well as in other glucose utilising isolates recovered from other chickens. Are chickens indeed a hostile environment for these strains and is glucose utilisation a disadvantage, making the *glc* locus unstable in the chicken? Were these different mutations in the *glc* locus secondary to some common earlier mutation? Understanding more about the benefits of the *glc* locus to these *C. jejuni* strains and factors that influence niche and host preference will contribute to understanding the biology of this important pathogen and how it survives in the environment.

To understand more, further investigation and future work is needed in several aspects;

1. Investigate the metabolic products during growth on glucose by NMR analysis. In addition to comparing strains grown with and without glucose, compare products during growth in different media and different concentration of oxygen.

2. In laboratory, surprisingly there was no evidence of a difference in growth between Dg43 which has three copies of *glc* locus and Dg275 which has two copies of *glc* loci. This point would be interesting to further investigate, and could involve insertion of a third copy of the *glc* locus in *rrnC* of Dg275 and removing the entire *glc* locus from *rrnC* in Dg43.

3. Use of genetic manipulation to create a Δ *pgi* construct, to determine the importance of the role of this gene in catabolism for glucose. While *pgi*2 belongs to ED pathway and converts fructose6P to glucose6P, it does not play a main role in the ED pathway and has no direct function in glucose metabolism. Importantly, this could also include comparison of the impact of ED positive and this and other genetically manipulated ED negative strains on rat caecal

microbiome. The two Ch95-95 *glk* mutants indicate that glucose metabolism may not be beneficial for *C. jejuni* when in the chicken caecum. The WGS of the other isolates should also be analysed. Studies involving rat and chicken caeum model systems as well as further live animal studies could be used to further investigate the influence of rat / chicken gut environment on these strains and also glucose metabolism by *C. jejuni* on the caecum microbiota.

4-*In vitro*, study the possibility and frequency of transmission of *glc* locus to ED-negative recipient of *C. jejuni* by natural transformation, and functionality in recipients.

Appendix 1. List of Chemical and Consumable

Chemical	Company	Code Number
Agar Bacteriological	Thermo Scientific™ Oxoid™	10351303
Agarose Genetic Analysis Grade	Fisher Scientific™	75000500
Blood Agar Base No. 2	Thermo Scientific™ Oxoid™	10269522
Bolton broth selective supplement	Thermo Scientific™ Oxoid™	11474219
Brain heart infusion (BHI)	Thermo Scientific™ Oxoid™	11061680
Bacterial viability assay kit	Abcam	ab189818
Carbol fuschin	TCS Bioscience	HS180-250
Campylobacter Blood-Free Selective Agar Base (CCDA)	Thermo Scientific™ Oxoid™	10699744
Dulbecco's Modified Eagle Medium. DMEM, No Glucose	Thermo Scientific™ Oxoid™	11520416 10188142
Columbia Blood Agar Base	Thermo Scientific™ Oxoid™	
D-Glucose (> 99.5%)	Sigma	G8270
Horse Blood Defibrinated (200ml)	TCS Bioscience	HB030
Iron (II) L- ascorbate	Sigma	24808-52-4
Lactose (AnalaR)	Fisher Scientific™	101394S
Maltose	Fisher Scientific™	10796291
Mueller-Hinton agar	Thermo Scientific™ Oxoid™	10452453
Mueller-Hinton broth	Thermo Scientific™ Oxoid™	10681675
GeneJET DNA Purification Kit	Fisher Scientific™	10304400
Glycerol	Thermo Scientific™ Pierce™	10618294
GeneJET PCR Purification Kit	Thermo Scientific™	10400450
Dream Taq polymerase	Thermo Scientific™	11896833

Bolton broth selective supplement	Thermo Scientific™Oxoid	11474219
CloneAmp™ HiFi PCR Premix	Clontech	639298
GeneRuler 1 kb DNA	Thermo Scientific	11823963
CloneJET PCR Cloning Kit	Thermo Scientific	10809720
GeneJET Plasmid Miniprep kit	Thermo Scientific	10319699
Monarch genomic DNA purification	New England Biolabs	T3010S
In-fusion HD Cloning Plus	TaKaRa	638910
GeneJET PCR Purification Kit	Thermo Scientific ™	K0701
PCR clean-up, Gel extraction kit	MACHEREY-NAGEL	740609.50
SYBR Safe DNA Gel Stain	Invitrogen	S33102
GelRed® Nucleic Acid Gel Stain.	Cambridge bioscience	BT41003
DeNovix dsDNA Broad Range Kit	Cambridge bioscience	DSDNA-BROAD-
1M Hydrogen perioxidi	Sigma-Aldrich	H1009
Maltose and Glucose Assay Kit	Abcam	ab65335
Fetal Bovine Serum, qualified, heat inactivated, E.U.-approved, South America Origin	Fisher-scientific	11543407

Consumable	**Company**	**Code Number**
CampyGen™ 2.5L (CN0025A)	Fisher-scientific	10108012
CampyGen™ 3.5L (CN0035A),	Fisher-scientific	10729393
Petri dish 3 vents sterile polystyrene clear 90mm Sterilin	Fisher-scientific	11309283
Disposable Cuvettes	Fisherbrand™	11904385
Syringe filter 0.2 um	Scientific – Fisher	15206869
Syringe filter 0.45 um	Scientific – Fisher	15216869
1ul Plastic Inoculation Loops 1000 (Sterile EtO)	Microspec	C4G
5ul Plastic Inoculation Loops 1000 (Sterile EtO)	Microspec	B4G

Thermo scientific PCR tubes	Fisher	11781108
Eppendorf Safe-lock (eppendorf tubes)	Fisher	15625367
MicroplaTE, 96 WELL, PS, F-BOTTOM, CLEAR	Greiner bio-one	655161
LID, PS, HIGH PROFILE (9 MM), CLEAR, STERILE.	Greiner bio-one	656171
Yellow tips, bagged	Fisher Scientific	739295
Polypropylene Yellow Graduated Beveled Pipet Tip	Fisherbrand™	11517452
10 ul filter tip (sterile)	Starlab	S1121-3810
10/20 ul filter tip (sterile)	Starlab	S1120-3810
200 ul filter sterile	Starlab	S1120-8810
1,000 filter sterile	Starlab	S1122-1830
Tips for CFU	Fisher – scientific	15760669
Cryogenic tubes	Fisher-scientific	12952431
Cryogenic storage boxes	Fisher-scientific	10149950
Carrousel Stand For 7 Pipettes Pk1	Gilson	F161401
Gilson Pipette PIPETMAN Classic P202 20uL	Gilson	F123600
Microtitre plates (sterile 96-well polystyrene plates	Greiner	655161
15-50ml Conical Polypropylene Centrifuge Tubes.	Fisherbrand™	11819650
Clear PCR tubes, TUBE-PCR-0.5-500 Quote.	Cambridge	CB-20-02-54670
microscope slide Corning Micro Slides Single	Thermo Fisher	171080
Coverslip	Thermo Fisher	180910
Electroporation Cuvettes	VWR	732-1135

Appendix 2. Preparation of media and solutions

Media	Component	gm/litre
Mueller Hinton agar (MHA).	Beef, dehydrated infusion from	300
	Casein hydrolysate	17.5
	Starch	1.5
	Agar	17
	pH 7.3 ± 0.1 @ 25°C	
	1 litre of DW containing 38g of MHB powder was sterilised by autoclaving at 121°C for 15 min, cooled to 45-50°C, and poured into sterile Petri dishes.	
Mueller Hinton broth (MHB).	Beef, dehydrated infusion from	300
	Casein hydrolysate	17.5
	Starch	1.5
	pH 7.3 ± 0.1 @ 25°C	
	1 litre of DW containing 38g of MHB powder was sterilised by autoclaving at 121°C for 15 min, cooled to 45-50°C, and poured into sterile Petri dishes.	
Blood agar (BA) and BA-2%	Proteose peptone	15.0
	Liver digest	2.5
	Yeast extract	5.0
	Sodium chloride	5.0
	Agar	12.0
	pH 7.4 ± 0.2 @ 25°C	
	1 litre of DW containing 40g of BA powder base no 2, was sterilised by autoclaving at 121°C for 15 min, cooled to 45-50°C and then defibrinated horse blood was added to 5% or 7% (v/v). BA was routinely prepared using an automatic plate pourer.BA-2% was prepared as above except that an additional 16g agar / litre was added to the BA powder.	
CCDA Media plate	Nutrient Broth No. 2	25g
	Charcoal	4g
	Casein Acid Hydrolysate	3g
	Sodium Desoxycholate	1g
	Ferrous Sulfate	0.25g
	Sodium Pyruvate	0.25g

Brain heart infusion (BHI)	Typical Formula	gm/litre
	Proteose peptone	10.0
	Brain infusion solids	12.5
	Beef heart infusion solids	5.0
	Glucose	2.0
	Sodium chloride	5.0
	Disodium phosphate	2.5
	pH 7.4 ± 0.2 @ 25°C	
	1 litre of DW containing 37g of BHI powder was poured into a universal, sterilised by autoclaving at 121°C for 15 min, before being cooled to 4550°C.	
DMEMf-FBS	DMEM medium was routinely supplemented with final concentration of 0.2mM iron (II) L-ascorbate (IA) and10 % FBS to support growth of C. jejuni isolates and designated as DMEMf-FBS for 10 ml DMEMf – FBS 1ml of FBS.In 50 ml conical flask, 15ml DMEMf-FBS (1.5 of FBS) Bolton Broth supplementary antibiotic (1%) and Foetal Bovine Serum (10%).	
DMEMf	For studying the effect of glucose on growth and monitoring glucose in a culture medium over a growth curve, the DMEM medium was supplemented with 0.2mM iron (II) L-ascorbate (IA) as described by Vorwerk et al. (2015). Bolton Broth supplementary antibiotic (1%) and Foetal Bovine Serum (10%) were added, where indicated, this medium was designated as DMEMf.	
DMEMf-Asp- FBS	DMEM medium was routinely supplemented with final concentration of 0.2mM iron (II) L-ascorbate (IA) and10 % FBS to support growth of C. jejuni isolates with 2mM aspartate, supplementary antibiotic (1%), were added 2mM aspartate and Foetal Bovine Serum (10%), where indicated, this medium was designated as DMEMf- asp-FBS.	
Ferrous iron ascorbate (30mM stock) – Always make fresh	Weigh 0.1218g, make up to 10ml with sterile DMEM in a falcon tube, sterilise 0.22um. Just before use dilute – add 660ul of 30mM ascorbate to 9.33ml sterile DMEM to give 2mM IA.	
DMEMf- pyruvate	DMEM medium was supplemented with 0.2mM iron (II) L-ascorbate (IA) as described by Vorwerk et al. (2015), 20mM pyruvate added.	
DMEMf+ glucose	DMEM medium was routinely supplemented with final concentration of 0.2mM iron (II) L-ascorbate (IA) and 20mM glucose in 50 ml conical flask, 15ml DMEMf.	
DMEMf+ Maltose	DMEM medium was routinely supplemented with final concentration of 0.2mM iron (II) L-ascorbate (IA) and 20mM Maltose in 50 ml conical flask, 15ml DMEMf.	

DMEMf+ lactose	DMEM medium was routinely supplemented with final concentration of 0.2mM iron (II) L-ascorbate (IA) and 20mM lactose in 25 ml conical flask, 10ml DMEMf.

DMEM	Components	Molecular Weight	Concentration mg/L)	mM
Dulbecco's Modified Eagle Medium.	L-Glutamine	146	584	4
	L-Serine	105	42	0.4
	L-Methionine	149	30	0.2
	Glycine	75	30	0.4
	L-Arginine hydrochloride	211	84	0.39
	L-cystine 2HCL	313	63	0.2
	L-Histidine hydrochloride H2o	210	42	0.2
	L-Isoleucine	131	105	0.8
	L-Leucine	131	105	0.8
	L-Lysine hydrochloride	183	146	0.79
	L-Phenylalanine	165	66	0.4
	L-Threonine	119	95	0.79
	L-Tryptophan	204	16	0.07
	L-Tyrosine disodium salt dihydrate	261	104	0.39
	L-Valine	117	94	0.8
	Folic Acid	441	4	0.009
	Choline chloride	140	4	0.02
	D-Calcium pantothenate	447	4	0.008
	Niacinamide	122	4	0.03
	Pyridoxine hydrochloride	206	4	0.019
	Riboflavin	376	0.4	0.001
	Thiamine hydrochloride	337	4	0.04
	i-Inositol	180	7.2	0.04
	Inorganic Salts			
	Ferric Nitrate	4.4	0.1	2.4
	Calcium Chloride	111	200	1.8
	Magnesium Sulfate	120	97.6	0.8
	Potassium Chloride	75	400	5.3
	Sodium Bicarbonate	83	3700	44
	Sodium Phosphate monobasic	138	125	0.9
	Sodium chloride	58	6400	110.3
	Phenol Res	376.4	15	0.039
	Typical formula	60	14	0.7
	Sodium chloride	78	8.0	0.46

Phosphate buffer saline (PBS)

Potassium chloride	**0.2**
Disodium hydrogen phosphate	1.15
Potassium dihydrogen phosphate	**0.2**
pH	**7.3**

Maximal recovery diluents (MRD)

Typical formula	**g/litre**
Peptone	**1.0**
Sodium chloride	**8.0**
pH	**7.0 ± 0.2 @ 25°C**

Following sterilization, MRD was stored at RT for a maximum of 8 weeks, peptone saline diluent, Oxoid, code: CM0733B.

TE buffer

5 mM Tris-HCl, 1 mM disodium ethylene diamine tetraacetic acid (EDTA), pH 8 (TE buffer) was prepared by mixing 0.1214g/l of Tris (Fisher Scientific, code BP1521-1) with 0.3722g/l of disodium ethylene diamine tetraacetic acid (EDTA, Fisher Scientific, code D/0700/53). The pH was adjusted to 8.0 through addition of HCl. Following sterilization, TE buffer was stored at room temperature, before using a 1/50 or 1/100 dilution for bacterial cell lysis.

15% glycerol stock medium

The storage medium was made from sterile BHI with 50% sterile glycerol to give a final concentration of 15% (v/v) glycerol. A full loopful of bacterial cells was suspended into the stock medium.

Bolton broth selective supplement.

Vial contents:

Cefoperazone	10.0mg
Vancomycin	10.0mg
Trimethoprim	10.0mg
Cycloheximide	25.0mg

For prepare Bolton broth selective supplement added 5ml of 50%ethanol to one vial of Bolton broth selective supplement, store at -20C.

Bacterial storage Medium

The storage medium was made from sterile BHI with 50% sterile glycerol to give a final concentration of 15% (v/v) glycerol. A full loopful of bacterial cells was suspended into the stock medium.

Campylobacter motility agar

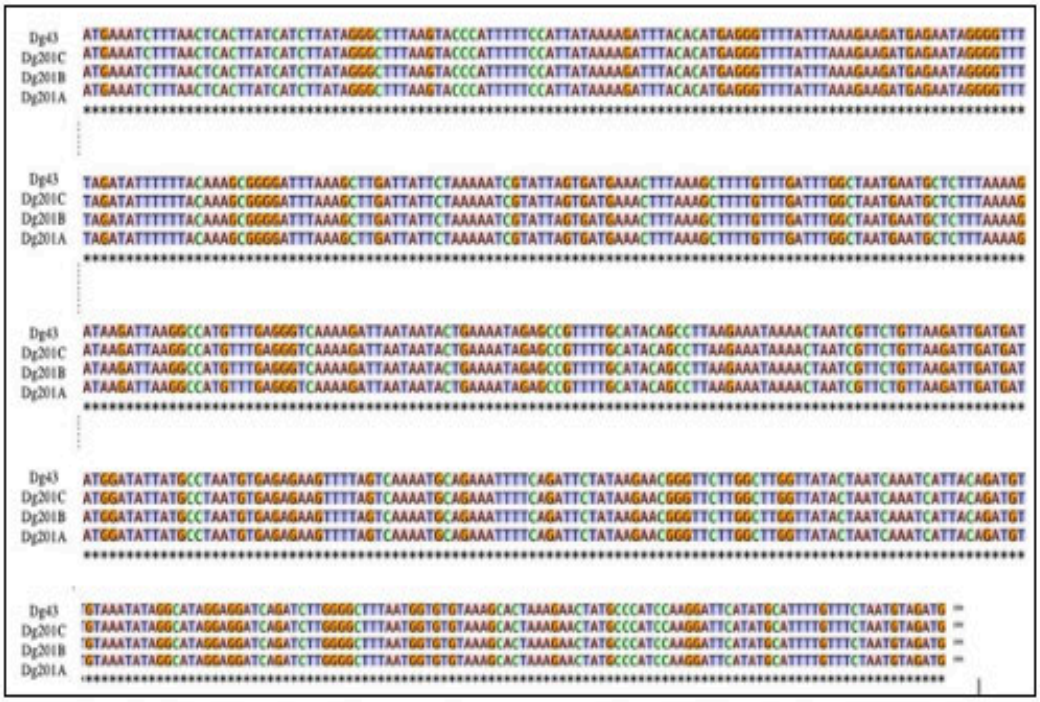
Mueller-Hinton broth (MHB) was supplemented with bacteriological agar No. 1 to a final concentration of 0.4% (w/v) and prepared according to manufacturer's instructions. 1 litre of motility agar contained 21g of MHB and 4g of agar.

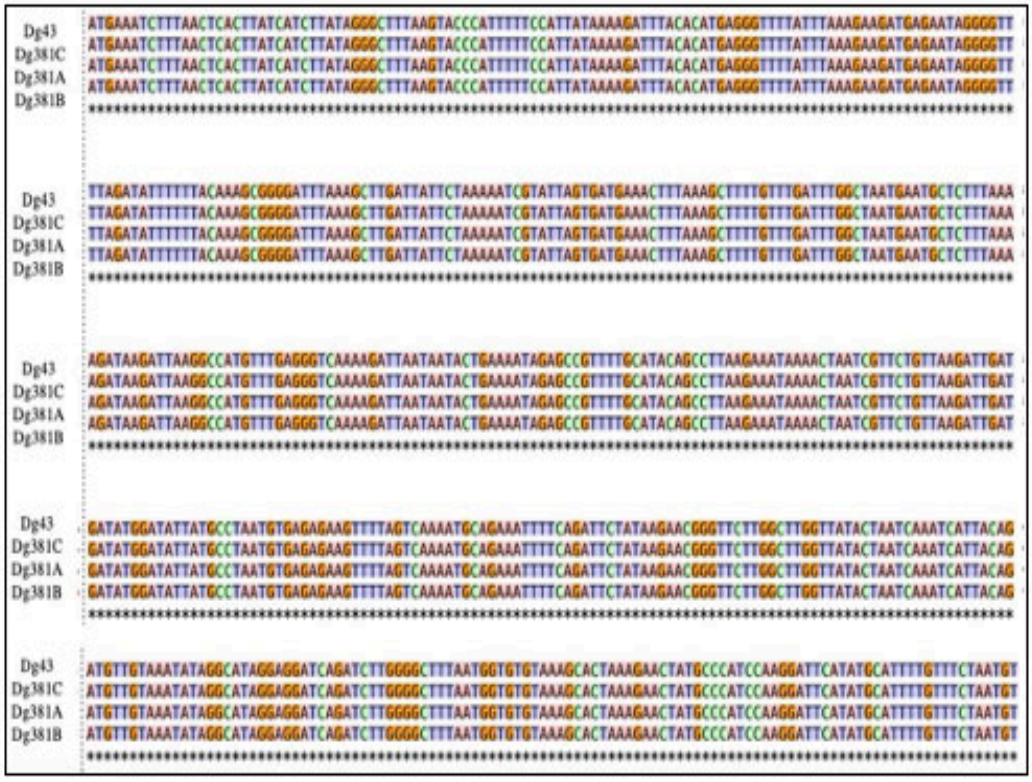
TAE buffer

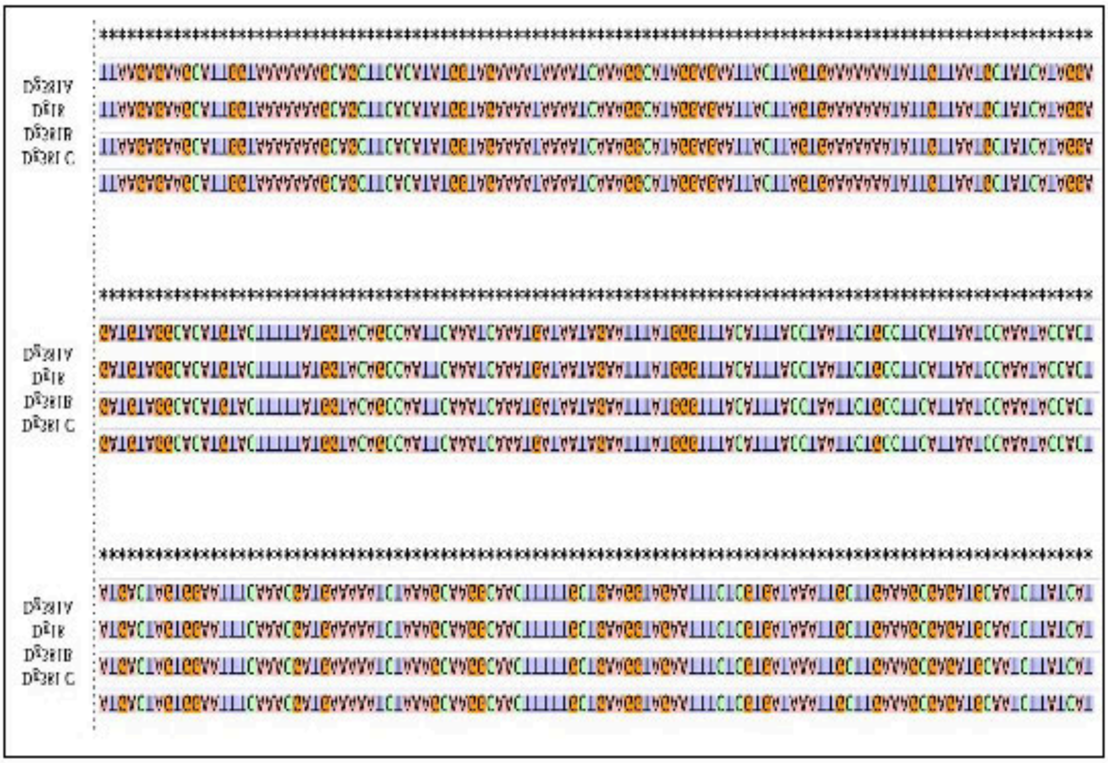
Component	Volume
Tris base	**242g**
Glacial acetic acid	**57.1ml**
5M EDTA pH 8.0	**100ml**

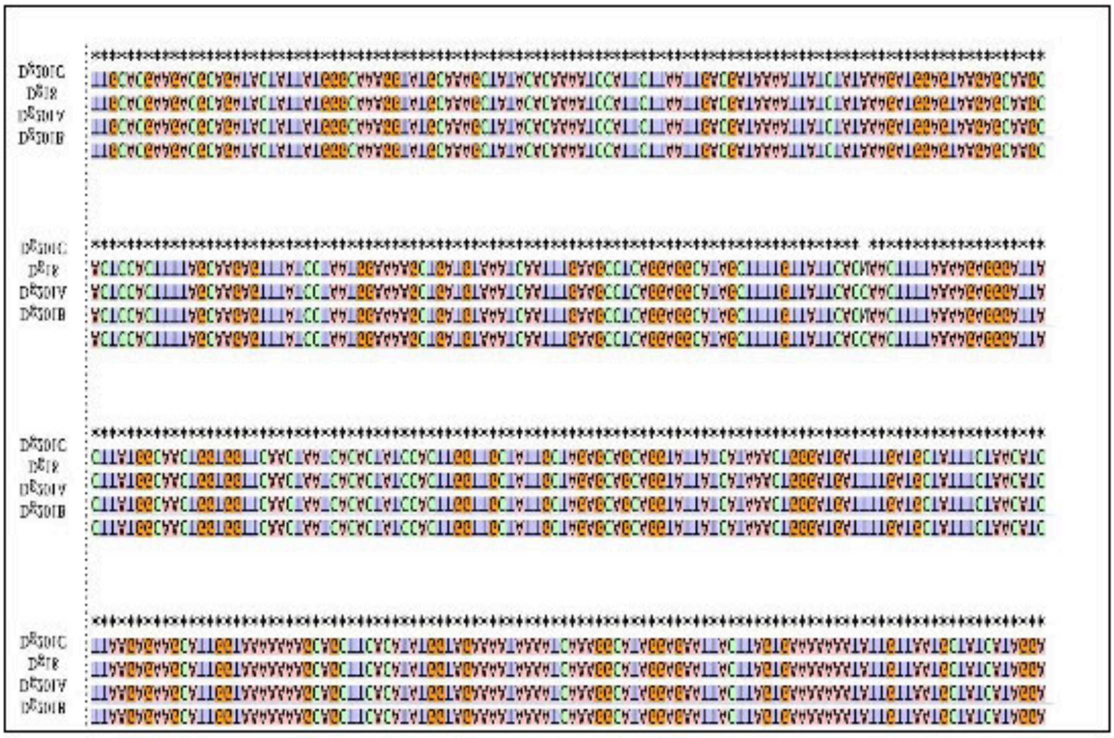
For preparation of 50x stock solution of TAE buffer, Tris base was dissolved in 750mL of Nano pure water. Add the acetic acid and EDTA, and adjusted the volume to 1L by adding water. The ultimate pH of the 50x TAE buffer should be about 8.5. For making the 1x TAE working buffer, 49 volume of water was added to 1 volume of 50x TAE buffer.

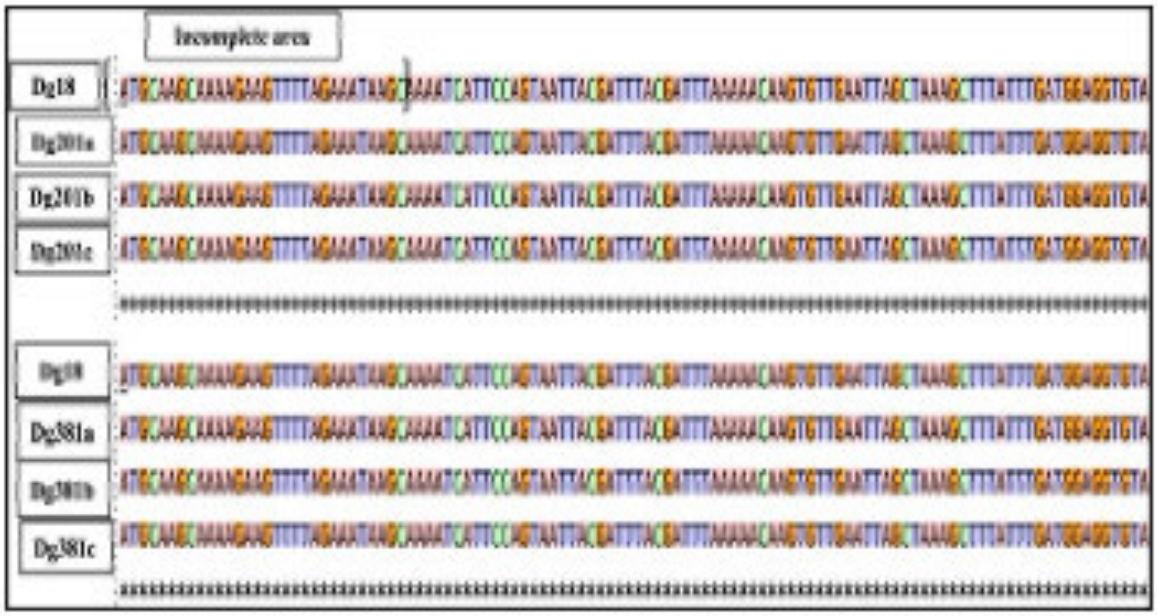
Appendix 3. Alignment for *pgi, edd and eda* gene for Dg201,Dg381 which Dg43 as reference











Appendix 4: Alignment for *glk* gene for RG2 group

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25978|Dg43a     ATGTCTATTTCTCAAAATAA A AAATCTTATCCAAG G CTTTTAGC T GATATTGGTGGAAC T            60
26009|Dg345     ATGTCTATTTCTCAAAATAA G AAATCTTATCCAAG A CTTTTAGC A GATATTGGTGGAAC A            60
26027|Dg210     ATGTCTATTTCTCAAAATAA G AAATCTTATCCAAG A CTTTTAGC A GATATTGGTGGAAC A            60
25974|Dg22      ATGTCTATTTCTCAAAATAA G AAATCTTATCCAAG A CTTTTAGC A GATATTGGTGGAAC A            60
26020|Dg95      ATGTCTATTTCTCAAAATAA G AAATCTTATCCAAG A CTTTTAGC A GATATTGGTGGAAC A            60
Ch95-95(1)      ATGTCTATTTCTCAAAATAA G AAATCTTATCCAAG A CTTTTAGC A GATATTGGTGGAAC A            60
Ch95-95(2)      ATGTCTATTTCTCAAAATAA G AAATCTTATCCAAG A CTTTTAGC A GATATTGGTGGAAC A            60
                ********************   **************   ********   ***************

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25978|Dg43a     AA T GCTCG G TTTGCA C TAGA G GT A GCAGCAAATA T TAT C A AAAA C ATAGAA A TTTT TCC T 120
26009|Dg345     AA C GCTCG T TTTGCA T TAGA A GT G GCAGCAAATA C TAT T G AAAA T ATAGAA G TTTT AGT T 120
26027|Dg210     AA C GCTCG T TTTGCA T TAGA A GT G GCAGCAAATA C TAT T G AAAA T ATAGAA G TTTT AGT T 120
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26020|Dg95      AA C GCTCG T TTTGCA T TAGA A GT G GCAGCAAATA C TAT T G AAAA T ATAGAA G TTTT AGT T 120
Ch95-95(1)      AA C GCTCG T TTTGCA T TAGA A GT G GCAGCAAATA C TAT T G AAAA T ATAGAA G TTTT AGT T 120
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25974|Dg22      TGTAATGATTATAA C AC C GTT A TAGATGC G GTTAA A G T G TATTTAAATAA G GTTGG C AAT     180
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Ch95-95(2)      TGTAATGATTATAA C AC C GTT A TAGATGC G GTTAA A G T G TATTTAAATAA G GTTGG C AAT     180
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25978|Dg43a     CCTA C A A TTAAATAT G GTGCTTTGCTAT A GCAAA T CCTGT A GTAGG A GATTGGGTGCA A      240
26009|Dg345     CCTA T A G TTAAATAT A GTGCTTTGCTAT T GCAAA C CCTGT G GTAGG G GATTGGGTGCA G      240
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Ch95-95(1)      CCTA T A G TTAAATAT A GTGCTTTGCTAT T GCAAA C CCTGT G GTAGG G GATTGGGTGCA G      240
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Ch95-95(1)      ATGACAAATCATCA C TGG A CTTTTTCTATAGAAACTACAA G ACAGGCTTTAAATTT G GAA            300
Ch95-95(2)      ATGACAAATCATCA C TGG A CTTTTTCTATAGAAACTACAA G ACAGGCTTTAAATTT G GAA            300
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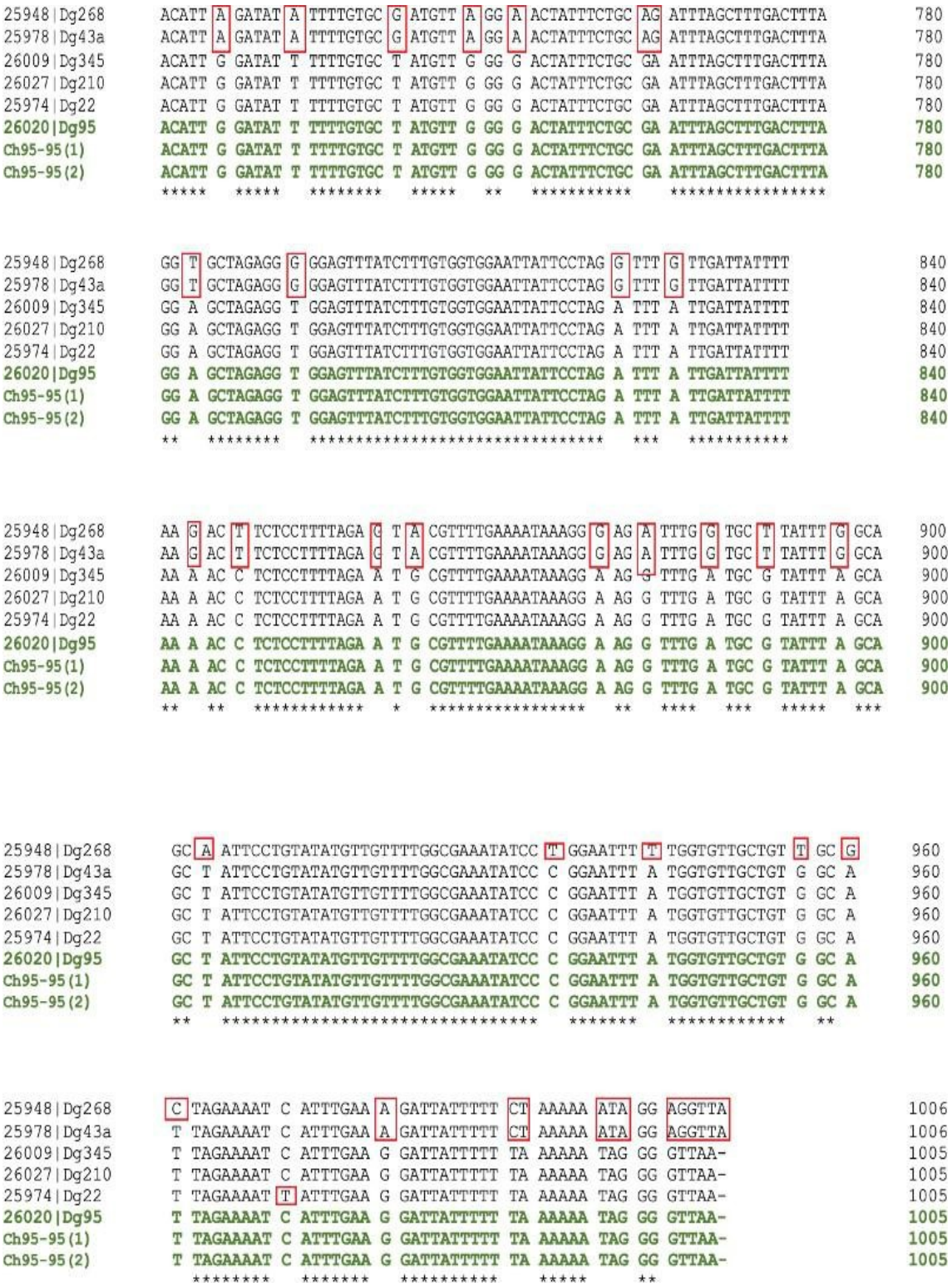
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25974|Dg22 AA A AC C TCTCCTTTTAGA A T G CGTTTTGAAAATAAAGG A AG G TTTG A TGC G TATTT A GCA 900
26020|Dg95 AA A AC C TCTCCTTTTAGA A T G CGTTTTGAAAATAAAGG A AG G TTTG A TGC G TATTT A GCA 900
Ch95-95(1) AA A AC C TCTCCTTTTAGA A T G CGTTTTGAAAATAAAGG A AG G TTTG A TGC G TATTT A GCA 900
Ch95-95(2) AA A AC C TCTCCTTTTAGA A T G CGTTTTGAAAATAAAGG A AG G TTTG A TGC G TATTT A GCA 900
25948|Dg268 GC A ATTCCTGTATATGTTGTTTTGGCGAAATATCC T GGAATTT T TGGTGTTGCTGT T GC G 960
25978|Dg43a GC T ATTCCTGTATATGTTGTTTTGGCGAAATATCC C GGAATTT A TGGTGTTGCTGT G GC A 960
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26027|Dg210 GC T ATTCCTGTATATGTTGTTTTGGCGAAATATCC C GGAATTT A TGGTGTTGCTGT G GC A 960
25974|Dg22 GC T ATTCCTGTATATGTTGTTTTGGCGAAATATCC C GGAATTT A TGGTGTTGCTGT G GC A 960
26020|Dg95 GC T ATTCCTGTATATGTTGTTTTGGCGAAATATCC C GGAATTT A TGGTGTTGCTGT G GC A 960
Ch95-95(1) GC T ATTCCTGTATATGTTGTTTTGGCGAAATATCC C GGAATTT A TGGTGTTGCTGT G GC A 960
Ch95-95(2) GC T ATTCCTGTATATGTTGTTTTGGCGAAATATCC C GGAATTT A TGGTGTTGCTGT G GC A 960
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25978|Dg43a T TAGAAAAT C ATTTGAA A GATTATTTTT CT AAAAA ATA GG AGGTTA 1006
26009|Dg345 T TAGAAAAT C ATTTGAA G GATTATTTTT TA AAAAA TAG GG GTTAA- 1005
26027|Dg210 T TAGAAAAT C ATTTGAA G GATTATTTTT TA AAAAA TAG GG GTTAA- 1005
25974|Dg22 T TAGAAAAT T ATTTGAA G GATTATTTTT TA AAAAA TAG GG GTTAA- 1005
26020|Dg95 T TAGAAAAT C ATTTGAA G GATTATTTTT TA AAAAA TAG GG GTTAA- 1005
Ch95-95(1) T TAGAAAAT C ATTTGAA G GATTATTTTT TA AAAAA TAG GG GTTAA- 1005
Ch95-95(2) T TAGAAAAT C ATTTGAA G GATTATTTTT TA AAAAA TAG GG GTTAA- 1005

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