

The role of biofilms in *Campylobacter jejuni* survival in the food chain

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Abstract

Campylobacter jejuni is the leading cause of bacterial foodborne poisoning in the developed world. The impact of infection is mainly economic, however the disease can lead to severe post infection complications, such as Guillain–Barré syndrome. Despite the fastidious nature of *C. jejuni*, it is able to survive food chain transit. One survival mechanism proposed to aid in *C. jejuni* food chain survival is its formation of, or incorporation into, biofilms.

Biofilms are defined as a surface attached microbial population, surrounded by a self-produced extracellular matrix. Previous work has shown that biofilm formation by *C. jejuni* is increased in food chain relevant conditions, but *C. jejuni* biofilms have received little attention compared to other foodborne pathogens. The work presented here investigated how biofilms may enable *C. jejuni* survival within the food chain. In order to achieve this aim, investigation was carried out into three complementary areas.

Initial investigations showed that supplementation of medium with chicken juice increased *C. jejuni* biofilm formation, due to the chicken juice conditioning the surface and allowing easier attachment of *C. jejuni*. In order to distinguish between the biofilm population and surface attached particulates, a novel method of staining *C. jejuni* biofilms, using a metabolic formazan dye, was developed and optimised. As biofilm formation by *C. jejuni* is relatively poorly understood, a broad investigation was performed to increase understanding of *C. jejuni* biofilm formation and structure. Finally, the role of extracellular DNA within the extracellular matrix was investigated.

The results presented here suggest that *C. jejuni* is able to utilise biofilm formation as a food chain survival mechanism. However biofilm persistence can be limited by enzymatic treatment of the biofilm and thorough cleaning of surfaces, limiting the potential for surface contamination.

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Abbreviations

Abbreviation	Description
A ₅₀₀ /A ₅₉₀	Measurement of absorbance at 500 and 590 nm wavelengths
AI-2	Autoinducer 2
BP	Base pair (genetic)
BSA	Bovine serum albumin
CAUTI	Catheter-associated urinary tract infections
CF	Cystic Fibrosis
CFU	Colony forming unit
CFW	Calcofluor white
CTB	<i>Campylobacter</i> transformation medium
DAPI	4',6-Diamidino-2-Phenylindole, Dihydrochloride
DTT	Dithiothreitol
ECM	Extracellular matrix
eDNA	Extracellular DNA
eDNase	Extracellular DNase
EDTA	Ethylenediaminetetraacetic acid
gDNA	Genomic DNA
FSA	Food Standards Agency
GFP	Green fluorescent protein
HUS	Haemolytic uremic syndrome
IBS	Irritable bowel syndrome
IFR	Institute of Food Research, Norwich, UK
IPTG	Isopropyl β -D-1-thiogalactopyranoside
LB	Luria broth
MPN	Minimum probable number
NEB	New England Biolabs
OD ₆₀₀	Measurement of cell concentration by assessing optical density at 600 nm wavelength
PCR	Polymerase chain reaction
PES	Polyethersulfone
PBS	Phosphate buffered saline (pH 7.4)
QS	Quorum sensing
RPM	Revolutions per minute
RT	Room temperature
SAM	S-Adenosyl methionine
SEM	Scanning electron microscopy
SE	Standard error of mean
SOC	Super optimised broth with catabolite repression
SOP	Standard operating procedure
TTC	2,3,5-Triphenyl-tetrazolium chloride
UV	Ultraviolet light
VAP	Ventilator associated pneumonia
VBNC	Viable but not culturable
WT	Wild type
X-gal	Bromo-chloro-indolyl-galactopyranoside
%CV	Coefficient of variance

Table 0-1 List of Abbreviations

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Chapter 1. Introduction

1.1 Bacterial biofilms

1.1.1 Definition of a biofilm

Biofilms are surface-, or self-attached bacterial colonies surrounded by, and embedded in, an extracellular matrix (ECM). This ECM is comprised of proteins, polysaccharides, nucleic acids and phospholipids (Hall-Stoodley et al., 2004). A graphical representation of a biofilm is shown in Figure 1-1. Biofilms have a highly organised structure, and can be found on a variety of biological and non-biological surfaces (Jayaraman, 2008), or free floating pellicles. They can be composed of a single bacterial species, although in environmental conditions it is more typical to see multiple species of bacteria within the biofilm (Abee et al., 2011).

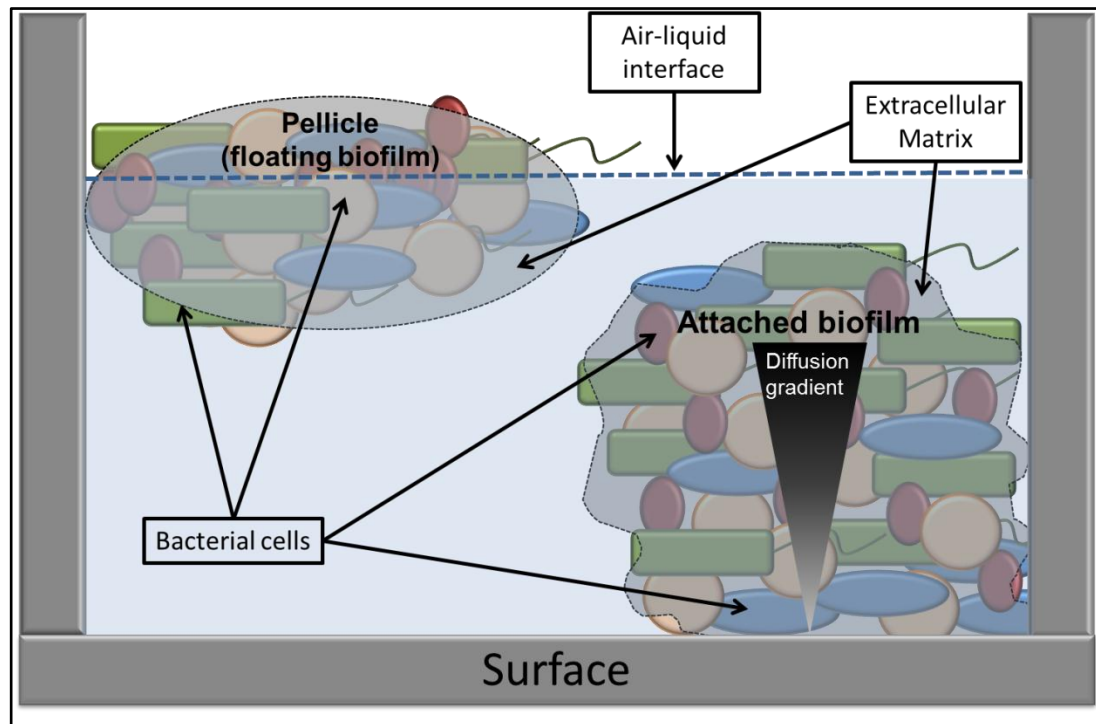


Figure 1-1 Graphical representation of attached and free floating biofilms

Representation of simple multispecies biofilms showing the architecture of the biofilm and its surrounding ECM. Biofilms are often found as either attached, free floating colonies (left of image) or attached colonies (right of image). Due to the density of many biofilms, a diffusion gradient of gases, nutrients and other components such as antimicrobials is frequently present, with concentrations decreasing from the outer to lower levels.

Several fungal species, such as *Aspergillus sp.* and *Candidia albicans*, have also been shown to be able to form *de novo* biofilms, and integrate into existing biofilms (Liu et al., 2014). Viruses, in particular bacteriophages, have also been found within biofilms, utilising the dense bacterial populations to assist in their spread (Chan and Abedon, 2015). Cells within the biofilm often have an altered metabolism with many entering a quiescent state (Williamson et al., 2012). This allows the cells not only to survive on the limited nutrients found in their immediate locality (Zhang et al., 2013), but also increases resistance to antimicrobials (Sawasdidoln et al., 2010), since many antimicrobials only act on actively growing cells. A diffusion gradient is also present within the biofilm, with the lower layers of the biofilm being less rich in metabolites and, in aerobic conditions, oxygen (Stewart, 2003).

The study of biofilms is a relatively new field of microbiology, developing over the last 30 years. It is now recognised that the majority of bacteria form biofilms in natural environments, rather than growing in the free swimming (planktonic) form, which is often used for maintenance of bacteria in laboratory conditions (Svensson et al., 2009, Sutherland, 2001). The term 'biofilm' has now become synonymous with all surface attached microbial growth. However, it should be noted that the term 'biofilm' is not always an accurate description of attached populations. In many situations, attached populations do not progress from an initial attachment phase to the complex, ECM producing biofilm (Otter et al., 2015). These 'attached but not biofilm' populations are no less detrimental to human health, or surface fouling, particularly if formed on medically (Otter et al., 2015) or food relevant surfaces (Herald and Zottola, 1988), however the term biofilm should still be used with caution in these cases. This is because their lack of structure distinguishes them from true biofilms. In these situations the term 'biotransfer potential' has been suggested, as it can be used to describe any microorganisms associated with a surface with the potential to cause contamination (Hood and Zottola, 1995). 'Biofouling' can also be used to describe both attachment and biofilm formation in areas where biofilm formation is considered a contamination, examples of which include ships hulls (Hunsucker et al., 2014), medical devices (Costerton et al., 1999) and food industry surfaces (Srey et al., 2013).

1.1.1.1 Milestones within the study of biofilms

Although oral biofilms were described by van Leeuwenhoek in the late seventeenth century (Hannig et al., 2010), it was not until the late 1970s that detailed investigation of biofilms was carried out. It is widely recognised that the pioneer of the biofilm field was Bill Costerton, initially describing biofilm communities within streams and rivers, but later carrying out research into biofilm formation in medical systems (Lappin-Scott et al., 2014). Biofilms rapidly became recognised as the way in which the majority of bacteria survive, and over the last 30 years research into biofilm growth and formation has increased dramatically (Figure 1-2).

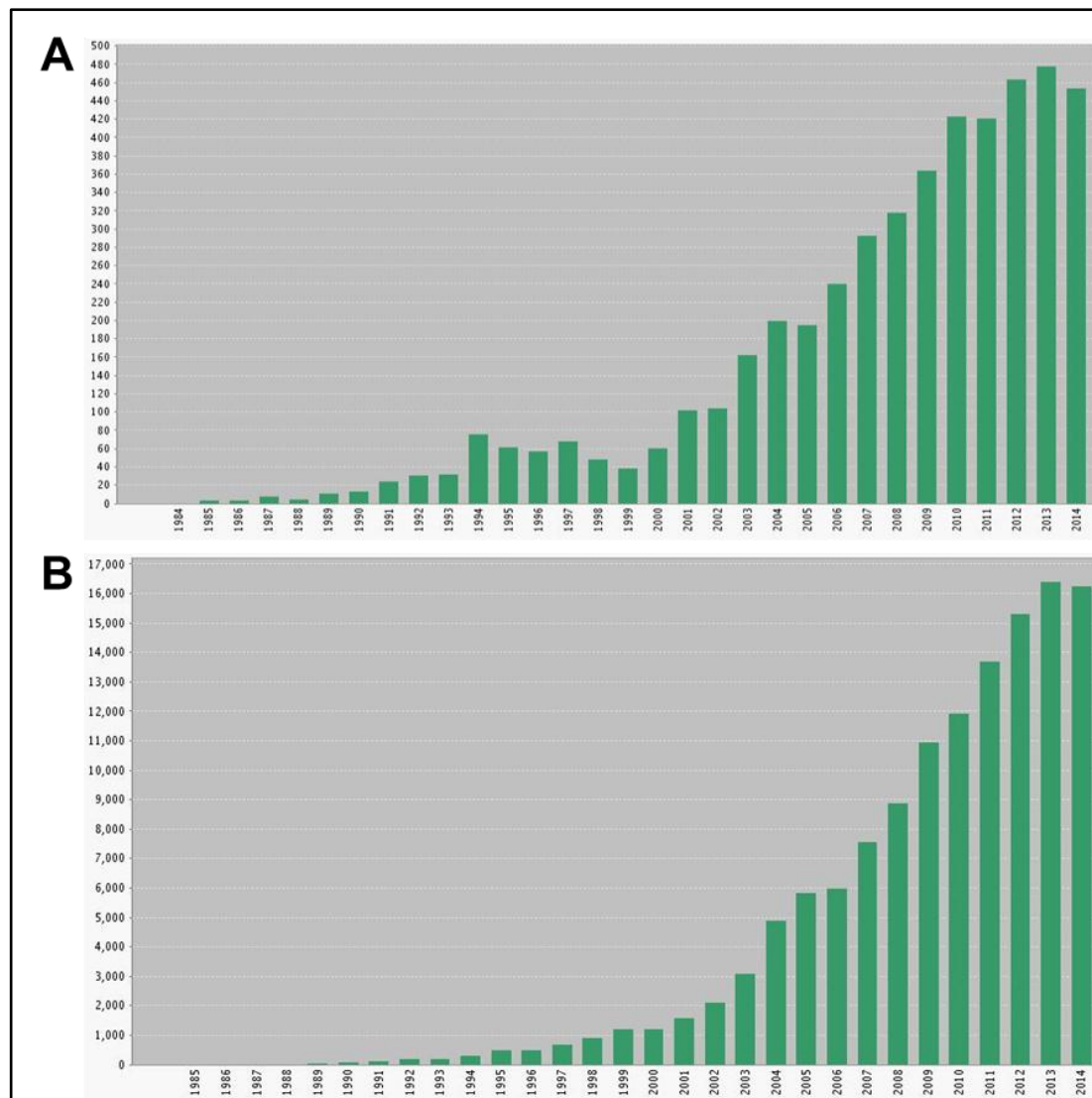


Figure 1-2 The study of biofilms has increased significantly over a 30 year period

Citation report for a Web of Science search for the term 'biofilm' within a journal article title. Graphs show the number of research papers published (A) and the number of citations within research articles (B) over a 30 year period (1984 to 2014). The Y axis indicates years in both graphs and the X axis indicated the numbers of papers published with this search term within the title.

1.1.1.2 Organisms of relevance

Two model organisms quickly emerged within the biofilm field: *Pseudomonas aeruginosa*, a Gram negative aerobe of interest due to its role in opportunistic human infections (Klausen et al., 2003b), and *Staphylococcus epidermidis*, a Gram positive bacteria able to cause nosocomial infections (Otto, 2014). These two model bacterium are of medical relevance, have well defined molecular protocols, are relatively easy to maintain *in vitro* and manipulate genetically, as well as being able to form biofilms in both static and flowing conditions. To date, many investigators have concentrated their studies on these two bacterial species, although within the last decade the study of other biofilm-forming human pathogens including *Listeria monocytogenes*, *Staphylococcus aureus*, *Burkholderia cepacia*, *Vibrio cholerae* and *Escherichia coli* has also increased.

1.1.1.3 Extracellular matrix

Biofilms are very complicated structures, requiring coordinated behavior from the bacteria within the structure in order to maintain the biofilms architecture and ECM (Stoodley et al., 2002). Since many biofilms are comprised of multiple species this organisation can be highly complex and is still relatively poorly understood. Advances in staining and imaging techniques have allowed investigators to gain an insight into the composition of the ECM and the architecture of the biofilm. It has long been known that the ECM contains polysaccharides, however work in 2002 by Whitchurch et al. (2002) indicated that extracellular DNA (eDNA) is also a component of the ECM in *P. aeruginosa*. During early stages of biofilm formation the degradation of this eDNA led to dispersal of the biofilm (Whitchurch et al., 2002). Since the publication of this seminal work, other studies have shown that eDNA plays a very important role in biofilm formation, structure and maintenance of the biofilms of other species (the role of eDNA in *Campylobacter jejuni* biofilms is discussed in more detail in 6 and 7).

1.1.1.4 Communication within the biofilm community

Quorum sensing (QS) is a well-recognised system of bacterial 'communication', allowing bacteria of the same species to sense population density and alter their gene expression in response (Miller and Bassler, 2001). The organisation of bacteria within the biofilm was proposed to require a form of sensing and communication due to its complexity. Most QS systems described are utilised for intra-species communication, producing unique molecules which are only recognised by other members of their species. Large multi-species biofilm communities require a global communication system which can be recognised by many different species. A system described by Bassler et al. (1994) utilises Autoinducer 2 (AI-2). Which is both produced and detected by multiple bacterial species. AI-2 is a by-product of the S-Adenosyl methionine cycle, and produced by the protein LuxS (Peixoto et al., 2014). It was first identified due to its ability to coordinate bioluminescence in the marine bacterium *Vibrio harveyi*. More recently investigators have focused on how AI-2 is able to be utilised by biofilm communities. Addition of exogenous AI-2, or inactivation of *luxS* has been shown to affect biofilm formation by *E. coli* (Niu et al., 2013), *L. monocytogenes* (Challan Belval et al., 2006) and *Salmonella typhimurium* (Miller et al., 2004). More recently investigators have attempted to utilise the AI-2 system to manipulate biofilm communities (discussed in Section 1.1.6.1). It should however be noted that although many bacterial species are able to produce AI-2 as part of their metabolism, its use as a QS molecule may be more limited, since not all bacterial species contain a homologue of the AI-2 receptor LuxR (Rezzonico and Duffy, 2008). Although it is possible that other, unknown, detection systems are utilised, investigators have proceeded more cautiously when investigating the effect of AI-2, its quenchers, or inactivation of the LuxS protein on biofilm formation. *C. jejuni* is one example of a bacterium which contains LuxS, and is able to produce AI-2 (Elvers and Park, 2002), but to date no receptor has been found and the alterations in phenotype following inactivation of the *luxS* gene cannot wholly be attributed to QS, since deletion also leads to metabolic alterations (Holmes et al., 2009, Adler et al., 2014).

1.1.1.5 Experimental models of biofilm formation

To date, many biofilm studies have been carried out using single species models. However, in environmental niches such as the mouth, food processing plants and water systems, multispecies, or even multi-kingdom, biofilms are the dominant microbial lifestyle (Jahid and Ha, 2014). Until recently, it was necessary to focus investigation on single species biofilms, since the complexity of multispecies models confounded effective data analysis and interpretation. Recent advances in omics technologies (including metagenomics,

transcriptomics, metabolomics and proteomics) and analytical software has allowed biofilms of increasing complexity to be analysed. Mathematical modelling has also allowed multispecies behaviour to be better predicted, allowing bench scientists to more effectively design multispecies biofilm experiments. These advances have allowed investigation of more complex biofilm communities, of which one well studied model system is the oral biofilm community. Oral communities can be comprised of up to 700 different species, both commensal and pathogenic (Zijngel et al., 2010). Dominant members of the oral community are from the Bacilli, Gammaproteobacteria, Clostridia and Bacteroides families (Belda-Ferre et al., 2012), although the exact species composition and ratio is variable depending on the individual host. Recent metabolic analysis of multispecies oral biofilms by Frias-Lopez and Duran-Pinedo (2012) indicated that the presence of dental pathogens such as *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* lead to altered expression of ABC transporters and chaperones in oral mixed species commensal bacteria communities. Work such as this would not be possible without the foundation of single species analysis, or the recent advances in technology and data analysis software.

As well as increasing the complexity of biofilm models, it has also now become possible to analyse single cell interactions within the biofilm. Connell et al. (2013) have developed a method of trapping single cells within a permeable gel 'cage'. This allows the cell to receive and deliver soluble signals but not directly interact with other bacterial cells. Since cells within the biofilm are often immobilised within the ECM this work closely models the interactions possible within the biofilm. Examples such as these show that the field of biofilm study is both rapidly expanding and evolving, allowing more refined analysis of the complex cell-cell interactions to be performed.

1.1.2 Biofilm formation and dispersal

The biofilm is formed in four stages; initial attachment, microcolony formation, maturation, and cell shedding or dispersal. Full maturation of the biofilm can take several weeks, however initial attachment of bacteria to a surface may be complete within a few seconds (Monroe, 2007). The mature biofilms' shape is characterised by the species it contains, the turbidity and flow of the surrounding liquid, and the composition of the ECM (Pamp et al., 2009). Figure 1-3 shows a typical example of biofilm initiation and maturation.

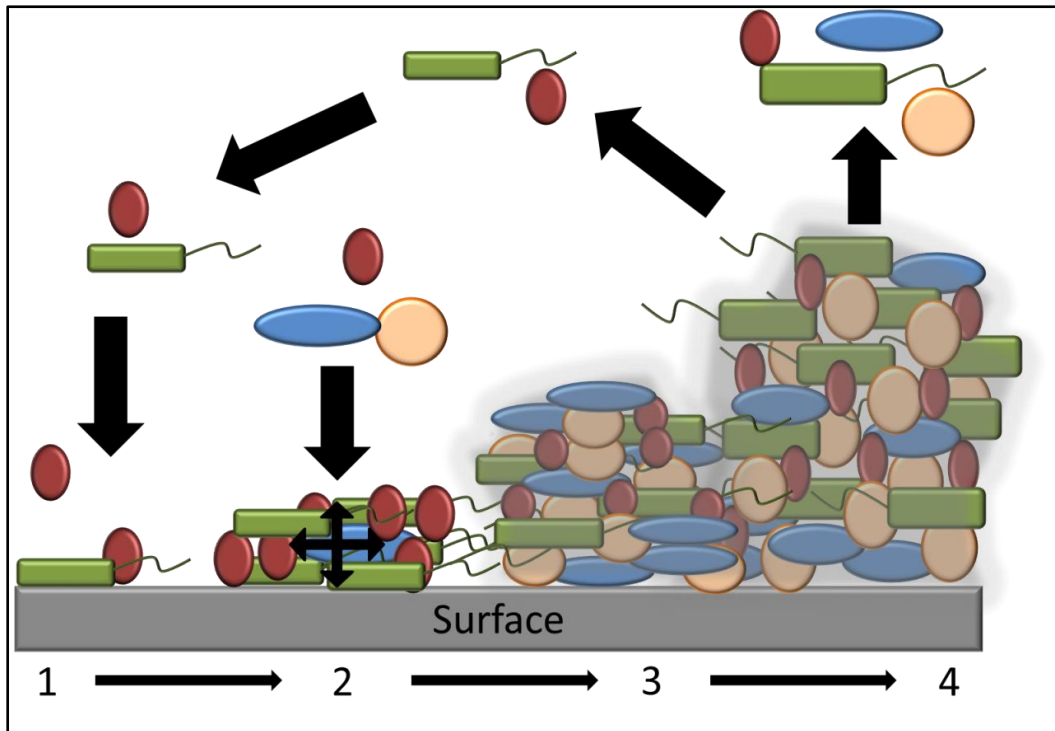


Figure 1-3 Diagrammatic representation of the four stages of biofilm formation.

Diagrammatic representation of the development of a simple multispecies biofilm. At stage 1 single cells become attached to the surface and begin to divide, forming micro-colonies (stage 2). Additionally, planktonic bacteria are able to integrate into the biofilm, further increasing its biomass. As the biofilm matures (stage 3) it develops a complex architecture and ECM is produced, providing structural support for the cells within the biofilm. Cells can be shed from the biofilm (stage 4), either actively or passively, moving back into the planktonic phase, potentially attaching in other areas or joining other, pre-existing biofilms.

1.1.2.1 Initial surface attachment

Biofilm formation is initiated when cells flowing across the surface are able to attach to the surface. Many abiotic surfaces are naturally repellent to bacteria since they have hydrophilic properties, similar to that of the bacterial cells themselves (Chmielewski and Frank, 2003). Initial attachments are weak, and as such attachment is likely to be temporary. This temporary attachment process will occur on multiple occasions before a firm attachment can be established. Firm attachment to the surface can be achieved by the secretion of ECM, effectively used as a type of 'glue' by many bacterial species during initial attachment. The flagella and fimbriae are also useful tools during initial attachment (Utada et al., 2014), allowing cells to move towards the surface, overcome fluid flow and surface repellence, as well as assisting in forming both temporary and permanent surface attachments.

1.1.2.2 Microcolony formation

Once the bacterial cells have become permanently attached to a surface they are able to initiate biofilm formation itself. Initially cells attached to the surface begin to divide, forming microcolonies (Sriramulu et al., 2005). In addition planktonic cells are able to integrate into the microcolonies, increasing their size. As the microcolonies increase in size they begin to form organised structures and produce ECM (Wilkins et al., 2014). One function of the ECM is to form a mechanical support for the cells (Abee et al., 2011). It is secreted by the bacteria within the biofilm and is typically comprised of polysaccharides, proteins, lipids and nucleic

acids (Abee et al., 2011), although specific composition and ratios of components is species and environment dependent. The majority of the mass of the ECM is water, and of the remaining components, proteins typically constitute the greatest fraction, comprising approximately 75% of the dry ECM (Sutherland, 2001). Polysaccharides, long molecules which are linear or branched (Mouw et al., 2014), are also a major component of the ECM, these are. Polysaccharides are very diverse, even within species, for example *P. aeruginosa* produces at least three distinct polysaccharides all of which contribute to biofilm development (Flemming and Wingender, 2010). The ECM accounts for up to 90% of the biofilm biomass (Yonezawa et al., 2011) and it is integral to the biofilms structure, providing a scaffold for the cells, maintaining the biofilms architecture and trapping nutrients, enzymes and allowing efficient cell to cell communication to take place (Flemming and Wingender, 2010). ECM composition affects the porosity, density, water content, charge, sorption properties, hydrophobicity, and mechanical stability of the biofilm (Flemming et al., 2007). Further to its function as an organic scaffold the ECM also provides protection to bacteria from external dangers such as phages, antimicrobial solutions and mechanical removal (Hunter, 2008).

1.1.2.3 Biofilm maturation

Mature biofilms are able to form a variety of shapes, the best described of which is the formation of 'mushroom-like' structures. These are typically formed within systems with liquid movement (Klausen et al., 2003a). This shape, with a wide, flattened upper section and narrower 'stalk' (Figure 1-4), allows the cells to remain fixed in a static position, while fluid is able to move through the biofilm, providing a source of fresh nutrients and removing waste products (Singh et al., 2006). Liquid flow may also allow 'streamer' formation. Streamers are sections of the biofilm which form long fronds able to move in the current. These streamers are able to rapidly clog pipelines in liquid systems (Drescher et al., 2013), or blood vessels in systemic *in vivo* infections (Kim et al., 2014).

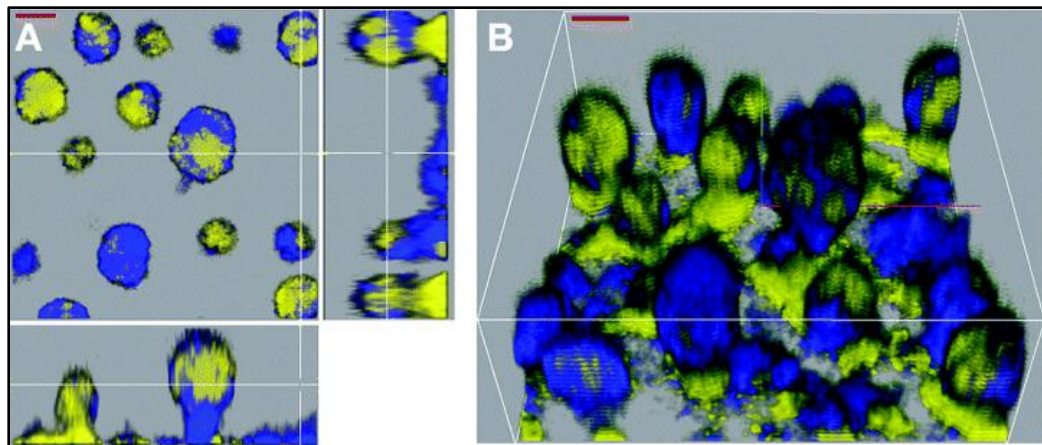


Figure 1-4 Image of 'mushroom like' biofilm structures

Confocal laser scanning microscope image from Klausen et al. (2003a) showing *P. aeruginosa* biofilms formed under flow conditions and displaying the mushroom like architecture. The scale bar indicates 20 μm .

To date, much of the work on biofilm structure has been carried out in flow cell systems, allowing liquid movement, and effective nutrient flow. It should however be noted that biofilms formed under static conditions form different shapes to those in flowing systems.

Biofilms formed on agar, or as pellicles in static liquid systems, often form large circular colonies with wrinkles (Figure 1-5). These biofilm types are particularly well studied in *Bacillus subtilis* (Trejo et al., 2013) and *Pseudomonas sp.* (Spiers et al., 2003) but can be formed by many different bacterial species including *C. jejuni* (Joshua et al., 2006).

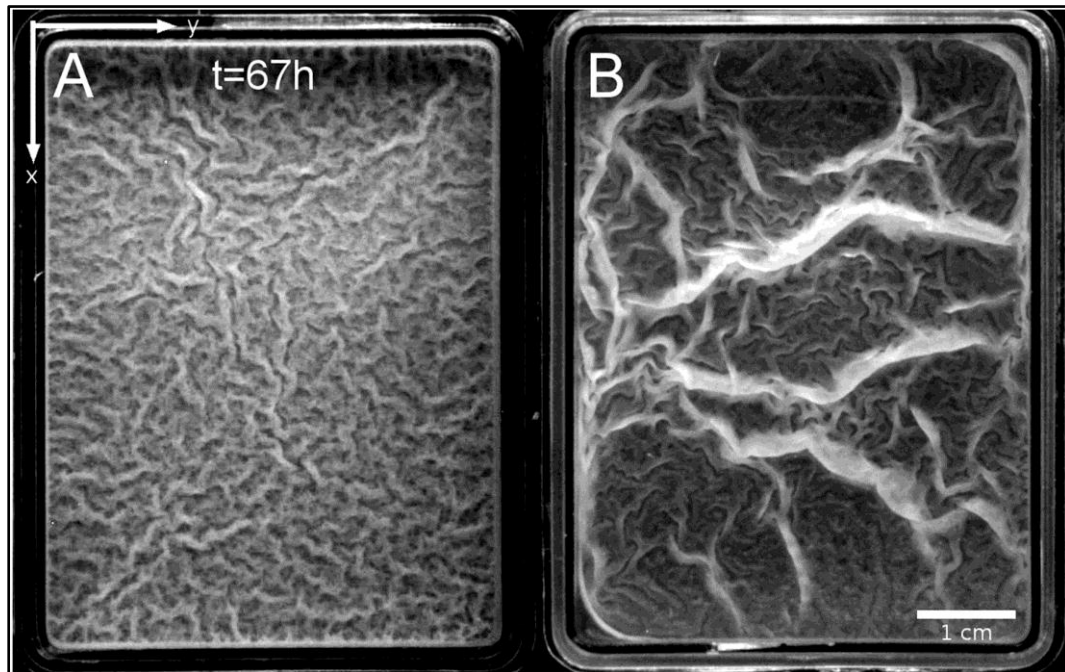


Figure 1-5 *B. subtilis* biofilms formed in static conditions displaying a wrinkled pellicle morphology.

Image of *B. subtilis* strains NCIB 3610 (A) and DV1 (B) biofilms formed following ~67 h of incubation at 23°C. The images show pellicles floating at the surface and displaying a wrinkled morphology typical of biofilms formed following static incubation. Image taken from Trejo et al. (2013).

1.1.2.4 *Biofilm dispersal*

The final stage of biofilm formation is the dispersal stage. Here cells are either passively shed, or actively dispersed from mature biofilms. The exact mechanism of dispersal is dependent on the species within the biofilm. Shedding can occur for various reasons, including mechanical abrasion (Verkaik et al., 2010), shearing by fluid flow (Ymele-Leki and Ross, 2007), or grazing by predators (Monnappa et al., 2014). In contrast, dispersal occurs when the bacteria within the biofilm respond to self-produced or environmental signals and initiate degradation of the ECM allowing their release from the biofilm. Dispersal has been reported in *S. aureus* (Mann et al., 2009), *P. aeruginosa* (Howlin et al., 2011), *Haemophilus influenzae* (Cho et al., 2014) and *V. cholerae* (Warner et al., 2014). Active dispersal is controlled by small molecules such as AI-2 (Abee et al., 2011), nitric oxide (Howlin et al., 2011) or environmental cues (Sawyer and Hermanowicz, 1998, Hunt et al., 2004, Jager et al., 2005). Cells within the biofilm are able to respond to such stimuli, producing enzymes such as DNase (Mann et al., 2009) or dispersin B (Ramasubbu et al., 2005) which degrade the ECM and release them from the biofilm. The dispersal stage of biofilm formation is arguably the most important life stage as it allows colonisation and contamination of new areas or, where biofilms form *in vivo*, the development of systemic infections.

1.1.3 Biofilms in environmental settings

Study of many environmental biofilm systems has been confounded by their complexity and lack of ability to cultivate many of the species within the communities (Davey and O'Toole, 2000). Recent advances in 'omics technologies have allowed better analysis of these complex communities. Well described environmental communities include: drinking water biofilms (Wingender and Flemming, 2011), the hot spring systems in Yellowstone park (Bowen De Leon et al., 2013) and bacterial communities found in acidic mine drainage water (Yelton et al., 2013). It should be noted that many pathogenic bacteria able to form biofilms are opportunistic pathogens, typically found in environmental communities. Examples include *P. aeruginosa*, a widespread bacterium found in soil (Gans et al., 2005) and waterways (Rusin et al., 1997), and *V. cholerae*, which forms biofilms on chitinous insect exoskeletons (Lutz et al., 2013). Environmental biofilms are also integral to procedures such as waste water treatment (Sutherland, 2001, Hunter, 2008), making their study and maintenance of great important to human health.

1.1.4 The role of biofilms in microbial pathogenesis

The biofilms of pathogenic bacteria are of great interest, as biofilm formation naturally confers a resistance phenotype (Jolivet-Gougeon and Bonnaure-Mallet, 2014). The ECM, although not the main source of antimicrobial resistance (Patel, 2005), also contributes to increased resistance phenotypes. It is able to decrease antimicrobial penetration and in some cases inactivate antimicrobials (Billings et al., 2013). Genetic material can be effectively exchanged within biofilms due to the close proximity of the bacteria, exposure of cells to sub-lethal doses of antimicrobials and their uptake of DNA directly from the ECM. These factors contribute to the spread of resistance phenotypes throughout the biofilm population (Madsen et al., 2012).

Biofilms also provide a reservoir, which pathogenic bacteria are able to utilise, moving from the biofilm to colonise new areas. Bacteria are also able to integrate into the biofilm community during periods of antimicrobial treatment, utilising the biofilms protective environment. Many important human pathogens are able to form biofilms. Three important areas of medical biofilm investigation will be reviewed in Sections 1.1.4.1 to 1.1.4.3: biofilm formation on implants, the contribution of biofilms to cystic fibrosis infections and wound infection biofilms.

1.1.4.1 Biofilm formation on implants

As surgical procedures increase in complexity, patients often require immobilisation and support of vital functions for extended periods of time (Bunker, 2001). In these instances patients are also at higher risk of infection and two well recognised biofilm infection reservoirs include ventilators (Browne et al., 2014) and catheters (Iacovelli et al., 2014). These not only are able to introduce bacteria into the body of potentially immunosuppressed patients, but also provide bacteria within the body with a surface on which they are able to attach and form a biofilm, therefore evading antimicrobial treatment and attack by the hosts immune system.

Implant biofilms are frequently found to contain only a single species, typically an opportunistic pathogen. Ventilator associated pneumonia (VAP) is a significant risk for patients undergoing mechanical ventilation. VAP occurs in up to 30% of patients (Chastre and Fagon, 2002), with an increasing incidence as the time ventilation is required is increased. Contraction of VAP increases patient morbidity and mortality and incurs a significant treatment cost as patients require longer hospitalisation and antibiotic treatment (Chastre and Fagon, 2002). VAP can be caused by various bacterial species, including *P.*

aeruginosa, *S. aureus* and *Klebsiella pneumoniae*. A link has been identified between patients' oral health and VAP, and a recent study has shown that better oral hygiene and monitoring of patients undergoing mechanical ventilation significantly reduced the incidence of VAP (Rello et al., 2010). This suggests colonisation of the ventilation tubing occurs via an oral route, with pathogenic bacteria utilising the oral biofilm prior to VAP infection.

Catheter-associated urinary tract infections (CAUTIs) are the most common hospital acquired (nosocomial) infection (Jacobsen et al., 2008), and biofilm involvement in CAUTIs leads to infection recurrence and complications in treatment of infection. *E. coli*, a member of the healthy urinary tract flora, is a frequent cause of urinary tract infections (Vollmerhausen et al., 2014), accounting for up to 80% of urinary tract infections (Stamm and Hooton, 1993). *E. coli*'s ability to form biofilms is a significant contributor to its persistence and pathogenicity (Bielecki et al., 2014).

1.1.4.2 Biofilm formation in the Cystic Fibrosis lung

Cystic fibrosis (CF) biofilms have received significant attention over the last two decades due to their role in increasing patient mortality (Ciofu et al., 2013) and the involvement of the model biofilm organism *P. aeruginosa* in CF pathology (Hoiby et al., 2010b). CF patients produce thick, viscous mucus, which in their lungs is easily colonised by airborne bacterial and fungal species (Kreda et al., 2012). Infections are usually life-long and contribute significantly to morbidity and mortality. Many CF pathogens are opportunistic, able to cause infection in only susceptible, immuno-compromised individuals (Mahenthiralingam et al., 2008, Gomez and Prince, 2007). Pathogenic, biofilm forming bacteria such as *P. aeruginosa*, *S. aureus*, *B. cepacia*, *H. influenzae*, *Stenotrophomonas maltophilia* and *Mycobacterium abscessus* are frequently isolated from patient sputum samples (Coutinho et al., 2013).

Research into CF pathogens is focused on two areas: treatments for the dispersal and killing of biofilm cells within the lungs, or discovery of the molecular mechanisms of pathogenesis and resistance. Some of the novel treatments developed will be discussed in more detail in Section 1.1.6, however a review of the extensive field of pathogenesis and resistance mechanism investigation is outside the scope of this work. Key research within the field is highlighted in reviews by Joo and Otto (2012) and Ciofu et al. (2014).

1.1.4.3 Biofilm formation in wounds

The formation of biofilms in wounds leads to chronic and persistent infections which can often only be cleared by debridement of the infection site (Cowan et al., 2013). Up to 90% of chronic wound infections contain biofilms (Attinger and Wolcott, 2012) and biofilm involvement has significant cost implications, recently estimated at \$20 billion each year in the USA (Cowan et al., 2013). Although biofilm-associated wound infections have the potential to develop in any wound, they are frequently encountered in diabetic patients, following the development of ulcers on the feet and lower legs. Diabetic patients account for 80% of all non-traumatic lower extremities amputation around the world (Berlanga-Acosta et al., 2014), and biofilm associated infections negatively impact treatment outcomes (Zubair et al., 2012). Although infections are typically multispecies in nature (Dalton et al., 2011), they are often dominated by Gram positive bacteria such as *Staphylococcus sp.* (James et al., 2008).

Treatment of the wound infections focuses around debridement, draining, and packing with antimicrobial materials. Specialized dressings containing honey or antimicrobial metals such as silver are frequently used (discussed in more detail in Sections 1.1.6.2 and 1.1.6.4

respectively). Debridement can be mechanical, although more recently insect larvae have been used with great success (Menon, 2012). Laval treatment, particularly using the maggots of the blow fly, is successful since it utilises four synergistic factors: the action of the maggots feeding stimulates wound drainage, the maggots destroy necrotic tissue and encourage formation of granulation tissue, and finally, the secretions of the maggots are antimicrobial, reducing the potential for further infection (Jaklic et al., 2008, Cazander et al., 2013). Studies have shown that maggot treatment was effective in over 80% of patients, and bacteria such as group G and C *Streptococcus*, *Klebsiella* sp., *Serratia marcescens*, *S. aureus* and *P. aeruginosa* were susceptible to the antibiotic properties of the maggot secretions (Jaklic et al., 2008).

1.1.5 Biofilm formation within the food chain

Biofilms are frequently detected in many different areas of poultry processing plants, from conveyor belts (Lindsay et al., 1996) and stainless steel surfaces (Sanders et al., 2008) to floor sealant (Blackman and Frank, 1996). Biofilms have also been detected in many areas of the home (Marshall et al., 2012), including the kitchen sink (Furuhata et al., 2010). The proximity of biofilms to areas of food preparation and human habitation contributes to the risk of food pathogen consumption. Although it should be acknowledged that many of the bacterial species found within these biofilms will not impact on human health, there is a possibility that these biofilms will contain species able to cause human infection. Foodborne pathogens such as *C. jejuni*, *E. coli*, *Salmonella* sp. and *L. monocytogenes* have all been shown to be capable of either forming biofilms or integrating into existing biofilms. *C. jejuni* biofilm formation will be discussed in more detail in Section 1.6. Sections 1.1.5.1 to 1.1.5.3 discuss biofilm formation by three other common foodborne, biofilm forming bacterial pathogens.

1.1.5.1 Biofilm formation by *E. coli*

Infection by foodborne *E. coli* has a higher hospitalisation rate than *Salmonella* sp. or *Campylobacter* sp. infection (Lim et al., 2010) due to the severity of the illness and the ability of some *E. coli* strains to produce shiga toxin, which is associated with the development of hemolytic uremic syndrome (HUS) and renal failure (Belongia et al., 2003). Shiga toxin producing strains such as those of serotype O157 and O104 have been responsible for several outbreaks, the most recent of which was in Germany in 2011. This outbreak, attributed to contamination of bean sprouts with bovine faeces, had a total of 3043 confirmed cases, of which 877 developed HUS and 48 patients died (Rubino et al., 2011).

The biofilm forming potential of *E. coli* is well recognised, and its ability to form biofilms during urinary tract infection has already been discussed in Section 1.1.4.1. In relation to food chain persistence, *E. coli* has been shown to form biofilms on glass, polystyrene and stainless steel at various temperatures, although some strain specificity can be observed (Nesse et al., 2014). Strains of *E. coli* O157:H7 has been shown to produce thick biofilms in response to environmental stresses such as nutrient limitation (Sharma et al., 2005), acidic and alkaline conditions, high temperatures and high hydrostatic pressure treatments (Alvarez-Ordóñez et al., 2013).

1.1.5.2 Biofilm formation by *Salmonella* sp.

There were 1.6 million confirmed cases of *Salmonella* infection in the EU between 1999 and 2008, the majority of which were attributed to consumption of undercooked or contaminated food stuffs (Le Hello et al., 2011). Two serotypes, Typhimurium and Enteritidis, are most commonly associated with disease in humans, although other serotypes are reported to cause disease in both poultry and humans (Barrow, 2000).

Biofilm formation is recognised as a survival mechanism of *Salmonella sp.*, with biofilm forming strains more able to survive desiccation (Iribuchi et al., 2010), low nutrient conditions also promote increased biofilm formation (Stepanovic et al., 2004). It is known that *Salmonella sp.* are able to form single species biofilms on various abiotic surfaces including plastic (Stepanovic et al., 2004), stainless steel (Giaouris et al., 2013), and food stuffs such as cantaloupe melon rinds (Annous et al., 2005). They also have the ability to integrate into preformed biofilms in bathrooms (Barker and Bloomfield, 2000) as well as forming biofilms on chitinous surfaces (Brandl et al., 2011). The *Salmonella* ECM is particularly rich in cellulose (Giaouris et al., 2013), and strains unable to form cellulose show reduced surface attachment and biofilm formation (Brandl et al., 2011).

1.1.5.3 Biofilm formation by *L. monocytogenes*

L. monocytogenes is typically only infectious to those with a suppressed immune-function, such as the elderly, very young, pregnant or immuno-compromised patients (Ouyang et al., 2012). Infection is particularly severe and frequently progresses from a gastrointestinal pathology to systemic infection, causing meningitis, septicaemia, central nervous system infections and abortion in pregnant women (Gandhi and Chikindas, 2007). *L. monocytogenes* is particularly difficult to eradicate from foodstuffs as it is extremely tolerant to low temperatures, desiccation, and pH fluctuations. The bacterium is able to grow at temperatures as low as 0°C, and pH levels between 4.6 and 9.5 (Carpentier and Cerf, 2011), as well as surviving in desiccating conditions for up to 91 days (Hansen and Vogel, 2011). These characteristics allow *L. monocytogenes* to persist within the food chain for an extended period of time, with some food processing plants showing survival of specific strains for years (Szlavik et al., 2012, Pan et al., 2010).

As with many other pathogens, the ability of *L. monocytogenes* to form biofilms shows strain specificity (Harmsen et al., 2010). Single species *L. monocytogenes* biofilms form a thin, homogenous layer in static conditions, and ball-shaped microcolonies when subjected to flow conditions. These attached colonies should be considered as true biofilms, rather than 'attached populations', since they are shown to produce an ECM which contains eDNA (da Silva and De Martinis, 2013, Nguyen and Burrows, 2014, Harmsen et al., 2010). Biofilm formation also appears to be linked to low nutrient, high salt conditions (Pan et al., 2010), and *L. monocytogenes* is able to attach to stainless steel (Nguyen and Burrows, 2014), glass, plastic, and conveyor belts (Midelet and Carpentier, 2002). Biofilm formation has been shown to contribute to *L. monocytogenes* cross contamination, with biofilms providing a source of viable cells (Hansen and Vogel, 2011) which when detached, are able to contaminate ready-to-eat foods or other processing areas (Pan et al., 2010).

1.1.6 Biofilm treatment and inhibition

Most currently available antibiotics are only tested on planktonic bacterial cultures, and minimum inhibitory concentrations obtained from these experiments are used to inform clinical treatments (Keays et al., 2009). Biofilms are widely acknowledged to be up to 1000 times more resistant to antimicrobial treatments than their planktonic counterparts (Olson et al., 2002). This, combined with the lack of novel antibiotics in the drug development pipeline (Clarke, 2003, Morel and Mossialos, 2010), has decreased the options for inactivation and removal of biofilms by traditional, antibiotic treatment.

Recently, the problem of antimicrobial resistances has become an increasing part of the public consciousness (Murphy, 2013), and there has been a move towards novel antibiotic discovery and development. Reform of regulatory guidelines has now been undertaken in the USA, allowing safety trials to be completed more easily (Gupta and Nayak, 2014).

However, products developed as part of this drive will not be available for many years. Additionally antibiotics may still show decreased activity against biofilm infections compared to planktonic cells, and bacteria still have the potential to become resistant to new antibiotic treatments, as they have done with existing antibiotics. This problem has led to the investigation of novel biofilm treatment strategies, aimed at dispersal of the biofilm or inhibition of biofilm initiation (Brooks and Brooks, 2014). Although cells in biofilms are more resistant to antimicrobials than planktonic cells, once cells are released from the biofilm they frequently revert back to their previous levels of susceptibility (Kaplan, 2010). In Sections 1.1.6.1 to 1.1.6.5 some of these novel approaches will be discussed in more detail.

1.1.6.1 Biofilm dispersal

Quorum sensing (discussed briefly in Section 1.1.1.4) has been shown to be responsible for several coordinated biofilm community behaviours, so its reduction or inhibition is a promising target. Treatments involve adding 'quorum quenching' molecules into the system and allowing them to either competitively bind the QS receptors on the cells, or inactivate the QS molecules themselves. The biofilms' response to the addition of quorum quenching molecules depends on the exact QS system targeted. The small molecule cis-2-decenoic acid is able to inhibit *de novo* biofilm formation by *P. aeruginosa*, and promote biofilm dispersal (Davies and Marques, 2009). Similarly, N-acyl homo-serine lactone molecules, in conjunction with nutritional and environmental cues, were found to regulate biofilm formation and dispersal (Rice et al., 2005).

Enzymatic treatment of biofilms has also received attention. Since many different biofilm forming species have been shown to contain eDNA within their ECM, several treatments utilising DNase enzymes have been used with some success. Impregnation of the biomaterial polymethylmethacrylate with DNase I decreased adherence of *P. aeruginosa* and *S. aureus*, without a detrimental effect on adhesion and proliferation of human cells (Swartjes et al., 2013). Human recombinant DNase dornase alpha (brand name Pulmozyme[®]) is used in the management of cystic fibrosis (Konstan and Ratjen, 2012), and it has also been shown to be a useful treatment for biofilms isolated from children with recurrent acute otitis media (Thornton et al., 2013). DNase I treatment has also been shown to reduce established *Bordetella bronchiseptica* and *Bordetella pertussis* biofilms from the mouse respiratory tract (Conover et al., 2011). *In vitro* treatment of Non-typeable *H. influenzae* biofilms with DNase I also allowed increased bacterial killing by β defensins (Jones et al., 2013), highlighting that even in biofilms where DNase I treatment does not have a direct biofilm reducing effect it can still be a useful addition to a treatment regimen. Although DNase has received a great deal of attention and has proven to be effective in a wide range of treatments, other enzymes have also recently been tested for their effectiveness in biofilm dispersal. Proteinase K has been shown to be able to degrade *L. monocytogenes* biofilms, both alone and in conjunction with DNase I treatment (Nguyen and Burrows, 2014).

1.1.6.2 Novel compound discovery

Several foodstuffs contain antimicrobial compounds, and recently there has been significant interest in the use of substances such as honey, garlic and essential oils in biofilm treatments. These treatments are of particular interest to the food industry as some, potentially antimicrobial, ingredients are already in use as flavourings or marinades, and as such are well accepted by the public. 'Antimicrobial marinades' using combinations of honey, mustard, pomegranate, red wine, lemon juice, vinegar and soy sauce have been shown to reduce *C. jejuni* numbers on chicken meat (Birk et al., 2010). Recent work by Hernández-Ochoa et al. (2011) has also shown that the essential oils of clove and cumin showed

antimicrobial activity against *E. coli*, *L. monocytogenes*, *Salmonella enterica*, *C. jejuni*, *S. aureus* and *Bacillus cereus*.

Honey, in particular manuka honey, is recognised as an effective method of treating medical biofilms, although to date there has been little study of its potential applications within the food chain. Honey impregnated dressings have been shown to decrease healing time for burn wounds (Moore et al., 2001) and it has also been shown to have antimicrobial activity against *P. aeruginosa*, and methicillin resistant strains of *S. aureus* (Lu et al., 2014). When used in combination with antibiotic treatments honey has been shown to increase antibiotic killing (Jenkins and Cooper, 2012, Campeau and Patel, 2014). This, in combination with its relatively low cost of production, has made honey an attractive novel antimicrobial treatment for biofilm infections.

1.1.6.3 Phage therapy

'Phage therapy' is defined as the use of lytic bacteriophages (hereafter referred to as 'phages') to eliminate bacteria (Donlan, 2009). Following the first descriptions of phages in the late 19th century (Sulakvelidze et al., 2001), they have been used to treat bacterial infections, particularly before antibiotic treatments were widely available (Sulakvelidze et al., 2001). Recently, due to increased recognition of the problem of antibiotic resistance, and the recognition of the role of biofilms in chronic infections there has been a renewal in interest in the use of phage in bacterial infection management. Phage therapy has been shown to be effective *in vitro* against several biofilm forming pathogens including *S. aureus* (Drilling et al., 2014), *P. aeruginosa* (Fu et al., 2010) and *C. jejuni* (Siringan et al., 2011).

Although promising results have been reported, particularly against biofilms, phage therapy has two disadvantages: firstly phages show a high degree of species and isolate specificity and secondly bacteria are able to develop resistance. To address specificity concerns, careful consideration is required when selecting phages for use in treatment. Several researchers have highlighted the need for potential bacteriophage therapeutics to be tested against many different bacterial isolates, in order to ensure efficacy in the clinic (Drilling et al., 2014, Chhibber et al., 2014, Melo et al., 2014). Novel rapid diagnostic techniques, such as those described in the review by Afshari et al. (2012), allow rapid identification of the bacteria causing infections meaning informed choices can be quickly made about phage selection and appropriate treatment regimes.

Resistance of bacteria to phages is more often encountered in *in vitro* systems than *in vivo*. A study by Carvalho et al. (2010) showed that bacterial populations did not recover following phage treatment of chickens experimentally infected with *Campylobacter jejuni* and *C. coli*. Despite studies such as this one, the development of resistance is still of concern to investigators and regulatory bodies and so has been addressed by several investigators. In order to combat the issue of resistance, bacteriophage 'cocktails' have been developed, which contain a mixture of different bacteriophages or bacteriophage and antibiotic combinations. Combinations of tobramycin and the bacteriophages T4 or PB1 were shown to effectively treat *E. coli* and *P. aeruginosa* biofilms respectively. Combination treatment displayed less resistance than single antibiotic or bacteriophage treatments either to phages or antibiotics (Coulter et al., 2014).

1.1.6.4 Nanoparticle technology

Nanoparticles are defined as particles which are less than 100 nm in diameter (Lu et al., 2012b). They have previously been used for many different applications including drug delivery (Manzoor et al., 2012) and stabilisation of emulsions in food (for a review see

Dickinson (2012)). The antimicrobial properties of silver, zinc oxide and gold nanoparticles are well described (for reviews see Wei et al. (2014), Shi et al. (2014) and Shah et al. (2014) respectively) and their incorporation into surgical wares and food packaging is a promising area of biofilm infection control. The success of nanoparticles in treating and preventing biofilm infections is in part due to their ability to penetrate the biofilm, which many currently available antimicrobial therapies are not able to achieve (Melo et al., 2013). Covering catheter surfaces with silver nanoparticles has been shown to inhibit growth and biofilm formation of several medically relevant bacteria, including *E. coli*, *S. aureus* and *P. aeruginosa* (Roe et al., 2008).

Nanoparticle technology is also a promising area of biofilm control within food industries, although currently there is still debate about the safety of nanoparticle consumption (Schilling et al., 2010, Chen et al., 2014) and consumer acceptance (Frewer et al., 2014). Two significant areas of research have emerged within food manufacture: the use of nanotechnology to detect alterations in food quality or pathogen presence, and incorporation of nanoparticles into packaging to extend shelf life. Quantum dots, nanoparticles 1 to 10 nm in radius, which contain fluorescently labelled antibodies, have been used to detect *E. coli*, and *Salmonella sp.* in minced beef (Wang et al., 2012). Nanoclay, which is comprised of mineral silicate nanoparticles, is frequently used in food packaging due to its ability to reduce gas permeability of packaging materials, increasing shelf life and inhibiting growth of aerobic food spoilage bacteria (Silvestre et al., 2011)

1.1.6.5 Development of reduced-biofilm surfaces

The topography and hydrophobic properties of surfaces is also an important consideration for limiting bacterial attachment. Within the food industry materials were historically selected due to their durability, strength and chemical stability (Chmielewski and Frank, 2003, Singh et al., 2011), rather than their antimicrobial or anti-attachment properties. When selecting materials, consideration of surface topography and, where required, surface coating is very important since these factors are able to influence bacterial attachment to a significant extent.

Stainless steel is a widely used material which can be polished to give a smooth finish with little variation in surface micro-topography. It would be expected that a decrease in surface micro-topography would reduce the ability of bacteria to attach to a surface, and indeed this has been reported by several investigators (Agle, 2007, Jullien et al., 2003). Conversely, other investigators have shown that managed surface roughening is able to decrease bacterial attachment, since it reduces the available surface area which bacteria are able to attach to, meaning that attachments are not as strong as would be found on smooth surfaces. Coating of surfaces with polyurethane urea films, containing micro-pillars, significantly reduced that attachment of *S. aureus* and *S. epidermis* in low fluid flow conditions (Xu and Siedlecki, 2012). Singh et al. (2011) also reported that nano-texturing of titanium oxide surfaces decreased *E. coli* attachment, as the texture allowed a greater absorption of protein to the surface, suppressing *E.coli* attachment.

Surfaces can also be coated with anti-biofilm materials, which either decrease the potential for initial attachment or have antimicrobial properties. An example of the former is polyethylene glycol (PEG) which when used as a surface coating is able to reduce bacterial attachment (Kingshott et al., 2003). Slippery Liquid-Infused Porous Surfaces (SLIPS) have also been shown to have very promising anti-biofilm activity. Results shown that they are able to inhibit >95% of attachment by *P. aeruginosa*, *S. aureus* and *E. coli*, making SLIPS even more effective in reducing bacterial attachment than PEG coated surfaces (Epstein et

al., 2012). Although materials such as these are useful tools for decreasing bacterial adhesion, they are not antimicrobial (Banerjee et al., 2011), so any bacteria which are able to attach to the surface will persist. To combat this, surface coverings, which also have antimicrobial properties are an appealing alternative. Antifouling paints have been used to minimise marine biofouling for many years, although their inclusion of heavy metals and other toxic materials has made them unsuitable for use in food and medical applications (Stowe et al., 2011). Recently, novel antimicrobial coatings have been investigated, which have less toxicity but retain antimicrobial activity. Coatings containing chitosan, a material derived from chitin, have been shown to have antimicrobial activity against *S. epidermidis*, *S. aureus*, *K. pneumoniae*, *P. aeruginosa* and the biofilm forming fungal pathogen *Candida albicans* (Carlson et al., 2008). Terpenes, isolated from sea sponges, have also shown promising anti-biofilm activity (Stowe et al., 2011).

The choice of surface is particularly important within food processing plants, where biofilm formation is hypothesised to allow bacterial persistence for extended periods of time. *L. monocytogenes* in particular is able to persist within the food chain for several years (Ferreira et al., 2014). These 'persistent strains' are shown to have increased ability to attach to stainless steel and show some resistance to antimicrobial treatments such as quaternary ammonium compounds (Lunden et al., 2002). Biofilm formation is also hypothesised to assist in *C. jejuni* food chain persistence, and this will be discussed in more detail in the following sections.

1.2 The genus *Campylobacter*

The genus *Campylobacter* is a Gram-negative member of the ϵ subdivision of the Proteobacteria (Young et al., 2007). This class is poorly characterised compared to species such as *E. coli*, since many species within the genera are still unculturable in the laboratory. The class is split into two orders; Nautiliales and Campylobacterales (Campbell et al., 2006). The division of the class is shown in Figure 1-6.

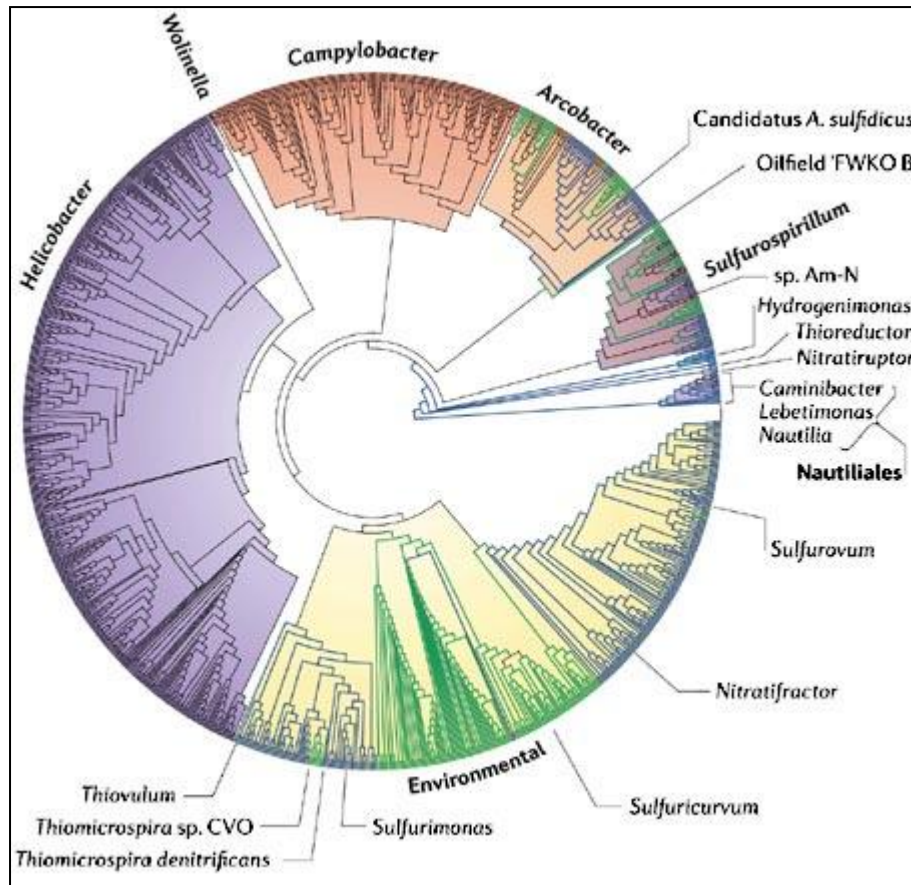


Figure 1-6 Circular phylogram of the ϵ Proteobacteria class

Image shows the diverse nature of the bacteria within the ϵ Proteobacteria class. Species colonies many different niches, from colonisation of animals and humans (i.e. *Campylobacter* and *Helicobacter*) to existing as part of the microflora of undersea vents (i.e. *Sulfurovum*). Image from Campbell et al. (2006).

RNA analysis of deep sea hydrothermal vent communities indicates that up to 90% of the total rRNA in a habitat belongs to ϵ Proteobacteria class. The majority of ϵ Proteobacteria in the vent communities are chemolithoautotrophs, able to oxidise hydrogen and sulphur compounds as well as reducing compounds containing oxygen, nitrate and sulphur (Nakagawa et al., 2007).

Of the two ϵ Proteobacteria orders, it is the order Campylobacterales which are of particular interest to medicine, since this class contains *Helicobacter*, *Campylobacter*, and *Arcobacter* species (Young et al., 2007). All these species have the potential to be pathogenic, however *Helicobacter* sp. and *Campylobacter* sp. are the most prevalent infectious members of the class. *Helicobacter pylori* has a particularly high prevalence in humans, being found in approximately 50% of the global population (Suerbaum and Achtman, 2004). Colonisation with *H. pylori* can lead to development of gastric ulcers and cancer (Nakagawa et al., 2007). Infection with thermotolerant *Campylobacter* sp., most commonly *C. jejuni* or *C. coli*, leads to the development of a serious but self-limiting gastroenteritis (Kapperud et al., 1992). Again, prevalence is high within human populations, and these two species are the most common global cause of intestinal infection (Moore et al., 2005).

The genus *Campylobacter* currently includes 18 species and 6 subspecies (Young et al., 2007). Morphologically, *Campylobacter sp.* are slim, between 1.5 and 6µm long (Ketley, 1997), with a single unsheathed polar flagella at either one or both ends of the bacterium (Balaban and Hendrixson, 2011), the flagella makes the bacterium highly motile, with rapid, darting or spinning motions (Shigematsu et al., 1998). Many *Campylobacter sp.*, including *C. jejuni* and *C. coli* are not only thermophilic, requiring temperatures above 35°C for growth, but are also capnophilic (Gaynor et al., 2005). *Campylobacter sp.* also lack homologues of many well-known toxins, adhesins, invasins, protein secretion systems and pathogenicity islands found in other foodborne pathogens such as *E. coli* and *Salmonella sp.* (Bereswill and Kist, 2003). Early investigation into *Campylobacter sp.* focused on veterinary medicine, since *C. fetus* is responsible for spontaneous abortion in domestic ungulates (Solomon and Hoover, 1999), and it was not until the 1970s that *Campylobacter sp.* were also recognised as human pathogens (Skirrow, 1977).

1.2.1 *Campylobacter sp.* as human pathogens

C. jejuni, *C. coli*, *C. upsaliensis*, *C. lari*, *C. concisus*, *C. fetus* subsp *fetus*, *C. jejuni* subsp *doylei*, *C. hyintestinalis* and the related *Arcobacter butzleri* are all recognised as human pathogens (Butzler, 2004). Hereafter the term '*Campylobacter sp.*' will be used to describe these pathogenic, thermophilic members of the genus (excluding *A. butzleri*). Of these species *C. jejuni* and *C. coli* are the most prevalent human pathogens, accounting for 95% of *Campylobacter sp.* infections. Within the genus, hippurate hydrolysis is able to distinguish *C. jejuni* from other members of the group (Butzler, 2004), and the two subspecies of *C. jejuni* (subsp *jejuni* and *doylei*) are separated on the basis of nitrate reduction and cephalothin susceptibility (Snelling et al., 2005).

Campylobacter sp. colonises the gut causing either an asymptomatic or symptomatic infection. Invasion of the intestinal mucosa leads to changes such as superficial ulceration and neutrophil infiltration of the epithelium leading to the production of bloody diarrhoea (due to the destruction of epithelial cells) (Cogan et al., 2007). *C. jejuni* has a preference for colonizing the distal ileum and colon, residing in the mucosal layer and disrupting the epithelial barrier, causing the development of an immune response.

1.2.1.1 Symptoms of infection

Acute infection is characterised as having diarrhoea preceded by a febrile period with malaise, myalgia, abdominal pain and a fever of approximately 40°C (Butzler, 2004). Vomiting is rare and diarrhoea typically resolves within 2-3 days, although abdominal pain and other symptoms may persist for longer (Butzler, 2004, de Zoete et al., 2007). Following resolution of symptoms, individuals can excrete *Campylobacter sp.* for up to seven weeks (Ekdahl and Andersson, 2004). Generally *Campylobacter sp.* infection has a good prognosis, typically being self-limiting (de Zoete et al., 2007), and rarely requires antimicrobial therapy (Allos, 2001), although in immunocompromised or acutely ill patients antibiotic treatment may be considered (Altekruse et al., 1999).

1.2.1.2 Post infection consequences

Due to the typically self-limiting nature of *Campylobacter sp.* infection, illness cause by *C. jejuni* only accounts for 5% of estimated food related deaths and 17% of foodborne infection hospitalisations within the EU (Dasti et al., 2010). Severe post infection consequences can occur though. It has been reported that 5 to 30% of patients who suffer from an episode of acute infectious gastroenteritis then report chronic irritable bowel syndrome, termed post-infection irritable bowel syndrome (IBS), following clearance of the pathogen (Schwille-Kiuntke et al., 2011). Although post infection IBS can be developed following several other

diseases, it has been linked to *C. jejuni* infection (Marshall et al., 2006). Risk factors for the development of IBS following *C. jejuni* infection include increased disease severity with the presence of abdominal cramps, increased duration of diarrhoea and a weight loss of less than 10 lb. Gender and age were also risk factors, with young girls being the most at risk of developing IBS (Marshall et al., 2006).

The most severe sequelae in terms of disability and risk of fatality is Guillain Barré Syndrome (GBS). This is an acute autoimmune disease caused by the molecular mimicry of ganglioside epitopes within the nervous system by the *C. jejuni* lipooligosaccharide (Dasti et al., 2010). The syndrome is characterised by acute symmetrical, descending and progressive paralysis and areflexia (Nyati and Nyati, 2013). Paralysis can range in severity from muscle weakness to complete paralysis of muscles. Although patients with GBS usually recover, recovery is protracted and may require extensive medical support and rehabilitation (Hoffmann et al., 2012).

1.2.1.3 The economic burden of infection

In the developed world the main burden of *Campylobacter sp.* infection is economic, as infected individuals are often not able to attend work or school during the symptomatic phase of the infection. In the European Union, infection costs approximately €500 per illness, with an annual cost burden of approximately €300 million (Ingmer, 2011). In the United States the cost of illness is estimated at \$1.2 billion annually (Batz et al., 2012).

In the UK, despite repeated government drives to reduce *Campylobacter sp.* little reduction can be seen in numbers of the bacterium in either chicken flocks, or in retail-ready packaged chicken products (Robyn et al., 2015). This is in direct contrast to other foodborne pathogens previously prevalent in the UK, such as *S. enterica*. *S. enterica* was responsible for a large UK outbreak in the 1980s, but numbers of the bacterium within the food chain have significantly decreased following the implementation of increased biosecurity measures and vaccination of egg laying flocks (Cogan and Humphrey, 2003).

Within the last decade *C. jejuni* 'epidemics' have been recorded in New Zealand (Sears et al., 2011), Iceland (Tustin et al., 2011), Norway (Hofshagen and Kruse, 2003) and the Netherlands (Bouwknegt et al., 2004). To date the most effective, and consumer acceptable, method of reducing *Campylobacter sp.* numbers on carcasses is freezing before sale (Georgsson et al., 2006, Sandberg et al., 2005, Harrison et al., 2013). This solution, although effective, has a significant impact on the poultry industry since fresh chicken meat commands much higher prices than that of frozen meat. Because of this, alternative control solutions are still being sought to reduce *Campylobacter sp.* numbers throughout the whole food chain.

1.3 *Campylobacter sp.* infection routes and potential reservoirs

Campylobacter sp. have been isolated from many different animals and environmental sources. Figure 1-7 shows some of the most common infection sources for humans. These, and other important reservoirs are described in Sections 1.3.1 and 1.3.2.

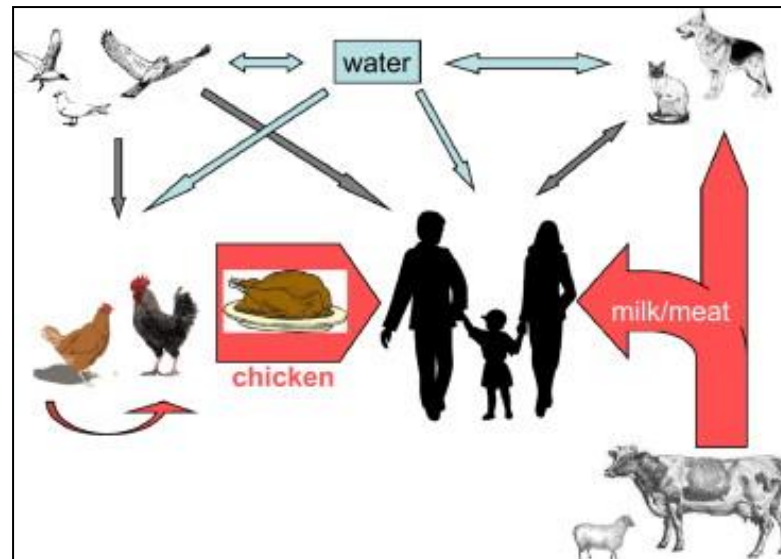


Figure 1-7 Potential *Campylobacter sp.* infection routes

Graphical representation of the proposed major *Campylobacter sp.* infection routes from the environment to man. Infection is hypothesised to occur primarily due to the consumption of contaminated poultry, or cross contamination of other foodstuffs from raw poultry meat. However other infection routes are also known, such as contact with contaminated environmental sources (e.g. open water bathing) or by contact/consumption of other animals. Image taken from DASTI et al 2010

1.3.1 Environmental sources of *Campylobacter sp.*

1.3.1.1 Wild bird populations

Wild bird populations are often found to contain various *Campylobacter sp.*, particularly *C. lari* (Ryu et al., 2014). Although direct contact with these animals is not considered to be a major infection route, faecal contamination by wild bird populations is possible. In 2008, a *C. jejuni* outbreak in Alaska was traced back to wild bird faecal contamination of raw peas (Kwan et al., 2014). Wild bird populations also potentially are able to contaminate water sources and migratory bird populations contribute to infection spread, providing an important reservoir for *Campylobacter sp.* (Ryu et al., 2014).

1.3.1.2 Water sources

Campylobacter sp. infection rates are highly seasonal in temperate climates, with approximately double the number of infections in summer compared to winter (Butzler, 2004), and the peak incidence rate occurring between May and October (Schlundt et al., 2004). This peak also correlates with the times when the public are more likely to be outdoors, potentially bringing them into contact with environmentally based infection sources. Contaminated water sources, either environmental or potable, have previously been identified as responsible for *Campylobacter sp.* outbreaks. In the US, water was identified as the second most common source of *Campylobacter sp.* outbreaks, accounting for 9% of outbreak sources (Taylor et al., 2013). Although this is still low compared to the number of cases attributed to consumption of contaminated foodstuffs (86% in the above study), it is still a significant source of infections.

Campylobacter sp. contamination of environmental water sources has been well documented, particularly in areas close to livestock or where groundwater containing effluent is likely to wash into the water source. *Campylobacter sp.* have been shown to be present in

both the water and sediment of UK rivers (Obiri-Danso et al., 2001). In countries where outdoor bathing is popular, such as Finland, *C. jejuni* outbreaks from water sources are relatively common, accounting for 19% of all waterborne outbreaks (Revez et al., 2014). Interestingly, several studies have observed an inverse seasonal peak, in which *Campylobacter sp.* numbers drop in the summer as the water temperature rises (Obiri-Danso et al., 2001, Hokajarvi et al., 2013, Brennhovd et al., 1992). This suggests that bathing or contact with contaminated environmental water sources may not wholly account for the seasonal peak observed in human cases.

1.3.2 Domestic sources of infection

Many domesticated animals are a reservoir for various thermophilic *Campylobacter sp.* Pets such as dogs (Mughini Gras et al., 2013), cats (Acke et al., 2011) and even reptiles (Giacomelli and Piccirillo, 2014) have been found to be colonised with *Campylobacter sp.* Houseflies are known vectors for many diseases, for example shigellosis, typhoid fever, and *E coli* infection (Ek Dahl et al., 2005). Domestic flies have also been shown to carry *Campylobacter sp.* (Nichols, 2005), and their presence within domestic environments could lead to spread of *Campylobacter sp.* infection or transmission to humans. Although humans frequently come into contact with these animals, they are not the main infection source in humans, this being the consumption of contaminated, poorly cooked meats or other contaminated foodstuffs.

The majority of domestic food animals (for example cows, pigs and poultry such as ducks, chickens and turkeys) carry *Campylobacter sp.* and shed it in high numbers in their faeces (Ogden et al., 2009). Use of this faeces on crops can in turn lead to contamination of water sources (as described in Section 1.3.1.2) or contamination of crops such as fruit and vegetables (Chai et al., 2009). The vast majority of human *Campylobacter sp.* cases are attributed to the consumption of contaminated meat. Poultry meat is by far the most common source of infection, however unpasteurised (raw) milk has been identified to be an important source of *C. jejuni* outbreaks (Evans et al., 1996, Taylor et al., 2013, Bianchini et al., 2014). Since poultry, particularly chicken meat, is such an important source of *Campylobacter sp.* infection the rest of this chapter will focus on chicken and its slaughter process.

1.3.2.1 Poultry

Campylobacter sp. are part of the normal flora in the poultry gut, forming densely packed parcels of cells within luminal crypts, attached to the mucus rather than the epithelium (Beery et al., 1988). Human *C. jejuni* infection is frequently associated with poultry (Quinones et al., 2007) and in the Netherlands 20-40% of laboratory confirmed cases are attributed to the consumption of undercooked poultry (Jore et al., 2010).

C. jejuni colonises the chicken gut, with the highest bacterial numbers seen in the large intestine, caecum and cloaca. Once individuals within a flock are infected, spread of infection through the flock is rapid and extensive (Corry and Atabay, 2001), with *C. jejuni* levels of between 10^5 and 10^9 CFU/g faeces (Hanning et al., 2008). Flock infection rates vary depending on season (Kovats et al., 2005) and sampling method (Jorgensen et al., 2002). In Ireland, 80% of flocks are reported to be infected (Anon, 2011) and a recent study by the FSA showed that 70% of fresh, commercially available, whole chickens in UK supermarkets were *Campylobacter sp.* positive (Anon, 2014).

C. jejuni is often thought of as a commensal of the chicken gut, with infected chickens usually showing no visible sign of infection (Shanker et al., 1990). However, a link between *C. jejuni* and vibriotic hepatitis has been described (Corry and Atabay, 2001). Vibriotic

hepatitis causes liver necrosis and a drop in egg production (Jennings et al., 2011). Recent work by Humphrey et al. (2014) has also shown that *C. jejuni* infection can lead to pathological changes within commercial broiler chicken breeds, although the pathology varies depending on the breed of bird.

1.4 *Campylobacter* sp. transmission throughout the food chain

Unlike bacterial species such as *L. monocytogenes*, it is not the ability of *Campylobacter* sp. to grow outside the host, but its ability to survive which makes it a successful human pathogen (Balamurugan et al., 2011). It is still debated which areas of the food chain *Campylobacter* sp. are able to contaminate most effectively. In Sections 1.4.1 to 1.4.2 potential routes of *C. jejuni* infection and cross contamination will be discussed.

1.4.1 Spread of *Campylobacter* sp. within flocks prior to slaughter

C. jejuni penetration of the egg has only been demonstrated at very low levels so, unlike in *Salmonella* sp., vertical transmission of *C. jejuni* is not considered a likely route of poultry contamination. In a study of 257 eggs challenged with *C. jejuni*, 63% of the eggs showed evidence of infection, although all chicks were *C. jejuni* free (Shanker et al., 1986).

Some farms show continual infection of flocks by one *C. jejuni* serotype, indicating that contamination likely occurs from a source within the farm. This effect may be exacerbated if the farm maintains a rolling flock policy, containing several flocks at different stages of development (Berndtson et al., 1996). Transmission of *C. jejuni* throughout flocks appears to have a horizontal origin, although the exact mechanism has yet to be established. Several hypothesis of how infection spreads within flocks have been suggested and risk assessment models have shown that the source of infection is likely multi-factorial (Newell and Fearnley, 2003). Flies may play a role in transmission of *Campylobacter* sp. between flocks of chickens (Nichols, 2005). Studies have shown that flies found near broiler houses show contamination with *C. jejuni* however other risk factors such as food and litter, water, other animals, house cleaning and disinfection, ventilation, contamination via human activities and, finally, poultry management process have also been considered as possible routes of transmission (Newell and Fearnley, 2003).

1.4.1.1 Environmental sources of contamination

Farms rearing broiler chickens are open systems in which, despite stringent hygiene measures, microbiological carriage is frequently observed. *C. jejuni* may come into contact with chicken through various interactions with humans and other animals. Food, water, human, animal and insect contacts have all been suggested as potential *Campylobacter* sp. transmission sources. To date no single source of environmental contamination has been discovered and it is likely that due to the ubiquitous nature of *Campylobacter* sp., multiple factors are responsible for the transmission from the environment to flocks.

Animal feeds are not considered to be a major source of *C. jejuni* persistence and contamination. Most chicken feeds are dried, and since *C. jejuni* is very susceptible to desiccation (Fernandez et al., 1985), it is unable to survive for prolonged periods within desiccated foods. Survival of *C. jejuni* in water systems is much more likely (Buswell et al., 1998). Untreated drinking water has been found to be a significant source of infection both in animals and humans, with flocks using untreated water more likely to be *Campylobacter* sp. positive (Kapperud et al., 1993). Treatment of water with lactic acid leads to a significant reduction in numbers of both *Salmonella* sp. and *Campylobacter* sp. (Byrd et al., 2001). In their risk assessment study, Kapperud et al. (1993) found that the treatment of flock drinking

water was the factor which would have the biggest impact on *C. jejuni* prevention within flocks.

Flies are recognised as a vector for several diseases (Ekdahl et al., 2005) and several investigators have hypothesised that they, along with other pests such as rats, may also play a role in the transmission of *C. jejuni* between flocks (Kapperud et al., 1993). Flies have been found to be able to transmit *C. jejuni* to chickens in experimental models (Hald et al., 2004), however it is still unclear if they are able to do this effectively within farm settings. Only a small percentage of flies (8.2%) captured during a transmission study tested positive for *C. jejuni* (Hald et al., 2004) and contradictory research has shown that although flies are frequently captured within broiler houses, *C. jejuni* could only be isolated from flies following infection of the flock (Berndtson et al., 1996). The presence of rats on a broiler farm was linked to increased risk of *Campylobacter* sp. infection of flocks, although the increase was not statistically significant (Kapperud et al., 1993). This perhaps suggests that pests such as flies and rats have a synergistic relationship with the broilers, potentially contributing to *C. jejuni* transmission between houses, but unlikely to be the primary source of infection (Meerburg et al., 2006).

Finally, the implementation of biosecurity and adherence to protocols is essential for the rearing of *Campylobacter*-free flocks. Several studies have highlighted the need for all staff on broiler farms to strictly follow biosecurity routines, since poor adherence can rapidly lead to contamination of the flocks (Berndtson et al., 1996, Wassenaar, 2011, Evans and Sayers, 2000). It is interesting to note that although the public perceive organic and free range broilers to be 'healthier and more ethically reared' (Harper and Makatouni, 2002) due to their extended growth time and the encouragement of natural behaviours (Allen et al., 2008), strict biosecurity measures are much more difficult to maintain in these flocks (Huneau-Salaun et al., 2007). Several studies have shown that this rearing method has led to increased prevalence of *C. jejuni*, although bacterial load per bird appears not to be increased (Cui et al., 2005, Rosenquist et al., 2013).

1.4.2 *Campylobacter* sp. within processing plants

As controlling *C. jejuni* infection on the farm is complex and difficult to achieve, it has been suggested that there should be a greater focus on prevention of cross contamination at the processing plant, or decontamination of carcasses following processing (Corry and Atabay, 2001). Plant automation has increased the number of birds a plant is able to process, which in turn has increased the potential for contamination (Arnold and Silvers, 2000). Plants typically deal with 12,000 birds per hour and run continually, stopping only to clean and disinfect machinery. Poultry are eviscerated without opening the carcass and skin is typically not removed. Feathers are loosened by submerging carcasses in warm water, the temperature of which is usually between 50°C and 60°C. Plucking machines have rubbery fingers attached to rotating disks, and plucking is aided by spraying water on to the carcass while the feathers are removed. The most important contamination control step is the wash before chilling. The carcass should be thoroughly washed inside and out at this point, as microbial contamination of meat is typically a surface phenomenon (Corry and Atabay, 2001).

Many studies on *Campylobacter* sp. within processing plants have now been completed and cross contamination of *C. jejuni* negative flocks is frequently found (Allen et al., 2007). A recent systematic review identified 1,716 papers when using the search terms '*Campylobacter*', 'chicken' and 'processing' (Guerin et al., 2010). Results from many of the papers appear contradictory, and total concentrations of *Campylobacter* sp. vary greatly

between studies. In part this variation is due to the different enumeration and isolation techniques used (Bai et al., 2014) or sampling sites chosen (Baré et al., 2013, Luber and Bartelt, 2007). Other factors effecting *Campylobacter sp.* cell numbers include slaughter processing methods, prevalence within the flocks before slaughter (Guerin et al., 2010) and season that the sampling took place (Jore et al., 2010, Kovats et al., 2005, Meldrum et al., 2005). Although studies show great variability, some conclusions are able to be drawn. It has been noted by several researchers that *Campylobacter sp.* numbers typically reduce following the scalding and chilling stages of processing (Rosenquist et al., 2006, Duffy et al., 2014, Guerin et al., 2010). There was also a trend of increased *Campylobacter sp.* prevalence following the defeathering process (Guerin et al., 2010), which occurs directly after scalding. A study of *E. coli*, *Campylobacter sp.* and Coliforms showed that the scalding process reduced the bacterial load on the production line from an initial mean value of 4.7 log CFU/ml of rinse fluid to 1.8 log CFU/ml rinse fluid at the post scald stage, increasing back to 3.7 log¹⁰ CFU/ml rinse fluid following plucking (Berrang and Dickens, 2000). The primary cause of this increase is an escape of gut contents when carcasses passed through the defeathering machine (Berrang et al., 2011).

Carcass chilling, one of the final stages of processing, can be carried out in two ways: air or, in the USA, water cooling (Demirok et al., 2013). Both methods provided a reduction in *Campylobacter sp.* numbers (Rosenquist et al., 2006), although the water tanks used during water cooling represent a major cross contamination risk (Guerin et al., 2010). The use of water treatments such as chlorine or gamma radiation has been shown to be effective in reducing bacterial contamination of the chill water (Corry and Atabay, 2001), however there is low consumer acceptance of these interventions (MacRitchie et al., 2014) and they are currently not used within the EU.

Following processing, carcasses are typically either refrigerated or frozen before sale. Freezing has been shown to reduce *Campylobacter sp.* numbers significantly (Wassenaar, 2011). This effect is so consistent that freezing of *Campylobacter sp.* contaminated poultry has become one of the main infection reduction strategies in countries such as Iceland (Tustin et al., 2011). Chilling, compared to freezing, allows greater *Campylobacter sp.* survival, although an overall reduction is still observed during storage. Chicken breast inoculated with *C. jejuni* and stored at 4°C showed a 1-2 log decline in CFU over 17 days (Blankenship and Craven, 1982).

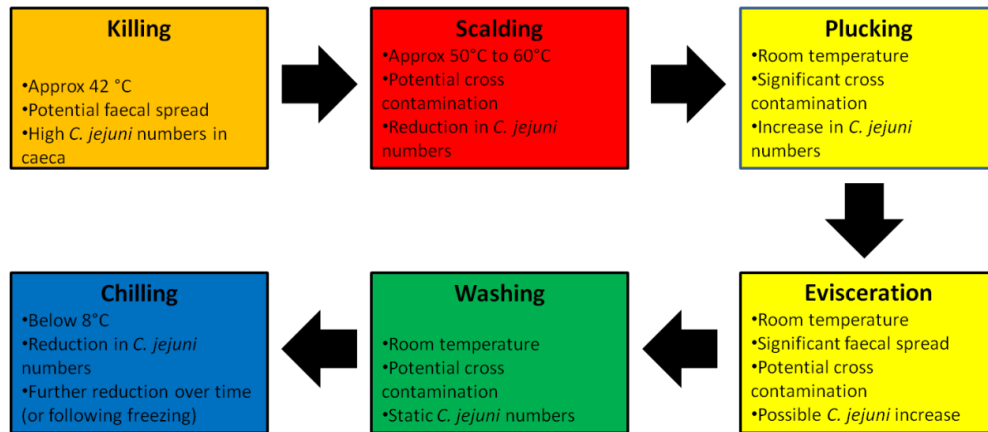


Figure 1-8 Flow diagram of slaughter process indicating potential contamination and *C. jejuni* release points

The diagram shows the main points of the poultry slaughter process. A brief description of the temperature the processing stage is carried out at, it's potential to increase/decrease or spread *C. jejuni*, and the net increase or decrease in *C. jejuni* numbers is shown. The colour of the boxes indicates the temperature the carcasses are at during the stage, with red being hottest, yellow indicating a mid-point, and blue being the coolest temperature in the process.

1.4.2.1 Cross contamination within the processing plant

During processing there are several points where cross contamination may occur. Several researchers have suggested that cross contamination is possible within processing factories, and food chain persistence is well recognised in other bacterial foodborne pathogens such as *L. monocytogenes* (discussed in Section 1.1.5.3). A study by Berghaus et al. (2013) showed that 63.6% of flocks leaving farms during their study tested positive for *Campylobacter sp.* Following processing 87.3% were positive, suggesting cross contamination occurred during this study. Allen et al. (2007) typed *Campylobacter sp.* strains isolated from chickens prior to slaughter and throughout the processing plant. They observed that the strains found on carcasses did not correlate closely with the strains found in the caeca prior to slaughter, again highlighting the potential for cross contamination and *Campylobacter sp.* persistence within the food chain. During this study *Campylobacter sp.* were isolated from aerosols, particles and droplets throughout the processing plant, although not in chilled storage areas, hinting at a possible cross contamination method (Allen et al., 2007).

In contrast, a study by (Elvers et al., 2011) found that the strains identified on the birds entering the processing plant, remained predominant throughout the processing procedure. This was also observed by Kudirkiene et al. (2011), who found that one genotype was predominant from farm to end of slaughter. Although these studies suggest that *Campylobacter sp.* strains are not able to persist for extended periods within processing plants, there is still the potential for short term persistence. An assessment of the transfer of *Campylobacter sp.* from positive to negative flocks was carried out by processing *Campylobacter sp.* negative flocks directly after positive flocks had been slaughtered. Several strains were isolated from previously negative flocks, and results showed that the isolates were of the same type as those found in the positive flocks (Elvers et al., 2011). Taken together these studies highlight that *Campylobacter sp.* are able to persist within food processing plants and contaminate other carcasses and the environment.

1.5 *C. jejuni* food chain survival mechanisms

Although *C. jejuni* is frequently described as been poorly adapted to survival in the food chain, its high isolation rate from poultry products suggests that in fact it is able to successfully survive in: low temperatures, atmospheric oxygen levels and impoverished medium. Although *C. jejuni* does indeed lack many of the canonical survival mechanisms found in pathogens such as *Salmonella sp.* and *E. coli* (Parkhill et al., 2000), it does contain other stress response mechanisms which allow it to survive in sub-optimal conditions. Those mechanisms with relevance to the food chain are described in more detail in Sections 1.5.1 to 1.5.3.

1.5.1 Oxidative stress

C. jejuni is widely recognised as a obligate microaerophile (Chan et al., 2001, Stead and Park, 2000), which makes it distinct from other pathogens such as *E. coli*, *L. monocytogenes* and *Salmonella sp.* (Handley et al., 2014). Despite this, *C. jejuni* is able to tolerate aerobic conditions during transmission to humans, invasion and attack by host immune cells such as macrophages (van Vliet et al., 2002). To date no dedicated aero-tolerance system, has been identified in *C. jejuni* (Dasti et al., 2010), although proteins involved in oxidative stress have been identified. The *C. jejuni* genome contains only one a superoxide dismutase (SOD) (Purdy and Park, 1994), which catalyses the formation of oxygen and hydrogen peroxide from superoxide (van Vliet et al., 2002). Alkyl hydroperoxidase reductase (AhpC) is also present and its deletion leads to increased sensitivity to aerobic conditions (Baillon et al., 1999). Also of interest is the hydrogen peroxide reductase Tpx. Tpx expression in *C. jejuni* has been shown to be increased in biofilms and its deletion leads to reduced growth when exposed to atmospheric conditions (Atack et al., 2008).

Oxygen tolerance is also reported to be medium-specific, with the addition of different tryptones leading to growth at O₂ concentrations of up to 21% (Hodge and Krieg, 1994). *In vitro* growth has been reported at between 15 and 21% O₂, particularly where medium is supplemented with antioxidants, for example SOD, catalase, sodium dithionite or histidine (Kaakoush et al., 2007), or where CO₂ concentrations are increased from 5% to 10% (Reuter et al., 2010). Increasing cell density also appears to provide oxidative stress protection with cells at a concentration of 10⁷ CFU ml⁻¹ observed to grow in aerobic conditions (19% O₂/10% CO₂) (Kaakoush et al., 2007). This was confirmed in a second study in which no significant difference in growth was observed in *C. jejuni* following 5 and 15 hours exposure to aerobic conditions, although clinical isolates did have reduced invasion ability following the five hour exposure (Mihaljevic et al., 2007). It should be noted though, that some strains did not show comparable growth in aerobic conditions and microaerobic conditions, survival was also reduced (Chynoweth et al., 1998).

Temperature also appears to affect the *C. jejuni* response to oxidative stress. *C. jejuni* suspensions exposed to oxidative conditions and 4°C had a much greater survival rate than those incubated at 25 or 42°C (1 log and 5 log reductions respectively). Following seven days of exposure to atmospheric oxygen concentrations no viable cells remained in cultures incubated at 25 or 37°C, where as those incubated at 4°C maintained a concentration of approximately 1 x 10⁷ (Garenaux et al., 2008).

1.5.2 Osmotic shock

Water is used throughout the poultry slaughter process and so *Campylobacter sp.* either transiting, or persisting within the food chain, must be able to mount a response to alterations in osmotic potential. Early work showed that *C. jejuni* was much less resistant to

osmotic shock than *E. coli*. A concentration of 2% NaCl in Muller Hinton medium is lethal to *C. jejuni*, whereas *E. coli* is able to tolerate concentrations of up to 30 % (Doyle and Roman, 1982). Osmotic stress led to alterations in *C. jejuni* cell morphology, with cells becoming elongated following exposure to 1.5 % NaCl, showing reduced or absence of (dependent on the concentration of NaCl) logarithmic growth (Cameron et al., 2012). Two genes have been associated with the *C. jejuni* osmotic shock response in strain 81-176: *cjj0263* and *cjj1025*. Both are predicted to be mechanosensitive channels, the function of which is to open in response to alterations in osmotic potential, allowing fluid to flow in and out of the cells and thereby prevent cell lysis. Deletion of *cjj0263* led to a severely defective hyperosmotic response, however the *cjj1025* deletion mutant responded in a similar manner to the WT, suggesting this gene, despite being predicted to be involved in the osmotic shock response, is not essential for osmotic shock response (Kakuda et al., 2012). *C. jejuni* biofilm formation has also been shown to be affected by osmotic conditions. In a study by Reeser et al. (2007) the addition of osmolytes such as NaCl, glucose and sucrose decreased biofilm formation.

1.5.3 Survival during temperature fluctuations

C. jejuni requires a temperature range of 34 to 44°C for optimal growth (Blaser et al., 1979). Outside this temperature range the bacterium becomes stressed and must respond in order to protect itself. *C. jejuni* transcribes 24 proteins in response to heat shock, and mutants deficient in proteins such as GroESL, DnaJ and Lon protease have reduced growth at 46°C and were unable to be isolated from chickens following experimental infection. This indicates that heat shock proteins may also have a role in invasion. The RacRS system also has a role in differential expression of proteins at 37°C and 42°C (Dasti et al., 2010). There is a very rapid drop in *C. jejuni* growth at 31°C. It was previously speculated that this was due to the lack of RNA chaperone CspA, which in *E. coli* is designated as a cold shock protein (Goldstein et al., 1990). Although *C. jejuni* growth is inhibited below 30°C, ATP production, chemotaxis and catalase production are still observed, indicating metabolic functions are maintained, although growth is halted (Hazeleger et al., 1998).

An important cold shock response is the ability to alter outer membrane composition, maintaining homoviscosity during temperature fluctuations. In a study investigating the effects of various heat treatments on *C. jejuni* and *E. coli* it was found that the lack of cold shock response in *C. jejuni* conferred a survival advantage when compared to *E. coli*. Pre-exposure of bacteria to 6°C significantly affected heat tolerance of *C. jejuni* NCTC 11168, leading to prolonged survival upon rapid temperature increase to 52°C (Hughes et al., 2010). The opposite was true of *E. coli*, which alters its membrane composition in response to cold shock, decreasing dodecanoic, triecanoic, tetradecanoic, pentadecanoic, hexadecanoic and heptadecanoic acids, and increasing in cis-vaccenic and palmitoleic acid. These changes increased membrane fluidity at 6°C. The lack of adaption means that *C. jejuni* membranes become less fluid following exposure to low temperatures, preventing membrane leakage when temperatures rapidly increase, for example during the cooking process (Hughes et al., 2009).

1.6 *C. jejuni* biofilm formation

Although the volume of information about biofilm formation by foodborne pathogens still lags behind that of bacteria causing life threatening wound or respiratory infections, it has now started to increase. The investigation of *C. jejuni* biofilm formation highlights this recent trend. Although investigation of *C. jejuni* biofilms is still in its early stages, a trend in increased awareness and study can be observed (Figure 1-9). A search of Web of Science

for research articles containing the word '*Campylobacter*' within their title and the keyword 'biofilm' as a topic produced a total of 118 papers, including review articles.

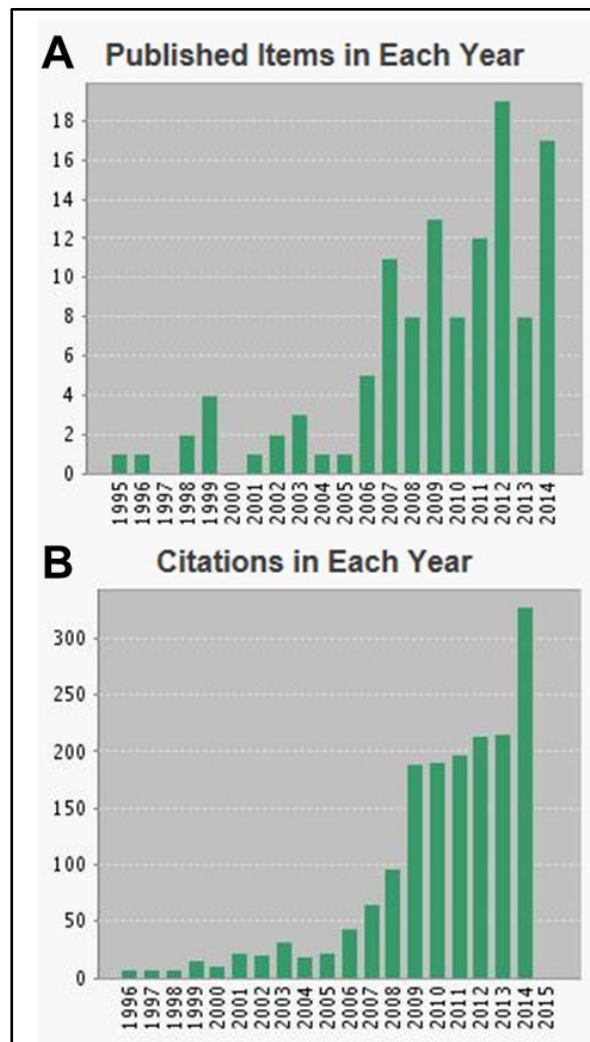


Figure 1-9 Investigation of *Campylobacter sp.* biofilms follows the same trend as that observed for the field of biofilm research.

Using the key words '*Campylobacter*' (within the papers title) and 'Biofilm' (within the topic), a total of 118 papers are found. Graphical representation of publications (A) and citations (B) by year shows a similar trend to that observed in the general field of biofilm study, although slightly delayed and with fewer numbers of papers. For both graphs the Y axis indicates years and x axis indicates the number of publications with the search terms in their title.

A review of the literature related to *Campylobacter sp.* biofilm formation allows some interesting conclusions to be drawn. It should be noted that biofilm formation has become part of the standard phenotypic testing which mutant strains undergo, along with measurement of growth, autoagglutination, swarming, and morphological variation compared to wild type strains. The addition of biofilm formation to the standard battery of phenotypic tests has contributed to the increase in papers published which discuss *C. jejuni* biofilm formation, however in the following Sections only papers with a significant focus on biofilm formation will be discussed. At the time of writing, approximately 90 original research articles on the subject of *Campylobacter sp.* biofilms are available on the Web of Science

website. Papers focusing primarily on biofilm formation can typically be divided into three areas of research: identification of biofilm forming capacity, investigation of factors involved in biofilm formation or identification of genes influencing biofilm formation.

Early *C. jejuni* biofilm studies focused on the identification of biofilm forming species and strains and the factors responsible for biofilm formation. A wide range of factors have been investigated including temperature (Dykes et al., 2003), interaction with other species (Trachoo et al., 2002), atmosphere (Reuter et al., 2010), nutritional or osmotic stress (Reeser et al., 2007) and surface type (Kalmokoff et al., 2006). More recently, the focus of research has moved to genes involved in biofilm formation. Researchers commonly focus on *C. jejuni* biofilm formation, rather than other *Campylobacter* sp., and two *C. jejuni* strains are overrepresented in the literature: NCTC 11168 and 81-176. This is to be expected, since *C. jejuni* is the most commonly isolated pathogenic *Campylobacter* sp. (Dasti et al., 2010), and so of particular relevance to investigators. The two most commonly used *C. jejuni* strains, NCTC 11168 (Parkhill et al., 2000) and 81-176 (Hofreuter et al., 2006) are well characterised, with well-established genetic manipulation protocols (van Vliet et al., 1998).

1.6.1 The biofilm forming ability of *Campylobacter* sp.

Campylobacter sp. are now widely recognised to be able to form biofilms (Wyss, 1995, Joshua et al., 2006, Gunther and Chen, 2009, Hanning and Slavik, 2009), although much variation in biofilm forming ability is present both between and within species (Sulaeman et al., 2010, Revez et al., 2011, Kudirkiene et al., 2012). Several studies have shown that *Campylobacter* sp. are also able to form biofilms in the presence of other bacterial species. This is of great importance to food chain relevant research, since within processing plants, biofilms are typically comprised of several species. Species used in the mixed biofilm experiments included *P. aeruginosa* (Trachoo and Frank, 2002, Teh et al., 2010), *Enterococcus* sp. (Trachoo and Brooks, 2005), and mixed species biofilms from various environmental or food processing sources (Sanders et al., 2007, Sanders et al., 2008, Maal-Bared et al., 2012, Hanning et al., 2008). Generally, biofilm formation with other bacterial species increases *C. jejuni* survival, however this increase is affected by the species present and, potentially, the environmental conditions the biofilm is exposed to.

1.6.2 The environmental factors influencing *Campylobacter* sp. biofilm formation

Although the majority of the biofilm formation assays have been carried out using plastic microtitre plates, glass slides, or test tubes, other materials, many of which are food chain relevant, have also been shown to support *C. jejuni* biofilms. Abiotic surfaces such as stainless steel (Trachoo and Brooks, 2005, Kalmokoff et al., 2006, Sanders et al., 2007, Gunther and Chen, 2009, Duffy and Dykes, 2009), poultry house drinking water systems (Zimmer et al., 2003, Hanning et al., 2008), river rock, slate, wood or sediment (Maal-Bared et al., 2012), PVC (Reeser et al., 2007), Polyethylene terephthalate (PET) (Tatchou-Nyamsi-Konig et al., 2008), nitrocellulose and glass fibre (Kalmokoff et al., 2006) have all been shown to support *Campylobacter* sp. attachment and biofilm growth. Interestingly, intestinal tissue (Haddock et al., 2010) was also shown to allow biofilm formation, suggesting a possible mode of persistence within chickens.

Other environmental factors have been shown to either increase biofilm formation or increase *C. jejuni* survival within biofilms. As reported for planktonic cells (Section 1.5.3), lower temperatures, such as those found during chilled storage, appear to prolong cell viability within biofilms. A study by Buswell et al. (1998) showed that *C. jejuni* survival within biofilms was enhanced at temperatures of 4°C or 10°C, compared to biofilms incubated at 22°C or 37°C. This study also showed that atmospheric conditions effected survival, with

aerobic conditions leading to increased survival in low temperatures. Reuter et al. (2010) expanded this work, showing that biofilm formation was more rapid in aerobic conditions and mature biofilms were able to shed viable cells into the planktonic phase regardless of atmospheric conditions. An increase in surface attachment in aerobic conditions was also noted by Sulaeman et al. (2012) in their study looking at *C. jejuni* membrane changes following oxidative shock and acclimatisation. Taken together these studies strongly suggest that biofilm formation is able to impact on the food chain survival and persistence of *C. jejuni*.

Integration of *C. jejuni* into mixed species biofilms has also been investigated. *C. jejuni* can be found in natural biofilms within waterways (Maal-Bared et al., 2012), and on biofilms formed within poultry house drinking water nipples. Interestingly *C. jejuni* could only be detected in these biofilms following colonisation of the flock, indicating that the water supply was not responsible for the initial flock colonisation in this instance (Zimmer et al., 2003). The presence of bacterial species such as *Enterococcus* and *Staphylococcus simulans* appear to increase *C. jejuni* biofilm formation (Sanders et al., 2007, Teh et al., 2010), whereas several studies have reported that the presence of *P. aeruginosa* leads to either decreased *C. jejuni* biofilm formation (Teh et al., 2010) or reduced *C. jejuni* viability within the biofilm (Trachoo et al., 2002).

1.6.3 The genetic basis of *Campylobacter* sp. biofilm formation

There is genomic and phenotypic instability within *Campylobacter* sp. both due to the frequent DNA rearrangement, transfer, presence of phase variable genes (de Zoete et al., 2007) and hyper-variable regions (Parkhill et al., 2000), all of which lead to pathogenic diversity between strains (Hofreuter et al., 2006). *Campylobacter* sp. have small, approximately 1.6 to 2 mega base long (Taylor et al., 1992), A and T rich genomes, with a GC ratio of approximately 30% (Parkhill et al., 2000). The genome is densely packed, with 94% of the *C. jejuni* NCTC 11168 genome coding functional regions. Organisation of the genome into functionally related operons and clusters is not common within *C. jejuni*.

The quantity of *C. jejuni* biofilm formation reported between studies is very varied, even where the same strain is used. This variation is likely in part to the different growth conditions, biofilm formation methods, mediums and attachment surfaces used. However despite this variation, several conclusions can be drawn from the current body of literature. Genes involved in motility, stress response, polysaccharide production, or the release of extracellular material have all been shown to affect biofilm formation. Each of these areas will be discussed in more detail in Sections 1.6.3.1 to 1.6.3.4.

1.6.3.1 The role of motility in biofilm formation

The factor with the greatest impact on *C. jejuni* biofilm formation appears to be cell motility. Mutations affecting flagella construction or chemotaxis are consistently reported to reduce biofilm formation. Several investigators have reported that deletion of genes involved in flagella biosynthesis led to delayed and/or reduced biofilm formation (Reuter et al., 2010, Moe et al., 2010, Svensson et al., 2014, Joshua et al., 2006). Although inactivation of several other genes, including the global repressor CsrA (Fields and Thompson, 2012), a peptidoglycan DL-carboxypeptidase named Pgp2 (Firdich et al., 2014), or the formate dehydrogenase subunit *fdhA* (Kassem et al., 2012) have been shown to reduce biofilm formation. In each case, motility was also affected by the inactivation. This makes it hard to determine if it is the deletion of the gene that is the 'primary' cause of the biofilm reduction, or a secondary symptom of the reduction in motility. In contrast to this observation Revez et

al. (2011) reported a lack of correlation between the ability of isolates to swarm, a frequently used measure of motility, and their ability to form biofilms.

Defects in chemotaxis systems also appear to effect biofilm formation. Deletion of the group A chemoreceptor *tlp3* was reported to decrease biofilm formation (Rahman et al., 2014), and chemotaxis has been shown to be upregulated in biofilms (Kalmokoff et al., 2006). Chemotaxis appears to be a promising area of research but is not as widely studied as motility. Further investigation is required before firm conclusions can be drawn about the role of chemotaxis in *C. jejuni* biofilm formation.

1.6.3.2 The role of the stress response in biofilm formation

Proteomic analysis of *C. jejuni* biofilms has indicated that many genes are differentially expressed in biofilms compared to planktonic lifestyles. Gene groups highly upregulated include those involved in iron metabolism, oxidative defence, energy metabolism and membrane modification (Sampathkumar et al., 2006).

Global regulators of stress responses, such as *spoT*, *csrA*, and *marA* have been linked to biofilm regulation. A *C. jejuni* 81-176 *spoT* deletion mutant was shown to have increased biofilm formation, producing thick mushroom like biofilms (McLennan et al., 2008). Deletion of the global regulator *csrA*, which in *E. coli* is involved in stationary phase regulation, has been shown to decrease *C. jejuni* 81-176 biofilm formation, motility and sensitivity to oxidative stress (Fields and Thompson, 2008). The deletion of *marA* from *C. jejuni* strain 11168H had the same effect (Gundogdu et al., 2011). Deletion of enzymes involved in the creation of poly-P, a molecule involved in several stress responses, has also been shown to affect biofilm formation, and biofilms formed by *ppk1* and *ppk2* deletion mutants (both involved in poly-P production) have been shown to have increased biofilm formation compared to WT strains (Candon et al., 2007, Gangaiah et al., 2010).

Two-dimensional gel electrophoresis has shown that there is an upregulation of oxidative stress proteins in *C. jejuni* biofilms (Kalmokoff et al., 2006) it is unsurprising that deletion of genes involved in oxidative stress led to alterations in biofilm formation. Deletion of *ahpC* (alkyl hydroperoxidase reductase) and *kataA* (catalase) increased *C. jejuni* NCTC 11168 biofilm formation. In the *ahpC* mutant, WT biofilm quantities could be restored by addition of antioxidants to the medium, or deletion of the oxidative stress regulator *perR*, allowing de-regulated *ahpC* transcription. Similarly, overexpression of *ahpC* decreased biofilm formation compared to WT (Oh and Jeon, 2014). These results again highlight the importance of biofilm formation as an oxidative stress response.

1.6.3.3 The role of polysaccharide production in biofilm formation

Polysaccharide production is an important aspect of biofilm maturation in many bacteria, since polysaccharides are a common component of the ECM. *P. aeruginosa* contains three polysaccharides within its ECM: alginate, Pel and Psl (Franklin et al., 2011), homologues of which cannot be found in *C. jejuni*. Instead, the production of alternative polysaccharides has been investigated. *C. jejuni* is able to produce at least three polysaccharides, all of which appear to be growth phase dependent to some extent (Corcoran and Moran, 2007). The role these polysaccharides, and the proteins involved in their production, have on biofilm formation has previously been investigated. Calcofluor white (CFW) is used extensively to study polysaccharides since it is able to bind to β 1-3 and β 1-4 carbohydrate linkages (McLennan et al., 2008). Deletion of genes involved in lipooligosaccharide production, such as *galT*, *cskIII* and *waaF* has also been shown to increase biofilm formation and CFW reactivity (Naito et al., 2010). Similarly, deletion of *cj1324*, part of the *C. jejuni*

NCTC 11168 O-linked flagellin glycosylation island, led to decreased biofilm formation with no loss of motility (Howard et al., 2009). The two peptidoglycan DL-carboxypeptidase enzymes, *pgp1* and *pgp2*, also appear to effect biofilm formation, and their deletion led to a reduced biofilm mass and CFW binding (Firdich et al., 2014). It should be noted however that deletion of these two genes also led to altered bacterial shape and reduced motility (Firdich et al., 2012, Firdich et al., 2014), which may have contributed to the biofilm phenotype observed. Alterations to the major outer membrane protein (MOMP) also increase biofilm formation, although in this mutant motility was increased (Mahdavi et al., 2014), suggesting that here an alteration in motility may not have contributed to the alterations observed in biofilm phenotype.

1.6.3.4 The role of ECM and signaling molecules in biofilm formation

SEM analysis of *C. jejuni* biofilms has shown the presence of fibre-like ECM products (Kalmokoff et al., 2006, Lu et al., 2012b, Moe et al., 2010) but to date few studies into the composition of the ECM have been carried out. A recent study by (Svensson et al., 2014) showed that eDNA was present within biofilms, and planktonic suspensions from 24 hour old cultures of *C. jejuni* strain 81-176. The presence of eDNA in supernatant coincided with the presence of cytosolic proteins, leading authors to suggest that eDNA release was via cell lysis rather than active secretion. Biofilm formation was significantly reduced following DNase I treatment, and the supplementation of biofilm cultures with additional genomic DNA led to an increase in biofilm quantity (Svensson et al., 2014).

Signalling molecules such as AI-2 have been shown to coordinate biofilm behaviour in several bacterial species (see Section 1.1.1.1 and 1.1.6.1 for details). *C. jejuni* does contain a *luxS* homologue and is able to produce AI-2, leading several investigators to study the role of QS in *C. jejuni* biofilm formation. Deletion of the *luxS* gene decreased biofilm formation (Reeser et al., 2007). Interestingly, one study has shown that biofilm formation by *luxS* deletion mutants may be returned to WT levels by supplementation of medium with cell free supernatant containing AI-2 (Reeser et al., 2007). Caution should however be used when assessing the role of AI-2 in *C. jejuni* biofilm formation since AI-2 receptors have still not been identified in *C. jejuni* (Golz et al., 2012). Since LuxS is involved in metabolic activity it is possible that any phenotypic changes observed within *luxS* deletion mutants are a result not of defective signaling but increased stress on the bacterium due to alterations in its metabolism (Adler et al., 2014). A study of five *Campylobacter sp.* by Tazumi et al. (2011) showed that *luxS* genes, although widely distributed in *Campylobacter sp.*, were not always present in biofilm forming species such as *C. lari* (Tazumi et al., 2011).

Other QS molecules have also been assessed for their effect on *C. jejuni* biofilm formation. Treatment of *C. jejuni* strains 81-176 and cj11 with the homoserine lactone 3OH-C4-HSL led to decreased biofilm formation, although surface attachment could still be observed, suggesting that this molecule inhibited biofilm maturation (Moorhead and Griffiths, 2011). One study has also shown that whole citrus extracts, which contain quorum quenching molecules, are able to decrease *C. jejuni* biofilm formation, motility and AI-2 production (Castillo et al., 2014), although it is unclear if the observed effects were due solely to the quorum quenching molecules, or other factors such as lowered pH. These studies highlight that further investigation is required before *C. jejuni* can be conclusively said to use QS molecules to coordinate its biofilm behaviour.

1.6.4 Treatment of *Campylobacter sp.* containing biofilms

C. jejuni biofilm eradication or inactivation studies have been carried out using several antimicrobials as well as nanoparticles and bacteriophage treatment. It is reported that many

bacterial species show increased antimicrobial resistance within biofilms (Fux et al., 2005). This is also observed in *C. jejuni*, although *C. jejuni* biofilms appear to still be able to be killed by recommended antimicrobial concentrations. Trisodium phosphate was shown to be effective against biofilms of *C. jejuni*, *E. coli*, *L. monocytogenes* and *S. typhimurium* (Somers et al., 1994). Diallyl-sulphide has also shown to be effective against *C. jejuni* biofilms since it is able to penetrate into the biofilm more effectively than ciprofloxacin and erythromycin (Lu et al., 2012a). An alternative to antibiotic inactivation of biofilms is the use of nanoparticle treatment (discussed in Section 1.1.6.4). Zinc oxide nanoparticles have been shown to be effective against *C. jejuni* strains F38011 and NCTC 11168 in both their planktonic and biofilm forms (Lu et al., 2012b).

C. jejuni biofilms have also been shown to be susceptible to treatment with CP8 and CP30 lytic bacteriophages. A significant reduction in the volume of mature *C. jejuni* NCTC 11168 and PT14 biofilms was observed following a two hour treatment with bacteriophage, although biofilm mass did increase slightly after treatment. Microscopy of treated biofilms showed disruption to the biofilm and the development of coccoid cells. Although some resistance to bacteriophage treatment was observed, resistance was specific to strain NCTC 11168 (Siringan et al., 2011). This study suggests that bacteriophage treatment of *C. jejuni* biofilms could provide a promising alternative to conventional antimicrobial treatments.

1.7 Conclusions

Campylobacter sp. is very different from other foodborne zoonotic pathogens such as *E. coli* and *Salmonella* making the study of *Campylobacter sp.* essential (Bereswill and Kist, 2003), since conclusions cannot be drawn about *C. jejuni* survival and transmission using data obtained from investigation of other bacterial pathogens. The importance of *Campylobacter sp.* specific investigation has been highlighted within the poultry industry, where containment measures implemented to reduce the spread of *Salmonella* have been unsuccessful in controlling the spread of *Campylobacter sp.* (Newell and Fearnley, 2003).

Since *in vitro* growth of *Campylobacter sp.* was made possible in the 1970s there has been an exponential growth in knowledge about *Campylobacter sp.* but there are still many questions about the bacteria's survival within the food chain. Consideration of food chain conditions must be considered during experimental design to ensure that studies are able to be used to inform novel *Campylobacter sp.* control measures.

Biofilm formation by *Campylobacter sp.* is able to increase persistence in sub-optimal environments (Lehtola et al., 2006). Biofilm formation has also been shown to increase in aerobic (Reuter et al., 2010) and low temperature conditions (Buswell et al., 1998). This, in combination with *C. jejuni*'s ability to integrate into mixed species food chain biofilms (Hanning et al., 2008) or form single species biofilms on materials such as stainless steel indicates that biofilm growth modes are likely to contribute significantly to *C. jejuni* food chain persistence, and potentially food chain transmission.

1.8 Aims and objectives of this project

Although biofilm formation by *C. jejuni* is now a well-recognised phenomenon, formation within the food chain environment is less well studied. A recent review by Teh et al. (2014) has suggested that *de novo* biofilm formation may not in fact be an important food chain survival mechanism for *C. jejuni*. The authors speculate that *C. jejuni* more likely integrates in to existing multispecies biofilms, or forms simple monolayer attachments, which do not progress to true biofilm formation.

The aim of this project was to investigate how biofilm formation by *C. jejuni* is able to contribute to its survival and transmission within the food chain. This is a complex and broad subject area, and so three objectives covering complementary areas were developed. The objectives are described in Sections 1.8.1 to 1.8.3.

1.8.1 Objective 1: Development of food chain relevant models of *C. jejuni* biofilm formation

In order to effectively study both attachment and/or biofilm formation within food chain relevant conditions, the use of food chain relevant models and suitable imaging techniques is required. In this work, incubation in aerobic conditions was combined with the use of food chain relevant surfaces and substrates to develop a greater understanding of *C. jejuni* biofilm formation within the food chain.

1.8.2 Objective 2: Understand how *C. jejuni* biofilms are formed

There is still little understanding of *C. jejuni* biofilm structure and the environmental factors which contribute to biofilm formation. Here, the biofilms' structure has been examined using SEM, fluorescent and light microscopy. Environmental factors such as temperature, cell density and surface type have all been investigated to gain a better understanding of how *C. jejuni* biofilm formation is initiated and progresses.

1.8.3 Objective 3: Understand the role of eDNA within the *C. jejuni* biofilm

Extracellular DNA has been highlighted as an important ECM component for many bacterial species, but to date investigations of *C. jejuni* biofilms has focused primarily on polysaccharide production. Since eDNA has been shown to be an integral ECM component of species such as *P. aeruginosa*, *V. cholera* and *S. aureus*, and is a promising target for biofilm eradication treatments, its role within the *C. jejuni* biofilms was further investigated.

2 Materials and Methods

2.1 General Techniques

2.1.1 Sterilisation and washing

All solutions, lab consumables and glassware required for aseptic assays were sterilised by autoclaving at 69Kpa for 20 minutes or sterile-filtering of solutions using a 0.22 µM Minisart filter (Satorius). All microbiological work was carried out following standard IFR SOPs and in a class II Microbiological safety cabinet (Walker BSW 5726).

2.2 Medium, buffers and solutions

2.2.1 Standard growth mediums

All recipes below (2.2.1.1 to 2.2.1.5) were sterilised using an autoclave. For plates 1.5% agar (set plates) or 0.4% agar (swarm plates) was added to the mixture before autoclaving. All antibiotics, dyes and X-gal were added as required following autoclaving. Ultrapure water (Millipore) was used unless otherwise stated.

2.2.1.1 *Blood medium*

15 g Proteose peptone
2.5 g liver digest
1 % yeast extract
5 g NaCl
5% horse blood
1000 ml water

2.2.1.2 *Brucella medium*

10 g Bacto™ Pancreatic digest of casin
10 g Bacto™ Peptic digest of animal tissue
1 g Dextrose
5 g Yeast extract
5 g NaCl
0.1 g Sodium bisulphide
1000 ml water

2.2.1.3 *DNase agar*

DNase agar was purchased from Oxoid and prepared following manufacturer's instructions with the addition of 0.005% v/v Toluidine Blue (Sigma).

2.2.1.4 *Luria Broth (LB) medium*

10 g Bacto™ tryptone
5 g Bacto™ yeast extract
10 g NaCl
1000 ml water

2.2.1.5 *Skirrow medium*

15 g Proteose peptone

2.5 g liver digest
5 g yeast extract
5 g NaCl
1000ml water

After autoclaving and cooling, *Campylobacter* selective supplement (Vancomycin 0.1 mg/ml Trimethoprim 50 µg/ml and Polymyxin B2 500 IU/ml) was added.

2.2.2 Buffers and solutions

2.2.2.1 *Antibody Dilution Buffer*

1% BSA in Natt buffer (Section 2.2.4)

2.2.2.2 *Blocking Buffer*

5% BSA in Natt buffer (Section 2.2.4)

2.2.2.3 Bromo-chloro-indolyl-galactopyranoside (X-gal)

X-gal stock solution was diluted to a 20 mg/ml stock solution in dimethyl formamide and stored in the dark at -20°C until use. A final concentration of 20 µg/ml was used in medium.

2.2.2.4 Campylobacter competent cell buffer

270 mM Sucrose

15 % v/v glycerol

H₂O

Before use the solution was filter sterilised and aliquotted into 10 ml single use volumes.

2.2.2.5 Chicken serum

Chicken serum (Sigma) was thawed at 2 – 8°C, sterile filtered and aliquotted into 10 ml volumes. The stock was then frozen at -20°C until required.

2.2.2.6 Isopropyl β-D-1-thiogalactopyranoside (IPTG)

IPTG was dissolved in H₂O to a stock concentration of 100 mM, filter sterilised and stored at -20°C in single use aliquots until use. For induction, the stock solution was diluted to 1 mM v/v within the cell suspension.

2.2.2.6.1 Meat juices

Frozen whole chickens, turkey, duck, goose, chicken portions or pork portions were purchased from UK supermarkets. The meat was thawed overnight at room temperature (RT), and the exudate was collected, pooled (where several birds were defrosted at once), centrifuged to remove debris and sterilised by using a 0.2 µm sterile polyethersulfone (PES) syringe filter (Millipore). Juices were aliquotted in to 10 ml volumes and stored at -20°C until use. Juice was diluted v/v in Brucella medium unless stated otherwise.

2.2.2.6.2 Assessment of chicken juice batch equivalency

On each occasion that chicken juice was prepared it was assessed to ensure it gave a standard experimental effect before experimental use. Each new pool was used to supplement Brucella medium (5% v/v) and a standard *C. jejuni* NCTC 11168 growth curve (Section 2.3.4), performed. The OD₆₀₀ values of the cell suspensions were compared to the new batch to cells grown in Brucella medium only or Brucella medium supplemented with an existing batch of chicken juice to ensure equivalency (coefficient of variance falling within 25%).

2.2.2.6.3 Proteinase K treatment of chicken juice

Chicken juice aliquots were incubated with Proteinase K (Sigma) following the manufacturers instructions. Incubations were carried out for one hour at 37°C before use in biofilm assays.

2.2.2.6.4 Pre-incubation of chicken juice

The required volume of chicken juice was placed in a 37°C incubator overnight to allow particulate formation within the chicken juice. Following incubation the juice was centrifuged at 3220 xg for 30 minutes to pellet any particulates formed, the resulting supernatant was then used to supplement medium for use in assays.

2.2.3 Methylation Buffer

20 mM Tris Acetate

50 mM Potassium Acetate

5 mM EDTA

1 mM Dithiothreitol

Protease inhibitor cocktail tablet (Sigma)
Water

2.2.4 Natt Buffer

20 mM Tris base
150 mM NaCl
0.5 ml Tween 20
1000 ml Ultrapure water
Adjust pH to 7.4 before use

2.2.4.1 Phosphate buffered saline (PBS)

1 mM Phosphate buffer
0.27 mM KCl
13.7 mM NaCl
Water

all contained in pre-made PBS tablets purchased from Sigma and diluted following manufacturers guidelines. The solution was adjusted to pH 7.4 before sterilisation.

2.2.4.2 Skimmed milk powder solutions

A 5% (w/v) skimmed milk powder solution was prepared by mixing commercially available skimmed milk powder (Co-operative instant dried skimmed milk powder, UK) into Brucella medium and dissolved completely before filtering.

2.2.4.3 Sterile spent medium

Bacterial cultures were grown overnight as described in Section 2.3.4. Following overnight growth, cell suspensions were centrifuged at 3000 g for 30 minutes and the supernatant removed and retained. Supernatant was sterile filtered using a 0.22 μ m PES filter (Millipore) and stored at -20°C until use.

2.2.4.4 Super optimised broth with catabolite repression (SOC)

2% Tryptone
0.5% yeast extract
8.56 mM NaCl
2.5 mM KCl
10 mM MgCl₂
10 mM MgSO₄
20 mM glucose
H₂O
Adjust to pH 7.0

2.2.4.5 TBE buffer

890 mM Tris
7 mM Boric acid
20 mM EDTA
H₂O

2.2.4.6 Transfer Buffer

25 mM Tris base
190 mM Glycine
5% w/v SDS
20% v/v Methanol
in Ultrapure water

2.2.4.7 0.5x Tris buffer

10x concentrate Tris-borate-EDTA buffer (Sigma)

Diluted to a 0.5% stock in H₂O

2.2.4.8 10mM Tris HCL Buffer (pH 7.4)

10mM Trizma hydrochloride base

H₂O

Adjust solution to pH 7.4 before use.

2.2.4.9 2,3,5-Triphenyl-tetrazolium chloride (TTC)

A 1% w/v stock solution of TTC was prepared in demineralised H₂O and sterile filtered before use. The stock solution was further diluted for use in assay as described in Section 2.9.3. TTC stock solutions were stored in the dark at 5°C between uses.

2.2.5 Fractionation of solutions using spin-columns

Spin-columns allowing collection of suspensions containing ≥ 5, 10, 50 and 100 kDa sized particulates (Millipore) were used following manufacturers guidelines. Once collected the suspensions were stored at -20°C until use.

2.2.6 Antibiotic concentration and preparation

Antibiotic	Dilution reagent	Usual stock concentration	Final concentration for <i>E. coli</i>	Final concentration for <i>C. jejuni</i>
Ampicillin	H ₂ O	100 mg/ml	100 µl/ml	-
Carbenicillin	H ₂ O	100 mg/ml	100 µl/ml	-
Chloramphenicol	96% ethanol	30 mg/ml	30 µl/ml	10 µl/ml
Kanamycin	H ₂ O	30 mg/ml	30 µl/ml	50 µl/ml
Streptomycin	H ₂ O	64 mg/ml	NA	64 µl/ml

Table 2-1 Antibiotics used and standard concentrations for medium

2.3 Routine bacterial cell maintenance**2.3.1 Preparation of *Campylobacter sp.* stocks**

All bacterial isolates were routinely cultured from glycerol stocks. To prepare glycerol stock cultures, either from an *Campylobacter* Research group master cell bank or from stocks provided by the ATCC, cells were thawed onto Skirrow agar and grown overnight in microaerobic conditions at 37°C. Following overnight incubation the lawn was resuspended in Brucella medium containing 10% sterile glycerol stock. Single use aliquots were prepared and stored at -80°C.

2.3.2 Preparation of *E.coli* stocks

All bacterial suspensions were routinely cultured from glycerol stocks. Frozen cell suspensions, either from an *Campylobacter* Research group master cell bank or cell lines provided by manufacturers, were thawed onto LB agar and grown overnight in aerobic conditions at 37°C. Following overnight incubation the lawn was suspended using LB medium containing 10% sterile glycerol stock. Single use aliquots were prepared and stored at -80°C until required.

2.3.3 Bacterial strains used

	Name	Description	Reference/ supplier
	NCTC 11168	Wild type human isolate	(Parkhill et al., 2000)
	NCTC 11168 GFP	11168 cj0046::GFP+ ^{porA} Cat ^R	This work
	NCTC 11168 Δ <i>flaAB</i>	NCTC 11168 (Δ <i>cj1338</i> , Δ <i>cj1339c</i>) ::kan ^R	(Reuter et al., 2010)
<i>C. jejuni</i>	81116	Wild type human isolate	(Pearson et al., 2007)
	81-176	Wild type human isolate	(Hofreuter et al., 2006)
	RM1221	Wild type chicken isolate	(Fouts et al., 2005b)
	RM1221 Δ <i>cje1441</i>	RM1221 Δ <i>cje1441</i> ::cat ^R	This Work
<i>C. coli</i>	15-537360	Wild type human isolate	(Pearson et al., 2013)
	Top 10	F ⁻ <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) 80 <i>lacZ</i> Δ M15 Δ <i>lacX74</i> <i>recA1</i> <i>araD139</i> Δ (<i>ara-leu</i>) 7697 <i>galJ</i> <i>galK</i> <i>rpsL</i> (Str ^R) <i>endA1</i> <i>nupG</i> λ -	Invitrogen
	NEB5 α	<i>fhuA2</i> Δ (<i>argF-lacZ</i>)U169 <i>phoA</i> <i>glnV44</i> Φ 80 Δ (<i>lacZ</i>)M15 <i>gyrA96</i> <i>recA1</i> <i>relA1</i> <i>endA1</i> <i>thi-1</i> <i>hsdR17</i>	New England Biolabs
<i>E. coli</i>	C41 (DE3)	F ⁻ <i>ompT</i> <i>hsdSB</i> (<i>rB- mB-</i>) <i>gal dcm</i> (DE3)	Lucigen
	C41 (DE3) pLysS	F ⁻ <i>ompT</i> <i>hsdSB</i> (<i>rB- mB-</i>) <i>gal dcm</i> (DE3) pLysS (CmR)	Lucigen
	C43 (DE3)	F ⁻ <i>ompT</i> <i>hsdSB</i> (<i>rB- mB-</i>) <i>gal dcm</i> (DE3)	Lucigen
	C43 (DE3) pLysS	F ⁻ <i>ompT</i> <i>hsdSB</i> (<i>rB- mB-</i>) <i>gal dcm</i> (DE3) pLysS (CmR)	Lucigen
	BL21 DE3	F ⁻ <i>ompT</i> <i>hsdSB</i> (<i>rB- mB-</i>) <i>gal dcm</i> (DE3)	Novagen

Table 2-2 List of bacterial strains used, indicating their name, genotype and source**2.3.4 *Campylobacter* sp. routine maintenance**

C. jejuni and *C. coli* strains were routinely cultured in a MACS-MG-1000 controlled atmosphere cabinet (Don Whitley Scientific) under microaerobic conditions (85% N₂, 5% O₂, and 10% CO₂) at 37°C.

2.3.4.1 Revival of frozen *Campylobacter sp.* suspensions

Glycerol stocks were freshly thawed for use in all assays. Thawed cell suspensions were spread onto Skirrow agar to create a lawn and incubated as described in Section 2.3.4 overnight (approximately 30 hours).

2.3.4.2 Preparation of *Campylobacter sp.* suspensions for use in assays

Two ml of Brucella medium was added to Skirrow plates with a *Campylobacter sp.* lawn and the cell lawn suspended using a sterile plastic loop. The resulting suspension was diluted 100x in fresh Brucella medium (typically 150 µl of cell suspension into a T₂₅ flask (Corning) containing 15 ml of Brucella medium), before further microaerobic incubation at 37°C for approximately 16 hours. This cell suspension was routinely used, following further dilution, in both growth (Section 2.6.1) and biofilm (Section 2.8) assays.

2.3.5 Revival of frozen *E. coli* suspensions

E. coli from glycerol stocks was grown overnight on LB agar plates in aerobic conditions at 37°C. One colony was transferred from the plate into 5 ml LB broth and further incubated overnight with 200 rpm shaking at 37°C, in aerobic conditions.

2.4 Bacterial viability assessment

2.4.1 Assessment of cell viability by culture

To determine the number of viable cells, a method based on the Miles-Misra method (Miles et al., 1938) was used (referred to hereafter as minimum probable number (MPN)). Briefly, a sample of the planktonic cell suspension (typically 10 µl from a 1 ml suspension) was serially tenfold diluted eight times in phosphate buffered saline (PBS, pH 7.5) in a sterile 96 well plate. This gave a range of dilutions between 10×10^{-1} and 10×10^{-8} , and 5 µl of each dilution was spotted onto Brucella agar plates and incubated at 37°C for 48h (hereafter referred to as the 'spot plate'). After 2 days of growth, CFU ml⁻¹ was calculated by observing the growth of colonies on the agar plates. Where two or more colonies were present for each dilution cell growth was recorded. If all tests were positive (i.e. two or more colonies for each of the eight tenfold dilutions) a value of 10×10^{-8} was recorded, as no end point could be accurately determined. Due to the small volumes of liquid used in this method, absolute viable cell concentrations could not be determined and the limit of detection is high, with a *C. jejuni* NCTC 11168 suspension diluted to OD₆₀₀ = 0.05 showing growth to approximately 10×10^{-7} .

This method of calculating cell viability was used to complement biofilm and growth experiments. As a minimum during biofilm assays spot plates were prepared from cell suspensions at the start and end of static incubations. Where TTC, rather than crystal violet or Congo red, was used for biofilm visualisation, an additional spot plate was prepared at the end of the secondary (TTC) incubation. For monitoring of cell viability during growth experiments, spot plates were prepared at each time point where optical density was measured. Where additional cell viability was undertaken the exact time points or samples assessed are described within the body of the thesis.

2.4.2 Use of TTC as indicator of bacterial growth

Cultures were grown as described in Section 2.3.1 and diluted in Brucella medium supplemented with 0.05% TTC, to reach a starting OD₆₀₀ of between 0.001 and 1.5, and incubated at 37°C in microaerobic conditions for 30 minutes. For analysis of 48 hour growth, cultures were diluted to an OD₆₀₀ of 0.05 in 0.05% TTC Brucella medium and incubated for 48 hours in microaerobic conditions at 37°C with shaking at 220 rpm.

Formazan crystals, produced following the reduction of TTC by metabolically active cells, were dissolved by removing a 1 ml aliquot of the culture and adding an equal volume of 20% acetone/80% ethanol before incubating at RT for 30 minutes to allow the formazan crystals to dissolve. The OD₆₀₀ of the aliquot was recorded before whole cells and debris were pelleted by centrifugation (16,000 x g, 10 minutes at RT). The supernatant was then carefully removed and the A₅₀₀ measured.

For assessment of *C. jejuni* MPN using TTC containing plates, Brucella agar plates supplemented with 0.05% TTC were prepared and stored at 5 °C in the dark until use. A sterile cotton wool bud was used to swab cultures and then streaked onto the plate. Plates were incubated at 37 °C for 48 hours in microaerobic conditions to allow TTC conversion by metabolically active cells. Cultures were considered to be metabolically active if any formazan crystals, indicating TTC conversion, were present following incubation.

2.5 Creation of *C. jejuni* mutant strains

2.5.1 Genomic DNA extraction from bacterial cultures

C. jejuni cells were grown as previously described in Section 2.3.1 before centrifugation at 4500 xg for 10 minutes. Following centrifugation the supernatant was removed from the pellet and cell pellets were stored at -20°C, to improve cell lysis. Cell pellets were thawed on ice and DNA extracted using the QIAGEN DNeasy Blood and Tissue Kit. Following resuspension of the pellet in 180 µl buffer ATL, 20 µl proteinase K (600mAU/ml) and 5 µl of RNase (20 mg/ml), genomic DNA was purified following the manufacturers instructions. The final elution was carried out with 100 µl buffer AE. Following the first elution the eluate was used for a second elution step to ensure the highest possible yield of DNA.

2.5.2 Primers used throughout the study

Name	Primer use	Sequence (5' to 3')
CJE0256fwd_B spHI	Preparation of pHB001, pHB004 plasmids	GGAGAATTCATGAAAAAATAATAAGCG
CJE0256rev_Bs pHI	Preparation of pHB001, pHB004 plasmids	GCTAAAGTTTGTCATGAATTGAGTAATGC
CJE1441fwd_B spHI	Preparation of pHB002, pHB005 plasmids	GGTATGAAACTCATGAAAAACTTATAATC
CJE1441rev_Bs pHI	Preparation of pHB002, pHB005 plasmids	CCATTTTTACTCATGATTAATTAATAATTGTC
CJE1440fwd_N col	Preparation of pHB003, pHB006 plasmids	GGTTATATCCATGGAAAATTATTATG
CJE1441rev_N col	Preparation of pHB003, pHB006 plasmids	CCATTTTTACTCCATGGTAATTAATAATTGTC
Dns_fwd_Ndel	Preparation of pHB007 plasmid	GGAGAACATATGAAAAAATAATAAGC
dns_rev_BamHI	Preparation of pHB007 plasmid	GCTAAAGGATCCAAAGAATTACAGTAATGC
566_fwd_Ndel	Preparation of pHB008 plasmid	GCAACAAGTTAAACCATATGAAAAACTC
566_rev_BamHI	Preparation of pHB008 plasmid	CAAAGATTAATAAAATGGATCCAAAAAGCACCC

Name	Primer use	Sequence (5' to 3')
1441_fwd_NdeI	Preparation of pHB009 plasmid	GGTATGAAACATATGAAAAAAGCTTATAATC
1441_rev_BAmHI	Preparation of pHB009 plasmid	GCAAAGCTAGAGTGG
dnsKO_FDEcoRI	Preparation of pHB010a plasmid	GGAAGATGTGAGCTTATTGGCGTGAATTCAGAGC
dnsKO_RVPstI	Preparation of pHB010a plasmid	GGGCTACAAAAAAGCAACTGCAGATGATACAAGC
dnsKO_2_fwd	Preparation of pHB010b plasmid	ATAGGATCCAACAATACCCTATGGATGAG
dnsKO_2_rev	Preparation of pHB010b plasmid	ATAGGATCCCAATAAAAAATCATACCAGTAAGAG
1441KO_FDEcoRI	Preparation of pHB012a plasmid	GCATTGAAAGAATTCTATGAGTTAAAAAAGG
1441KO_RVPstI	Preparation of pHB012a plasmid	GCTTTTTAACGCTGCAGTTGATAGGTTGT
1441KO_2_fwd	Preparation of pHB012b plasmid	ATAGGATCCGTTACCAAGTGCCTAATCAC
1441KO_2_rev	Preparation of pHB012b plasmid	ATAGGATCCGTTTTGTATTGTGTATAATC
Dnsreadin_fwd	pHB001, pHB004, pHB007 confirmation	CGTTTTAATACTTGCTTTAAGCTTATTAATGC
Dnsreadin_rev	pHB001, pHB004, pHB007 confirmation	GCTCTAATTCTTTTTCTTTCTCATCC
Cje0566readin_fw	pHB008 confirmation	CCACTCTAGCTTTTGCTGACTATACG
Cje0566readin_rev	pHB008 confirmation	GGTACTTGGTAACATTCTTTAAAATTCTCACC
Cje1441readin_fwd	pHB009 confirmation	GGCGAACAAATCAAAAAACGCCACG
Cje1441readin_rev	pHB009 confirmation	CCTTTTAAAATCTTAGTGTAAGAGCTTGG
Kan_pr_readout	pHB007, pHB008, pHB009 confirmation	GCGATATCTTCTATATAAGCGTACCG

Name	Primer use	Sequence (5' to 3')
Kan_readout	pHB007, pHB008, pHB009 confirmation	CGGGGAAGAACAGTATGTCTCGAGC
T7 promoter	pHB007, pHB008, pHB009 confirmation	TAATACGACTCACTATAGG
T7 terminator	pHB007, pHB008, pHB009 confirmation	CCCGTTTAGAGGCCCAAGG
Dnsreadin_rev	pHB007 confirmation	GCTCTAATTCTTTTTCTTTCTCATCC
Cje0566readin_rev	pHB008 confirmation	GGTACTTGTAACATTCTTTAAAATTCTCACC
1441readin_fwd	pHB009 confirmation	GGCGAACAAATCAAAAAACGCCACG
1441readin_rev	pHB009 confirmation	CCTTTTAAAATCTTAGTGTAAGAGCTTGG
M13 read in	pHB010a, pHB011a, pHB012a, pHB010b, pHB011b, pHB012b confirmation	CGCCAGGGTTTTCCCAGTCACGAC
M13 read out	pHB010a, pHB011a, pHB012a, pHB010b, pHB011b, pHB012b confirmation	TCACACAGGAAACAGCTATGAC
1441_fwd_schk 2	Confirmation of pHB012b insertion into RM1221	GGAAAATTATTATGAATTAG
1441_rev_schk 2	Confirmation of pHB012b insertion into RM1221	GCCAATAGCAAAAAATGAAC
cat fwd readin	<ul style="list-style-type: none"> Confirmation of pCporAGFP⁺ insertion into NCTC 11168 Confirmation of pHB012b insertion into RM1221 	GGACACGAAAAGAGTATTTTCGACC

Name	Primer use	Sequence (5' to 3')
cat rev readin	<ul style="list-style-type: none"> Confirmation of pCporAGFP insertion into NCTC 11168 Confirmation of pHB012b insertion into RM1221 	GCATGATGCACTTGAATCGATAAGG
GFP fwdreadin	Confirmation of pCporAGFP insertion into NCTC 11168	GGAGAAGAAGCTTTTCACTGGAGTTG
GFP revreadin	Confirmation of pCporAGFP insertion into NCTC 11168	GCAGTTACAAACTCAAGAAGGACC
0046Fcheck3	Confirmation of pCporAGFP ⁺ insertion	GCAGAGCACTTGATTTTAGTGTGTGC
0046Rcheck2	Confirmation of pCporAGFP ⁺ insertion	GCAAAAATCATCCTAAAAGATCC
Cje1441 readin_fwd	pHB006 confirmation	GGCGAACAAATCAAAAAACGCCACG

Table 2-3 List of primers used within this study.

The table shows the primers name, sequence and for what purpose it was used.

2.5.3 Polymerase Chain Reaction (PCR)

PCR reactions were performed using a Multigene OptiMax Thermo Cycler (Labnet international). Unless otherwise stated reactions were carried out using the methods stated in Table 2-4.

PCR type	Reagents used	Protocol
Hotstart	25 µl Hotstart master mix (QIAGEN)	Initial: 98°C for 15 min
Premixed solution of buffer and Taq polymerase, used routinely to confirm DNA recombination events	2.5 µl of each primer (at a concentration of 10µM) 1 µl of DNA template (10x dilution) RNase free water to a final volume of 50 µl	Cycles (30x): 98°C 30 sec, 50°C 30 sec, 72°C (60 sec per kilobase) Final extension: 72°C for 10 min
Phusion	10 µl HF buffer (NEB)	Initial: 98°C for 1 min
High fidelity polymerase used for cloning, and routine production of PCR fragments of ≥ 2kb	0.5 µl dNTPS (at a concentration of 50mM) 0.5 µl Phusion (NEB) 5 µl of each primer (at a concentration of 10µM) 1 µl of DNA template (10x dilution) RNase free water to a final volume of 50 µl	Cycles (30x): 98°C 30 sec, 50°C 30 sec, 72°C (30 sec per kilobase) Final extension: 72°C for 10 min
Colony PCR	25 µl Hotstart master mix (QIAGEN)	Initial: 98°C for 15 min
	2.5 µl of each primer (at a concentration of 10µM) 1 µl of bacterial cell suspension RNase free water to a final volume of 50 µl	Cycles (30x): 98°C 30 sec, 50°C 30 sec, 72°C (60 sec per kilobase) Final extension: 72°C for 10 min

Table 2-4 PCR reaction names and details

2.5.4 Agarose Gel Electrophoresis

Unless otherwise stated, 0.9% Agarose gels were prepared using Agarose powder (Sigma) and 0.5x Tris buffer. The solution was heated in order to fully dissolve, before allowing to cool and pouring into a mould. The gels were allowed to set before storage in deionised water at 2-8 °C until use. Prior to loading, samples were mixed with a volume of 6x gel loading dye (NEB). Electrophoresis took place at voltages of between 50 and 150 V (typically 100 V) for between 15 and 60 minutes (typically 30 minutes). Variations in voltage and time were required for better band separation and visibility. Following electrophoresis, gels were incubated at RT in deionised water containing ethidium bromide before visualisation under UV light.

2.5.5 Gel extraction and purification of PCR products/digested fragments

For purification of PCR fragments the QIAGEN QiaQuick PCR purification kit was used as recommended by the manufacturer. The final elution of the PCR product was in 30 µl of buffer EB. Purified products were either used immediately or stored at -20°C until required.

DNA fragments were separated on a 0.9% agarose gel before the desired band was excised from the gel using a scalpel. The mass of the fragment was calculated to identify the correct fragment. The QIAGEN QiaQuick gel extraction kit was used as recommended by the manufacturer. The final elution of the DNA fragment was in 30 μ l of buffer EB. Purified DNA were either used immediately or stored at -20 °C until required.

2.5.6 Digestion by restriction enzymes

Restriction digests were carried out using manufacturer's guidelines and recommended buffers. Unless otherwise stated the following quantities were used:

Reagent	Quantity (μ l)
Enzyme	1
10 x Buffer	5
10 x BSA (if required)	5
10 x DTT (if required)	5
Sample	30
H ₂ O	To a final volume of 50

Table 2-5 Representative restriction enzyme digest protocol

All incubations (unless otherwise stated) were carried out in a 37°C water bath for a minimum of 60 minutes. A list of restriction enzymes used throughout this study is give below:

Restriction enzyme name	Use	Buffer used	Manufacturer
<i>Bam</i> HI	Preparation of pHB007, pHB008 and pHB009 Digestion of inverse PCR of pHB010a – 012a	NEB 3 + BSA/ React 3	NEB/ Invitrogen
<i>Bsp</i> HI	<ul style="list-style-type: none"> Digestion of <i>cje1441</i> PCR fragment for insertion into pCfdxA plasmids Confirmation of insertion of pHB007-9 into pET28α plasmid 	NEB 4	NEB
<i>Eco</i> RI	Digestion of pNEB193	Buffer H	Promega

Restriction enzyme name	Use	Buffer used	Manufacturer
	plasmids		
<i>Esp3I</i>	Digestion of pCfdxA and pCmetK plasmids	Tango Buffer + DTT	Thermo
<i>HindIII</i>	Digestion of pHB002, pHB003, pHB010a and pHB012a to confirm correct insert orientation within plasmid	NEB 2	NEB
<i>NcoI</i>	Digestion of <i>cje1440</i> – 1441 PCR fragments for insertion into pCfdxA plasmids	NEB 3	NEB
<i>NdeI</i>	Preparation of pHB007, pHB008 and pHB009	NEB 4	NEB
<i>PstI</i>	Digestion of pNEB193 plasmids	Buffer H	Promega
<i>SspI</i>	Confirmation of correct insert orientation within pHB002 Confirmation of insertion of pHB007-9 into pET28 α	React6/NEB 2	Invitrogen/NEB

Table 2-6 List of restriction enzymes used in this study.

2.5.7 Dephosphorylation of digested plasmid fragments

Dephosphorylation of plasmid fragments was carried out using Antarctic Dephosphatase (NEB) following manufacturers guidelines. Following incubation, the Antarctic Phosphatase was inactivated by heating to 65°C for 10 minutes followed by DNA purification carried out as described in Section 2.5.4.

2.5.8 Plasmid Ligation

All routine ligations were carried out using T4 DNA ligase (NEB) in the following reaction:

Reagent	Quantity (μ l)
T4 Ligase	1
T4 Ligase buffer	2
Digested plasmid	2.5
Purified DNA insert	Calculated based on size and concentration
Water	To a total of 20 μ l

Table 2-7 Representative ligation protocol

Following preparation, the reaction was incubated for 30 minutes on ice, 30 minutes in a waterbath at RT before a final incubation of 30 minutes on ice. The ligation product was either used directly in a transformation reaction or stored at -20°C until required.

2.5.9 Methylation of Plasmids

Plasmid methylation was carried out as described in (Donahue et al., 2000). *C. jejuni* RM1221 cell suspensions grown as described in Section 2.3.4.1 and centrifuged to form a cell pellet, the pellet was resuspended in methylation buffer, aliquotted into 500 μ l volumes and the cells lysed by sonication (Soniprep 150 MSE; Sanyo), using 3 x 30 second pulses on ice. Following sonication, the suspensions were centrifuged at 15000 xg for 15 minutes, supernatant was retained, and stored at -20°C in single use aliquots. Plasmid methylation was carried out using the following reaction:

Reagent	Quantity (μ l)
Plasmid	57
Cell free methylation extract	43
SAM (100 μ M/ml)	0.7

Table 2-8 Representative methylation protocol

The reaction was incubated for 60 min in a 37°C water bath. Following incubation the plasmid was extracted using the QIAGEN PCR clean-up kit (Section 2.5.4).

2.5.10 Transformation procedures

2.5.10.1 Routine electroporation of *C. jejuni*

Lawns from overnight growth on agar plates (Section 2.3.4.1) were resuspended and centrifuged at 20000 xg for 5 min to pellet cells. Supernatant was removed and the cells were resuspended in 1 ml of cold CTB, this washing process was repeated twice with cells being resuspended in 500 μ l CTB following the final centrifugation. Cell suspensions were aliquotted in to 100 μ l aliquots, mixed with 2 μ l of plasmid and placed in chilled electroporation cuvettes (Cell Projects). Electroporation was carried out using the following parameters: 2.5kV, 200 Ω load resistance and 25 μ F capacitance. Following electroporation, 250 μ l of fresh Brucella medium was added to the cuvettes and the whole suspension plated on to blood agar plates and incubated microaerobically at 37°C for a minimum of 5 hours.

Following this incubation the lawn was resuspended and 100 µl of cells plated onto selective agar and incubated for a further 48 to 72 hours to allow growth of colonies.

2.5.10.2 Electroporation of *C. jejuni* RM1221 cells

Lawns from overnight growth on agar plates (Section 2.3.4.1) were resuspended and pelleted by centrifugation at 4°C, 20000 xg for 5 min. Supernatant was removed and the cells were resuspended in 1 ml of ice cold CTB containing 10 mM EDTA. Resuspended pellets were incubated on ice before progressing to the second wash step. The washing process was repeated twice more, with cells being resuspended in 500 µl CTB (not containing EDTA) following the final centrifugation step.

Cell suspensions were aliquotted in to 100 µl aliquots, mixed with 2 µl of plasmid and placed in ice cold electroporation cuvettes (Cell Projects). Electroporation was carried out using the following parameters: 2.5kV, 200 Ω load resistance and 25 µF capacitance. Following electroporation, 250 µl of fresh Brucella medium was added to the cuvettes and the whole suspension plated on to blood agar plates and incubated microaerobically at 37°C for a minimum of 5 hours. Following this incubation the lawn was resuspended and 100 µl of cells plated onto selective agar and incubated for a further 48 to 72 hours to allow growth of colonies.

2.5.10.3 Chemical transformation of *E. coli* cells

E. coli Top 10 (Invitrogen) suspensions were removed from -80°C storage and thawed on ice. A volume of 2 µl of plasmid was added to the cell suspensions and gently mixed. The suspensions were incubated on ice for 30 min, transferred to a water bath at 42°C for 30 sec and then replaced on ice for 2 min. Following the incubation stages, 250 µl of SOC medium was added to each tube of cell suspension and the tubes were incubated at 37°C for one hour with shaking. Suspensions were then plated on to selective medium and incubated at 37°C for approximately 15-24 hours to allow colony growth. For *E. coli* Overexpress™ C41 and C43 cells (Lucigen) the above method was followed with the exception that 950 µl of SOC medium was added.

To continue the growth of transformants, colonies were picked from selective agar plates using sterile tooth picks and added to 5 ml LB with appropriate antibiotics. The suspensions were then grown in aerobic conditions at 37°C, overnight, with shaking before harvesting and further manipulation as required.

2.5.10.4 Plasmids created during this study

Table 2-9 shows a list of all plasmids created during this study. The plasmids pCfdxA, pCmetK, pET28α and pNEB193 were used as backbones for all the listed plasmids. Plasmid maps of all plasmids, including the 'backbone plasmids' can be found in Section 10.2.

Plasmid Name	Construction
pHB001	pCfdxA with cje0256 insert
pHB002	pCfdxA with cje1441 insert
pHB003	pCfdxA with cje1440-1441 insert
pHB004	pCmetK with cje0256 insert
pHB005	pCmetK with cje1441 insert
pHB006	pCmetK with cje1440-1441 insert
pHB007	pET28α with cje0256 insert
pHB008	pET28α with cje0566 insert
pHB009	pET28α with cje1441 insert
pHB010a	pNEB193 with cje0256 insert
pHB010b	pNEB193 with Kanamycin resistance inserted into cje0256
pHB012a	pNEB193 with cje1441 insert
pHB012b	pNEB193 with Chloramphenicol resistance inserted into cje1441
pCporAGFP ⁺	pCporA with GFP ⁺ insert
pCMark09	Plasmid containing Kanamycin resistance cassette
pCASO040	pET28α with <i>cj1388</i> insert

Table 2-9 Details of the plasmids created, or used, in this study

2.5.11 Plasmid extraction from *E.coli*

E.coli strains containing the desired plasmid were grown overnight in LB broth containing selection supplements at 37 °C, 200 rpm shaking, and in aerobic conditions. Following overnight growth the culture was centrifuged to pellet cells and the plasmid extracted using the QIAGEN Miniprep kit and following manufacturers guidelines. The plasmid was eluted in 30 µl of buffer EB.

2.5.12 Sequencing of *C. jejuni* and *E. coli* fragments

Purified plasmids and PCR products were sequenced offsite by Eurofins Genomics. The sequences were assembled using BioEdit and compared to expected *in silico* sequences constructed in pDRAW (Acaclone) or Artemis (Carver et al., 2005).

2.5.13 Creation of a *C. jejuni* strain expressing a green fluorescent protein

To make a strain of *C. jejuni* NCTC 11168 that constitutively expressed GFP protein, cells were transformed with the plasmid pCporAGFP⁺ (a gift from Duncan Gaskin, IFR) using standard protocols (van Vliet et al., 1998). Plasmid pCporAGFP⁺ contains GFP⁺ from pWM1007 (Miller et al., 2000) under control of the *C. jejuni* *porA* promoter and a chloramphenicol resistance cassette, flanked by the 5' and 3' sequences of the *cj0046*

pseudogene (Reuter and van Vliet, 2013). Insertion of the plasmid into the *cj0046* pseudogene was confirmed using the primers indicated in Table 2-3.

2.5.14 Creation of *E. coli* expressing DNase containing pET28 α constructs

Fragments containing the *dns*, *cje0566* and *cje1441* genes were amplified from the *C. jejuni* RM1221 genome using the primers indicated in Table 2-3. The fragments and plasmid pET28 α were cut as described in Table 2-6 and ligated to form pHB007, pHB008 and pHB009. Following confirmation that the plasmids contained the correct genetic sequences, they were transformed into *E. coli* BL21 (DE3) or C41/C43 cells as described in Section 2.5.10.3.

2.5.15 Creation of the *C. jejuni* RM1221 Δ *cje1441* mutant

A *C. jejuni* RM1221 *cje1441* deletion mutant (Δ *cje1441*) was created by insertional inactivation of the *cje1441* gene using a chloramphenicol resistance cassette. The *cje1441* gene and flanking regions were PCR amplified using the primers indicated in Table 2-3 and cloned into the pNEB193 plasmid (NEB) to form pHB012a. Subsequently the *cje1441* gene was excised and replaced with the *cat* cassette from pAV35 (van Vliet et al., 1998) by inverse PCR using primers indicated in Table 2-3 to form pHB012b. The plasmid sequence was confirmed to be correct and plasmids were inserted into strain RM1221 as described in Section 2.5.10.2.

2.6 Strain Characterisation Assays

In order to assess the comparability of *Campylobacter* strains, or mutants with their WT parent strain, several phenotype tests were carried out. Since this was a study of *C. jejuni* biofilm formation, tests focused on factors which could affect capacity to form biofilms, such as growth rate, eDNA release, and the ability to swarm or autoagglutinate. This information, combined with data on biofilm forming ability, allowed conclusions to be made about what effect, if any, deletion of genes or genetic variability between strains had on biofilm formation.

2.6.1 Autoagglutination

The ability of cells to aggregate was tested by growing cells as described in Section 2.3.4 before measuring the optical density at 600 nm and diluting to an OD₆₀₀ of 0.5. Two ml of culture was added to a cuvette and incubated at RT in aerobic conditions for 24 hours. An OD₆₀₀ reading was taken at the start and end of the incubation. All test samples were compared to NCTC 11168 WT and its $\Delta flaAB$ mutant, which is deficient in its ability to autoagglutinate.

2.6.2 Degradation of extracellular DNA by *C. jejuni* RM1221

Degradation of exogenous DNA was investigated using three separate experimental approaches: assessment of a) DNase activity using DNA containing agar plates, b) *C. jejuni* RM1221 eDNA degradation during growth and c) the ability of *C. jejuni* RM1221 to degrade DNA over a three hour time period.

2.6.2.1 Measurement of DNase activity using DNase plates

The DNase activity of *C. jejuni* strain RM1221, its $\Delta cje1441$ mutant, and *E. coli* expressing the RM1221 eDNase genes via the pET28 α expression system, was assessed by spotting cell suspension on to DNase plates (Oxoid) containing 0.005% v/v Toluidine Blue. *E. coli* suspensions were spotted directly on to the agar plates with no further manipulation, whereas *C. jejuni* suspensions were typically condensed to approximately 10x their original volume prior to spotting. Plates were incubated at 37°C in appropriate atmospheric conditions for 24 (*E. coli*) or 48 (RM1221) hours to allow development of a halo around the cell spots (indicating degradation). Plates were imaged using a GS800 Calibrated Densitometer (BioRad).

2.6.2.2 Measurement of genomic DNA degradation by *C. jejuni* RM1221

Degradation of DNA by *C. jejuni* strains NCTC 11168, RM1221 and the RM1221 $\Delta cje1441$ mutant was also assessed. *C. jejuni* RM1221 cells were allowed to form a lawn on Skirrow plates. The cells were removed from the plate and suspended in 2 ml Brucella medium before pelleting and washing twice in sterile PBS. Following washing the cell concentration was measured and the culture diluted to an OD₆₀₀ of 0.5 in sterile PBS. Fifty μ l of cell suspension was added to approximately 2 μ g of genomic *C. jejuni* NCTC 11168 DNA, and incubated at 37°C in a water bath for up to three hours. At 30 minute intervals, an aliquot was taken, the cells pelleted and the supernatant removed and frozen at -20°C until analysis. Following thawing, samples were visualised using agarose gel electrophoresis (described in Section 2.5.4).

2.6.3 Measuring extracellular DNA in cell supernatants

Shaking cell suspensions were grown as described in Section 2.6.1. At two hour intervals (for the first ten hours), 24 and 48 hours, a 1 ml volume of cell suspension was removed, pelleted and the supernatant added to an equal volume of phenol: chloroform: isoamyl

alcohol (in a ratio of 25:24:1 respectively) (Sigma). The mixture was vortexed and centrifuged at 20,000 × *g*, for 15 min at 4°C. The top fraction of the centrifuged liquid (approx. 500 µl) was removed and mixed with 50 µl sodium acetate (Invitrogen) and 1380 µl chilled absolute ethanol (Sigma) before centrifugation at 20,000 × *g*, for 30 min at 4°C. Supernatant was removed and 200 µl of 70% ethanol (sigma) was added before centrifugation at 20,000 × *g*, for 30 min at 4°C. Following removal of the supernatant the DNA was air dried and resuspended in 50 µl buffer EB (QIAGEN). DNA was stored at -20°C until use. DNA quantification was carried out by measuring the absorbance at 260 nm using the NanoDrop (Thermo Scientific), following manufactures guidelines.

2.6.4 Measurement of GFP expression

Levels of GFP expression in *E. coli* NEB5α cells containing pCporAGFP⁺ was assessed in cultures grown overnight in aerobic conditions at 37 °C. Cultures were centrifuged at 3000 x *g* for 30 minutes, the supernatant removed, and the cell pellets resuspended in sterile 1 ml PBS. Fluorescence analysis was performed using the FluoStar OPTIMA plate reader (excitation 485 nm, emission 520 nm). OD₆₀₀ measurements of the suspension were also obtained in order to calculate relative fluorescence.

2.6.5 Growth

C. jejuni was cultured as described in Section 2.3.4 before measuring the OD₆₀₀ and diluting to a OD₆₀₀ of 0.05 in Brucella medium. Suspensions were then cultured at 37°C, 220 rpm shaking, in microaerobic conditions for 48 hours. Unless stated a OD₆₀₀ reading was taken every 2 hours for the first 8 hours and then at 24 and 48 hours. MPN was also assessed at these time points (described in Section 2.4.1). Test strains or mediums were compared to NCTC 11168 WT and Brucella medium respectively. For cell growth in chicken juice containing medium, TTC staining was carried out as described in Section 2.4.2.

2.6.5.1 Growth measurement by Omega microplate reader

For comparison of *C. jejuni* RM1221 Δ*cje1441* to WT strain, the FLUOstar Omega microplate reader (BMG Labtech) was used. A 200 µl volume of cell suspension, diluted to an OD₆₀₀ of 0.05, was added in triplicate to the wells of a flat bottomed, clear 96 well plate (Sigma) before sealing with a FluidX adhesive pre-pierced polyolefin film to maintain sterility. Plates were incubated in the Omega machine for a 24 hour period with microaerobic conditions, 37°C, and 400 rpm shaking. OD₆₀₀ measurements were taken every 60 minutes throughout the experiments time course.

2.6.6 Swarming Motility

The ability of cells to swarm was tested by placing cells on 0.4% Brucella agar, supplemented with 0.05% TTC. *C. jejuni* was cultured as described in Section 2.3.4 before measuring the optical density at 600 nm and diluting to an OD₆₀₀ of 0.5. A 5 µl aliquot of diluted cell suspension was placed below the surface of the agar plate and incubated at 37 °C, microaerobic conditions for 48 hours. Images were photographed following incubation and the size of swarming halos calculated in cm² using Image J (RSB). All test samples were compared to *C. jejuni* NCTC 11168 WT and its Δ*flaAB* mutant, which is non-motile (Reuter et al., 2010).

2.7 Protein expression and purification

2.7.1 Induction of protein expression

Plasmids (pHB007, pHB008, pHB009 and pET28 α) were introduced into the *E.coli* strains as described in Section 2.5.10.3. Induction of *E. coli* C41/C43 and BL21 (DE3) cells was carried out following manufacturers guidelines. Briefly plasmid containing cells were thawed on LB agar containing selective antibiotics and incubated at 37°C for 15 - 24 hours. A colony was picked from the plate using a sterile tooth pick and added to 5 ml of LB containing 30 μ g/ml kanamycin and 0.2% (v/v) glucose and incubated at 37°C in shaking aerobic conditions overnight. Following overnight growth, 50 ml of fresh LB containing 0.2 % (v/v) glucose and 30 μ g/ml kanamycin was inoculated with 0.5 ml of overnight suspension and incubated at 37°C in shaking, aerobic conditions until a OD₆₀₀ of above 0.8 (or 0.5 for the *E. coli* BL21 (DE3) background) was achieved. A final concentration of 1 mM IPTG was added to initiate induction of the target protein from the T7 promoter, and cell suspensions were further incubated for up to 6 hours. Cell concentration and MPN was monitored throughout. Following termination of the IPTG incubation, cell suspensions were centrifuged and the pellet and supernatant retained for further analysis.

2.7.2 Measurement of protein concentration by Bradford assay

Bradford reagent (BioRad) was allowed to equilibrate to RT and diluted 1:5 in water before use. Samples were also diluted in water as required, unless otherwise stated, the dilutions used shown below:

Dilution (name)	Volume of sample (μ l)	Volume of water (μ l)
1:10 (A)	100	900
1:100 (B)	100 of A	900
1:1000	100 of B	900

Table 2-10 Sample dilution for Bradford analysis

A standard curve using known concentrations of BSA was also prepared as shown:

Concentration (mg/ml)	Volume of sample (μ l)	Volume of water (μ l)
10	100	0
9	90	10
8	80	20
7	70	30
6	60	40
5	50	50
4	40	60
3	30	70
2	20	80
1 (A)	200	1800
0.75	75 (A)	25
0.5	50 (A)	50
0.25	25 (A)	75
0.1	10 (A)	90
0	0	100

Table 2-11 Standard curve dilution for Bradford analysis. (A) represents a 1:10 dilution of the BSA stock (as shown in Table 2-10)

A 5 μ l sample of either standard or sample was added to 995 μ l of diluted Bradford reagent and incubated for 10 minutes before measuring the absorbance of the sample at 595 nm. Values from the known BSA concentrations were used to construct a standard curve, from which the unknown concentrations of the samples could be calculated.

2.7.3 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE) analysis

Samples were resuspended in 10 mM Tris HCL pH 7.4 and then supplemented with 4x NuPAGE LDS sample buffer (Invitrogen), and β mercaptoethanol (2.5 % final volume) before vortexing and boiling for 5 minutes. Samples were loaded onto 4-20 % RunBlue SDS precast gels (Expedeon) with 1 x NuPAGE MOPS running buffer (Invitrogen). Benchmark pre-stained molecular weight marker (Novex) was included on the gels as a size marker. Gels were run for 90 minutes at 135 V before overnight staining with Instant Blue stain (Expedeon). Gels were scanned using a GS800 Calibrated Densitometer (BioRad).

2.7.4 Western Blotting

SDS PAGE analysis was carried out as described in Section 2.7.3. Following gel electroporation, instead of staining the gel with Instant Blue it was transferred from the SDS PAGE gel to a nitrocellulose membrane using a X cell sure lock tank (Life Technologies), set up according to the manufactures instructions and filled with transfer buffer at 100V for 60

min. Following transfer, the membrane was rinsed with napp buffer before addition of blocking buffer and incubation on a shaking platform for 60 min at 4°C. A 1000x dilution of the anti-His antibody (GE Healthcare) was prepared using antibody dilution buffer. The membrane and antibody solution were carefully placed in a sealed plastic bag, ensuring that no air bubbles were present before incubation on a rocking platform for 60 min at RT. Following incubation, the membrane was rinsed with napp buffer and Goat-anti-mouse HRP conjugate antibody (diluted 10000 x in antibody dilution buffer) was added for 60 min at RT. The secondary antibody was removed and the membrane washed 6 times for 60 sec in napp buffer. His containing samples were visualised using the Supersignal West Pico Rabbit IgG detection kit (Pierce) following manufacturers guidelines and imaged using FluorChem E (Protein Simple).

2.8 **Biofilm assays**

Various methods were used for the formation of biofilms during this investigation, a brief overview of which are presented in Figure 2-1. Detailed descriptions of the methods are given in Sections 2.8.1 to 2.8.11.
















Method	Day				
	-2	-1	1	2	3-5
Standard Biofilm Method	<p>Thaw cells and incubate at 37 °C in microaerobic conditions</p> 	<p>Resuspend cells, dilute and incubate at 37 °C in microaerobic conditions, shaking</p> 	<p>Dilute overnight cell culture to required concentration, add to abiotic surface and incubate statically at 37°C.</p> 		<p>Gently remove the cell suspension, assess viability of the planktonic fraction, and stain as required</p> 
Co-culture Method	<p>Thaw cells and incubate at 37 °C in microaerobic conditions</p> 	<p>Resuspend cells, dilute and incubate at 37 °C in microaerobic conditions, shaking</p>  <p>Thaw cells and incubate at 37 °C in microaerobic conditions</p> 	<p>Dilute overnight cell culture to required concentration, add to abiotic surface and incubate statically at 37 °C.</p>  <p>Resuspend cells, dilute and incubate at 37 °C in microaerobic conditions, shaking</p> 	<p>Dilute overnight cell culture to required concentration, add to abiotic surfaces treated on Day 1. Incubate statically at 37 °C.</p> 	<p>Gently remove the cell suspension, assess viability of the planktonic fraction, and stain as required</p> 
Conditioning method	<p>Thaw cells and incubate at 37 °C in microaerobic conditions</p> 	<p>Resuspend cells, dilute and incubate at 37 °C in microaerobic conditions, shaking. Add conditioning solution to abiotic surfaces, incubate at 37 °C in aerobic conditions</p> 	<p>Remove conditioning solution and rinse once with sterile PBS. Dilute overnight cell culture to required concentration, add to abiotic surface and incubate statically at 37 °C</p> 		<p>Gently remove the cell suspension, assess viability of the planktonic fraction, and stain as required</p> 

Figure 2-1 Brief description of the major assay types used throughout this work

2.8.1 Biofilm formation in borosilicate test tubes

Cell cultures were grown as described in Section 2.3.4 and typically adjusted to an OD₆₀₀ of 0.05 (alternative dilutions are stated as required in the following chapters). Unless otherwise stated, 1 ml of this solution was added to a sterile borosilicate glass test tube (Corning) containing a cotton wool stopper. Tubes were incubated without shaking for the required time period (approximately 48 hours unless otherwise stated) at 37°C in either microaerobic or atmospheric air conditions. Following incubation, biofilms were stained, photographed and cell MPN of the planktonic fraction was assessed.

This method was adapted to allow growth of biofilms in a 100ml volume of Brucella medium. Medium bottles containing 100 ml of sterile Brucella medium were inoculated with *C. jejuni* to a final OD₆₀₀ of 0.05. Bottles were incubated with loosened lids to allow air exchange. Each week bottles were photographed and MPN determined using TTC containing agar (Section 2.4.2).

2.8.2 Growth of biofilms in 24 well plates

Cell cultures were grown as described in Section 2.3.4 and adjusted to an OD₆₀₀ of 0.05 in fresh Brucella medium. A 1.5 ml volume of this solution was added to a sterile polystyrene 24 well plate (Corning). Plates were incubated with lid and without shaking for approximately 48 hours at 37°C in either microaerobic or atmospheric air conditions. Following incubation, biofilms were stained as required, photographed and MPN assessed.

2.8.3 Growth of biofilms on stainless steel coupons

Cell cultures were grown as described in Section 2.3.4 and adjusted to an OD₆₀₀ of 0.05. Sterile stainless steel coupons (Stainless steel type 1.4301 according to EN 10088-1, with a Type 2B finish according to EN 10088-2) were placed in a six-well polystyrene tissue culture plate (Corning) and incubated statically with 4 ml of cell suspension in either Brucella medium, Brucella medium containing 5-90 % chicken juice, or 100% chicken juice. Following incubation, biofilms were stained as required, photographed and MPN assessed.

2.8.4 Growth of biofilms of glass slides

For biofilm formation on glass slides, 20 ml of cell suspension at a concentration of OD₆₀₀ 0.05 was added to a 50 ml falcon tube (Corning) containing a sterile twin frost borosilicate glass microscope slide (VWR) and incubated statically at 37°C for 48 hours in either aerobic or microaerobic conditions. Following incubation the slide was gently washed in sterile water and fixed either by incubation at RT in 4% formalin for 15 minutes before drying (for fluorescent microscopy) or by drying at 60°C for 30 minutes (where the light microscopy was and crystal violet staining was performed). Slides were stored at 4°C (for fluorescent microscopy) or RT (for light microscopy analysis), in the dark until use.

2.8.5 Growth of biofilms on cover slips

Cell cultures were grown as described in Section 2.3.4 and adjusted to an OD₆₀₀ of 0.05. One ml of this solution was added to a sterile borosilicate glass test tube (Corning) containing a cotton wool stopper, in which a sterile Thermanox coverslip (Agar Scientific, Stansted, UK) had been placed. Tubes were incubated for approximately 48 hours at 37°C in either microaerobic or atmospheric air conditions. Following incubation the cover slip was removed and prepared for SEM imaging as described in Section 2.9.8.2.

2.8.6 Pre-coating of abiotic surfaces

Chicken juice was diluted to the desired concentration in Brucella medium, or used without dilution, and added to borosilicate test tubes (1 ml total volume added) or six well plates

containing a sterile stainless steel coupon (4 ml total volume added). Tubes/plates were incubated overnight at 37°C in aerobic conditions to allow pre-coating. The medium was subsequently removed and surfaces were washed with an equal volume of PBS (1 ml for test tubes, 4 ml for six-well plates with stainless steel coupons), and immediately used in biofilm assays.

2.8.7 Proteinase K treatment of biofilms

Following dilution of the cell suspension and addition to borosilicate test tubes as described in Section 2.8.1, 20 µl of proteinase K (>600 U/ml concentration, supplied by QIAGEN) was added to each tube prior to the 48 hour incubation. For assays requiring heat inactivated enzyme, the required volume of proteinase K was heated to 95°C for 10 minutes and allowed to cool before use.

2.8.8 DNase I treatment of biofilms

Following dilution of the cell suspension and addition to borosilicate test tubes or six well plates containing stainless steel coupons, 4 µl of DNase I (1 U/ml concentration, supplied by Thermo Scientific) and 4 µl of DNase I buffer (Thermo Scientific), or 16 µl of each where six well plates were used, was added to each biofilm tube prior to the 48 hour incubation. For assays requiring heat inactivated enzyme, the required volume of DNase I was heated to 95°C for 10 minutes before use. Several alterations to this method are listed below:

2.8.8.1 Addition of DNase at various biofilm maturity stages

In order to assess the impact of DNase I addition to biofilms at various stages of maturation, biofilms were prepared as described in Section 2.8.1. Following 0, 12, 24, 36 or 48 hours of static incubation, 4 µl of DNase I (1 U/ml concentration, supplied by Thermo Scientific) and 4 µl of DNase I buffer (Thermo Scientific) were added to each biofilm tube. Following a total of 48 hours static incubation, test tubes were stained as required, photographed and MPN assessed.

2.8.8.2 DNase addition prior to staining

Biofilms were prepared as described in Section 2.8.1. Following the static 48 hours incubation 4 µl of DNase I (1 U/ml concentration, supplied by Thermo Scientific) and 4 µl of DNase I buffer (Thermo Scientific) were added to each biofilm tube and the test tubes replaced at 37°C. Tubes were removed, stained and MPN assessed following a 5, 15, 30, 45, 60 or 120 min incubation with the DNase I. This allowed the rapidity of the biofilm degradation to be assessed.

2.8.8.3 DNase I enzyme dilution

In order to determine the volume of DNase I required to degrade a *C. jejuni* NCTC 11168 biofilm, biofilms were prepared as described in Section 2.8.1. To each test tube 4 µl of DNase I buffer (Thermo Scientific) was added and then DNase I at concentrations of 5, 2.5, 1.25, 0.63, 0.31, 0.16, 0.08, 0.04, 0.02, 0.01 and 0 U/ml was also added to the test tubes. Once DNase I was added the test tubes were replaced at 37°C. Following a total of 48 hours static incubation test tubes were stained, photographed and MPN assessed.

2.8.9 Restriction enzyme treatment of biofilms

In order to establish the cut pattern of *C. jejuni* NCTC 11168 and 81116 gDNA, a 1 µl volume of restriction enzyme (*Bam*HI, *B*lpl, *Hae*III, *H*indIII, *M*seI or *R*saI, all supplied by NEB), DNase I (Fermentas), or RNase (QIAGEN) was added to a mixture containing 5 µl of *C. jejuni* NCTC 11168 or 81116 gDNA, prepared using a QIAGEN DNeasy Blood and Tissue kit following manufactures guidelines, 1 µl of 10x enzyme buffer (if required), 1 µl of BSA (if

required) and molecular grade water to a final volume of 10 μ l. Table 2-12 indicates which buffers were required for each enzyme:

Enzyme	Recognition site	Buffer(s) required
<i>Bam</i> HI	G [▼] GATC C C CTAG [▲] G	NEBuffer 3 + BSA
<i>B</i> lpI	GC [▼] TNA GC CG ANT [▲] CG	NEBuffer 4
<i>Ha</i> eIII	GG [▼] CC CC [▲] GG	NEBuffer 4
<i>H</i> indIII	A [▼] AGCT T T TCGA [▲] A	NEBuffer 2
<i>M</i> seI	TT [▼] AA AA [▲] TT	NEBuffer 4 + BSA
<i>R</i> saI	GT [▼] AC CA [▲] TG	NEBuffer 4
DNase I	NA	DNase I buffer (Fermentas)
RNase	NA	NA

Table 2-12 Restriction sites of enzymes used throughout the project. Arrows indicate enzyme cut sites (Code N = any base).

Biofilms were prepared as described in Section 2.8.1 and incubated statically at 37°C in aerobic conditions for approximately 48 hours. A 4 μ l volume of restriction enzyme and 4 μ l of its respective buffer(s) (Table 2-12) were added to the test tubes at either the start of the 48 hours incubation or for the final hour of the 48 hours incubation. Following the 48 hours static incubation test tubes were stained as required, photographed and MPN assessed.

In order to assess the activity of the enzymes against genomic DNA, samples were incubated for 60 minutes in a 37°C water bath to allow digestion of the gDNA before visualisation of the degradation products by gel electrophoresis.

2.8.10 Assessment of genetic transfer within the biofilm

Genomic DNA (gDNA) was extracted from the 11168 GFP⁺ mutant using the QIAGEN DNeasy kit following manufacturers guidelines. DNA concentration was calculated following the final elution. Purified DNA was stored at -20°C until use. The standard 48 hour static biofilm incubation was carried out as described in Section 2.8.1. In addition a total of 2 μ g gDNA was added to test tubes in duplicate either prior to the start of biofilm incubation, or following 24 hours of static incubation. Following a total of 48 hours of incubation one test tube of each condition was stained using crystal violet and the second tube washed and the biofilm population released using a sterile cotton bud. Both the initial supernatant and scraped biofilm populations were retained for MPN assessment. MPN in both populations was assessed using both Brucella agar and Brucella agar containing 10 μ l/ml chloramphenicol (for assessment of antibiotic resistance cassette transfer from the 11168 GFP gDNA to either the planktonic or biofilm cell populations).

2.8.11 Biofilm co-culture method

Biofilm formation was initiated as described in Section 2.8.1 and a primary incubation of 24 hours carried out at 37°C. Following 24 hours of static incubation, a second 1 ml volume of either fresh cell suspension (diluted to a OD₆₀₀ of 0.05), fresh sterile medium or cell-free spent medium was added to the test tube and a further (secondary) incubation carried out in

the same conditions as the primary incubation. Following completion of the secondary incubation MPN was assessed and the test tubes crystal violet stained as described in Section 2.9.2.

2.9 Biofilm staining methods

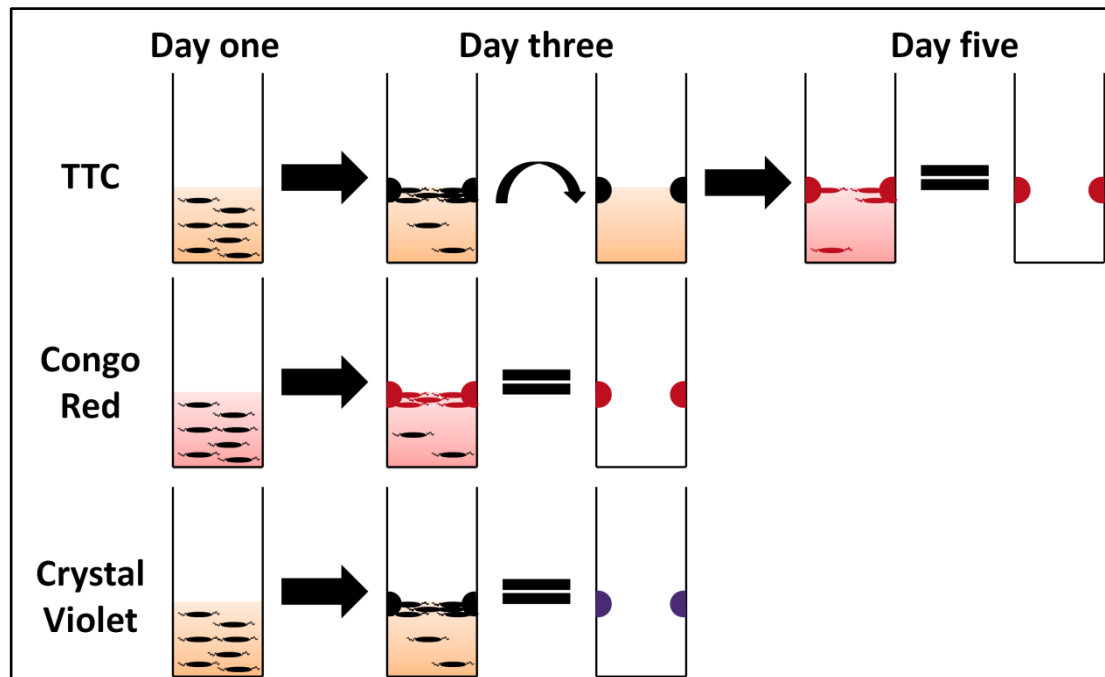


Figure 2-2 Graphical representation of the basic assay methods for TTC, Congo red and crystal violet staining.

TTC, Congo red and crystal violet were the three most commonly used assay techniques during this project. For TTC staining: cell suspensions were incubated statically for (typically) 48 hours to allow biofilm formation. Medium and planktonic cells were removed before addition of fresh, TTC containing medium and further incubation in microaerobic conditions for (typically) 72 hours to allow staining to develop. For Congo red staining: cell suspensions supplemented with Congo red dye were incubated statically for (typically) 48 hours to allow biofilm formation. Cell suspension was removed and the biofilms (including bound Congo red) were allowed to dry. For crystal violet staining: cell suspensions were incubated statically for (typically) 48 hours to allow biofilm formation. Following incubation, cell suspension was removed and the biofilms fixed before addition of crystal violet dye. Excess dye was removed and the stained biofilms allowed to dry. Once dried all biofilms could be imaged, and further analysis carried out.

2.9.1 Congo Red staining

A 0.1% v/v concentration of Congo Red was added to Brucella medium, Brucella medium supplemented with 5% v/v chicken juice or 100% chicken juice with or without *C. jejuni*, before static incubation at 37°C for 48 hours. At the end of the incubation period, the medium was removed from the tube before washing with 1 ml of PBS and drying at 37°C.

2.9.2 Crystal violet staining of biofilm cultures

Cell suspensions were removed from the test tubes, MPN assessed as described in Section 2.4.1, and the tubes were subsequently washed once with de-mineralized water and dried at 60°C for 30 minutes. One ml of 1% w/v crystal violet solution in de-mineralized water was added and tubes were further incubated on a rocker at RT for 30 minutes. After incubation the non-bound dye was removed from the tubes by thorough washing with demineralized water followed by drying at 37°C.

2.9.2.1 Crystal violet staining of slides

Following drying at 60°C for 30 minutes, slides were placed on a horizontally on a surface and 1 ml of 1% crystal violet was added to each slide and distributed over the slides surface. Slides were incubated, without movement, for approximately 10 minutes before excess crystal violet was removed by washing with demineralised water followed by drying at 37°C.

2.9.3 TTC staining of biofilm cultures

TTC staining was performed as described with minor adaptations during the method optimization process (Sections 2.9.3.1 to 2.9.3.5). Following the 48 hour incubation the cell suspension was removed, MPN assessed as described in Section 2.4.1, and tubes were washed twice by adding 1 ml of sterile PBS to gently rinse the attached biofilm, and remove unbound cells. A 1% TTC stock solution was diluted in fresh Brucella medium to a final concentration of 0.05% and 1.2 ml added to each tube before incubating at 37°C in microaerobic conditions for 72 hours. Following incubation MPN was assessed and the remaining Brucella medium/TTC solution was then removed and the tubes air dried.

2.9.3.1 Standard TTC staining method during optimisation process

Following the 48 hour static biofilm incubation, cell suspension was removed and tubes were washed twice by adding 0.6 ml of sterile PBS and swirled gently. TTC stock solution (1%) was diluted in fresh Brucella medium to a final concentration of 0.1% w/v), before incubating at 37°C in microaerobic conditions for 24 hours. Following incubation the TTC solution was removed and the tubes air dried.

2.9.3.2 Assessment of TTC conversion in aerobic and microaerobic conditions

The extent of TTC conversion from the initial clear solution to red formazan crystals by *C. jejuni* biofilms was assessed in both aerobic and microaerobic conditions. This was carried out by performing the secondary (TTC incubation) described in Section 2.9.3.1 at 37°C in both aerobic and microaerobic atmospheric conditions.

2.9.3.3 Optimisation of TTC washing process

During assay optimization process the following biofilm washing procedures were tested:

- Removal of cell suspension and no further washing before addition of TTC containing Brucella medium
- Removal of cell suspension, one 0.5 ml gentle wash with sterile PBS before addition of TTC containing Brucella medium
- Removal of cell suspension, two 0.5 ml gentle washes with sterile PBS before addition of TTC containing Brucella medium
- Removal of cell suspension, one 1 ml gentle wash with sterile PBS before addition of TTC containing Brucella medium
- Removal of cell suspension, two 1 ml gentle washes with sterile PBS before addition of TTC containing Brucella medium

2.9.3.4 Optimisation of TTC concentration

Several TTC concentrations were tested during the TTC staining optimisation process. A 1% stock solution was diluted in fresh sterile Brucella medium to concentrations of 0.01%, 0.02%, 0.05%, 0.1% and 0.5% v/v before addition to biofilm containing test tubes.

2.9.3.5 Optimisation of secondary TTC incubation

Multiple secondary TTC incubation times were assessed during the TTC staining optimisation process. Following addition of the 0.1% TTC solution to the biofilm cultures,

incubations of 6, 12, 24, 48 and 72 hours were carried out at 37°C in microaerobic conditions.

2.9.4 Quantification of dyes using spectrophotometer

Biofilms formed in tubes, plates or on stainless steel coupons were routinely photographed on a black or white background using a FinePix S4200 14 megapixel digital camera (Fugi). To quantify bound Congo red, crystal violet and TTC, dyes were dissolved by adding 20% acetone/80% ethanol to borosilicate test tubes or tissue culture plates and incubating on a rocking platform for 15 minutes at RT. The absorbance levels of dissolved dye were measured at a wavelength of A_{500} (Congo red and TTC) or A_{590} (crystal violet) using a Biomate 5 spectrophotometer (Thermo).

2.9.5 DAPI staining of *C. jejuni* NCTC 11168 GFP biofilms

Biofilms grown on glass slides were allowed to equilibrate to RT in the dark, before staining with 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) using manufacturers guidelines (Invitrogen). Prior to addition of a coverslip, Slowfade Gold antifade reagent (Invitrogen) was added to the slide as recommended by the manufacturer. Slides were imaged using a Zeiss 200M fluorescent and light microscope with Axiovision software.

2.9.6 Visualisation of extracellular DNA

Following incubation to allow biofilm formation in both aerobic and microaerobic conditions, the supernatant was removed and the tubes were rinsed once with sterile PBS to remove loosely attached bacterial populations. After rinsing and removal of the rinse suspension, a second 1ml volume of sterile PBS was added to each test tube and a sterile cotton wool swab was used to gently remove the biofilm from the walls of the test tube. An aliquot of this supernatant was retained for further analysis. DNase I (Fermentas) and RNase (Sigma) treatments were carried out following manufactures guidelines and incubated at 37°C in a water bath for 1 hour. Aliquots were mixed with gel loading buffer (NEB) and added to a 0.9% agarose gel and run at 100 V for 45 minutes in 0.5% TBE buffer.

2.9.7 Microscopy methods

2.9.8 Light microscopy

To observe cell motility and shape, 10 μ l of culture was added to a microscope slide and covered with a coverslip. Cells were observed using an Eclipse 50i microscope using the x100 lens (x1000 including ocular lens). Images were captured using a Coolpix 4500 digital camera (Nikon).

Observation of biofilms was carried out following growth of the biofilm as described in Section 2.8.4. Slides were dried at 60°C for 30 minutes and followed by staining with crystal violet (Section 2.9.2). Imaging was carried out using an Eclipse 50i microscope using the x20, x40 or x100 lens (x200, x400 or x1000 including ocular lens). Images were captured using a Coolpix 4500 digital camera (Nikon).

2.9.8.1 Ryu Stain

C. jejuni were grown as described in Section 2.6.1 for approximately 4 hours, to allow cells to reach sufficient numbers for optimal imaging density. Flagella were visualised using the Ryu stain described by (Kodaka et al., 1982). Briefly, a solution of 5% phenol, 2 g tannic acid and 10 μ l saturated aluminium potassium sulphate was mixed in a 1:10 ratio with 6 g crystal violet in 50 ml ethanol. Microscopy slides were prepared with the cell cultures of interest, and 5 μ l of Ryu stain was added to the corner of the cover slip and allowed to move under

the cover slip by capillary action. Imaging was at 1000 x magnification using an Eclipse 50i microscope and photographed using a Nikon Coolpix E4500 camera.

2.9.8.2 Electron Microscopy

Sterile thermanox coverslips (Agar Scientific, Stansted, UK) were placed vertically into a borosilicate glass test tube and incubated statically at 37°C in both aerobic and microaerobic conditions to allow biofilm formation to occur. Following the primary biofilm incubation the slides were removed, washed once with sterile PBS and fixed with 2.5 % glutaraldehyde in 0.1 M PIPES buffer (pH 7.4) for 1 hour. The fixative was then replaced with 3 changes of 0.1M PIPES buffer. The cells, supported by the cover slips, were then dehydrated in a series of ethanol solutions (30, 40, 50, 60, 70, 80, 90, and 3 × 100%) for at least 20 minutes in each. Samples were critical point dried in a Polaron E3000 critical point drier using liquid carbon dioxide as the transition fluid. The cover slips were then mounted with the cell layer facing upwards on aluminium SEM stubs using sticky tabs. The samples were coated with gold in an Agar high resolution sputter-coater apparatus. Scanning electron microscopy was carried out by Louise Salt and Kathryn Cross (NRP bioimaging group) using a Zeiss Supra 55 VP FEG SEM, operating at 3kV.

2.10 Data analysis

The mean, standard deviation, standard error of mean (SE), and coefficient of variance (%CV) was routinely calculated for all experimental data. This was used to assess both inter-assay (biological replicates) and intra-assay (technical replicates) variability. Typically five biological replicates of each assay were completed. The N value for each assay is indicated in the figure legends.

2.10.1 Statistical analysis

Statistical analysis was carried out using GraphPad Prism software (GraphPad software). Significance was assessed by calculating Students' t-test, Tukey, and Bonferroni post-test probabilities. A *P* value below 0.05 was considered significant. Linear regression was used in the cell density measurements presented in Section 3.2.5.

2.10.2 Dot plot analysis

The *cje0556* and *cje1441* genes were compared using the dotpath software, available on the EMBOSS website (<http://emboss.bioinformatics.nl> accessed 18 December 2014) and following the software instructions.

3 Development of a novel staining method for assessment of *C. jejuni* biofilm formation in the presence of organic materials.

This chapter is based on the paper: Brown H L, van Vliet A H M, Betts R P, Reuter M (2013) Tetrazolium reduction allows assessment of biofilm formation by *Campylobacter jejuni* in a food matrix model. *Journal of Applied Microbiology*, 115, p1212-21

3.1 Introduction

Levels of biofilm formation are usually assessed by dye-based staining techniques, which broadly fall into two categories: non-specific dyes and dyes targeting specific molecules within the biofilm, such as fluorescent dyes, which can be visualised using confocal laser scanning microscopy (Lawrence and Neu, 1999). Such specific dyes are useful for detailed and dynamic imaging of biofilms (Baird et al., 2012), but are costly and have labour-intensive and time consuming methodologies, making them an inappropriate tool when rapid visualisation of biofilms is required. Conversely non-specific dyes, although rapid, may overestimate the quantity of viable biofilm cells present, due to their non-specific binding of the matrix or surface components.

The most commonly used method to detect and quantify biofilms is staining with crystal violet (Chavant et al., 2007). This dye is non-specific, as it binds to all surface molecules of a negative charge, which can be found on either the bacteria or ECM (Extremina et al., 2011, Pan et al., 2010). Although crystal violet is frequently used to detect and quantify biofilm formation, some authors have criticized crystal violet for its relatively high inter-assay variability, particularly when compared to other imaging methods (Li et al., 2003).

Tetrazolium salts are the basis of several redox sensitive dyes, and have been used previously to study cell growth and biofilm formation in various bacterial models (Tengerdy et al., 1967, Schaule et al., 1993), but not with *C. jejuni* biofilms. In this chapter it is demonstrated that crystal violet gives high levels of non-specific (false-positive) staining in the presence of organic food matrices. As an alternative to crystal violet the reduction of the metabolic stain TTC to insoluble, red crystals of 1,3,5-triphenylformazan (TFP) (Bakor and Fahselt, 2005, Berends et al., 2010) was tested, before optimisation for use with *C. jejuni* biofilms.

3.2 Results

3.2.1 Crystal violet is able to non-specifically stain chicken juice

Crystal violet staining is a frequently used method for detection and quantification of biofilm formation, and has been used previously with *C. jejuni* biofilms (Joshua et al., 2006, Reeser et al., 2007, Reuter et al., 2010). However, initial experiments with crystal violet, chicken juice (for details of the chicken juice method see 2.2.2.6.1 and 4) and skimmed milk showed high levels of non-specific, false-positive staining (Figure 3-1). Tubes incubated with chicken juice but no *C. jejuni* cells gave high levels of staining, visualized as both a diffuse stain below the air/liquid interface and a strongly stained ring at the air-liquid interface (Figure 3-1b). This staining is independent of the presence or absence of *C. jejuni*. Similar results were obtained when replacing the chicken juice with skimmed milk solution (Figure 3-1c), another food-relevant matrix that has previously been used (Chmielewski and Frank, 2003).

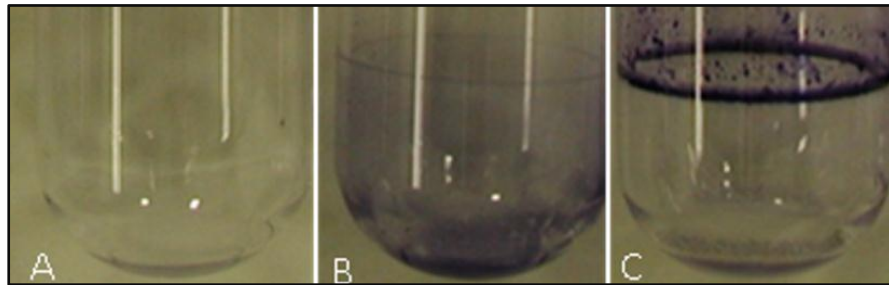


Figure 3-1 Representative images of CV stained chicken juice and skimmed milk

Images show crystal violet staining of test tubes incubated for 48 hours with Brucella medium only (A), 5% chicken juice (B) or 5% skimmed milk (C). No *C. jejuni* is added to these test tubes so staining shown reflects the ability of crystal violet to non-specifically stain the attached particulates.

This non-specific staining necessitated the development of an alternative method for measuring biofilm levels of *C. jejuni*. As shown in Figure 3-2, to allow TTC staining the standard biofilm assay was modified. In both the crystal violet and TTC methods, *C. jejuni* NCTC 11168 biofilms were cultivated using static culture (primary culture). In addition the TTC method required replacement of primary culture growth medium with fresh, TTC-supplemented media (secondary culture).

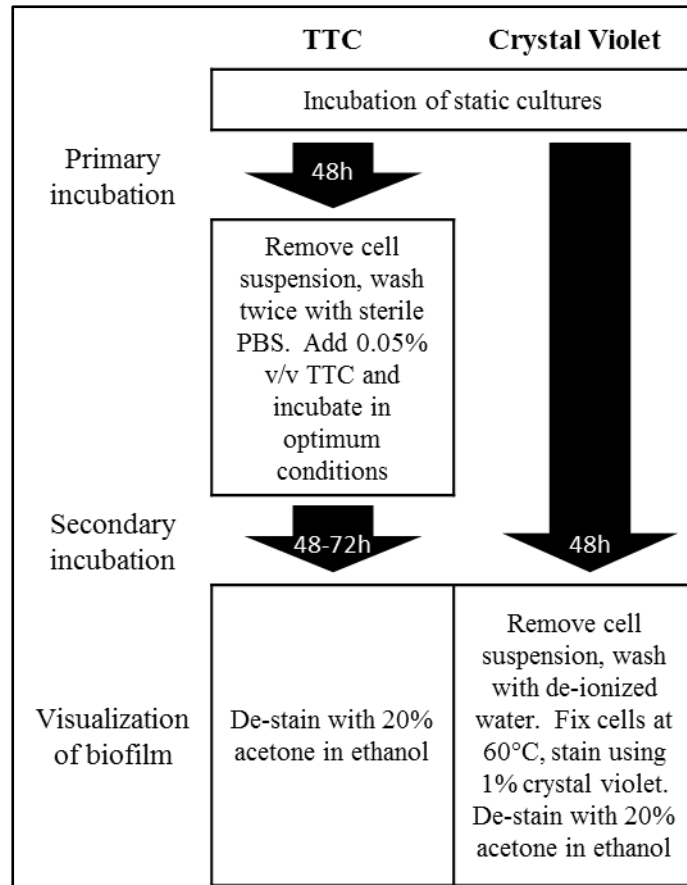


Figure 3-2 Diagrammatic representation of the staining assay methodologies showing incubation length and assay steps.

The workflow of both the TTC and crystal violet assays are shown to highlight both the methodological and time-span differences between the two methods.

During the secondary incubation, viable cells within the attached population were able to reduce the TTC, because the addition of the fresh medium allowed metabolic activity to increase, leading to conversion of TTC and the formation of a visible red formazan ring. Supplementation of the medium used during the primary incubation with TTC led to a diffuse red dye, which could not be collected and quantified, hence a secondary incubation step was added to the biofilm method, allowing quantification of the biofilm only. For the secondary incubation, only Brucella medium was tested as the *C. jejuni* cultures were known to be metabolically active and form biofilms within this medium. Since this medium is also widely used by this group and other groups working in the field, the results could be compared more easily to existing studies. The formazan crystals could be quantified by dissolving the bound dye and measuring absorbance at a wavelength of 500 nm (Figure 3-3). This formazan conversion allowed distinction between bacterial populations and attached organic material, a distinction which could not be achieved by use of non-specific dyes such as crystal violet (Figure 3-3).

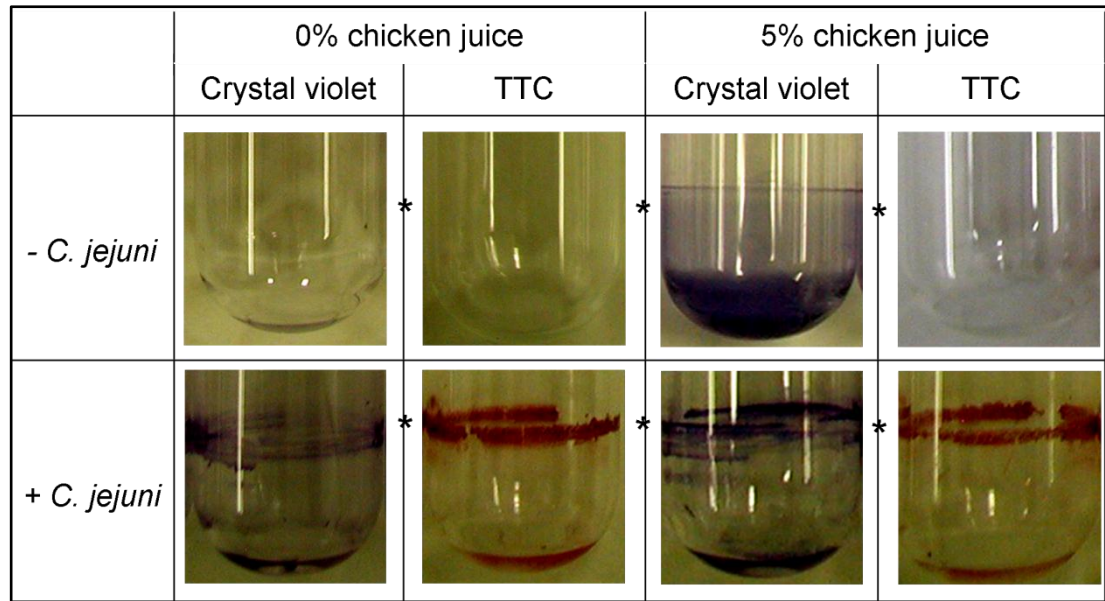


Figure 3-3 TTC does not non-specifically stain 5% chicken juice

Representative images of test tubes which have been incubated for 48 hours to allow biofilm formation before staining with either crystal violet (first and third columns) or TTC (second or last columns). The upper panel highlights that TTC is unable to convert to red formazan crystals without the presence of viable *C. jejuni*, whereas crystal violet confound biofilm quantification by staining both attached cellular populations and chicken juice (* denotes the approximate level of the meniscus following a 48 hour incubation). Chicken juice was selected for use here since BSA, another protein rich commonly used medium supplement, inhibited biofilm formation by *C. jejuni* NCTC 11168 as so could not be used in experiments where *C. jejuni* biofilm formation was required.

3.2.2 The reduction of TTC by *C. jejuni* is more effective under microaerobic conditions

It has been previously demonstrated that *C. jejuni* biofilm formation during incubation in aerobic conditions is increased compared to microaerobic conditions (Reuter et al., 2010). In order to ensure that the new TTC staining method was useful in both aerobic and microaerobic conditions, TTC conversion by aerobic *C. jejuni* biofilms grown without chicken juice was investigated and staining compared to crystal violet-stained biofilms. As can be seen in Figure 3-4 levels of TTC staining were significantly lower when compared to crystal violet, following secondary incubation in air. This suggests that TTC conversion by aerobically incubated *C. jejuni* does not correlate to the total level of biofilm, as determined by crystal violet staining.

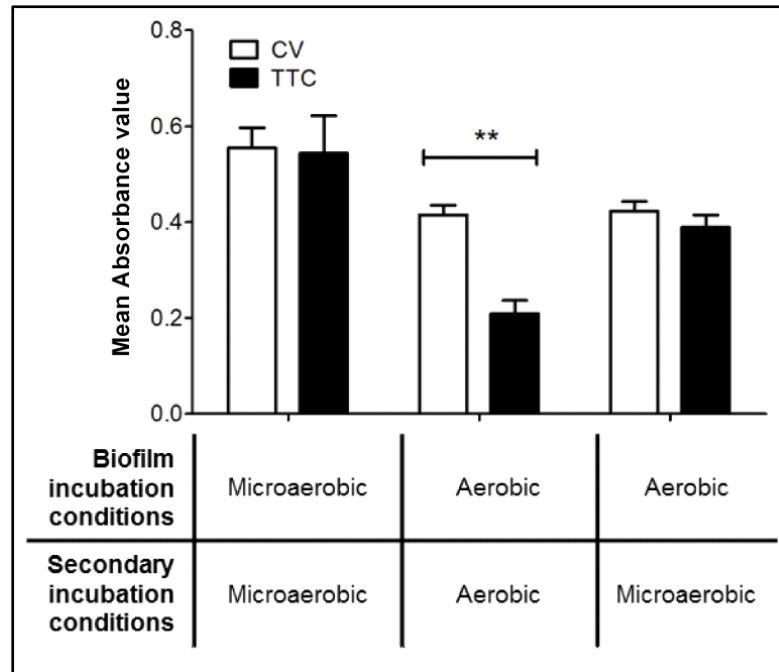


Figure 3-4 TTC staining required microaerobic incubation after biofilm formation

Comparison of TTC and crystal violet (CV) staining of *C. jejuni* biofilms incubated in microaerobic and aerobic conditions for biofilm formation and staining. White bars represent crystal violet staining (measured as A_{590}), black bars represent TTC staining (measured as A_{500}). The first two columns show the results of biofilm formation and staining in microaerobic conditions, columns 3 and 4 show results from both biofilm formation and staining in aerobic conditions, and columns 5 and 6 show results of biofilm formation in aerobic conditions but staining in microaerobic conditions. Error bars show the SE from three biological replicates, asterisks denote a statistically significant difference between crystal violet and TTC staining ($P < 0.01$, Students' T-test). $N = 5$

This difference was not observed in cultures where primary incubation in air was followed by secondary incubation in microaerobic conditions. Hence, all secondary incubations were performed in microaerobic conditions. This allowed optimum respiration and metabolism of the *C. jejuni* cells during the secondary incubation, while also maintaining the inhospitable environment which favours biofilm growth in the primary incubation.

3.2.3 Secondary incubation of biofilms does not lead to significant increases in biofilm biomass.

A lengthy secondary incubation was required to allow substantial TTC conversion by the attached NCTC 11168 population. There was a concern that this second incubation would allow significant further biofilm modification and so the impact of the secondary incubation on levels of biofilm was assessed. Biofilms were allowed to form in aerobic or microaerobic conditions during the primary incubation, before washing and addition of Brucella medium (without TTC) and the commencement of a secondary incubation in microaerobic conditions. Test tubes were removed following 24, 48 or 72 hours of secondary incubation and crystal violet stained to allow biofilm quantification. As can be seen in Figure 3-5, there is no statistically significant additional biofilm formation following the secondary incubation, although biomass does increase over the 72 hour additional incubation period.

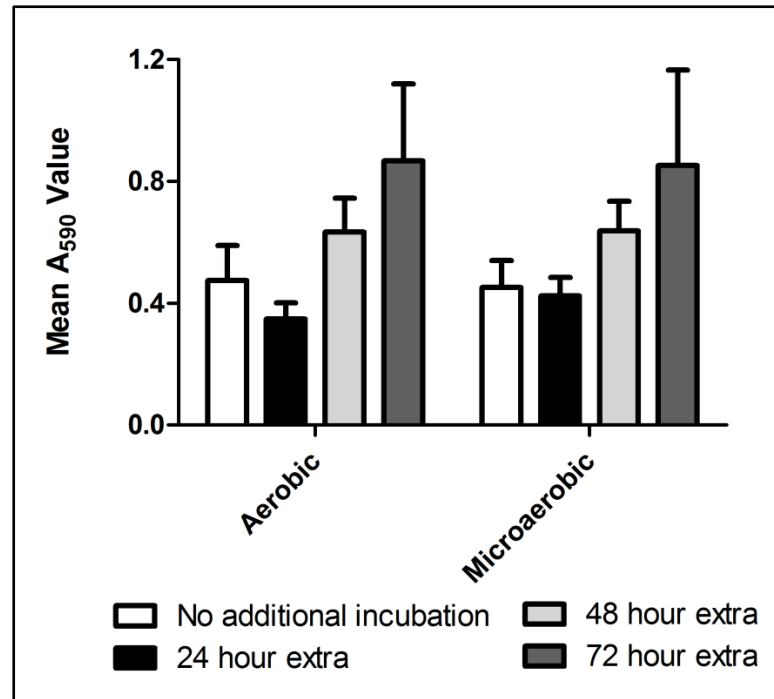


Figure 3-5 Additional growth during the secondary incubation is not significant.

Biofilm biomass was measured following a 24 hour (black bars), 48 hour (light grey bars) or 72 hour (dark grey bars) secondary microaerobic incubation of *C. jejuni* biofilms in Brucella medium and compared to a standard 48 hour primary incubation (white bars). Although a trend of increasing biofilm biomass was observed as time progressed, the difference was not statistically significant following ANOVA analysis. Bars show mean levels of crystal violet staining (N=5) and error bars show SE.

This suggests that a secondary incubation of up to 72 hours allows biofilm formation to be assessed without confounding results by allowing additional significant biofilm biomass increases. It was interesting to note that following 24 hours of secondary incubation the biofilm level was slightly reduced compared to biofilms which had undergone on further incubation. This could be due to minor mechanical disruption of the biofilm which may occur during the washing steps or addition of the secondary incubation medium.

3.2.4 Optimization of a TTC staining protocol to reduce assay variability

In order to optimize the TTC assay for use with *C. jejuni* and ensure that variability was minimised, several methodological variables of assay reproducibility were explored: rigor of the biofilm washing, TTC concentration, *C. jejuni* cell concentration, and finally TTC incubation time.

To investigate the effect of the washing procedure on quantification of biofilm formation and its impact on cell loss in the TTC assay, the following washing procedures were compared: no washing, wash once with 0.5 ml PBS, wash twice with 0.5 ml PBS, wash once with 1 ml of PBS, or wash twice with 1 ml of PBS. None of the washing methods resulted in a statistically significant difference in the A_{500} values obtained (Figure 3-6) suggesting that the washing procedure would not impact the final levels of biofilm staining. The coefficient of variance (%CV) was subsequently calculated and analysed to show the levels of reproducibility between replicates. Results were more reproducible (i.e. lower % CV) using two washes with 1 ml of PBS. Therefore, on all subsequent assays, tubes were washed twice with 1 ml sterile PBS.

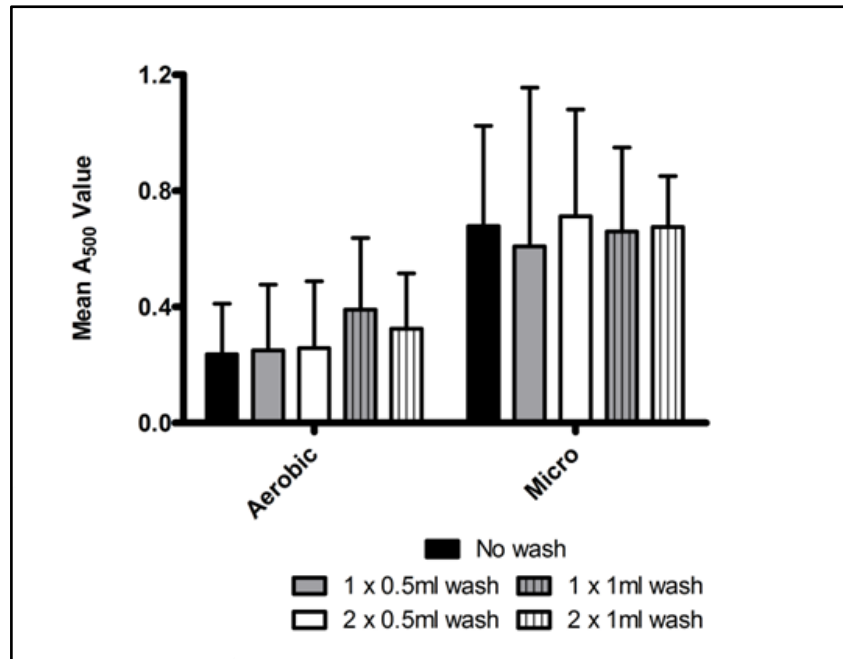


Figure 3-6 Biofilm formation is not significantly affected by alterations in the washing regimen.

Biofilm biomass was quantified by measuring A_{500} values following TTC staining for five different washing procedures. The washes tested were: no wash before staining (blank bars), one wash with 0.5 ml of sterile PBS (mid grey bars), two washes with 0.5 ml of sterile PBS (white bars), one wash with 1 ml of sterile PBS (mid grey striped bars) and two washes with 1 ml of sterile PBS (white striped bars). Both aerobic and microaerobic conditions are shown. (Error bars indicate SE, N = 5).

Tetrazolium dyes have previously been reported to be toxic to bacteria at high concentrations, both reducing the viability of bacterial populations and suppressing metabolic activity (Ullrich et al., 1996). Although this property has allowed TTC to be used as part of selective mediums (Weinberg, 1953), toxicity would be counter-productive in this assay. To address this potential problem a range of TTC concentrations were tested to assess optimal staining, while not affecting viability of *C. jejuni* during the incubation. Although there was a trend towards less TTC reduction at higher concentrations, there was no statistically significant difference between any of the TTC concentrations tested (from 0.01% to 0.5%), demonstrating that TTC reduction occurred at all tested concentrations (Figure 3-7a). However, MPN was drastically reduced at concentrations $\geq 0.1\%$ TTC (Figure 3-7b). To avoid artifacts resulting from such cytotoxicity, all future assays were performed with TTC at a final concentration of 0.05%.

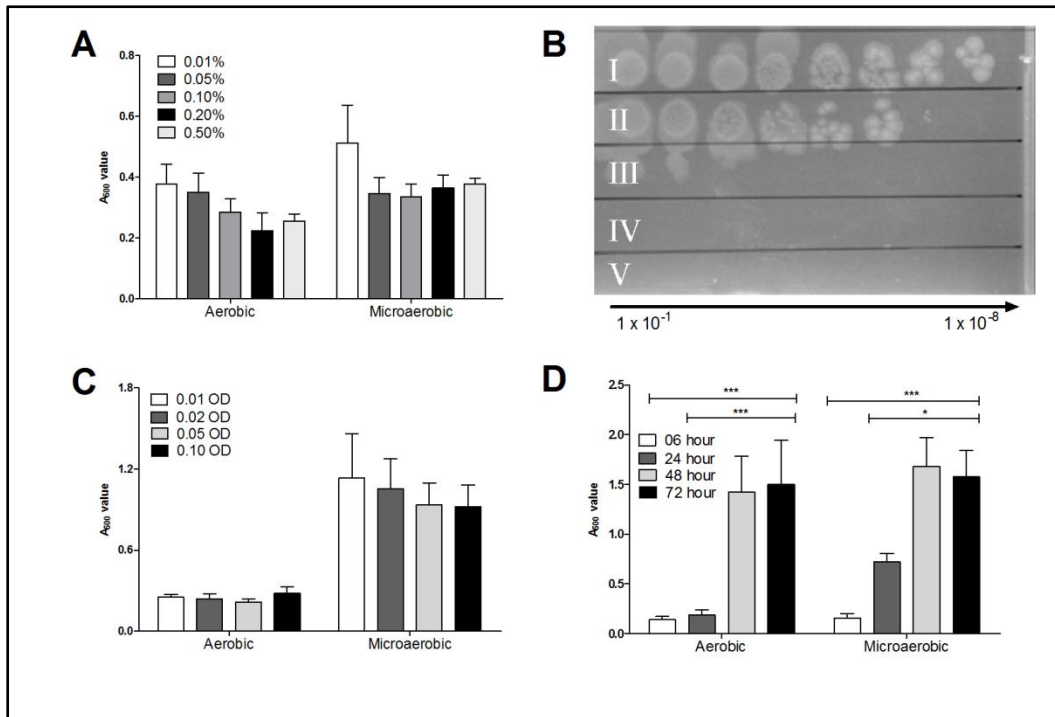


Figure 3-7 Optimization of the TTC staining assay.

Sections A, C and D show graphs with mean A_{500} values of TTC staining from optimization experiments (A) assaying the effect on biofilm biomass of different TTC concentrations; (C) initial *C. jejuni* NCTC 11168 inoculum concentrations; and (D) incubation time after addition of fresh TTC-supplemented medium. Each bar in the charts shows the average of three biological replicates, and error bars show SE ($N = 5$). Asterisks denote statistical significance: * = $P < 0.05$, *** = $P < 0.001$ (Students' T-test). Panel B shows a representative image of a *C. jejuni* MPN assessment, following 48 hours incubation with a range of TTC concentrations (I: 0.01%, II: 0.05%, III: 0.10%, IV: 0.20% and V: 0.50%). The picture shows (from left to right) a tenfold dilution series of cell suspension, with 5 μ l spotted per dilution, and the plate was incubated in microaerobic conditions at 37°C for 48 hours to allow cell growth. Following the 48 hour incubation no viable cells were observed in the planktonic phase of static cultures were concentrations of 0.01% TTC or higher were used.

To test if the total OD number of cells used to inoculate the static cultures influenced the final levels of TTC staining, overnight *C. jejuni* cultures were diluted to initial OD_{600} of 0.01, 0.02, 0.05 and 0.1 prior to inoculation. When assessing biofilm formation using the TTC method, no significant difference in TTC staining was seen over this range of inocula (Figure 3-7c), and thus set to a standard inoculum of $OD_{600} = 0.05$. Following the addition of fresh TTC-supplemented medium, various secondary incubation times, ranging from 6-72 hours, were compared in order to establish the optimal secondary incubation period. Although no significant increase in biofilm biomass was observed (Figure 3-5), staining was significantly increased 48 hours after the addition of TTC, with little further increase from 48 to 72 hours (Figure 3-7d). As such all further incubation were carried out for a minimum of 48 and a maximum of 72 hours.

3.2.5 TTC reduction in medium cultures correlates with OD_{600}

A high level of precipitation and aggregation in shaking cultures supplemented with chicken juice was observed (Figure 3-8). This hampered accurate tracking of growth both by measurement of OD_{600} values, or when performing serial dilutions and plating suspensions

for colony counting.

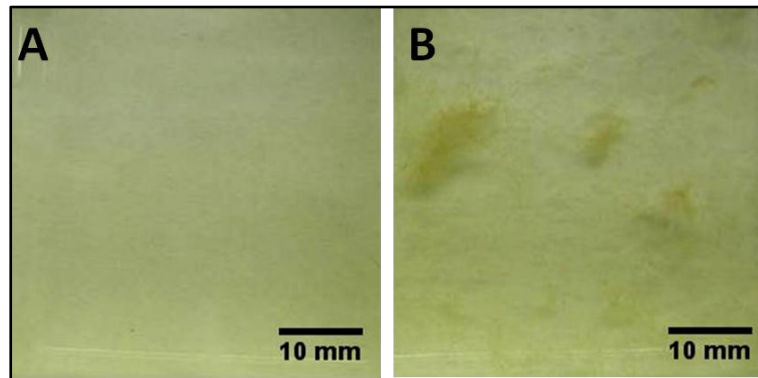


Figure 3-8 Representative images of chicken juice particulates.

Images were taken with Nikon Coolpix camera, without magnification, following four hours of shaking incubation in a T₂₅ tissue culture flask at 37°C in microaerobic conditions. The left image (A) shows a sterile Brucella only medium and the right image (B) shows sterile Brucella medium supplemented with 5% chicken juice. Although the solution in A appears clear, large aggregates can be seen in B, indicating that inclusion of chicken juice into solutions leads to the production of particulates. In both flasks no bacterial growth was observable following 48 hrs. of incubation, indicating that the particulates were not caused by the interaction of the chicken juice with bacteria.

as TTC conversion is dependent on metabolic activity, it is hypothesized that it can be used as an alternative to OD₆₀₀ measurement in medium supplemented with food matrices (for example chicken juice). In order to test this, a range of pre-set bacterial concentrations in Brucella medium were supplemented with TTC, and both the OD₆₀₀ and A₅₀₀ were determined following incubation (Figure 3-9).

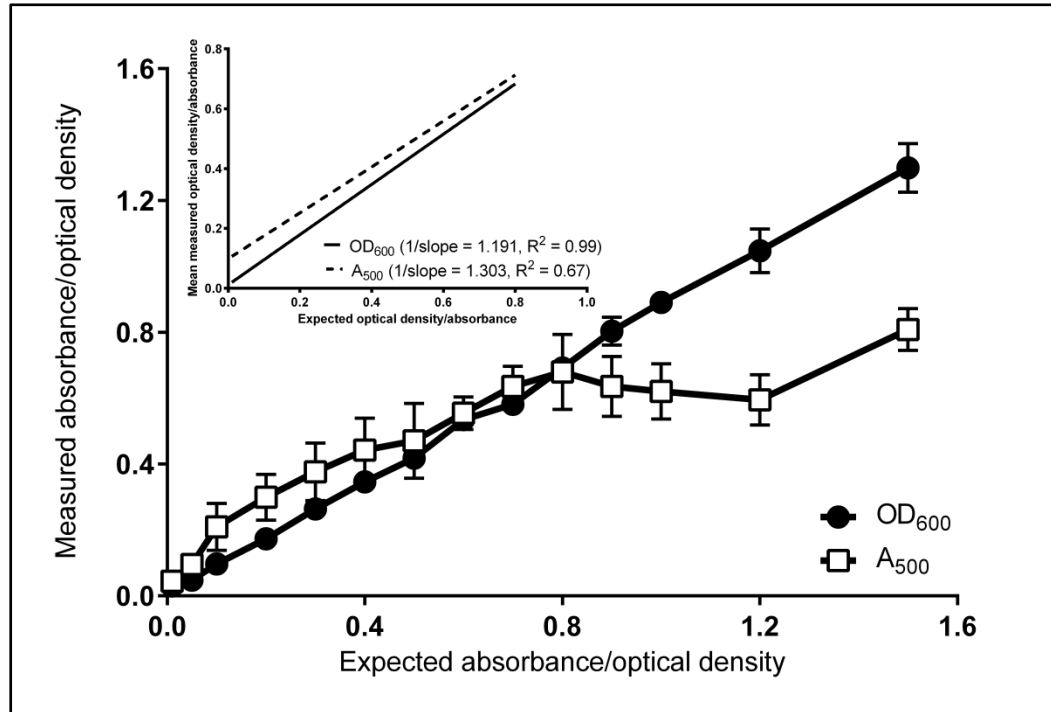


Figure 3-9 TTC staining can be used as alternative for OD₆₀₀ measurements to monitor growth of *C. jejuni*, at OD₆₀₀ values below 0.8

Graph showing comparison of absorbance values of the specific cells concentrations supplemented, as measured by OD₆₀₀ (black circles), and stained with TTC ((white squares). Cells were diluted to a known OD₆₀₀ value, and TTC was added and samples were incubated for 30 minutes. An equal volume of solvent was added and samples were centrifuged before measurement of absorbance or optical density. The supernatants were removed and the OD₆₀₀ and A₅₀₀ values measured. The Y axis indicates the expected optical density/absorbance of the diluted samples and the X axis plots the actual value obtained by measuring the optical density/absorbance. Error bars show the standard deviation from 5 biological replicates.

At an OD₆₀₀ ≤ 0.8, TTC conversion (as measured by A₅₀₀) showed a linear correlation with the OD₆₀₀ (Figure 3-9). The gradients of A₅₀₀ and measured OD₆₀₀ values did not significantly differ ($P = 0.377$). Above OD₆₀₀ = 0.8, OD₆₀₀ and A₅₀₀ measurements did not correlate, with A₅₀₀ values beginning to plateau. This could be due to maximal conversion of the dye, or poor recovery of the dissolved dye due to the high cell concentration. Thus, for tracking bacterial growth at OD₆₀₀ = 0 to 0.8, TTC supplementation can be used where OD₆₀₀ measurement is not possible, due to interfering components present in food matrices.

3.3 Discussion

In this chapter it is shown that TTC staining of *C. jejuni* biofilms is a suitable alternative to crystal violet dye, giving similar levels of sensitivity and assay reproducibility. TTC also has the advantage of allowing metabolic activity detection and visualization of biofilms in matrices relevant to the food chain, such as chicken juice. It was previously shown that viable cells could be recovered from an aerobically grown biofilm (Reuter et al., 2010). This optimized TTC staining methodology also demonstrates that viable cells contribute to the adhered population of aerobically incubated biofilms.

Crystal violet is commonly used in biofilm studies, however various authors have suggested that information gained from crystal violet staining alone may be misleading due to its non-specific staining (Pan et al., 2010, Skogman et al., 2012). Other techniques, such as cell enumeration, must be carried out alongside crystal violet staining, leading to increased study cost and time. Crystal violet is also not suitable for use in combination with high protein content matrices, or where non-specific binding to components in the growth matrix is expected. Calcofluor white has also been reported to stain *C. jejuni* biofilms (McLennan et al., 2008), however as this is another carbohydrate component stain, we anticipate similar issues in food matrix models. High levels of background are also expected with the protein stain Coomassie blue (Austin et al., 1998, Rogan et al., 2004).

The TTC method described in this chapter allows rapid, non-toxic and low cost quantification of metabolically active attached *C. jejuni* cells. TTC has been used to detect cell viability in a wide range of tissues (Steponkus and Lanphear, 1967, Adegboyega et al., 1997). A number of studies have reported the use of TTC in identification and distinction of *Campylobacter* species (Skirrow and Benjamin, 1980, Luechtefeld and Wang, 1982). TTC has been used to assess the levels of biofilm formation and growth of *Staphylococcus aureus* and *E. coli* (Skogman et al., 2012, Tengerdy et al., 1967), but a review of the literature shows that TTC has not been used to assess *C. jejuni* biofilm formation, in either monoculture or in a food matrix model.

As TTC is a metabolic dye, growth conditions should be optimised when using the TTC stain. In this chapter, sub-optimal growth or stressful conditions (such as aerobic incubation) led to inefficient reduction of TTC dye. Rapid loss of signal due to starvation conditions has also been observed for 5-cyano-2,3-ditoyl tetrazolium chloride-detection of *Pseudomonas putida* in a drinking water system (Schaule et al., 1993) and during assessment of antibiotic activity against *Streptomyces venezuelae* (Brooks et al., 2012). This suggests that conventional staining and microscopy still have a role to play in measuring biofilm formation in sub-optimal conditions.

TTC has also been suggested as a tool for allowing rapid enumeration of *Campylobacter* colonies in selective medium (Line, 2001). Here concentrations of 0.1, 0.5, 1, 2 and 5 mg ml⁻¹ were tested, and results were consistent with those of the previous authors (Butzler and Skirrow, 1979, Line, 2001, Skirrow and Benjamin, 1980, Veron and Chatelain, 1973), showing normal growth of *C. jejuni* at TTC concentrations of 0.2 and 0.4 mg/ml, but very weak growth at 1 mg/ml, and no growth observed above this concentration.

During this investigation it was noticed that the speed of TTC conversion was dependent on the conditions the *C. jejuni* was subjected to. For example, it is possible to measure the growth of *C. jejuni* in shaking cultures following a 30 minute treatment with TTC, however at least a 24 hour incubation must be performed with biofilm cultures in order to achieve the

same levels of staining. Many investigators have shown that bacteria in biofilms have reduced metabolic activity, due to gradients of nutrients and oxygen within the biofilm itself (Fux et al., 2005, Hoiby et al., 2010a), leading to differential conversion of TTC.

It is advisable to further optimize the TTC methodology presented here for specific applications other than *C. jejuni* static biofilm formation. This has previously been reported by (Klancnik et al., 2010), who found that *C. jejuni* and *C. coli* did not respond to the TTC staining protocol used for *Bacillus cereus*, *S. aureus* and *Salmonella infantis*. It is hoped however that this work will be able to provide a basis for optimisation of TTC with other biofilm forming species and different applications of *C. jejuni* biofilm study.

In conclusion, the method presented in this chapter offers a new low-cost technique suitable for use in biofilm analysis, allowing rapid and simple imaging of metabolically active cells. This method is able to specifically stain adhered and metabolically active *C. jejuni*. Moreover, the use of TTC conversion to monitor growth in matrices that do not allow measurement of OD₆₀₀ values is also demonstrated. Use of TTC is especially relevant in investigations where matrices are used that are likely to lead to high levels of non-specific staining by traditional dyes.

4 Chicken juice enhances surface attachment and biofilm formation of *Campylobacter jejuni*

This chapter is based on the paper: [Brown H L](#), Reuter M, Salt L J, Cross K L, Betts R P, van Vliet A H M (2014) Chicken juice enhances surface attachment and biofilm formation of *Campylobacter jejuni*. *Applied Environmental Microbiology*, 80, 22, 7053 – 7060

- SEM images by K Cross and L Salt (NRP bioimaging group)

4.1 Introduction

Insufficient or ineffective removal of organic material is a serious problem in food processing areas. Spilled foodstuffs or run-off from carcass eviscerations contain a complex blend of carbohydrates, proteins, lipids and sugars (Chmielewski and Frank, 2007), providing an ideal solution in which bacteria can thrive and survive. A build-up of these organic materials on a surface is hereafter referred to as a conditioning layer. Conditioning layers assist bacterial attachment to surfaces by altering the surface physio-chemical properties, and attracting the bacteria to the surface due to the increased nutrient availability (Hwang et al., 2012, Dat et al., 2010). One well studied example of a conditioning layer is the oral pellicle, which assists attachment of bacterial species such as *Streptococcus mutans* to the tooth surface and contributes to subsequent periodontal disease (Di Giulio et al., 2013). Surface conditioning layers have also been shown to be important for the initial attachment of food-borne pathogens, for example *L. monocytogenes* survival rates increase when biological soil is present on stainless steel surfaces (Van Houdt and Michiels, 2010), and milk proteins are able to increase attachment of *E. coli*, *L. monocytogenes* and *S. aureus* to stainless steel (Barnes et al., 1999).

To date, most studies on *C. jejuni* biofilms have been performed in laboratory conditions, which do not mimic the conditions encountered in the processing environment. It is important to ensure that studies are designed to allow accurate interpretation and extrapolation of laboratory obtained results to the food industry (Balamurugan et al., 2011). Various experimental systems have been used to mimic the conditions encountered by *C. jejuni* in the food chain. These models typically include the use of cooked or raw meat (Yoon et al., 2004), modelling relevant packaging conditions (Balamurugan et al., 2011), or use materials relevant to the food chain such as stainless steel (Sanders et al., 2008). One such model system is the “chicken juice” model (Birk et al., 2004). This model is based on the collection of exudate from defrosted, commercially obtained chicken carcasses, followed by supplementation or replacement of standard laboratory medium with this sterile filtered liquid. Supplementation of Brucella medium with chicken juice resulted in increased survival of planktonic cells of *C. jejuni* following both chilled and frozen storage (Birk et al., 2004, Birk et al., 2006).

In this chapter the effect of chicken juice on attachment of *C. jejuni* and *C. coli* to surfaces and their subsequent biofilm formation was investigated. It is shown that in the presence of chicken juice, *C. jejuni* biofilm formation is increased, and that this increase in biofilm levels is not simply due to increased cell numbers within the suspensions, but to an increase in attachment to abiotic surfaces. It is also shown that this increase in attachment is due to the ability of chicken juice to condition abiotic surfaces relevant to food processing environments.

4.2 Results

4.2.1 *C. jejuni* biofilm increases in the presence of chicken juice

Meat and meat exudates have been previously reported to allow for an increase in survival of *C. jejuni* (Balamurugan et al., 2011, Birk et al., 2004, Birk et al., 2006). To assess whether meat exudates affect *C. jejuni* biofilm formation, biofilm levels of static *C. jejuni* NCTC 11168 were measured using cultures supplemented with meat exudates recovered from defrosted chicken (chicken juice), turkey, duck and goose carcasses as well as pork steaks. As dyes such as crystal violet and Congo red aspecifically bind to meat exudate components (Figure 3-1), we measured biofilm formation via conversion of TTC (Brown et al., 2013). Supplementation of Brucella medium with chicken juice resulted in an increase in biofilm formation compared to Brucella medium alone, in both microaerobic and aerobic conditions (Figure 4-1).

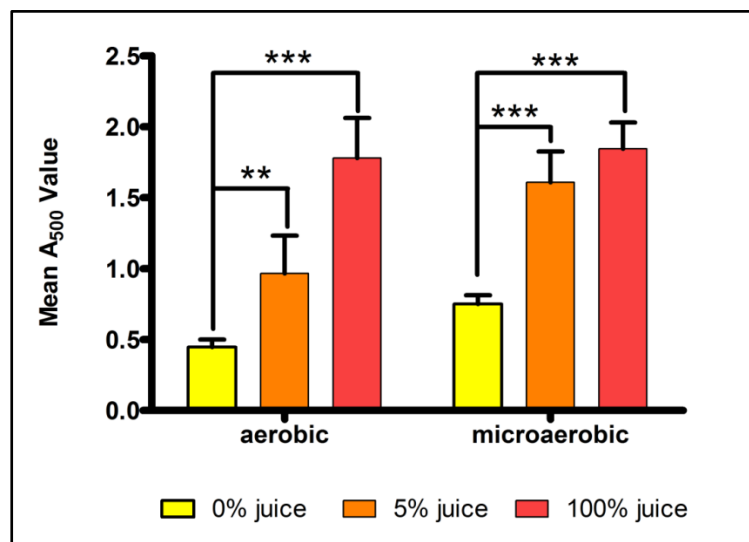


Figure 4-1 Biofilm formation of *C. jejuni* NCTC 11168 is increased in the presence of chicken juice.

Static incubation of *C. jejuni* in Brucella medium supplemented with chicken juice results in increased biofilm formation, as shown using the TTC biofilm assay. Error bars show SE (N = 5) and significance was measured using Bonferroni post-tests following ANOVA analysis (** = P<0.01, *** = P<0.001).

4.2.2 Other meat exudates are also able to alter the biofilm forming properties of *C. jejuni* NCTC 11168

Although the most important transmission route of *C. jejuni* to humans is via chicken meat (Batz et al., 2012), other meats are still considered to be a risk. In order to assess the universality of the findings in Section 4.2.1, juices prepared from chicken carcasses were purchased from several different UK supermarkets, other poultry animals such as duck, goose and turkey, as well as pork steaks were all tested for their ability to affect *C. jejuni* NCTC 11168 biofilm formation.

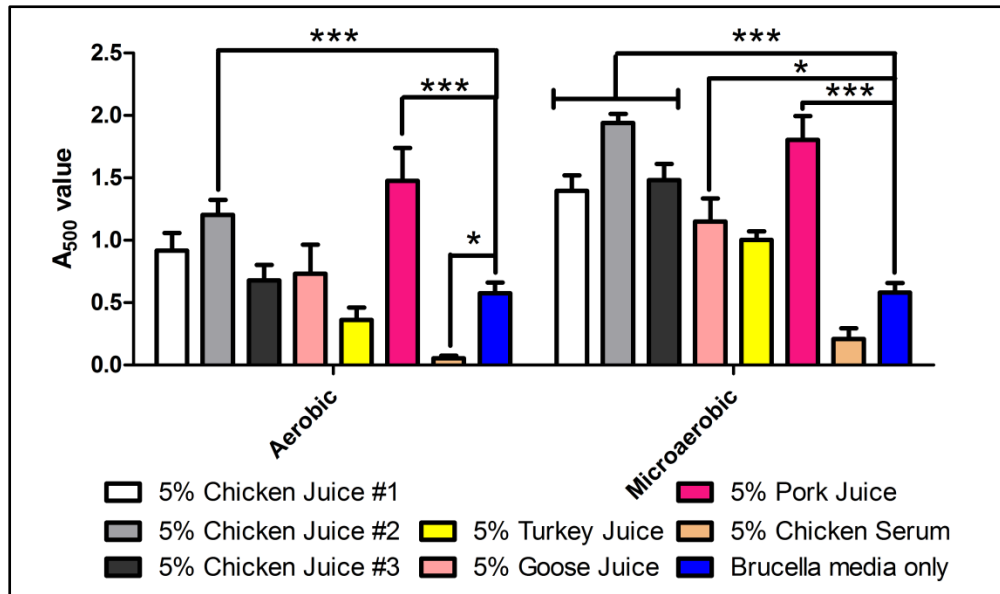


Figure 4-2 *C. jejuni* NCTC 11168 biofilm formation is affected by various meat juices.

Frozen chicken, turkey and goose carcasses, and frozen pork steaks were obtained from major UK supermarkets and defrosted to obtain the juice solution. Chicken serum was obtained from a scientific supplier. All juices were used to supplement *C. jejuni* NCTC 11168 biofilm assays (5% v/v in Brucella medium). Error bars show SE (N = 5) and significance was measured using Bonferroni post-test (* = P<0.05, *** = P<0.001) by comparing to the non-supplemented, 'Brucella medium only' sample. Chicken juice #1 to #3 indicates chicken juice prepared from frozen whole chickens purchased at three different UK supermarket chains.

As can be seen in Figure 4-2, supplementation of Brucella medium with chicken and pork juice leads to increased biofilm formation. This is particularly obvious in microaerobic conditions, where all three batches of chicken juice showed significantly increased biofilm formation compared to biofilms formed in un-supplemented Brucella medium. It is interesting to note that juice prepared from frozen chicken and goose carcasses do not show such significant increases in biofilm formation and chicken serum greatly reduces biofilm formation.

These differences could be in part due to differences in protein concentration (Figure 4-3). Although all the solutions did contain a high level of protein the exact composition differed and could contribute to alterations in particulate formation and aggregation, which in turn may affect surface conditioning.

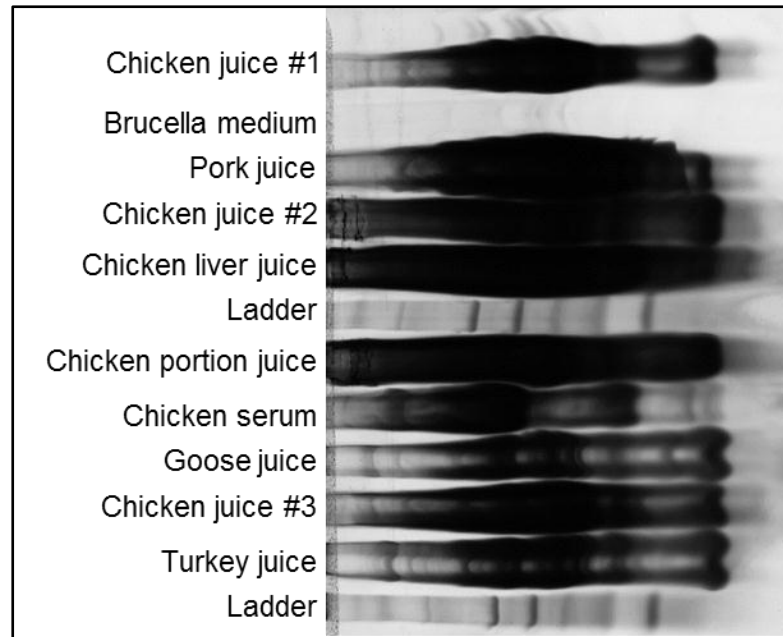


Figure 4-3 SDS PAGE analysis of juice used in this study

A representative SDS-PAGE gel image of the juices used within this study to show varying protein profiles and quantity of protein. Chicken juice #1 to #3 represent the whole chicken bought from three different national supermarkets, annotated in the same manner as in Figure 4-2.

4.2.3 Biofilm formation is increased in several isolates

In order to ensure that the effect observed in the glass test tubes was present on other abiotic surfaces and not specific to *C. jejuni* strain NCTC 11168, the previous assay was repeated using polystyrene plates as well as borosilicate test tubes, and the number of *C. jejuni* and *C. coli* isolates used was also increased. Three other *C. jejuni* isolates (81116, 81-176 and RM1221) and one *C. coli* isolate (15-537360) were selected as they were from both clinical and environmental sources and well characterised both within this study (Figure 4-4) and by other groups (Pearson et al., 2007, Pearson et al., 2013, Parkhill et al., 2000, Fouts et al., 2005b, Hofreuter et al., 2006).

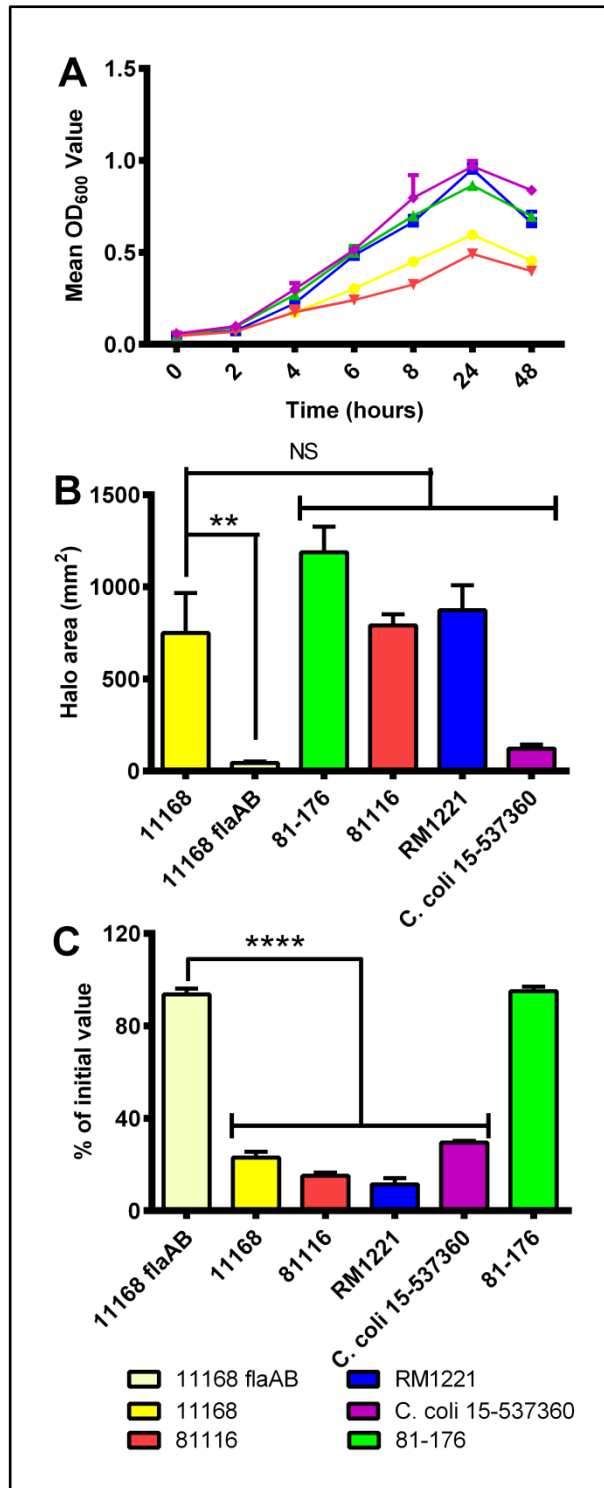


Figure 4-4 Swarming, autoagglutination and growth comparison for all *C. jejuni* and *C. coli* strains used

Comparison of *C. jejuni* strains NCTC 11168 (yellow), 11168 Δ flaAB (cream), 81-176 (green), 81116 (red), RM1221 (blue) and the *C. coli* strain 15-537360 (purple) growth (A), swarming (B) and ability to autoagglutinate (C) under standard conditions (for A and B: 37 °C in microaerobic conditions and for C room temperature in aerobic conditions) in Brucella medium. Error bars show SE (N = 5) and significance was measured using Bonferroni post-test (** = P<0.01, **** = P<0.0001).

As was noted by Joshua et al. (2006), *Campylobacter* shows strain specificity in its ability to form biofilms. This was also noted following phenotypic analysis of the strains selected for use in this study (Figure 4-4). These differences in biofilm formation could be linked to the altered growth, motility and autoagglutination phenotypes of the strains assessed. For the purposes for this study, strains NCTC 11168 and 81116 are classed as good biofilm formers, and the other three strains tested, RM1221, 81-176 and *C. coli* 15-537630, are considered to form negligible amounts of biofilm. All the tested strains showed a significant increase in biofilm formation when Brucella medium was supplemented with 5% chicken juice. This increase occurred in borosilicate test tubes and 24-wells polystyrene wells, in both atmospheric conditions (Figure 4-5). The chicken juice-dependent increase in biofilm formation was particularly clear in *C. jejuni* RM1221 and *C. coli* 15-537360, as these strains showed very low levels of biofilm formation in Brucella medium alone (Figure 4-5 blue and purple bars respectively).

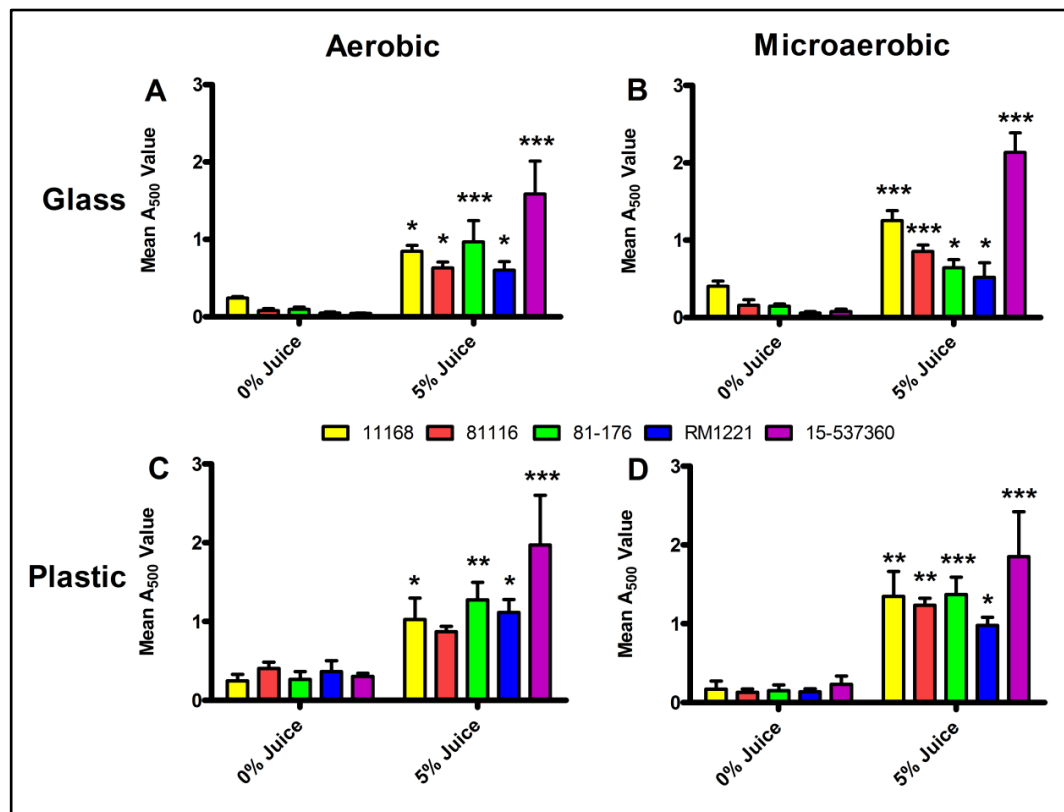


Figure 4-5 Static incubation of four strains of *C. jejuni* and one strain of *C. coli* in the presence of chicken juice leads to increased attachment and TTC staining.

Graphs A and C show data for biofilms incubated in atmospheric conditions and B and D show data from a comparable treatment in microaerobic conditions. Materials tested are (A, B) borosilicate glass and (C, D), Polystyrene. There was an increase in TTC conversion when Brucella medium was supplemented or replaced by chicken juice. Figure shows quantity of biofilm formation measured by TTC conversion. Error bars show SE (N = 5) and significance was measured using Bonferroni post-test (* = P<0.05, ** = P<0.01, *** = P<0.001).

4.2.4 Chicken juice is also able to support *C. jejuni* growth

Chicken juice is a complex medium and although the exact composition is unknown Bradford and SDS-PAGE analysis has indicated a high protein content (Figure 4-6), and phase separation of the thawed samples also suggests lipids are present within the solution.

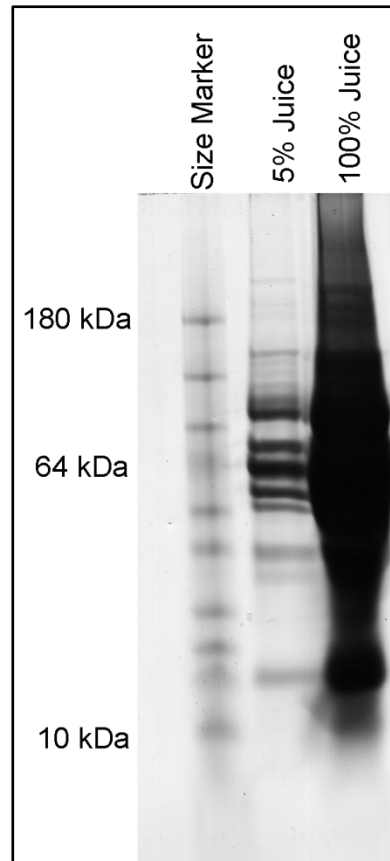


Figure 4-6 SDS-PAGE gel of 5% and 100% chicken juice samples

SDS-PAGE gel showing high protein content of both 5% chicken juice in Brucella medium and chicken juice alone

Figure 4-1 showed that replacement of medium by 100% chicken juice gave the highest level of biofilm formation. This difference was not due to differences in viability, as cultures incubated in Brucella medium and medium supplemented with 5% and in 100% chicken juice all had similar MPN values (Figure 4-7). It was however possible that the growth rate of the cell cultures may lead to increased biofilm initiation and more rapid biofilm maturation.

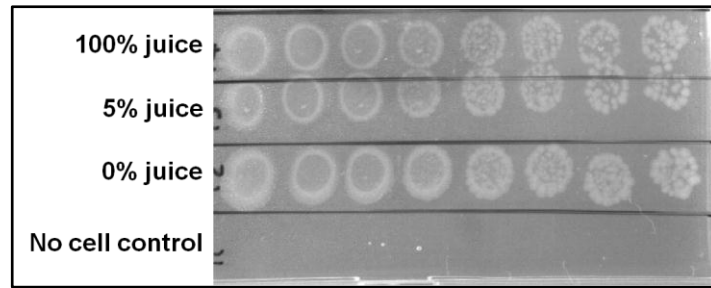


Figure 4-7 MPN of *C. jejuni* NCTC 11168 planktonic cells is not effected by chicken juice concentration

Representative image of spot plate following 48 hour static incubation in aerobic conditions to allow biofilm formation. Following incubation the planktonic phase was sampled as described in Section 2.4.1 before imaging and assessing the effect, if any, the chicken juice supplementation of Brucella medium had on viability.

To differentiate between growth and biofilm formation, growth of *C. jejuni* NCTC 11168 was assessed in Brucella medium, Brucella medium supplemented with 5% chicken juice and 100% chicken juice. There was no significant difference between growth in Brucella medium and medium supplemented with 5% chicken juice over a 24 hour period as measured by TTC conversion (Figure 4-8), unfortunately this data could not be confirmed by viable cell counting due to the confounding effect of chicken juice particulates in the cell suspension. This suggests that the increase in biofilm formation in a 5% chicken juice solution is solely due to increased attachment of *Campylobacter* to the abiotic surface. In 100% chicken juice, the mean A_{500} value of the 24 hour sample was significantly higher than the Brucella medium control, suggesting that the increased biofilm formation present in 100% chicken juice could in part be due to enhanced growth of the *C. jejuni*. These results also show that chicken juice supports *C. jejuni* growth.

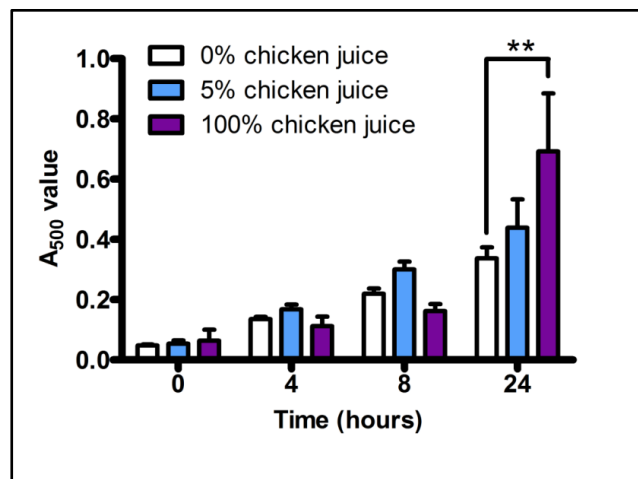


Figure 4-8 Growth of *C. jejuni* NCTC 11168 in the presence of chicken juice

100% chicken juice (purple bars) results in statistically significantly increased growth of *C. jejuni* only after 24 hours. The growth was measured by assessing the conversion of TTC to red formazan crystals by metabolically active bacterial cells in the shaking suspension. Growth in medium supplemented with 5% chicken juice (blue bars) is not significantly different from un-supplemented Brucella medium (white bars). Error bars show SE (N = 5) and significance was measured using Bonferroni post-test following ANOVA analysis (** = $P < 0.01$).

4.2.5 Chicken juice batches allowed uniform growth

As the chicken juice used throughout this study was prepared from several different frozen chicken carcasses on nine separate occasions it was important to ensure that the different batches of chicken juice gave a uniform response in assays. In order to assess this uniformity, *C. jejuni* NCTC 11168 growth was assessed using medium supplemented with each new batch of chicken juice (5% v/v). Growth in the new batch was then compared to previous batches to ensure inter-batch reproducibility. Each qualification experiment also included a *C. jejuni* NCTC 11168 culture grown in Brucella medium only, to ensure that the cells were growing as expected.

All batches of chicken juice showed similarity to each other and had a similar level of variability to the Brucella medium only control samples (Figure 4-9). This indicates that the chicken juice from each batch has a uniform mechanism of action, although its exact composition, which was not investigated, may have varied between batches.

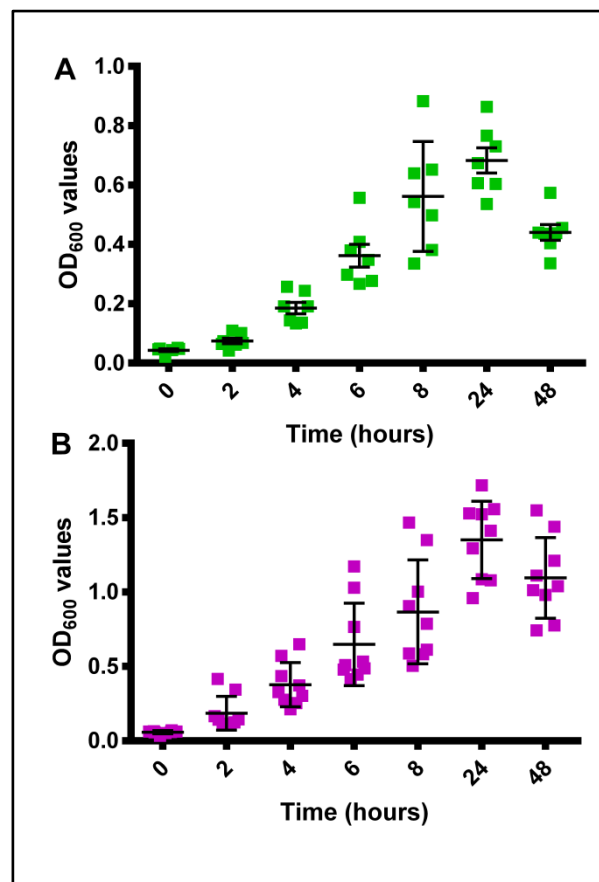


Figure 4-9 Assessment of variability within chicken juice batches

C. jejuni NCTC 11168 growth in Brucella medium only (A) and Brucella medium supplemented with 5% chicken juice (B) was assessed when each new chicken juice batch was prepared. The figure shows compiled data from all batch testing assays. The mid-line of each box and whisker plot indicates the mean values while the outer bars indicate one standard deviation from the mean (N = 5).

Although *C. jejuni* growth is not an ideal measure of chicken juice activity, it was used for comparison purposes as it could be easily measured for each batch of chicken juice used.

This was particularly important as earlier batches of chicken juice were not used in biofilm assays, meaning no data on their influence of biofilm culture was gathered, but had been included in preliminary growth experiments. Growth experiments also allowed some indication of the particulate/aggregation formation within each batch of chicken juice to be assessed. This was important as particulate formation plays an important role in both surface conditioning and increased biofilm formation.

4.2.6 *Campylobacter jejuni* preferentially attaches to chicken juice particulates

Since biofilm formation was increased by chicken juice the ability of chicken juice to bind to an abiotic surface was assessed. Brucella medium with and without 5% chicken juice, or 100% chicken juice were incubated in static cultures under the standard assay conditions (as Section 2.8.1, but without *C. jejuni* cells within the Brucella medium). Following incubation tubes were stained with TTC, crystal violet or Congo red and non-specific binding assessed (Figure 4-10).

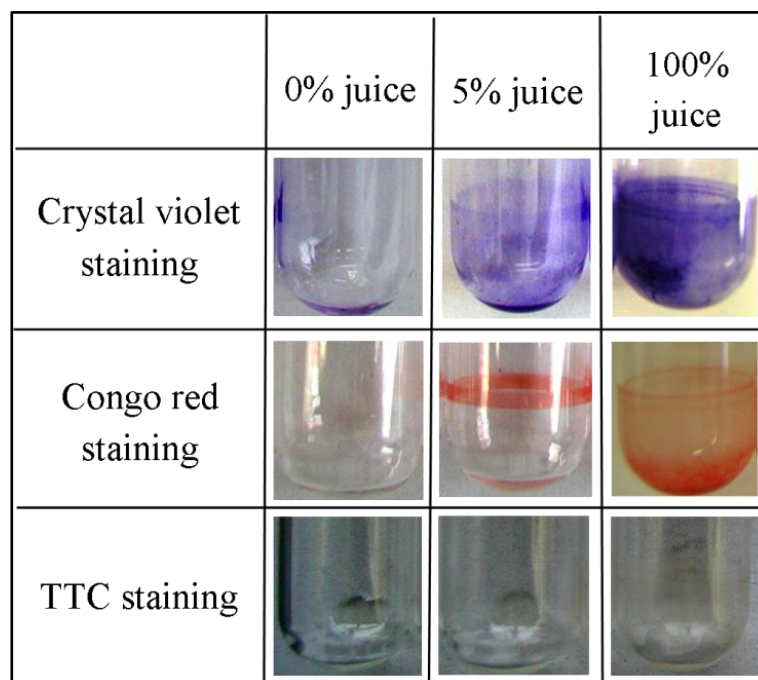


Figure 4-10 Non-specific staining of chicken juice components by Crystal violet and Congo red in the absence of bacteria.

Representative images of chicken juice stained test tubes following static incubation. Upper panel: crystal violet staining of glass tubes incubated with Brucella medium only, Brucella medium supplemented with 5% chicken juice, or 100% (undiluted) chicken juice. Central panel: Congo red staining of glass tubes incubated with Brucella medium only, Brucella medium supplemented with 5% chicken juice, or 100% chicken juice. Bottom panel: TTC-staining of glass tubes incubated with Brucella medium only, Brucella medium supplemented with 5% chicken juice, or 100% chicken juice.

There was a significant increase in crystal violet and Congo red staining in the presence of chicken juice, while staining with TTC (measuring bacterial respiration) was negative, demonstrating that components of chicken juice bind to the abiotic surface but remain sterile. The non-specific binding observed was similar to that seen when skimmed milk containing mediums were stained with crystal violet (Figure 3-1). As the formation of precipitates (particulates) was also observed, as shown in Figure 3-8, it is hypothesised that

chicken juice components may form a conditioning layer on the abiotic surface facilitating bacterial attachment.

In order to further investigate this phenomenon SEM was used. *C. jejuni* NCTC 11168 biofilms obtained following incubation in Brucella medium, Brucella medium supplemented with 5% chicken juice or 100% chicken juice, were investigated using SEM (Figure 4-11). In the presence of chicken juice, *C. jejuni* cells preferentially bind to the particulates rather than directly to the abiotic surface (Figure 4-11b and c). This is especially apparent in the 5% chicken juice image (Figure 4-11b) where only the chicken juice particulates, but not the abiotic surface, are bound by *C. jejuni* cells. Figure 4-11c also visually supports the observations in Figure 4-8 that the total number of cells within the biofilm is increased in 100% chicken juice. Hence chicken juice provides a highly adhesive environment supporting subsequent formation of a *C. jejuni* biofilm.

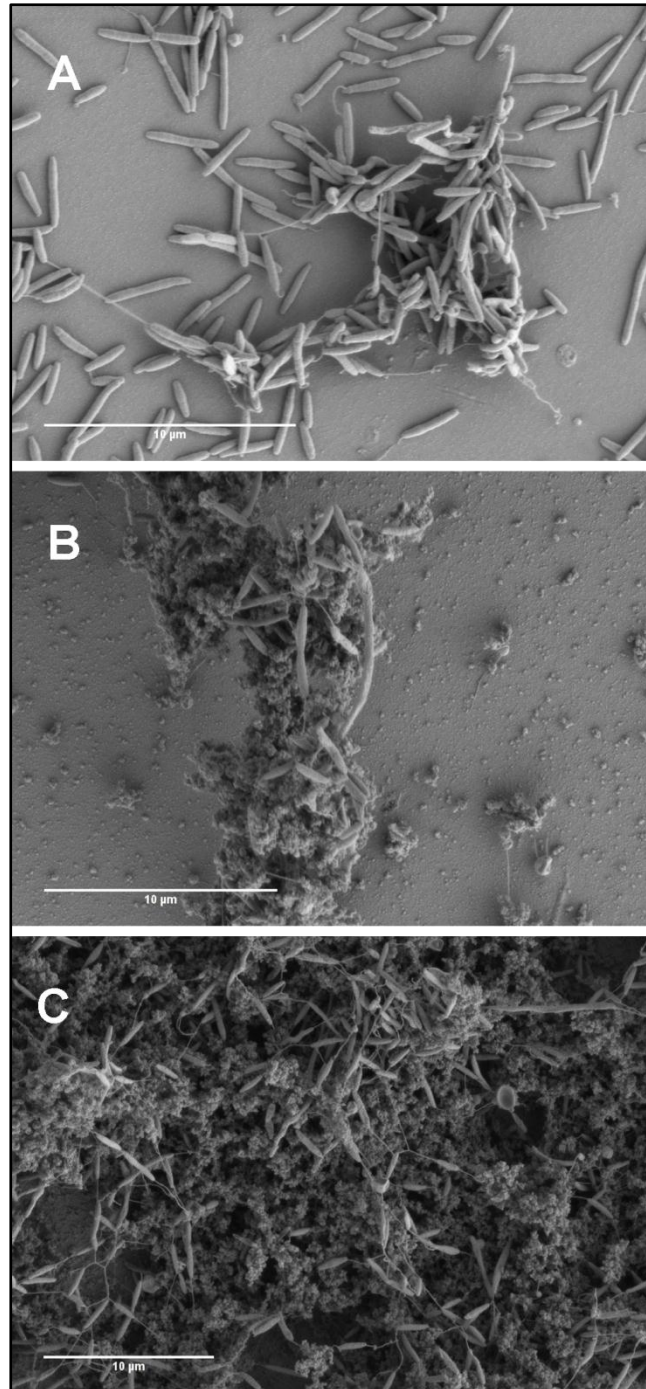


Figure 4-11 Chicken juice facilitates binding of *C. jejuni* NCTC 11168 via modification of abiotic surfaces

Representative SEM images of *C. jejuni* biofilms supplemented with 0% (A), 5% (B) or 100% (C) chicken juice on cover slips. In chicken juice containing samples, the *C. jejuni* can be seen to adhere to the juice particulates rather than the abiotic surface. A large chicken juice particulate can be seen adhered to the slide surface in (B), with *C. jejuni* attached to it in preference to the slide surface. In (C) particulates are densely packed and so cover the field of view. Scale bar = 10µm. Larger version of the images can be found in Appendix 3.

4.2.7 Pre-conditioning surfaces with chicken juice increases biofilm formation

All previous experiments within this chapter were performed with simultaneous addition of *C. jejuni* and chicken juice, followed by static incubation to allow biofilm formation. Since SEM images appeared to show preferential attachment of the *C. jejuni* to chicken juice aggregates it was hypothesised that attachment of these aggregates to a surface, rather than the presence of the chicken juice *per se*, was the main factor contributing to increased biofilm formation.

An experiment was therefore designed in which borosilicate test tubes were incubated overnight with Brucella medium supplemented with chicken juice. This solution was then removed and the tube rinsed to remove non-adhered aggregates before addition of cell suspension. The experimental set-up allowed assessment of the biofilm increasing potential of only attached chicken juice particulates. A range of chicken juice concentrations were tested during the pre-coating stage, from Brucella medium with 10-90% chicken juice and with 100% chicken juice. Subsequent addition of *C. jejuni* NCTC 11168 resulted in an increase in levels of biofilm formation (Figure 4-12) with all concentrations of chicken juice when compared to the Brucella medium, in both aerobic and microaerobic conditions.

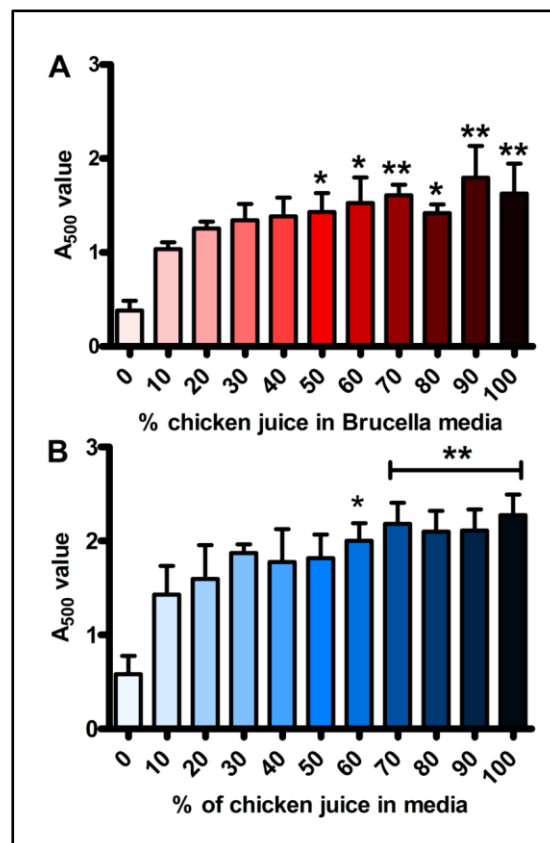


Figure 4-12 Pre-coating of test tubes with chicken juice increases biofilm formation by *C. jejuni* NCTC 11168.

Tubes were pre-coated with a range of chicken juice concentrations before being used in the standard TTC biofilm assay under both aerobic (A) and microaerobic (B) conditions, using unsupplemented Brucella medium. Error bars show SE (N = 5) and significance was measured using Bonferroni post-test following ANOVA analysis (* = P<0.05, ** = P<0.01).

4.2.8 The conditioning layer alters as the concentration of chicken juice within solution increases

In order to assess if the chicken juice was shed from the conditioning layer, providing a supplemental nutrient source for the *C. jejuni* cells, the experimental procedure was repeated with sterile Brucella medium used instead of *C. jejuni* cultures. At each step of the procedure the protein content of the supernatant was tested by both SDS PAGE analysis and quantification by Bradford reagent. The experimental set up is described in Table 4-1.

Day	Action	Bradford Assay	SDS-PAGE
1	1 ml cultures of Brucella medium containing 0, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100% chicken juice cultures placed in test tubes and incubated aerobically at 37°C overnight	performed for all concentrations	performed for 0, 5, 100% concentrations
2	Overnight cultures removed, tubes washed with 1 ml of sterile PBS before addition of 1 ml fresh Brucella medium. Tubes incubated aerobically for 48 hours at 37°C	performed for all concentrations (overnight samples and washing)	performed for 0, 5, 100% concentrations (overnight samples and washing)
4	48 hour cultures removed, tubes washed twice with 1 ml of sterile PBS before addition of 1.2 ml fresh Brucella medium. Tubes incubated aerobically for 72 hours at 37°C	performed for all concentrations (48 hour samples and washings)	performed for 0, 5, 100% concentrations (48 hour samples and washings)
7	72 hour cultures removed. Following removal tubes were either flooded with 1ml of Bradford reagent (to assess residual material in the tube), or 1 ml of sterile PBS added and the tube scraped.	performed for all concentrations (72 hour samples and tubes flooded)	performed for 0, 5, 100% concentrations (72 hour samples and tube scrape)

Table 4-1 Experimental workflow for the assessment of protein concentration within borosilicate glass test tubes pre-conditioned with sterile chicken juice.

Assessment of the protein concentration by SDS PAGE showed that samples containing 5% chicken juice displayed very little shedding from the conditioning layer following the first overnight incubation. However, when the test tubes were scraped on Day 7 some protein remained. This suggests that at low concentrations of chicken juice, a firm attachment to the abiotic surface occurs which can only be reversed by mechanical debridement. In 100% juice samples a more loose attachment with the surface was observed in which chicken juice protein could be detected in both incubation suspensions and washing solutions (Figure 4-13).

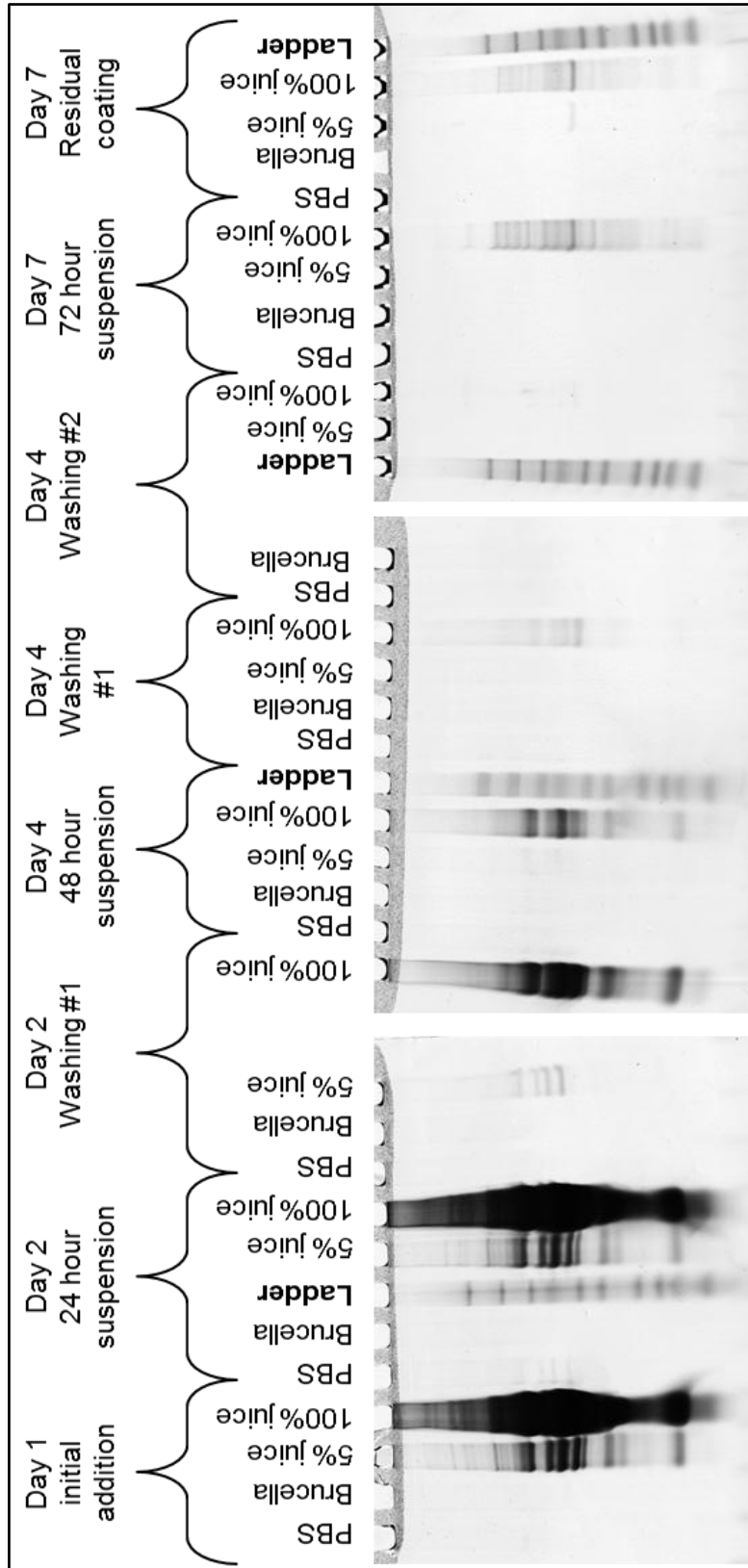


Figure 4-13 SDS-PAGE analysis of washing and incubation solutions from a pre-conditioning assay.

Representative SDS PAGE gels showing the protein detected in all the solutions of a pre-conditioning assay containing 0, 5 or 100% chicken juice solutions. No *C. jejuni* was included in this experimental set up. At all stages where cell suspensions would typically be added to the tubes, these solutions were replaced with sterile Brucella medium. This ensured that only protein from the chicken juice solutions was detected.

This observation was also confirmed by measuring protein concentration with Bradford reagent. Figure 4-14 shows a representative image of borosilicate test tubes following the final day 7, 72 hour incubation. It was observed that all the test tubes containing chicken juice (labeled 5 to 100 in Figure 4-14) contain an attached, protein rich, conditioning layer on their inner surfaces. It is however interesting to note that protein is only present in the solution of tubes which have concentrations of over 20% v/v chicken juice in Brucella medium. This suggests that at concentrations below 20%, the chicken juice has formed a tightly attached, protein rich, layer on the surface of the test tubes which is unable to be dislodged by washing or addition of new medium. At concentrations of 20% v/v juice and higher the chicken juice appears to be more loosely attached to the surface, allowing particulates to be released into the supernatant.

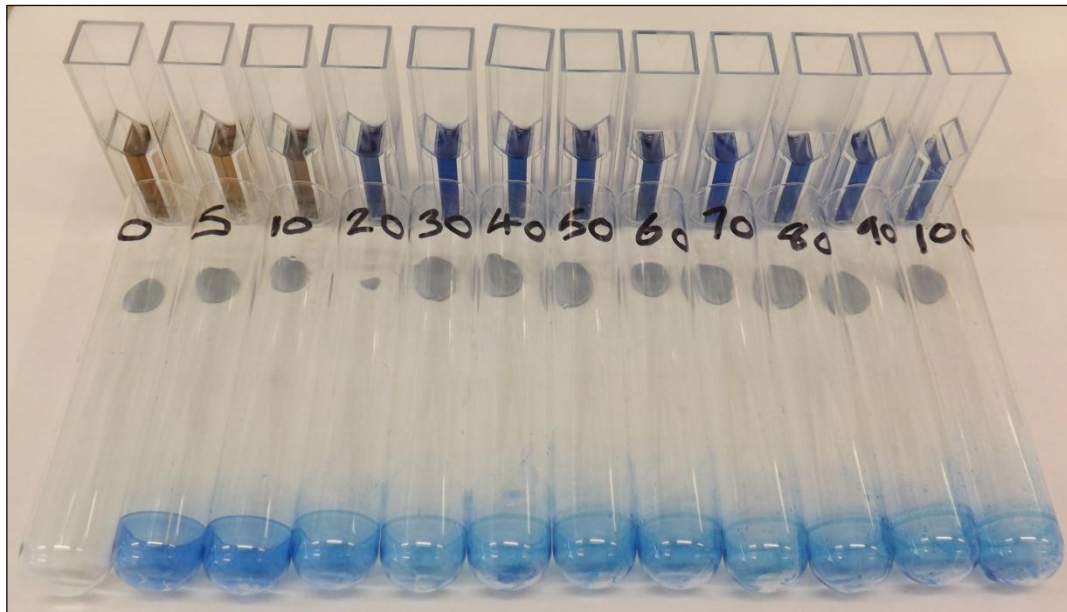


Figure 4-14 Representative image of pre-conditioned tubes containing Bradford reagent.

Image shows borosilicate test tubes following completion of the conditioning assay methodology (with sterile Brucella medium used instead of *C. jejuni* cultures). Bradford reagent was poured directly into the test tubes before removal of the suspension from the tube into a cuvette. Cuvettes contain the solution removed from the test tube below it.

4.2.9 Conditioning is also effective on stainless steel coupons

Stainless steel is a commonly used material within the food chain, and so is an important surface for bacterial attachment, biofilm formation and subsequent bacterial survival within processing plants. Sterile stainless steel coupons were incubated statically with *C. jejuni* NCTC 11168 cultures to allow biofilm formation. The cultures were either supplemented with 5% chicken juice at the start of the static incubation, or the stainless steel coupons were incubated with a 5% chicken juice solution prior to addition of the cell suspension (pre-conditioning).

In both aerobic and microaerobic conditions, supplementation of Brucella medium with chicken juice and pre-conditioning of the stainless steel coupons lead to increased biofilm formation when compared to Brucella medium alone (Figure 4-15). Since chicken juice and similar liquids are potentially present throughout the poultry processing plant this data

suggests that *C. jejuni* may be able to more easily form biofilms on soiled stainless steel surfaces than has been previously reported.

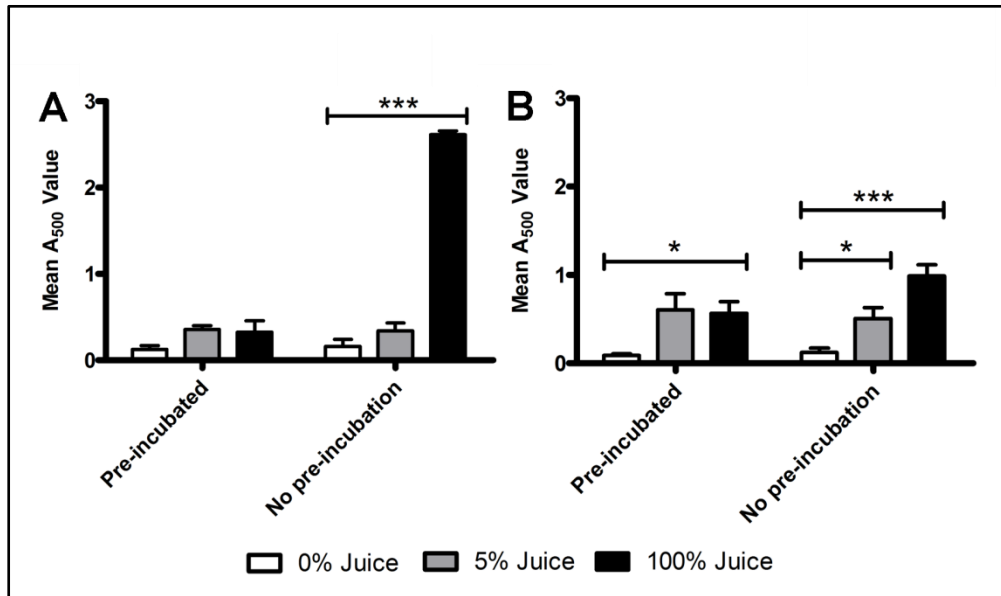


Figure 4-15 Chicken juice increases *C. jejuni* biofilm formation on stainless steel surfaces.

Biofilm formation on stainless steel coupons was measured by TTC conversion following either conditioning of the coupons with chicken juice (Pre-incubated) or by direct supplementation of the cell suspension medium with chicken juice (no pre-incubation). The experimental method was carried out in both aerobic (A) and microaerobic (B) conditions. Error bars show SE (N = 5) and significance was measured using Bonferroni post-test (* = P<0.05, *** = P<0.001).

4.2.10 Chicken juice increases biofilm formation by aflagellated *C. jejuni*

Flagella are known to contribute to attachment and biofilm formation in several bacterial pathogens (Lemon et al., 2007, O'Toole and Kolter, 1998), and previous work in *C. jejuni* has shown that an aflagellated *C. jejuni* Δ *flaAB* mutant produces significantly less biofilm than the wild-type NCTC 11168 strain (Reuter et al., 2010, Kalmokoff et al., 2006). Incubation with chicken juice or pre-coating of tubes with chicken juice both resulted in a significant increase of biofilm formation with the *C. jejuni* Δ *flaAB* mutant when compared to incubation in Brucella medium alone (Fig. 5). In the presence of chicken juice, biofilm levels were similar to that of wild-type *C. jejuni* NCTC 11168 (Fig. 5), showing that chicken juice can complement the lack of flagella and support biofilm formation by aflagellated strains. This also suggests that the effect of chicken juice is mediated through facilitating attachment, either to the abiotic surface, or chicken juice aggregates, and not via chemotactic motility.

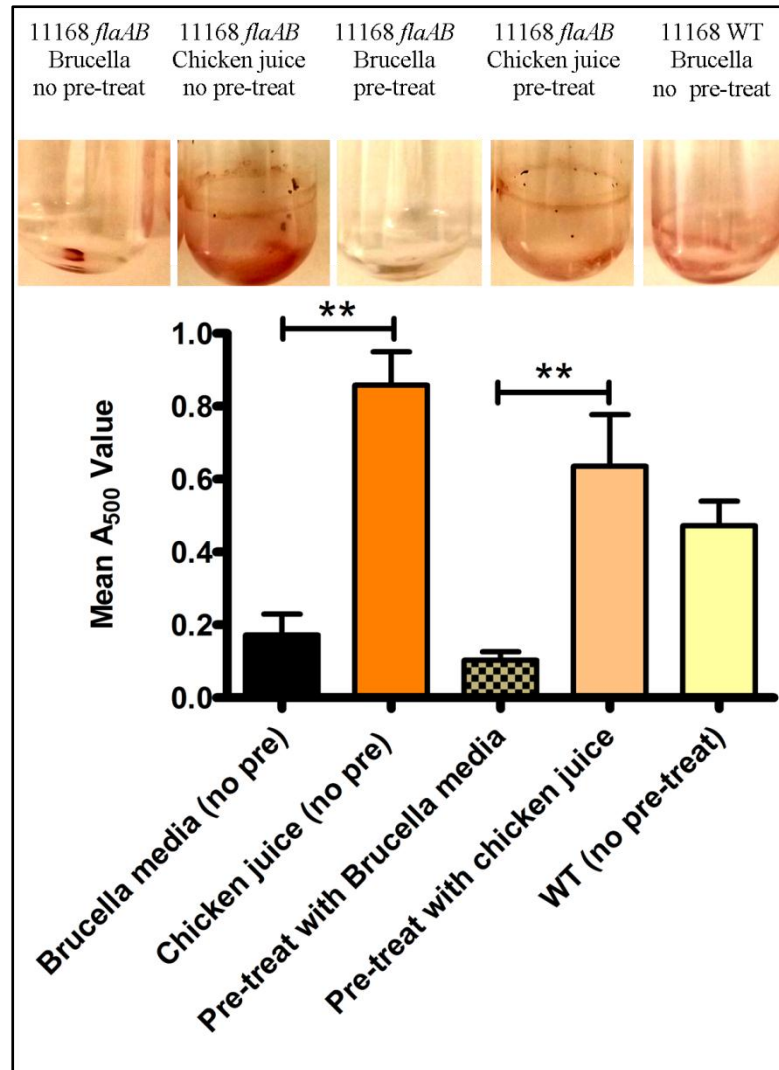


Figure 4-16 Chicken juice increases the ability of *C. jejuni* NCTC 11168 Δ *flaAB* mutants to form biofilms in static culture

Static suspensions of *C. jejuni* Δ *flaAB* mutants were incubated for 48 hours, to allow biofilm formation in various medium types, before TTC staining. Bar chart from left to right shows: *C. jejuni* Δ *flaAB* mutants in Brucella medium (with no pre-treating of the test tubes), *C. jejuni* Δ *flaAB* mutants in 100% chicken juice (with no pre-treating of the test tubes), *C. jejuni* Δ *flaAB* mutants in Brucella medium (with a 24 hour Brucella medium pre-treating of the test tubes), *C. jejuni* Δ *flaAB* mutants in Brucella medium (with a 24 hour 100% chicken juice pre-treating of the test tubes) and a *C. jejuni* NCTC 11168 wild type (WT) culture (with no pre-treating of the test tubes). Error bars show SE (N = 5) and images above the bar chart are representative of the TTC staining observed for each condition. Significance was measured using Bonferroni post-test following ANOVA analysis (** = P<0.01).

4.2.11 Pre-incubation of chicken juice to allow particulate formation reduces its ability to condition abiotic surfaces

Incubation of chicken juice at 37°C leads to the formation of large aggregates which can both confound measurement of growth (Section 3.2.5) and lead to conditioning of abiotic surfaces, as shown in this chapter. It was hypothesised that this aggregate formation was key to the surface conditioning effect. In order to test this hypothesis, assays were conducted in which chicken juice particulates were allowed to form prior to static incubation

with *C. jejuni* cells. Chicken juice was incubated for 24 hours at 37°C to allow particulate formation to occur. The juice was then centrifuged to pellet the aggregates and the supernatant used to supplement Brucella medium in static biofilm assays.

As can be seen in Figure 4-7 removal of the aggregates prior to static incubation leads to a significant reduction in biofilm formation. Pre-incubation of chicken juice not only negates its ability to increase biofilm formation but actually leads to a decrease in biofilm formation compared to that seen in Brucella medium alone.

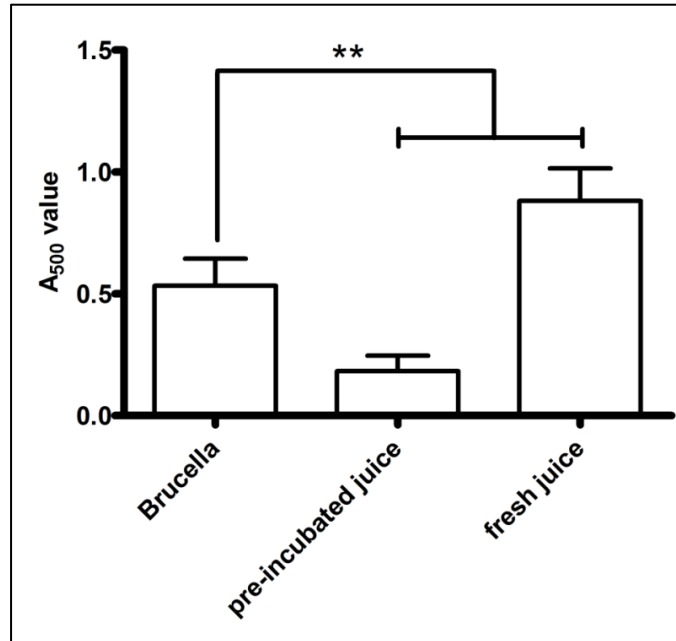


Figure 4-17 Pre-incubation of chicken juice reduces its conditioning ability.

Biofilm formation, measured by conversion of TTC to formazan crystals, is significantly reduced in biofilm cultures supplemented with pre-incubated chicken juice (incubated for 24 hours at 37 °C before use in assay) compared to biofilm formation in Brucella medium alone, or biofilm formation in cultures supplemented with 5% fresh chicken juice. Error bars show SE (N = 5) and significance was measured using Bonferroni post-test (** = P<0.01).

Secondly, chicken juice was filtered through 5, 10, 50 and 100 kDa filters to produce a solution deficient in some of the content the original juice contained. These 'juice fractions' were then used to supplement static biofilm cultures (5% v/v) and the subsequent biofilm formation was measured by TTC conversion. As can be seen in Figure 4-18 fractionation of the chicken juice lead to a reduced ability to increase biofilm formation, with fractionated juice showing no significant difference in biofilm formation compared to the Brucella medium only control. This effect could be reversed by re-combining the liquid retained above the membrane (too large to move through the membrane) with the fractionated juice, suggesting that the entire contents of the juice is required for increased biofilm formation.

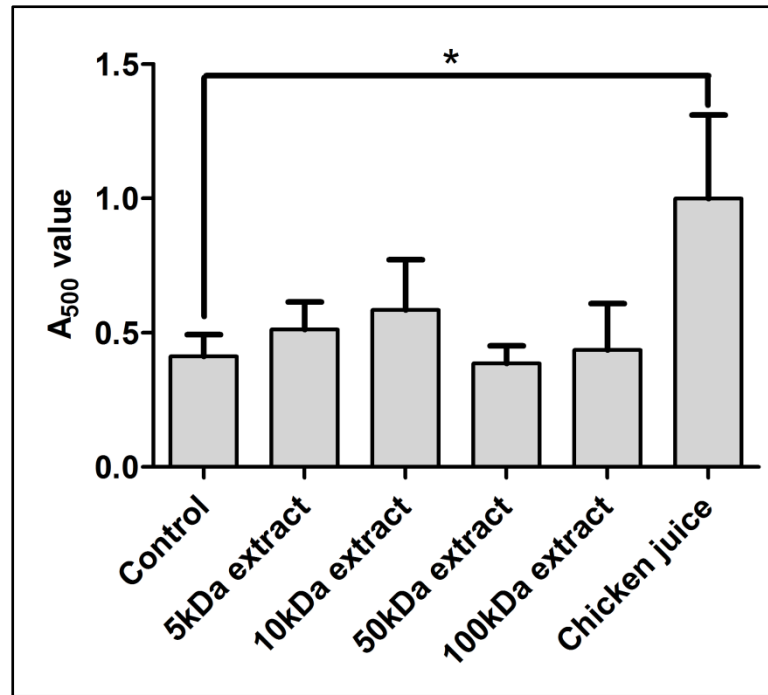


Figure 4-18 Size fractionation of chicken juice inhibits its effect on biofilm formation

Chicken juice was filtered through membranes with pore sizes of between 5 and 100 kDa and the flow through used to supplement biofilm cultures. Filtered chicken juice no longer exhibits an ability to increase biofilm formation. Graph shows data from aerobic biofilms only. Error bars show SE (N = 5) and significance was measured using Bonferroni post-test (* = $P < 0.05$), all statistical analysis was calculated by comparison to biofilms formed in Brucella medium only (control).

4.2.12 Proteinase K treatment decreases conditioning ability.

In this chapter it has been shown that chicken and other meat juices have a high protein content, which may be contributing to the conditioning effect that they have on surfaces. In order to address this question, chicken juice was treated with 30 mAU/ml of Proteinase K for three hours prior to its use in biofilm assays. Treatment of chicken juice with Proteinase K led to decreased biofilm formation. This decrease was not due to alteration of the chicken juice composition during the three hour incubation as a treatment control showed minimal difference to freshly defrosted chicken juice (Figure 4-19).

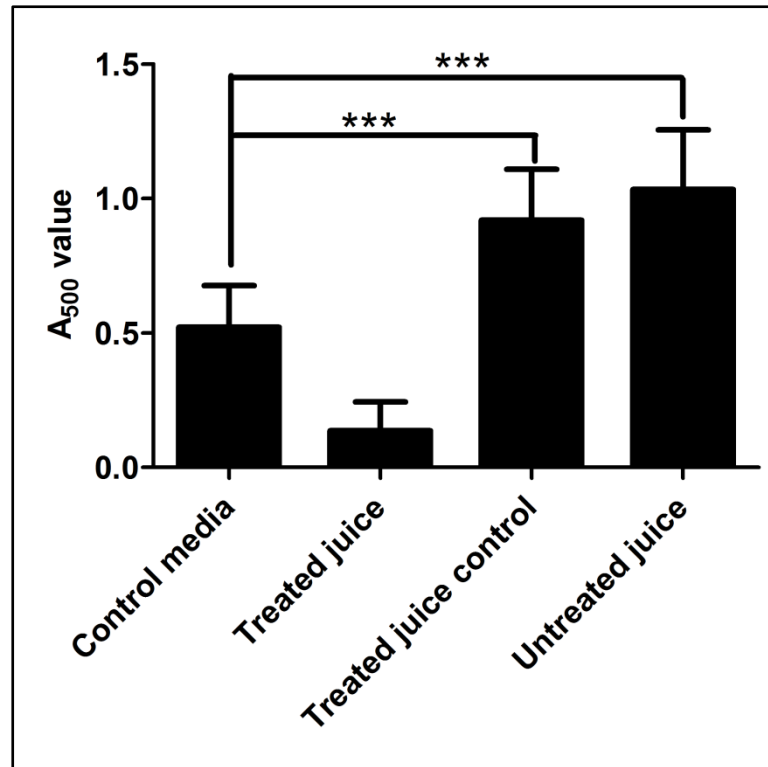


Figure 4-19 Treatment of chicken juice with Proteinase K decreases biofilm formation

C. jejuni biofilm formation in Brucella medium supplemented with untreated chicken juice, chicken juice treated with proteinase K or chicken juice undergoing a proteinase K treatment control (chicken juice with PBS added in place of Proteinase K) was measured. Figure shows data from aerobic conditions only. Error bars show SE (N = 5) and significance was measured using Bonferroni post-test (***) = $P < 0.001$). During statistical analysis, all data was compared to the biofilm formed in Brucella medium only (control).

Effective inactivation of Proteinase K is by heat inactivation at 65°C for 10 minutes. This could not be achieved for Proteinase K which had been added to the chicken juice as the heat inactivation led to the creation of large aggregates within the juice, which in turn affected its conditioning ability (Figure 4-17). This meant that once treated, the chicken juice was added to the biofilm containing a small volume of active Proteinase K (approximately 1.5 mAU total within the cell suspension). This proteinase K may also effect the ability of *C. jejuni* to form biofilms by directly interacting with the bacterial cells and any ECM produced by them, therefore confounding measurement of the effect of protein reduction in chicken juice on biofilm formation.

It should however be noted that the difference between biofilms grown in chicken juice treated with proteinase K and those grown in Brucella medium only were not statistically different. Figure 4-5 also shows that *C. jejuni* strain RM1221 also shows significant levels of biofilm formation in cultures supplemented with 5% chicken juice. This increase is despite its production of three predicted extracellular DNase proteins, which significantly inhibit its ability to form biofilms in un-supplemented Brucella medium (see 7 for further details of *C. jejuni* DNase genes). This suggests that the action of enzymes on the extracellular matrix may be reduced in chicken juice due to the conditioning of the surface and the integration of the *C. jejuni* cells into this conditioning layer, leaving the ECM less exposed. This is further addressed in Section 6.2.5.

4.3 Discussion

The potential role of meat exudates on *C. jejuni* biofilm formation was investigated in this chapter, and it was shown that chicken juice is able to enhance biofilm formation when compared to Brucella medium. In an industrial food setting, this means that the presence of meat exudates can aggravate the problem of contamination by food-borne pathogens such as *C. jejuni*. Within the food chain, biofouling is an important area of study as it contributes to increased biofilm formation, loss of heat transfer efficiency and reduced liquid flow in pipes (Agle, 2007). Meat exudates may play a significant role in enhancing survival of *C. jejuni* by increasing surface adhesion and by supplying a scaffold containing nutrients and materials on which the bacteria are able to form a biofilm.

The food chain is very complex and dynamic, containing various bacterial contamination sources, and a variety of environmental conditions and nutrient sources (Habimana et al., 2010). *In vitro* laboratory studies allow for a reductionist approach, controlling variables to assess the effect of specific conditions, material or genes on biofilm formation, however a middle ground must be found in which experimental set-up allows control but reflects the food chains complexity. The chicken juice system (Birk et al., 2004) is one method of experimenting with food-chain relevance in a laboratory setting. Chicken juice more accurately reflects the conditions in the food chain, but is easy to manipulate and reproducible.

This work also adds to the body of evidence that several food relevant compounds are able to form conditioning layers, by their ability to increase biofilm formation in various food relevant bacteria. Bacterial soil increases *L. monocytogenes* survival on surfaces (Van Houdt and Michiels, 2010), while milk residues and chicken fillet suspension increase survival of planktonic *S. enteritidis* and *C. jejuni* on stainless steel (Kusumaningrum et al., 2003) and whey protein and casein are important for *Cronobacter* biofilm formation (Healy et al., 2010). Although many animal macromolecules have been reported to be able to form a conditioning film, they are not always able to enhance biofilm formation. Bovine serum albumin reduces biofilm formation in *S. aureus* (Xu et al., 2008) and ***B. cepacia*** (Hwang et al., 2012) and skimmed milk and milk albumin inhibit biofilm formation by *Cronobacter* (Barnes et al., 1999).

The results in this chapter suggest that as the concentration of chicken juice increases the method of attachment to the surface alters. A two layer model is proposed (Figure 4-20) in which chicken juice is able to both firmly attached to the abiotic surface itself, but also form a second, more loosely attached, conditioning film on top of the firmly attached film. At low concentrations of chicken juice, only the firmly attached layer is present (Figure 4-20a). This layer covers the whole surface in contact with the chicken juice solution. *C. jejuni* is able to utilise this conditioning layer to more easily attach to the surface, increasing biofilm formation. As the concentration of chicken juice increases $\geq 20\%$ v/v, the surface binding sites are saturated and a second, more loosely attached, layer is formed on top of the firmly attached layer (Figure 4-20b). This layer is not able to form a firm attachment to the surface, as binding sites on the surface have reached saturation. This second layer also contributes to biofilm formation by *C. jejuni* but due to its looser association with the surface it is able to become dislodged from the surface and move into the liquid phase. Here it is able to not only provide a source of contamination, since *C. jejuni* is likely to still be attached to the detached particulates, but can also provide an additional food source for the planktonic bacteria, contributing to their increased growth.

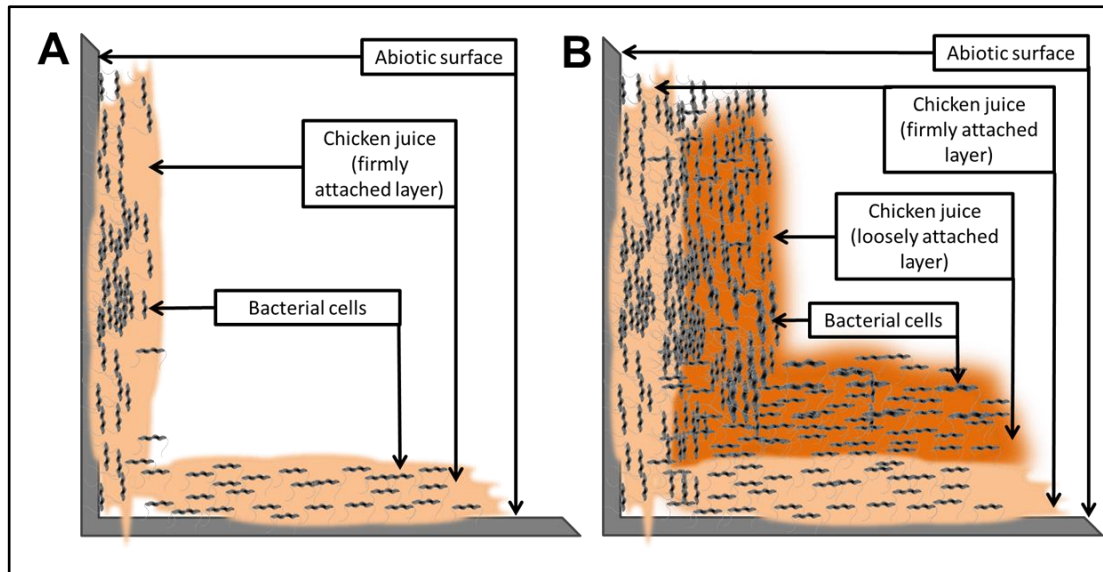


Figure 4-20 Diagrammatic representation of chicken juice attached to a surface and its impact on *C. jejuni* biofilm formation.

Chicken juice is able to form two layers on an abiotic surface. This first layer (A) is firmly attached to the surface and cannot be easily removed. *C. jejuni* is able to attach to the chicken juice on the surface more easily than the surface itself and so preferentially forms biofilms in areas where the chicken juice covers the surface. The second layer (B) is more loosely attached and can be shed into the supernatant both releasing cells from the biofilm and providing an additional food source for planktonic cells.

The exact composition of the chicken juice has not yet been investigated, but chicken juice fractions of <100 kDa, <50 kDa, <30 kDa, <10 kDa and <3 kDa did not individually increase biofilm formation, nor did juice which had been pre-incubated to allow particulate formation, or that treated with Proteinase K. This suggests that the conditioning layer produced by the chicken juice is not just comprised of a single monomeric protein or metabolite, but instead conditioning is a complex, multicomponent effect and further investigation is needed to identify the exact components of the chicken juice that contribute to surface conditioning and biofilm formation. It is also well reported that factors such as surface roughness and hydrophobicity also affect bacterial attachment and biofilm formation (Teughels et al., 2006). Hydrophilic surfaces, such as stainless steel and glass, increase the time required for bacterial attachment and biofilm formation (Agle, 2007). Surface microstructure is also capable of affecting protein absorption, as shown in a study by (Singh et al., 2011), again leading to variability in surface conditioning and subsequent biofilm formation.

It should also be noted that although the chicken juice was filtered to ensure its sterility before use, the pore size of the filter would have allowed the passage of phages. It has previously been reported that *C. jejuni* bacteriophages are found within the chicken ceca (Atterbury et al., 2005) and on carcasses (Atterbury et al., 2003), so their presence in chicken juice should also be assumed. No loss in *C. jejuni* viability was observed, with growth of *C. jejuni* NCTC 11168 cultures in chicken juice showing similar or higher A_{500} values compared to Brucella Medium only. This suggest that the lytic activity of *C. jejuni* specific lytic phages was minimal in the data presented here, but further investigation should be carried out to assess the quantity and effect of *C. jejuni* bacteriophages in chicken juice.

We have demonstrated that biofilm formation in *C. jejuni* NCTC 11168 Δ *flaAB* mutants is also enhanced following pre-conditioning of test tubes with chicken juice (Figure 4-16). These mutants are aflagellated and hence non-motile, so unable to migrate towards food sources. This means that any increase in their attachment, or subsequent biofilm formation, must be due to alteration of the glass surface properties by the conditioning layer from the chicken juice, rather than due to increased chemotactic or energy taxis-directed motility towards a food source. The flagellum is known to be important in biofilm formation of many food chain relevant bacteria. Examples include *L. monocytogenes*, where over the first four hours of biofilm formation, aflagellate mutants have a tenfold lower level of attachment to stainless steel coupons (Vatanyoopaisarn et al., 2000). The majority of biofilm studies assessing the importance of flagella in biofilm formation are performed without the presence of conditioning surfaces; however it is proposed that future studies may wish to consider the effect of conditioning layers on biofilm formation and bacterial attachment.

In conclusion, although the notion of conditioning layers within the food chain is not novel, investigation of the literature shows that this is the first study measuring the effects of chicken juice on *C. jejuni* and *C. coli* biofilm formation, as well as investigating the capacity of chicken juice to condition food-chain relevant abiotic surfaces. Chicken juice allows increased attachment of *C. jejuni* as it attaches to the surface of the test tubes, providing a conditioned surface for the bacteria to adhere to. This conditioning surface is still present following a simple washing procedure and able to increase biofilm formation if the subsequent incubation with bacteria lacks chicken juice in the medium. Chicken juice also provides a suitable laboratory model for the study of *C. jejuni* biofilm formation in the food chain; allowing investigators to more closely mimic the food chain conditions that lead to *C. jejuni* spread and cross contamination of carcasses. Furthermore, identification of the chicken juice components involved in surface conditioning and bacterial attachment may give the opportunity for targeted intervention and prevention strategies to reduce transmission of *C. jejuni* and *C. coli* through the food chain.

5 Development of *Campylobacter jejuni* NCTC 11168 biofilms in food chain relevant conditions

SEM images were produced by K Cross and L Salt, NRP Bioimaging group.

5.1 Introduction

Within the last ten years there has been a shift the focus of *C. jejuni* biofilm studies. The majority of investigations now focus on the genetic factors responsible for biofilm formation, rather than how the environment is able to contribute to the initiation of biofilm formation by *C. jejuni*. Investigation of environmental factors in *C. jejuni* biofilm formation to date has been by no means exhaustive, and environmental factors contributing to biofilm formation are of particular interest to those attempting to control biofilm formation within the food chain (Speranza et al., 2011). Previous studies have shown that *C. jejuni* biofilm formation is increased in aerobic conditions (Reuter et al., 2010), low temperatures (Buswell et al., 1998, Dykes et al., 2003), and under nutritional stress (Reeser et al., 2007). Biofilm initiation and shedding have also been neglected, although they remain important areas of study since biofilm initiation allows establishment of populations in previously uncontaminated areas (Brooks and Flint, 2008) and shedding from biofilms may contribute to contamination of food and other processing plant areas (Chmielewski and Frank, 2003).

Biofilm initiation, maturation and shedding has been investigated in this chapter, with particular reference to the contribution of environmental factors such as nutrient and aerobic stress which are encountered by *C. jejuni* within the food chain. *C. jejuni* strain NCTC 11168 was selected since it is a well characterised strain (Parkhill et al., 2000, Hong et al., 2014, Revez et al., 2012, Chaudhuri et al., 2011) which has previously been shown to exhibit biofilm formation in food chain relevant conditions (Reuter et al., 2010, Gunther and Chen, 2009, Hanning and Slavik, 2009, Howard et al., 2009, Siringan et al., 2011). Here it is shown that a minimum number of bacterial cells are required to initiate biofilm formation in aerobic conditions. Aerobic conditions also led to accelerated biofilm formation and biofilm lifestyle was shown to contribute to an extension of time that cells were metabolically active. Finally, shedding of viable cells from the biofilm was also observed following washing, indicating that biofilms are a source of bacteria which are potentially able to contaminate and persist within the food chain.

5.2 Results

5.2.1 Biofilm formation is effected by variations in initial cell density.

Although the number of *C. jejuni* is known to be high within the avian caecum (Bai et al., 2014), numbers throughout the processing plant and on carcasses are generally reported to be much lower, and *C. jejuni* numbers vary throughout the processing plant (Baré et al., 2013). It is therefore important to establish what cell density is required for the initiation of *C. jejuni* biofilm formation. *C. jejuni* NCTC 11168 was diluted to OD₆₀₀ values between 0.001 and 1 and incubated statically for 48 hours in both aerobic and microaerobic conditions. All of the cell dilutions were prepared following centrifugation of overnight *C. jejuni* cultures and resuspension in fresh Brucella medium. This additional step was performed in order to minimise the influence that nutrient starvation may play in establishment of biofilm (due to increased volumes of spent medium within the cell cultures as the cell numbers increased).

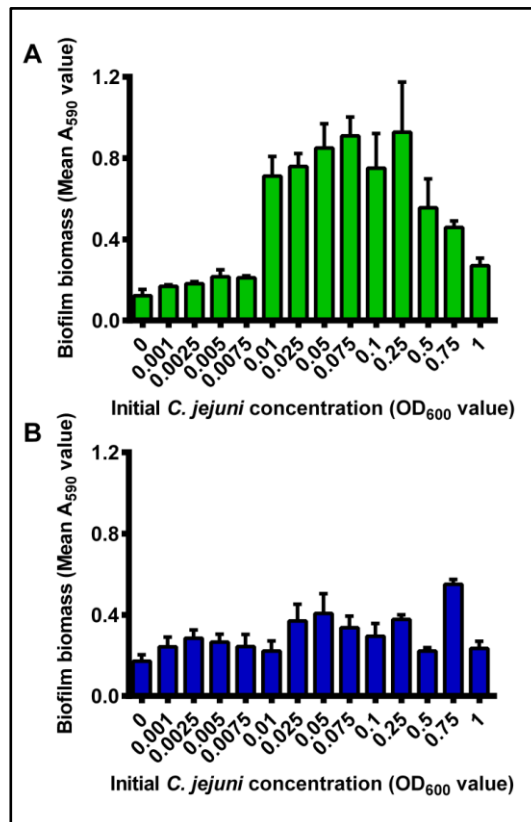


Figure 5-1 Initiation of biofilm formation in aerobic conditions is not observed at OD₆₀₀ concentrations below 0.01.

Overnight cultures of *C. jejuni* NCTC 11168 were diluted to OD₆₀₀ concentrations of between 0.001 and 1 before static incubation for 48 hours in either aerobic (A) or microaerobic (B) conditions. To allow quantification of biofilm biomass the biofilms were stained with crystal violet following incubation. Although in microaerobic conditions levels of crystal violet staining (assessed by measuring absorbance at 590 nm) were relatively static at all concentrations, a sharp increase in staining was observed in aerobic conditions at OD₆₀₀ concentrations of between 0.01 and 0.05. Error bars show SE (N = 5).

In aerobic conditions, statistically significant biofilm formation was not observed at OD₆₀₀ values below 0.01 (Figure 5-1a), and no visible crystal violet staining of test tubes could be

observed below this concentration either (Figure 5-2). A statistically significant increase in staining was observed between aerobic and microaerobic cultures ($P = \leq 0.05$) at OD_{600} values of between 0.01 and 0.05. In microaerobic conditions, although staining could clearly be seen at the air liquid interface of the test tubes at all concentrations (Figure 5-2), this did not translate to a statistically significant increase in crystal violet staining compared to a microaerobic Brucella medium only control (Figure 5-1b).

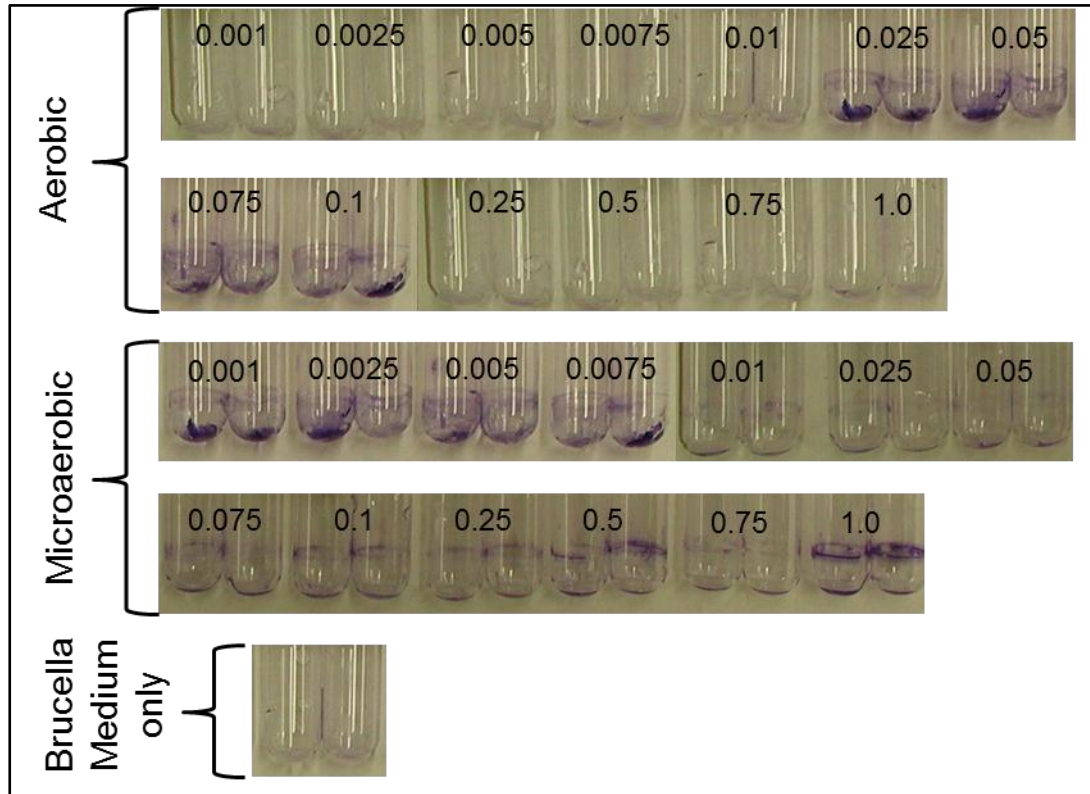


Figure 5-2 Biofilm formation is present at all *C. jejuni* concentrations in microaerobic conditions

Representative images of duplicate borosilicate test tubes stained with crystal violet following a 48 hour static incubation in either aerobic (top two panels) or microaerobic (middle two panels) conditions with varying concentration of initial *C. jejuni* suspension (digits above the duplicate tubes indicate the OD_{600} value of the cell suspension at the start of the static incubation). Crystal violet staining suggests biofilm has formed at the air liquid interface in all microaerobic tubes, regardless of concentration. Tubes incubated in aerobic conditions only appear to have biofilm formation where initial concentrations of between OD_{600} 0.025 and 0.1 were used.

Biofilm biomass in aerobic conditions remained relatively constant at OD_{600} concentrations of between 0.01 and 0.05, although at higher concentrations (above OD_{600} 0.75) a decrease in levels of biofilm staining was observed. This decreased biofilm biomass at higher concentrations was not due to a decline in MPN since assessment of the planktonic phase of the static cultures showed comparable MPN values to those found at concentrations which did allow increased biofilm formation (Figure 5-3). At OD_{600} concentrations of less than 0.01 cell viability did appear to influence biofilm formation, since few viable cells could be found following 48 hours static incubation in aerobic conditions.

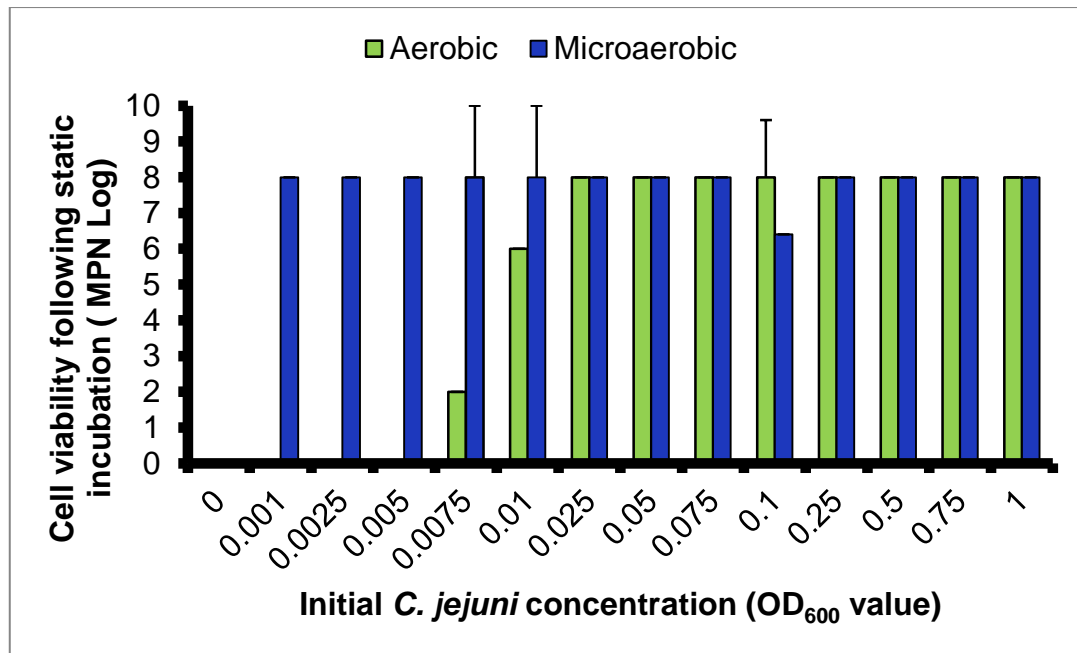


Figure 5-3 Viability of planktonic fraction following 48 hours of static incubation.

Overnight cultures of *C. jejuni* NCTC 11168 were diluted to OD₆₀₀ concentrations of between 0.001 and 1 and incubated statically for 48 hours before the MPN assessment of the planktonic cell suspension was assessed. MPN in both aerobic (green bars) and microaerobic (blue bars) conditions is displayed. Error bars show SE (N =5).

In microaerobic conditions a different trend in biofilm biomass was observed, with viable cells detected even at low concentrations and some biofilm, indicated by crystal violet staining at the air-liquid interface, apparent at all concentrations in microaerobic conditions (Figure 5-2). Taken together these results suggest that, although biofilm formation is ubiquitous in microaerobic conditions, a minimum cell density is required for survival in aerobic atmospheres. Once this concentration is reached, biofilm formation is initiated and enhanced compared to its microaerobic counterpart. Whereas in microaerobic conditions, although more cells are able to survive and attach to the surface, even where low numbers of *C. jejuni* are present, biomass is not as high as that found in aerobic conditions. This difference in biomass is potentially due to increased production of ECM by *C. jejuni* in aerobic conditions.

5.2.2 Oxygen concentration effects the structure of mature *C. jejuni* NCTC 11168 biofilms

SEM analysis was undertaken on mature (48 hour) biofilms grown on sterile plastic coverslips within borosilicate glass test tubes. Image analysis showed that in microaerobic conditions *C. jejuni* was able to attach to the surface, producing a single layer attachment. There was little evidence of ECM production, and micro-colony formation is also reduced (Figure 5-4a, b and c). In contrast, static incubation in aerobic conditions led to significant production of ECM, with microcolonies frequently observed (Figure 5-4d, e and f). Aerobic images appear to show micro-colonies growing in a 'honey-comb' grid-like structure, and it is hypothesised that as the micro-colonies mature and increase this grid closes up to form the single layer of biofilm observed in Figure 5-4d.

Spatial arrangement of cells, rather than the cell density, appears to be the major difference between aerobic and microaerobic conditions. Cells within aerobic cultures associate closely with each other, forming micro-colonies which contain 'string-like' particulates hypothesised to be ECM. In contrast the cells attached to surfaces incubated in microaerobic conditions are more sparsely spread across the abiotic surface, binding directly to it and showing little or no micro-colony formation. The images show in Figure 5-4 indicate that *C. jejuni* is able to attach to abiotic surfaces in microaerobic conditions, however it is still unclear from these images if microaerobic conditions allow a progression beyond this initial attachment stage to biofilm formation when incubated with Brucella medium at 37°C.

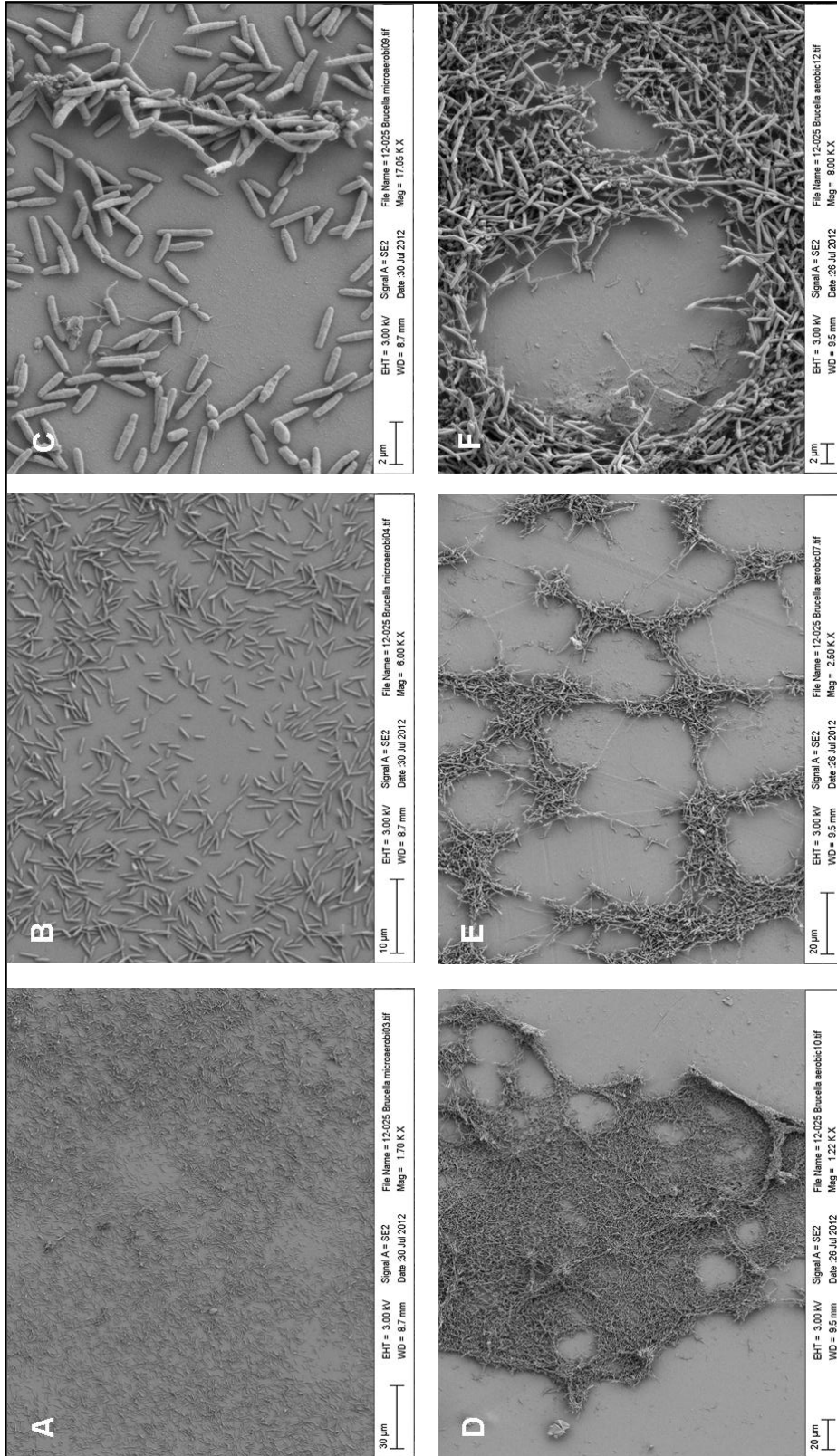


Figure 5-4 SEM imaging of mature *C. jejuni* NCTC 11168 biofilms in aerobic and microaerobic conditions show morphological differences

Comparison of representative SEM images of mature (48 hour) *C. jejuni* NCTC 11168 biofilms in microaerobic (images A, B and C) and aerobic (images D, E and F) conditions show several differences in morphology. In microaerobic conditions single cells can be observed on the surface, whereas in aerobic conditions structured microcolonies, indicative of a more mature biofilm, are present. Enlarged images can be found in Appendix 3.

It should however be noted that although SEM shows little evidence of biofilm formation in microaerobic conditions using Brucella medium only, supplementation of the static cultures with chicken juice (Figure 5-5a) or the presence of particulates on the abiotic surface (Figure 5-5b), allows biofilm formation to occur in a similar fashion to that seen in aerobic cultures. The observed increase is likely due to the altered surface conditions allowing easier attachment of *C. jejuni*, increasing the speed of microcolony formation and ECM production.

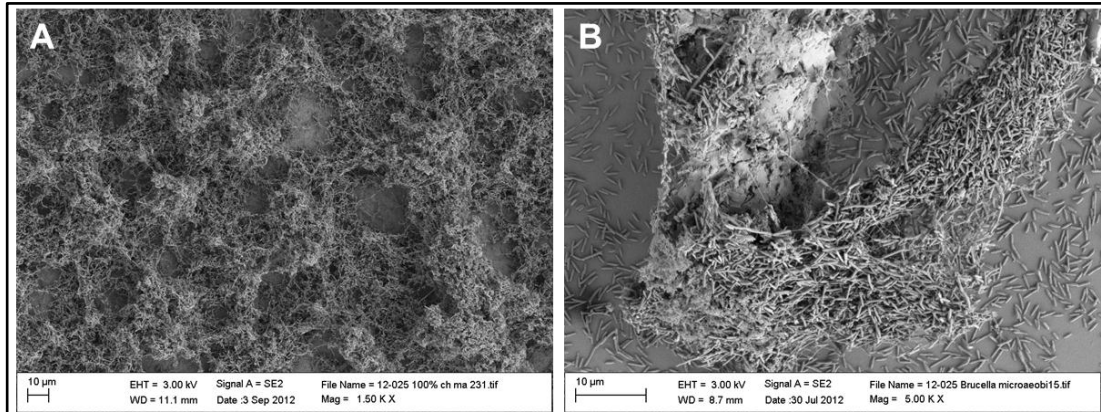


Figure 5-5 Representative images of biofilm formation in microaerobic conditions on enhanced surfaces.

Representative SEM images of biofilm formation by *C. jejuni* NCTC 11168 in chicken juice (A) or in the proximity of debris on the abiotic surface in Brucella medium static cultures (B). All static incubations were carried out at 37°C in microaerobic conditions. Biofilms were formed on plastic cover slips inserted vertically into a borosilicate test tube. In contrast to other microaerobic static cultures, here cells appear to be spatially organised and ECM can be observed. Enlarged images can be found in Appendix 3.

5.2.3 Quantification of biofilms indicates that its biomass does not increase in a linear fashion.

It is currently unknown if, following their formation, *C. jejuni* biofilms continually increase in mass over their lifespan or reach a state of equilibrium, where the amount of new biofilm created is equal to the volume of biomass lost via shedding and dispersal processes. Staining of the biofilm over an extended time course was carried out to assess this question. *C. jejuni* NCTC 11168 was incubated statically for 24, 48, 72 or 96 hours, to allow biofilm formation, before quantification of biomass by crystal violet staining (Figure 5-6).

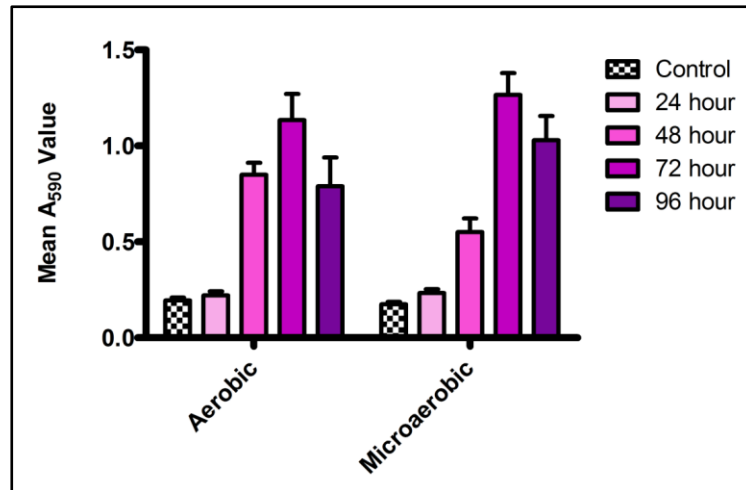


Figure 5-6 Biofilm quantity increases over a 96 hour period.

Quantification of biofilm formation by crystal violet staining suggests that in both aerobic and microaerobic conditions the biofilm biomass reaches a peak at 72 hours, after which a decline in biofilm biomass occurs. The control sample, included to show basal staining levels, contained sterile Brucella medium incubated for 96 hours. Error bars show SE (N = 5).

Following 24 hours of incubation there is no statistically significant difference in crystal violet quantification between a Brucella medium only control (control) and the 24 hour biofilm, although a thin band of staining can clearly be seen at the air liquid interface of tubes incubated in both aerobic and microaerobic conditions (Figure 5-7). These results indicate that biofilm is present at the air-liquid interface at 24 hours, but is not able to be determined by measurement of crystal violet stain density alone. This inability to measure the 24 hour samples is likely due to the high background staining level of crystal violet.

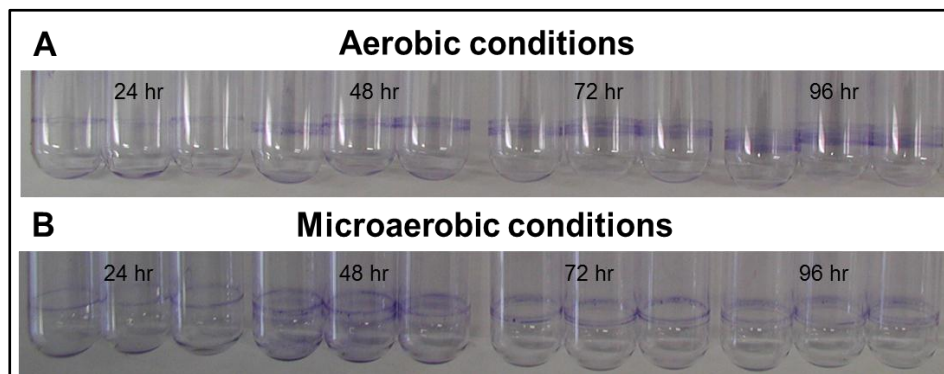


Figure 5-7 Biofilm formation can be observed at all time-points tested in both aerobic and microaerobic conditions.

Representative examples of crystal violet stained, biofilm containing borosilicate test tubes after either 24, 48, 72 or 96 hours of static incubation at 37 °C in either aerobic (A) or microaerobic (B) conditions. Crystal violet staining, indicating biofilm formation, can be observed at all time points and in all conditions, although staining is less apparent in the 24 hour samples than those of later time points.

Biofilm formation is greatly increased at 48 hours ($P = <0.01$ in aerobic conditions and $P = <0.05$ in microaerobic conditions) when compared to the Brucella medium control. Crystal violet staining of the biofilm peaked at 72 hours in both aerobic and microaerobic samples,

before reducing slightly at 96 hours. In aerobic conditions this decrease also correlated with a decrease in MPN (Figure 5-8), which could in part contribute to the lack of additional biofilm formation from 72 hours onwards.

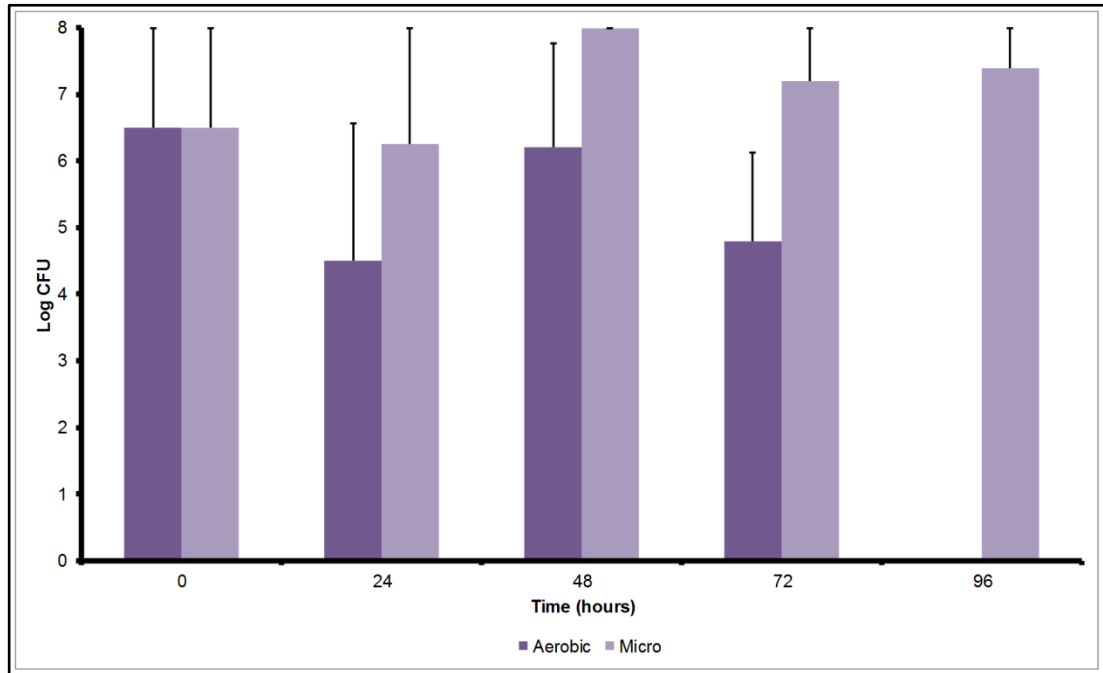


Figure 5-8 Quantification of MPN in biofilm suspensions indicates cell viability in aerobic conditions decreases after 72 hours

C. jejuni NCTC 11168 cultures were incubated statically for 24, 48, 72 and 96 hours at 37 °C in either aerobic (pale purple) or microaerobic (dark purple) conditions. At 24 hour intervals the viability of the culture was assessed by sampling the planktonic fraction. Although MPN in microaerobic cultures remains relatively constant, it decreases rapidly between 72 and 96 hours in biofilm cultures incubated aerobically, and no viable cells can be detected following a 96 hour incubation in aerobic conditions. Error bars show SE (N = 5).

5.2.4 *C. jejuni* biofilm formation progresses more rapidly in aerobic conditions

It has previously been reported that *C. jejuni* NCTC 11168 biofilm formation, measured by crystal violet staining of biofilms, is increased in aerobic conditions (Reuter et al., 2010). The same has also been observed here, where biofilms are cultured for 48 hours or less. As discussed in Section 3.2.1, crystal violet is a non-specific stain, able to stain cells, ECM and components of the medium. This means that information about alterations in cell densities or ECM production cannot be determined by crystal violet staining alone. In order to better define the observed increase in biofilm formation, static cultures were incubated for set times and investigated using light microscopy, alongside quantification of MPN within the planktonic phase.

In aerobic conditions biofilm formation appears to progress rapidly over the first 48 hours (Figure 5-9). Following 12 hours of incubation in aerobic conditions, both single cell attachments and progression to microcolony development can be observed (Figure 5-9 top right hand image). Cells and microcolonies show some spatial organisation, with microcolonies appearing to be elongated along the 'north-south axis'. In the images shown in Figure 5-9 and Figure 5-12, this axis corresponds to the air/liquid interface, with the air being on the right hand side of the image and the liquid on the left. Slides incubated in

microaerobic conditions show little evidence of cellular attachment to the glass surface until 24 hours (Figure 5-9 middle left hand image), at which point they show a very similar structure to aerobic biofilms at the 12 hour time point, containing attached single cells and small microcolonies.

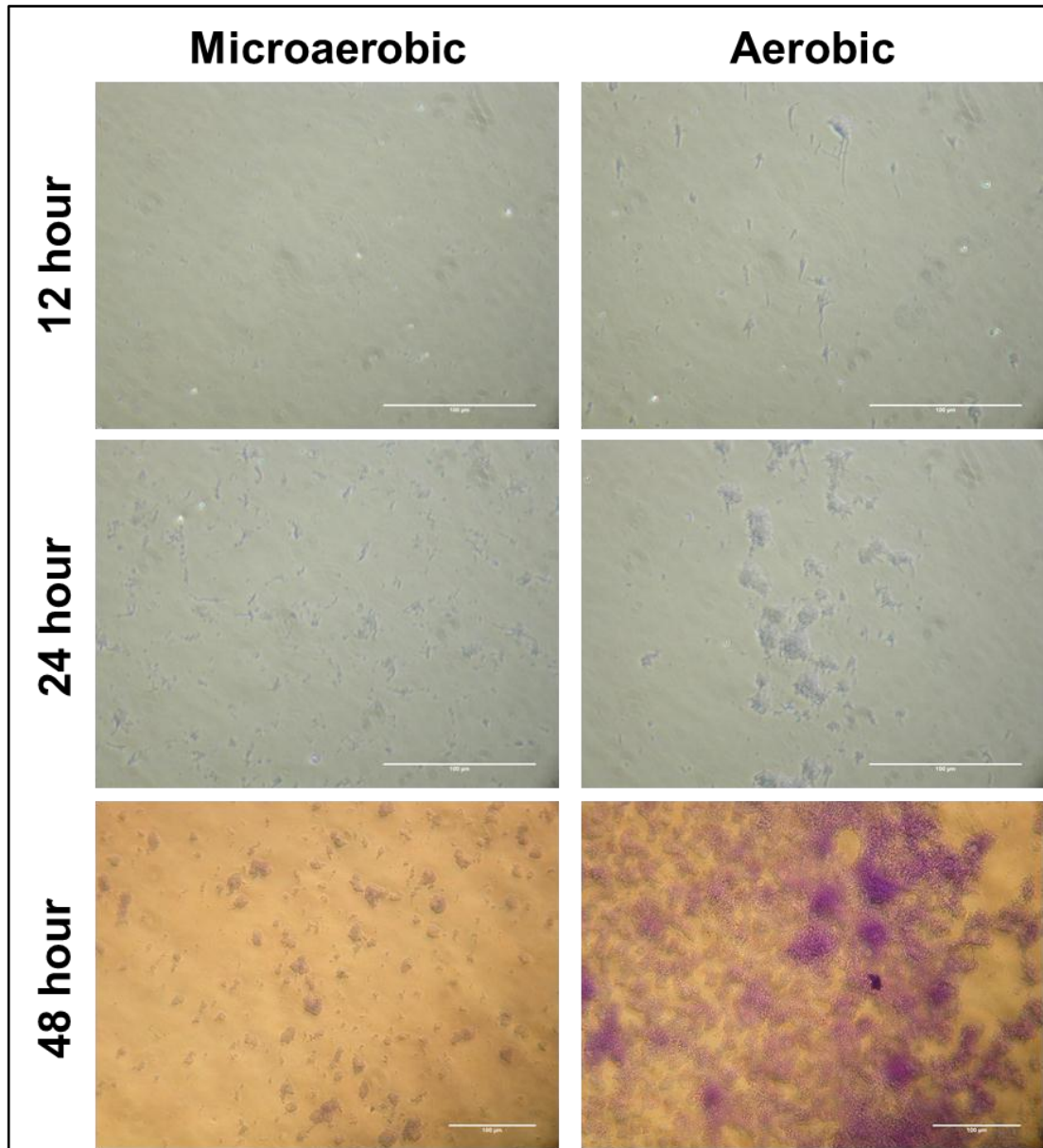


Figure 5-9 *C. jejuni* biofilm formation progresses more rapidly in aerobic conditions

Representative images of *C. jejuni* NCTC 11168 biofilms at the air/liquid interface following 12 (top row), 24 (middle row) or 48 (bottom row) hours of static incubation at 37°C in either microaerobic (left hand column) or aerobic (right hand column) conditions. All slides were dried and crystal violet stained before imaging. Images show a progression through the stages of biofilm maturation for both atmospheric conditions, although this progression appears to be accelerated where biofilms are cultured in aerobic conditions. Scale bar represents 100 µm. Enlarged images can be found in Appendix 3

At 24 hours (Figure 5-9 middle right image), large microcolonies are clearly visible in aerobic conditions, showing significant expansion and amalgamation compared to the 12 hour image. Although the immature biofilm is visible by light microscopy at 24 hours, it is not visible to the naked eye, even following crystal violet staining (Figure 5-10).

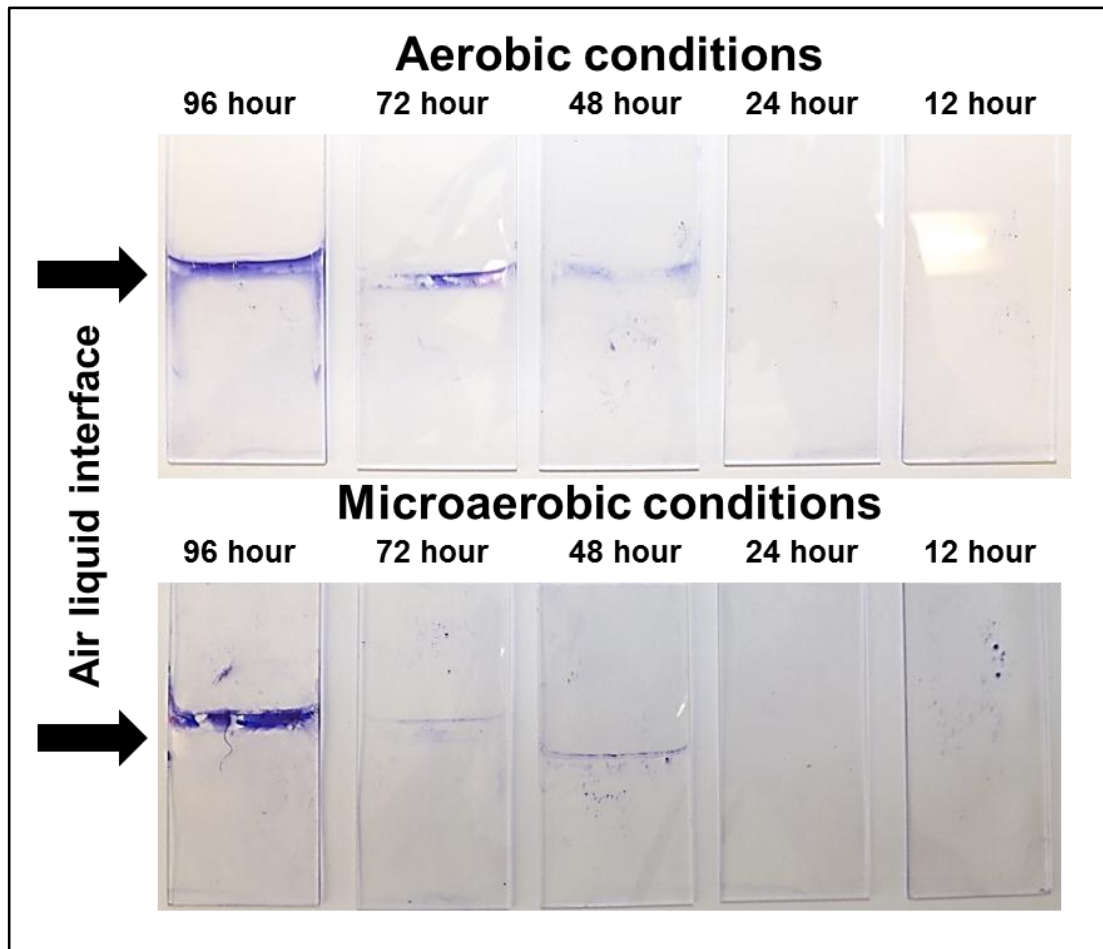


Figure 5-10 Biofilm formation is not visible by eye until static incubation has occurred for 48 hours or more.

Representative images of borosilicate glass slides following static incubation with *C. jejuni* NCTC 11168 cultures for between 12 and 96 hours in either aerobic (top panel) or microaerobic (bottom panel) conditions. No staining is visible to the naked eye on either the slides incubated for 12 or 24 hours (in either atmospheric condition), however crystal violet staining is clearly visible following a 48 or more hour incubation, indicating an increased biofilm biomass.

In aerobic conditions ECM can clearly be seen at 24 hours, appearing above the centre of the microcolonies rather than surrounding them. This positioning increases the density of the microcolony, rather than their diameter (Figure 5-11).



Figure 5-11 Image of 24 hour old *C. jejuni* NCTC 11168 microcolonies formed in aerobic conditions.

Representative image of a semi-mature (24 hour) biofilm formed on a sterile glass slide and incubated at 37°C in aerobic conditions. The image has been manipulated using IrfanView software to allow better visualisation of the microcolonies and positioning of the ECM within the microcolonies. The original image (inset) was converted to a negative grey scale version of the original. Enlarged version of the images can be found in Appendix 3. The images shows not only *C. jejuni* cells attached to the surface of the slide, but also appears to show ECM build-up, particularly on top of microcolonies.

Although Figure 5-6 clearly shows that biofilm formation is increased in aerobic conditions at 24 and 48 hours compared to microaerobic incubations, it is unclear from crystal violet staining of biofilm containing test tubes if this increase is due to additional ECM production,

attachment of bacteria or a combination of the two. The images in Figure 5-9 suggest that the increase observed is due to a combination of increased single cell attachment, microcolony formation, and ECM production in aerobic conditions. In addition, the biofilms appear much more structurally defined, with microcolonies forming and maturing in a narrow area. Microcolonies appear to increase in density (observable by their increased retention of the crystal violet stain and deeper colour) both due to expansion of the cellular population, and the production of ECM (Figure 5-9 and Figure 5-11). These observations complement those presented in Section 5.2.2, providing additional information about the production and placement of ECM. Information about the ECM could not be discerned from SEM images alone since the preparation of samples for SEM imaging leads to their dehydration, deforming the ECM.

At later time points, 48 hours onwards, biofilm formation in aerobic and microaerobic conditions is much more similar, with both conditions producing thick, mature biofilms at the air liquid interface (Figure 5-12). At 96 hours the biofilm had become so thick that its imaging by light microscopy was confounded, and distinguishing individual features of the biofilm became difficult in all areas except the edges of the air-liquid interface. Biofilm formation in microaerobic conditions progressed quickly between 48 and 72 hours, with a rapid expansion of microcolonies and large amounts of ECM produced (Figure 5-12 top and middle left images).

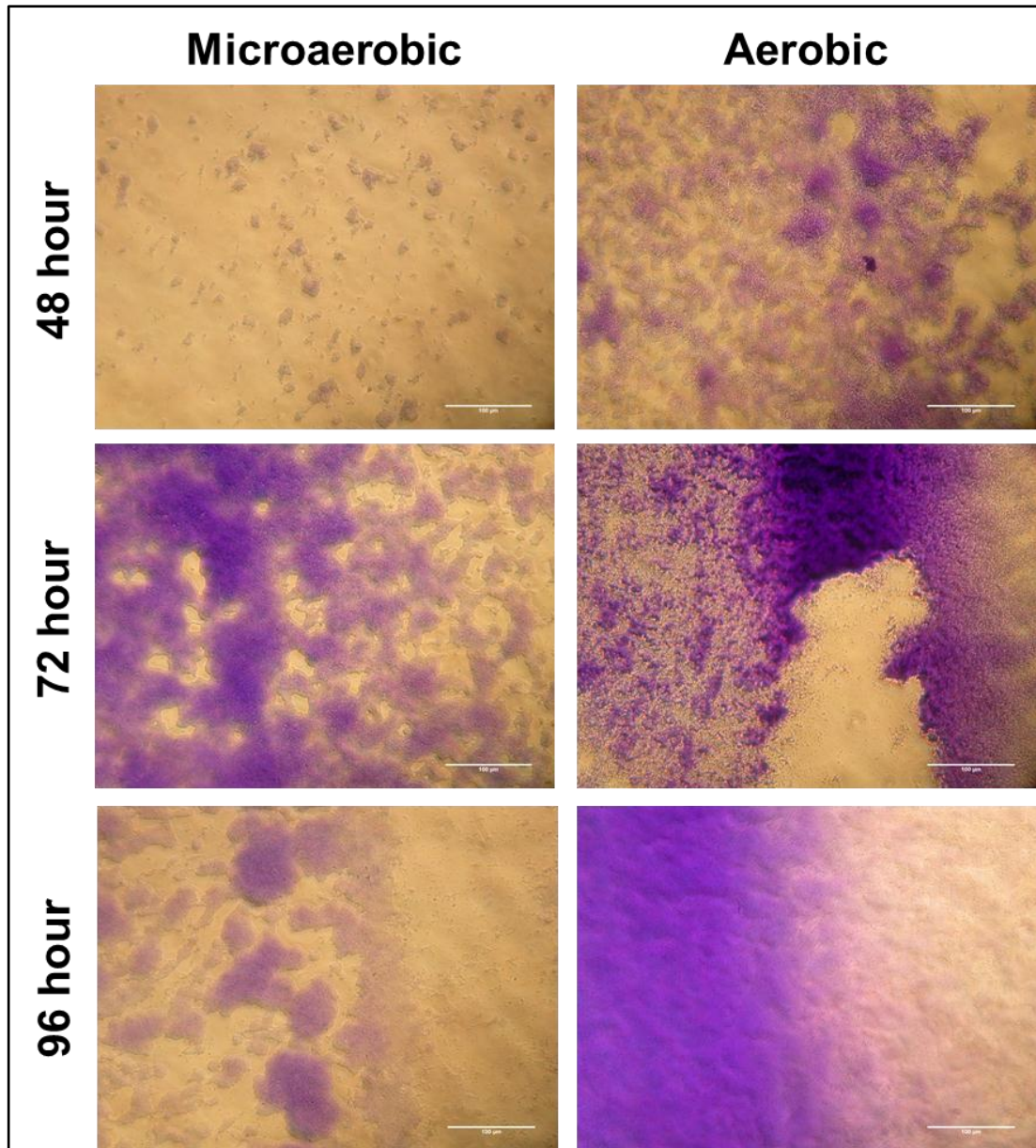


Figure 5-12 Biofilm formation is comparable in aerobic and microaerobic conditions following a 72 hour static incubation

Representative images of *C. jejuni* NCTC 11168 biofilms at the air/liquid interface following 48 (top row), 72 (middle row) or 96 (bottom row) hours of static incubation at 37°C in either microaerobic (left hand column) or aerobic (right hand column) conditions. All slides were crystal violet stained before imaging. Although there appear to be significant differences in biofilm maturity at 48 hours (with much smaller microcolonies where biofilms were incubated microaerobically), these differences are largely gone by 72 hours, and biofilms in both aerobic and microaerobic conditions appear comparable. Scale bar shows 100 µm. Enlarged images can be found in Appendix 3

In both aerobic and microaerobic conditions, biofilm formation appears limited to the air liquid interface (Figure 5-13). Although attached single cells and small microcolonies can be observed below the air liquid interface there appears to be no further maturation of these microcolonies, and the air liquid interface is the only area of the slide which contains a structured, ECM containing, biofilm.

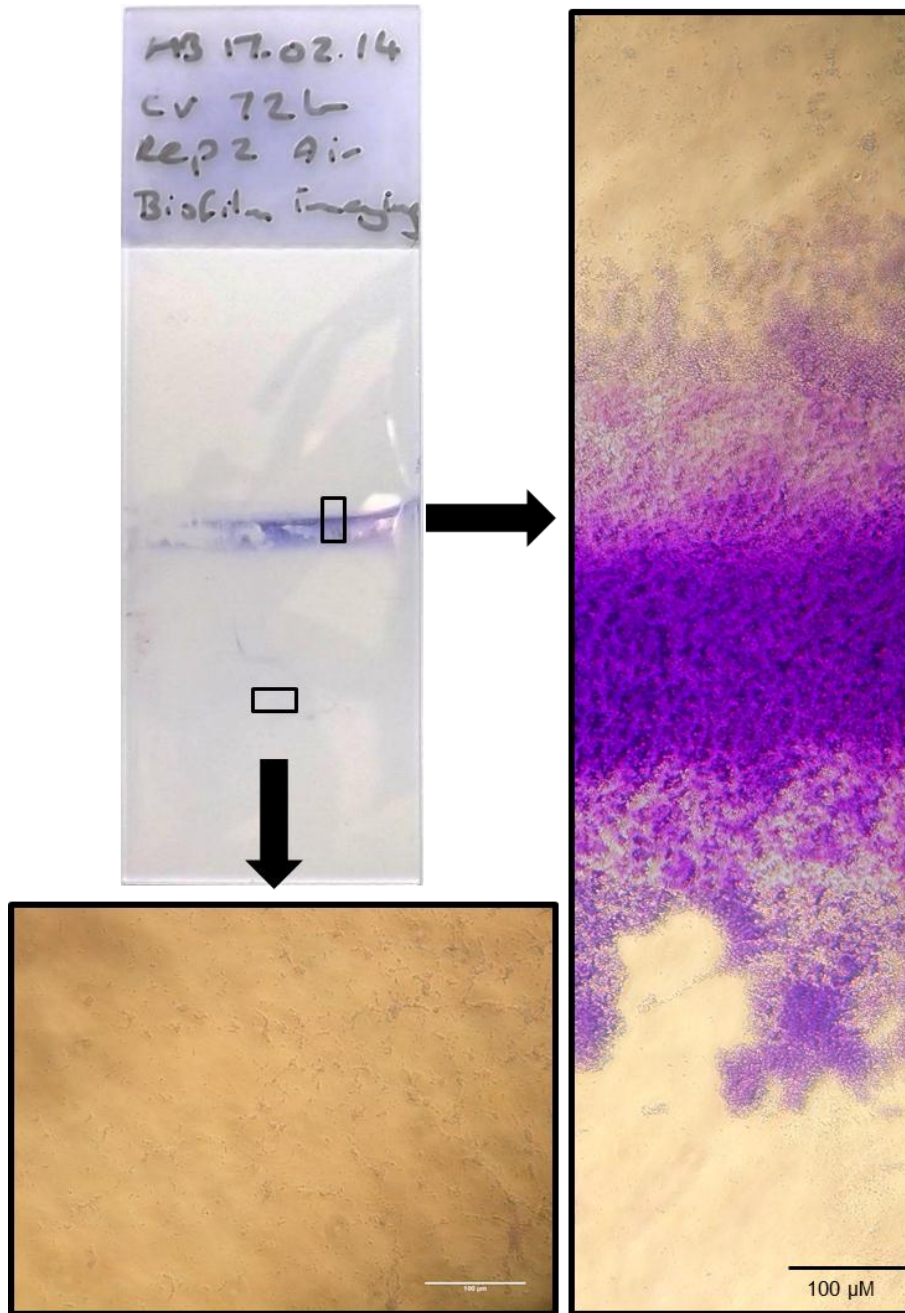


Figure 5-13 Minimal attachment of *C. jejuni* is seen above or below the air liquid interface

Representative cross-section of the *C. jejuni* air-liquid interface and area below the liquid shows preferential *C. jejuni* attachment and biofilms formation at the air-liquid interface. The Air-liquid interface cross section was compiled using Microsoft ICE software and following the manufacturers instructions. An enlarged image of the 'below air liquid' interface image can be found in Appendix 3.

It has previously been reported by Reuter et al. (2010) that biofilm formation is enhanced in aerobic conditions over a 48 hour period, when compared to incubation in microaerobic conditions. Additional statistical analysis of Figure 5-6 suggests that aerobic biofilms mature much more quickly than microaerobic ones. Whereas the greatest increase in biomass is between 24 and 48 hours in aerobic biofilms ($P = \leq 0.001$), the greatest increase in microaerobic biofilms is 24 hours later, between 48 and 72 hours ($P = \leq 0.0001$). All other

time point pairs show no statistically significant increase in biomass. Taken together the results from Sections 5.2.2 to 5.2.4 suggest that, over a 96 hour period, biofilm formation is accelerated in aerobic conditions, rather than enhanced.

5.2.5 Biofilm formation increases survival in microaerobic conditions

Biofilm formation is reported to enhance the survival of many bacterial species due to their ability to enter a 'semi-hibernating' state while residing within the biofilm. One example of this is found in *P. aeruginosa* biofilms, where transcriptomics has shown that in the upper layers of the biofilm cells are actively dividing, but in deeper biofilm layers transcription of hibernation factors is increased (Williamson et al., 2012). Biofilm formation has been shown to increase *C. jejuni* viability, although extended survival appears to be closely linked to temperature and nutritional conditions (Buswell et al., 1998).

Previous work measuring *C. jejuni* survival within biofilms has focused on the cultivation of cells rather than direct measurement of their viability. Many bacteria are able to enter a 'viable but not cultivable' state' (VBNC). VBNC cells are not able to be cultured, but other viability assessment methods, such as live/dead staining, show that they are metabolically active (Trevors, 2011). *C. jejuni* has previously been reported to be able to enter a VBNC state (Rollins and Colwell, 1986) in which despite cells being unculturable, invasion of CACO 2 cells and expression virulence factors is still possible (Chaisowwong et al., 2012). Recent work has also shown that *C. jejuni* biofilm cultures lose the ability to form colonies on Brucella agar (referred to as 'culturability' hereafter) more rapidly than cells in planktonic culture, although viability testing (using live/dead staining techniques) indicated that both planktonic and biofilm cultures remained viable for at least 60 days (Magajna and Schraft, 2015).

In order to assess metabolic activity rather than culturability, TTC was added to Brucella agar to give a final TTC concentration of 0.05% v/v. As described in 3, TTC is converted from a colourless solution to red formazan crystals by metabolically active cells. This allows viability of the biofilm cultures to be easily assessed over a longer period of time than could be measured by cell culture alone. Sterile glass bottles were filled with 100 ml of fresh sterile Brucella medium, containing *C. jejuni* NCTC 11168 diluted to an optical density of OD₆₀₀ 0.05. These were incubated, with loosened lids to allow air exchange, at either 5°C, RT (approx. 18°C), 37°C or at 42°C. All incubations were in aerobic conditions and compared to a positive control incubated at 37°C in microaerobic conditions. Bottles were inspected weekly to determine alterations, if any, in the pellicle. In addition a sterile cotton wool swab was placed into the bottle each week, and used to inoculate a TTC containing Brucella agar plate. The plates were incubated at 37°C in microaerobic conditions for 48 hours to allow TTC conversion by viable cells.

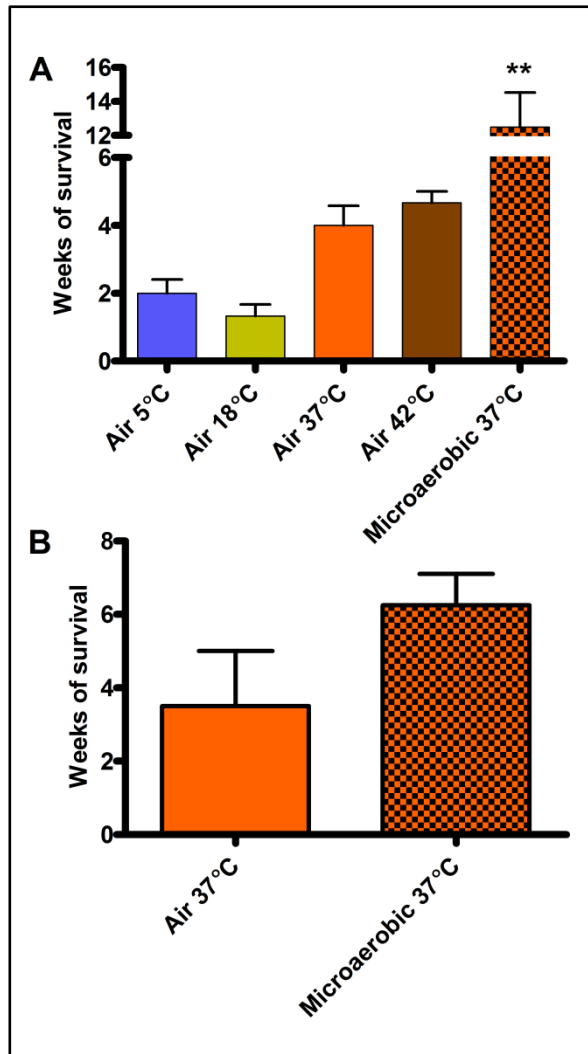


Figure 5-14 *C. jejuni* viability in biofilms is significantly increased at temperatures of 37°C and above.

Viability of *C. jejuni* NCTC 11168 was measured in static (A) and shaking (B) cultures over an extended time period and in various temperatures. Each week a swab of cells was removed from the static cultures and streaked onto a TTC containing Brucella agar plate, before incubation at 37 °C in microaerobic conditions for 72 hours. Cells were considered to be viable if conversion of TTC occurred (measured by the formation of red formazan crystals on the surface of the agar plates) and plates were scored as 'viable' or 'not viable' each week. Those samples incubated in aerobic conditions showed a prolonged period of viability compared to their aerobic counterparts. Similarly, an elevation in temperature to 37 °C or higher also conferred an increase in survival. Bars indicate the last week cultures provided a 'viable' sample. Error bars show SE (N = 3) and significance was measured using Bonferroni post-test following ANOVA analysis (** = $P \leq 0.01$).

Viable *C. jejuni* NCTC 11168 cells could not be detected following a two week static incubation at either RT (18°C) or 5°C (Figure 5-14a). Although, as reported by previous researchers (Buswell et al., 1998), a temperature of 5°C appeared to extend survival slightly compared to RT, this increase was not statistically significant ($P = 0.2856$). Attempts to extend survival of static cultures incubated at 5 and 18°C by incubating them at 37°C in aerobic conditions for either one or two weeks before transferring to the lower temperature were unsuccessful. Once the cultures were placed in the lower temperatures, recovery of

viable cells was limited to one or two weeks (Figure 5-13). Although survival is low compared to biofilms formed at 37°C or 42°C, this period of viability is significant within the food chain, and indicates that *C. jejuni* may be able to persist for extended periods, even when exposed to low temperatures and atmospheric oxygen levels.

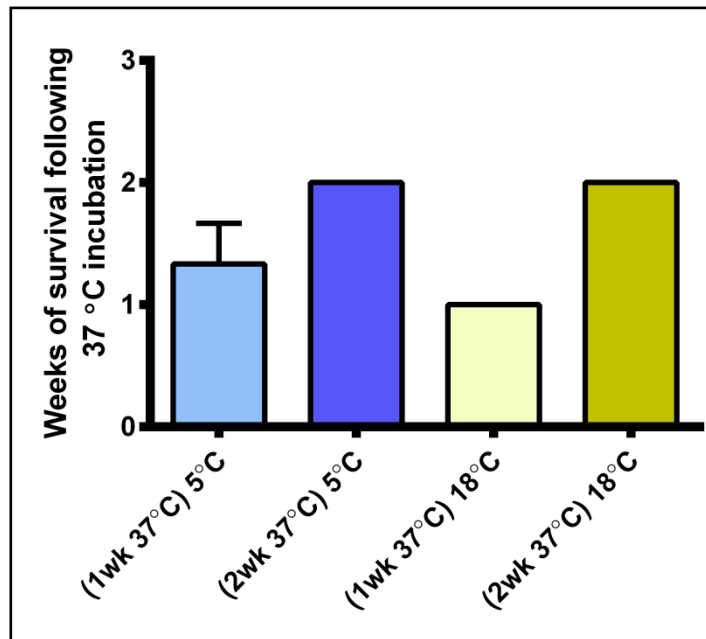


Figure 5-15 Pre-incubation of biofilms at 37°C does not improve *C. jejuni* survival at low temperatures

Viability of *C. jejuni* NCTC 11168 was measured in static cultures over an extended time period and in various temperatures. Static cultures were incubated at 37 °C for either one or two weeks before transferring to 5 or 25 °C to continue the incubation. After the cultures were transferred to lower temperatures a swab of cells was removed from the static cultures weekly and streaked onto a TTC containing Brucella agar plate, before incubation at 37 °C in microaerobic conditions for 72 hours. Cells were considered to be viable if conversion of TTC occurred (measured by the formation of red formazan crystals on the surface of the agar plates) and plates were scored as 'viable' or 'not viable' each week. Allowing biofilms to become established at 37 °C prior to their storage at a lower temperature did not increase survival beyond two weeks. Error bars show SE (N = 3) and significance was measured using Bonferroni post-test following ANOVA analysis (** = $P \leq 0.01$).

Increased survival was observed in aerobic conditions at 37 and 42°C, although this was not statistically different to survival at 5 or 18°C, nor was survival of cells within a biofilm statistically increased compared to shaking cultures when incubated at 37°C in aerobic conditions (Figure 5-14b). However, in microaerobic conditions biofilm formation conferred a statistically significant increase in survival compared to other tested temperatures ($P = \leq 0.01$) or shaking incubation ($P = \leq 0.05$).

5.2.6 Shedding from the biofilm

Arguably, the most important stage of the biofilms lifecycle is the final 'dispersal' step, in which bacterial cells are released from the biofilm, either actively or passively. Shedding could have particular impact within the food chain, where the release of pathogenic bacteria from biofilms may contribute to contamination of surfaces and foodstuffs (Chmielewski and Frank, 2003).

In order to assess the quantity of cells within the biofilm and supernatant, *C. jejuni* NCTC 11168 biofilms were allowed to form at 37°C in both aerobic and microaerobic conditions for 48 hours. Following incubation, the cells were gently removed from the surface with a cotton wool swab and re-suspended in sterile PBS before the optical density at a wavelength of 600 nm was measured.

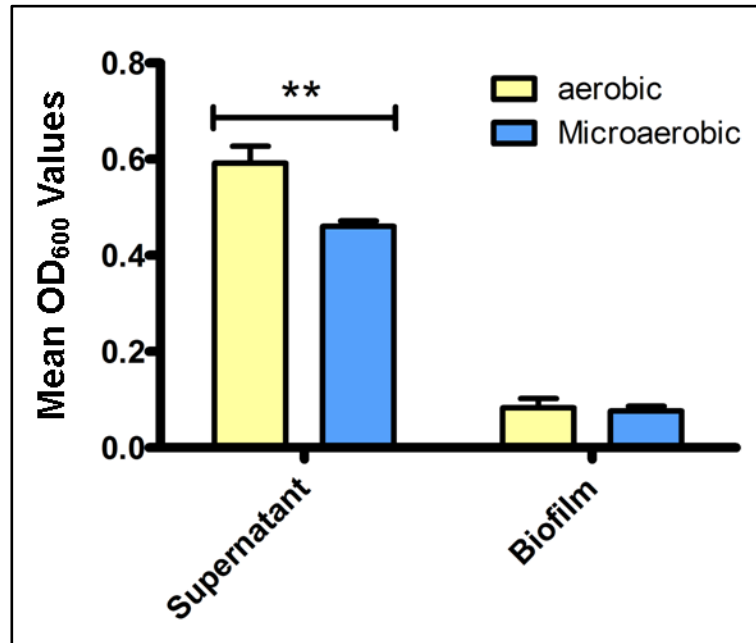


Figure 5-16 The OD₆₀₀ value of static cultures is significantly increased in aerobic conditions
 OD₆₀₀ values of both supernatant removed following static incubation and biofilms removed from the surface and resuspended in sterile PBS were measured following 48 hours of static incubation at 37 °C in either aerobic (yellow bars) or microaerobic (blue bars) conditions. Although a statistically significant difference could be observed in the density of the supernatant cultures, this was not replicated in the resuspended attached populations. Error bars show SE (N = 3) and significance was measured using Bonferroni post-test following ANOVA analysis (** = P≤0.01).

Measurement of the OD₆₀₀ values of supernatant and resuspended biofilms showed that although the OD₆₀₀ values of the biofilm cultures remained similar in both aerobic and microaerobic cultures, a significant increase in OD₆₀₀ value was observed in the supernatant of aerobic cultures compared to their microaerobic counterparts. Since the presence of ECM components may confound measurement of cell concentration by OD₆₀₀, as was observed when measuring OD₆₀₀ values of cell cultures grown in chicken juice containing medium (Section 3.2.5), cell viability assessment was also carried out.

Quantification of MPN within both the biofilm and planktonic phase suggests that there is little difference between MPN in either aerobic or microaerobic biofilms and planktonic cultures (Figure 5-17). Within the biofilm this trend is also observable in the images presented in Figure 5-4, where similar cell numbers can be observed in both aerobic and microaerobic conditions (Figure 5-4a, b, d and e).

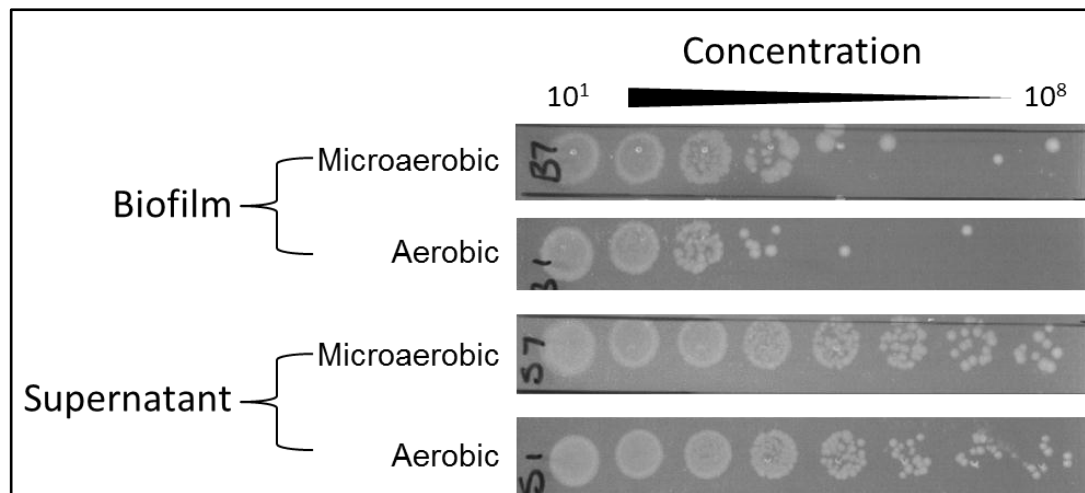


Figure 5-17 Representative image of MPN assessment from biofilms and static culture supernatant in variable O₂ conditions

Biofilms were allowed to form in both aerobic and microaerobic conditions for 48 hours before MPN of viable bacteria in both the supernatant and biofilm were assessed. Dilutions (1:10) were performed eight times and then placed on Brucella medium to allow growth of viable cells.

Following analysis of MPN in biofilm and planktonic phases the potential for cell release from the biofilm was assessed. Shedding of cells from mature (48 hour), *C. jejuni* NCTC 11168 biofilms was assessed by gentle washing of the biofilm, multiple times, using sterile PBS. Following each wash the PBS was recovered and MPN determined. Crystal violet staining was also carried out to assess the impact, if any, repeated washing had on the total biomass of the biofilm. A large reduction in biomass was observed following the first PBS wash (Figure 5-18a). This reduction is likely due to the removal of any loosely attached cells, and debris from the abiotic surface. No further reduction in crystal violet staining was observed as the wash cycles progressed, suggesting that the biofilm population is firmly attached to the surface, and cannot be dislodged by rinsing with PBS.

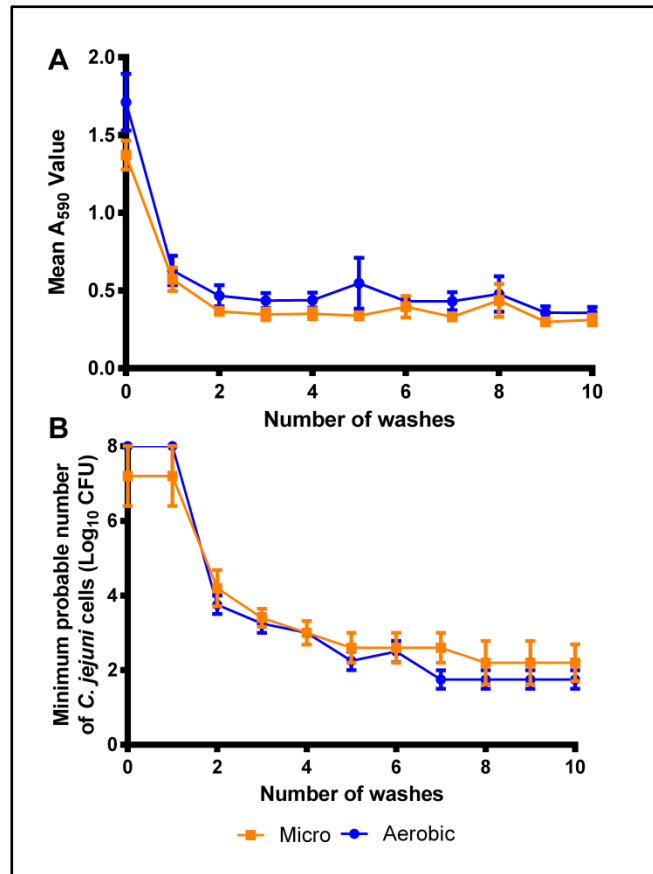


Figure 5-18 Release of cells from the biofilm rapidly decreases following washing

C. jejuni NCTC 11168 biofilms formed at 37 °C in either aerobic (blue) or microaerobic (orange) conditions were washed up to 10 times before crystal violet staining of the test tubes to quantify biofilm biomass (A). Following each rinse with PBS the washing solution was retained and MPN assessed (B). Both biofilm biomass and the number of cells ‘shed’ from the biofilm decreases rapidly between the first and second wash, after which biomass remains relatively stable. Cell shedding continues to decrease gradually from two to seven washes, after which the number of cells shed from the biofilm stabilises, but does not reach zero. Error bars show SE (N= 5).

Although the overall biomass of the biofilm remained unaltered as washing progressed, culturable cells were detected in the PBS solution following each of the ten washes (Figure 5-18a). Although the number of colony forming units decreased as the washing progressed, during the course of the experiment it did not reach zero. Although these results appear contrasting, it should be recognised that crystal violet is able to stain both the ECM and cells within the biofilm so it is possible that although the majority of the ECM remain attached to the surface during washing; individual cells, both from the biofilm or attached to the abiotic surface singly and in small microcolonies (as were observed in Figure 5-13), may become dislodged and move into the planktonic phase.

5.3 Discussion

In this chapter the progression of biofilm formation by *C. jejuni* NCTC 11168 has been described, with particular reference to the establishment and survival of the biofilm population. Data presented here that shows the formation and maturation of biofilms in aerobic and microaerobic conditions progresses at a different rate, with structural differences observed at key time points in the maturation process. In contrast, once the biofilms in both conditions reach maturity they become very similar, containing comparable amounts of cells, analogous structures and shedding similar levels of cells.

To date little analysis of the structure of *C. jejuni* NCTC 11168 single species biofilms has been carried out, particularly during the early stages of their maturation. Although individual elements of this research, such as the use of SEM analysis (Kalmokoff et al., 2006), aerobic conditions (Reuter et al., 2010) and assessment of biofilm survival (Hanning et al., 2008), have previously been utilised by other researchers, this is the first attempt to combine several biofilm measurement techniques to assess aspects of the whole *C. jejuni* biofilm lifecycle.

It has previously been reported by Reuter et al. (2010) that biofilm formation is enhanced in aerobic conditions over a 48 hour period, although not at 72 hours. The results presented in this chapter confirm this finding and in addition add data about biofilm formation at 12, and 96 hours. Alterations of biofilm structure and composition due to atmospheric condition have also been reported in other biofilm forming biofilm species. A recent study by Asai et al. (2015) indicated that the ECM composition of *S. aureus* and *S. epidermis* biofilms was altered in aerobic and microaerobic conditions and alterations in oxygen conditions were also found to effect biomass of several different *Salmonella sp.* (Stepanovic et al., 2004). Independent of any effect aerobic conditions have on biofilm formation, the results in Figure 5-1 also indicate that a minimum number of cells are required for *C. jejuni* NCTC 11168 biofilm initiation. In aerobic conditions a higher cell death rate, due to oxidative stress, is likely and so the initial cell concentration must be higher to cross the threshold required for biofilm formation. In microaerobic conditions less cell death would be expected and so this threshold will be achieved by addition of a lower number of bacteria in the initial culture. This may explain why, although biofilm formation was accelerated in aerobic conditions, it could not be observed where cell densities of less than OD₆₀₀ 0.01 were found in the initial cell culture.

Analysis of slides following a 12 hour static incubation indicated that cellular attachment took place prior to the production of extracellular matrix, with ECM production being found only where microcolony formation was also present. The evidence presented here does not allow firm conclusions to be drawn about if ECM production directly precedes microcolony formation, allowing progression from single cells attachments to microcolonies, or if ECM production is a response to microcolony formation, being secreted only once microcolonies have been established. Previously work using bacteria isolated from waterways (Allison and Sutherland, 1987) suggested that the production of ECM leads to microcolony formation. This was concluded since the bacterial species not able to produce a mucoid ECM, were also not able to form microcolonies. Both the light and SEM images presented in Figure 5-4 and Figure 5-9 appear to show that surface attached single cells are not associated with ECM, but further analysis is required before this can be confirmed beyond doubt.

Once ECM is present within microcolonies, biofilms rapidly progress from separated microcolonies to become dense, mature biofilms in both aerobic and microaerobic

conditions. This emphasises the importance of the ECM and its role in *C. jejuni* biofilm formation. In aerobic conditions, small quantities of ECM can be observed to associate with microcolonies containing several cells following only 12 hours of incubation. The action of the ECM during the early stages of biofilms formation is likely to be multifactorial: providing the expanding microcolonies with structural support (Flemming and Wingender, 2010), limiting oxygen and nutrient diffusion (Stewart, 2003) and providing protection to the cells within the structure (Nadell et al., 2015). Analysis of mixed species oral biofilm has shown that the ECM allows 'environmental pockets' to be formed within the biofilm. These pockets contain very specific environments, differing from the overall environmental conditions. This division of the biofilm allows greater survival of species where sub-optimal conditions are encountered (Xiao et al., 2012). The production of ECM can also be directly linked to exposure to stress. *B. subtilis* cells encountering low nutrient environments not only increase their production of ECM, but alter the shape of the microcolonies, moving towards a thinner wider shape than those not grown under nutrient limiting conditions (Wenbo et al., 2014). This structural alteration, assisted by the secretion of ECM, maximises the surface area of the microcolony, allowing more efficient nutrient acquisition. In *C. jejuni* biofilms the opposite appears to occur, with the microcolonies observed in aerobic conditions denser than their microaerobic counterparts. It is possible that this denser microcolony shape allows microaerobic conditions to predominate in the deeper layers of the biofilm, assisting cell survival.

The observed increases in *C. jejuni* NCTC 11168 biofilm formation at earlier stages in aerobic conditions led to speculation that biofilm formation was an adaptive response of *C. jejuni*, following exposure to high oxygen environments. This hypothesis is supported by the observation in Section 5.2.6 that *C. jejuni* biofilms in aerobic and microaerobic conditions contain similar levels of viable cells, and that *C. jejuni* cells are no longer viable following a 48 hour static aerobic incubation if no biofilm is formed (Section 5.2.1). It is likely that the observed increase in biofilm biomass in aerobic conditions is due to both increased cellular attachment and early production of ECM by aerobic biofilms. Previous work has also shown that inactivation of genes encoding AhpC, a protein involved in the oxidative stress response, increased biofilm formation (Oh and Jeon, 2014). Proteomic analysis has also shown that aerobic conditions increase the expression of membrane proteins involved in adhesion and biofilm formation (Sulaeman et al., 2012).

As described in Section 1.1.2.4, shedding from biofilms can either be due to active or passive processes. To date no *C. jejuni* shedding/dispersed method has been discovered. Previous work by Reuter et al. (2010) has shown that *C. jejuni* NCTC 11168 biofilms are able to release viable cells both during a single wash of the biofilm and following a 24 hours of incubation in fresh medium. The results presented in Section 5.2.6 have extended this work, indicating that although repeated washing of the biofilm leads to continued shedding of cells, a population of cells remained attached to the surface. This suggests that a proportion of the *C. jejuni* biofilm forms tight attachments to the abiotic surface, which cannot be overcome by the mild shearing forces generated during the washing stages. Although this phenomenon has not been reported previously in *C. jejuni*, it has been observed in *Pseudomonas fluorescens* biofilms where washing with a citrate buffer detergent removed less than 1% of the cell population from a mature biofilm (Simoes et al., 2008b). Similarly, strong attachment of *E. coli* to abiotic and biotic surfaces could not be reversed by repeated washing (Silagyi et al., 2009).

In conclusion the formation of biofilms by *C. jejuni* NCTC 11168 is accelerated by incubation in aerobic conditions, however successful establishment of the biofilm is only possible at

initial optical densities of between OD₆₀₀ 0.01 and 0.5. Although cellular attachment and biofilm maturation progresses more slowly in microaerobic conditions, the biofilm lifestyle is able to confer a significant increase in cell viability over time. Release of *C. jejuni* from mature biofilms is possible in both aerobic and microaerobic conditions. This, in combination with the acceleration of biofilm formation in aerobic conditions, and the ability of *C. jejuni* cells within biofilms to remain viable for several weeks all indicate that biofilm formation is not only a form of stress response to oxidative shock, but may contribute to the spread and persistence of *C. jejuni* within the food chain.

6 Removal of extracellular DNA from the of the *Campylobacter jejuni* biofilm leads to biofilm disruption

This chapter is based on the paper: [Brown H L](#), Reuter M, Hanman K, Betts R P, van Vliet A H M (2015) *Campylobacter jejuni* biofilms contain extracellular DNA and are sensitive to DNase I treatment. *Frontiers in Microbiology*. 6, article 699

6.1 Introduction

The ECM is an essential component of bacterial biofilms, and accounts for more than 90% of the dry mass of a biofilm (Flemming and Wingender, 2010). It allows cells to remain hydrated and metabolically active by trapping nutrients and liquid in close proximity to the bacterial populations. It also assists in inactivation of antimicrobials (Billings et al., 2013, Mulcahy et al., 2008), leading to increased bacterial persistence. ECM is also structurally important, maintaining the shape of the biofilm and ensuring the biofilms cohesion (Sutherland, 2001). Extracellular DNA (eDNA) appears to have a structural role in the biofilms of many different species, including *P. aeruginosa* (Chiang et al., 2013), *S. aureus* (Mann et al., 2009), *L. monocytogenes* (Harmsen et al., 2010) and *E. coli* (Zhao et al., 2013).

The structure and composition of the *C. jejuni* biofilm ECM is still relatively unknown. It has previously been shown that eDNA is present within the ECM of *C. jejuni* biofilms (Svensson et al., 2009) and that eDNA appears to have a role in biofilm maturation (Svensson et al., 2014). Since *C. jejuni* shows optimal viability in a microaerobic environment, the majority of investigations to date have been carried out in microaerobic conditions (approximately 5% O₂ and 10% CO₂). Within the food chain *C. jejuni* encounters aerobic conditions for extended periods of time, and to date little investigation of biofilm formation in aerobic conditions has been undertaken. A literature review shows that no investigation of eDNA in aerobic conditions has yet been carried out.

This chapter presents evidence that eDNA is present in biofilms of *C. jejuni* strains NCTC 11168 and 81116, when they are formed in aerobic conditions and or on food chain relevant materials. Degradation of this eDNA by DNase I can lead to a rapid loss of biofilm structure, releasing cells into the planktonic phase. Treatment of surfaces with DNase I also inhibits further biofilm formation. *C. jejuni* eDNA is also able to contribute to genetic transformation, allowing increased antimicrobial resistance within populations. This work suggests that *C. jejuni* biofilms within the food chain would respond to DNase I treatment, providing another potential treatment option to reduce *C. jejuni* food chain persistence.

6.2 Results

6.2.1 Extracellular DNA is present within the *C. jejuni* biofilm during both aerobic and microaerobic incubation

In order to confirm that eDNA was present in the biofilm, biofilms were grown for 48 hours in aerobic conditions. Following incubation, biofilms were removed from the surface by swabbing with a sterile cotton wool bud, and the MPN of both the planktonic and biofilm cultures was assessed. Samples were analysed by gel electrophoresis in order to visualise any eDNA present. Figure 6-1 shows agarose gels of NCTC 11168 and 81116 biofilms incubated for 48 hours, with eDNA present in both the planktonic and biofilm suspensions incubated in either aerobic and microaerobic conditions. It should however be noted that this method of DNA detection allows visualisation on only the soluble present within the biofilms. It is possible that other cross-linked molecules are also present, but not detected by this method.

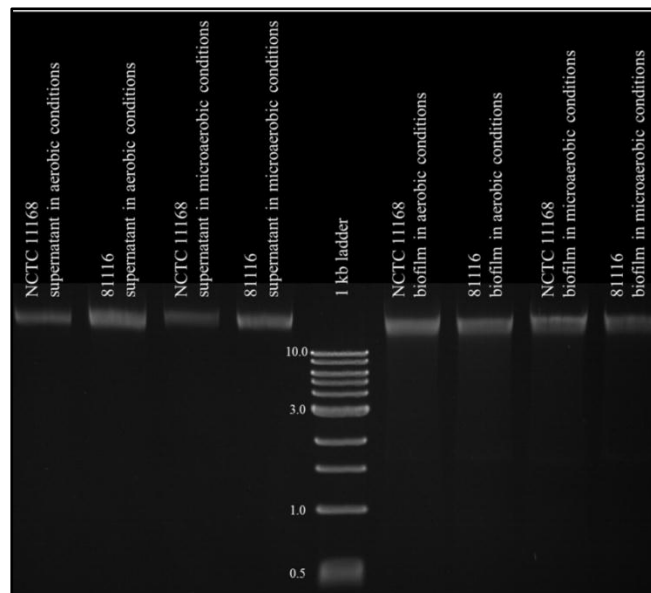
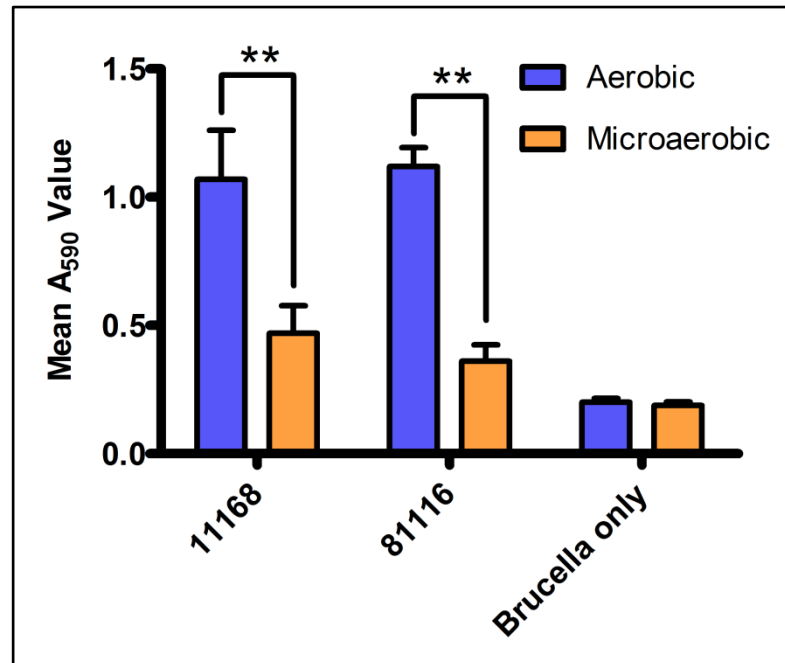


Figure 6-1 Extracellular DNA is present in both aerobic and microaerobic conditions following static incubation

A 48 hour static incubation of *C. jejuni* NCTC 11168 and 81116 was performed to allow biofilm formation. Following incubation, the supernatant and biofilm were recovered and eDNA presence or absence confirmed by gel electrophoresis. No distinction could be made between DNA quantities in either the biofilm or supernatant samples when the atmospheric conditions were compared, suggesting that eDNA is present in the biofilm ECM in both aerobic and microaerobic conditions. A distinction could however be made between the quantity of eDNA found in the supernatant samples of *C. jejuni* NCTC 11168 and 81116.

When the gel is observed there is no distinguishable difference between the eDNA bands produced by surface attached *C. jejuni* NCTC 11168 and 81116, although when the columns containing planktonic samples are observed the NCTC 11168 band appears fainter than 81116 in both atmospheric conditions. It is therefore hypothesised that slightly less genetic material is present within the supernatant of the NCTC 11168 planktonic phase than its

81116 counterpart. The atmospheric condition appears not to affect eDNA release, although, as previously reported (Reuter et al., 2010), total biofilm mass in aerobic conditions did increase during the 48 hour incubation (Figure 6-2).



6-2 Biofilm biomass is increased in aerobic conditions for both *C. jejuni* strains NCTC 11168 and 81116

Quantification of biofilm biomass by crystal violet staining indicates that following a 48 hour static incubation *C. jejuni* strains NCTC 11168 and 81116 produce comparable quantities of biofilm to each other in both aerobic and microaerobic conditions. However there is a significant increase in the biofilm biomass for both strains when static incubation takes place in aerobic conditions. Error bars show SE (N = 5) and significance was measured using Bonferroni post-test following ANOVA analysis (** = P<0.01).

In order to confirm the observation of eDNA within the biofilm, *C. jejuni* NCTC 11168 expressing GFP⁺ was allowed to form biofilms on glass slides and counter stained with DAPI, allowing eDNA visualisation. Imaging (Figure 6-3) showed a diffuse blue stain surrounding GFP⁺ expressing colonies.

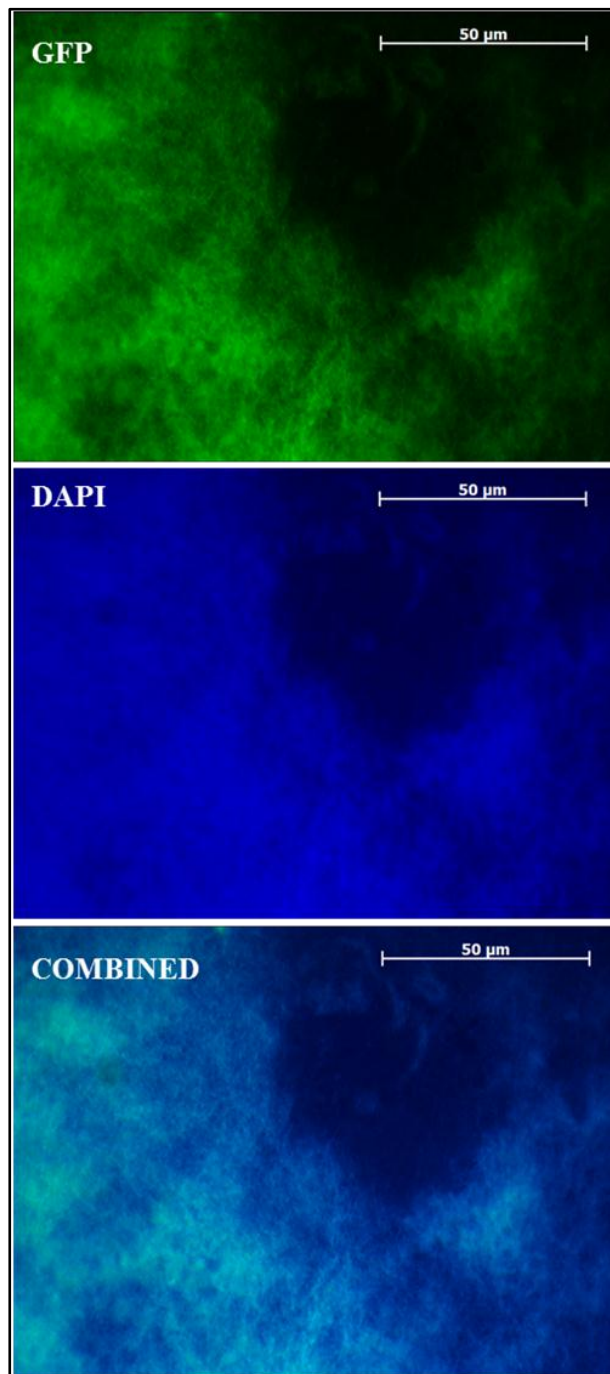


Figure 6-3 Extracellular DNA is present in the biofilms of *C. jejuni* NCTC 11168 biofilms.

Representative fluorescent microscopy images of GFP⁺ expressing NCTC 11168 biofilms counter stained with DAPI. The three images all show the same field of vision, with the top panel showing the GFP expressing cells only, the middle panel showing DNA stained with DAPI and the bottom panel showing the two images combined. A diffuse blue dye can be seen around the GFP expressing cells suggesting that there is a large quantity of eDNA present within the mature biofilm. Enlarged versions of these images can be found in Appendix 3.

Since the GFP⁺ expressing mutant of strain NCTC 11168 showed no observable differences in growth or biofilm formation (Figure 6-4) it can be assumed that eDNA is also present within the NCTC 11168 parent strain.

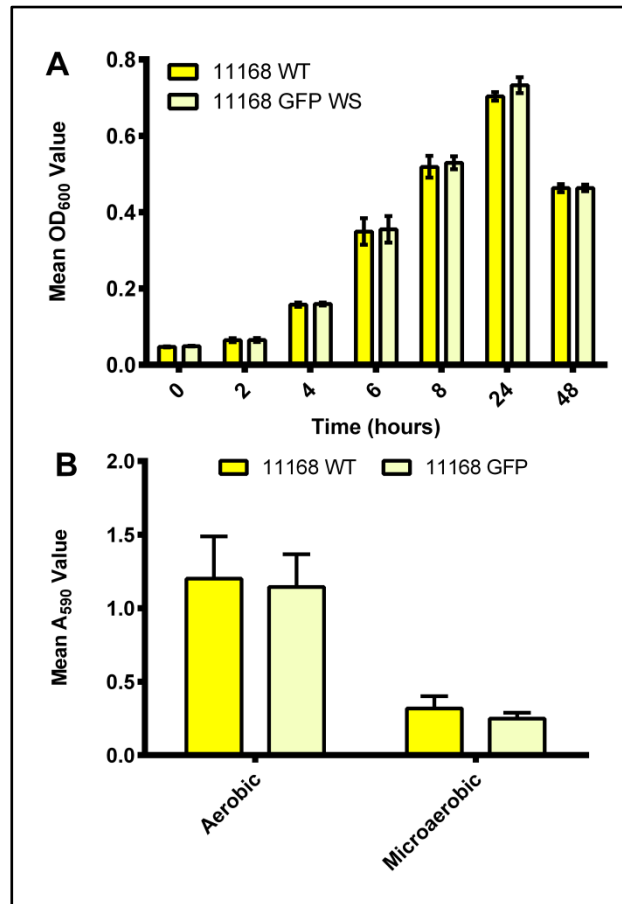


Figure 6-4 A GFP⁺ expressing mutant of *C. jejuni* NCTC 11168 shows similar growth and biofilm formation characteristics to the wild type strain.

A comparison of growth (A) and biofilm forming ability (B) of *C. jejuni* NCTC 11168 and its GFP⁺ expressing mutant showed that there were no statistically significant differences in either growth or biofilm formation between the parent and mutant strains. Error bars show SE (N = 5).

The eDNA appears to be present throughout the colonies, showing no preference for localisation to particular areas of the biofilm as has been observed in *P. aeruginosa* biofilms (Gloag et al., 2013). It should however be noted that no three dimensional analysis of the NCTC 11168 biofilm has been undertaken as part of this study and so although these preliminary investigations of biofilm structure and eDNA distribution appear to show no localisation a more detailed investigation, not within the remit of this work, is required in order to support this observation.

6.2.2 Treatment of the biofilm with exogenous DNase I leads to rapid and irreversible biofilm disruption

We next tested the possibility that degradation of eDNA in biofilms would lead to biofilm disintegration. Previous work in *P. aeruginosa* biofilms has shown that eDNA is important in early stages of biofilm formation, however DNase I treatment has less impact on biofilm structure as the biofilm matures (Whitchurch et al., 2002). In order to assess if this was also the case in *C. jejuni* NCTC 11168 biofilms, DNase I was added at 12 hour intervals over the total 48 hour aerobic incubation period. The *C. jejuni* biofilm was degraded following addition of 4 U/ml DNase I regardless of the biofilms maturity, indicating that eDNA is an important ECM component throughout the entire *C. jejuni* biofilm life cycle (Figure 6-5).

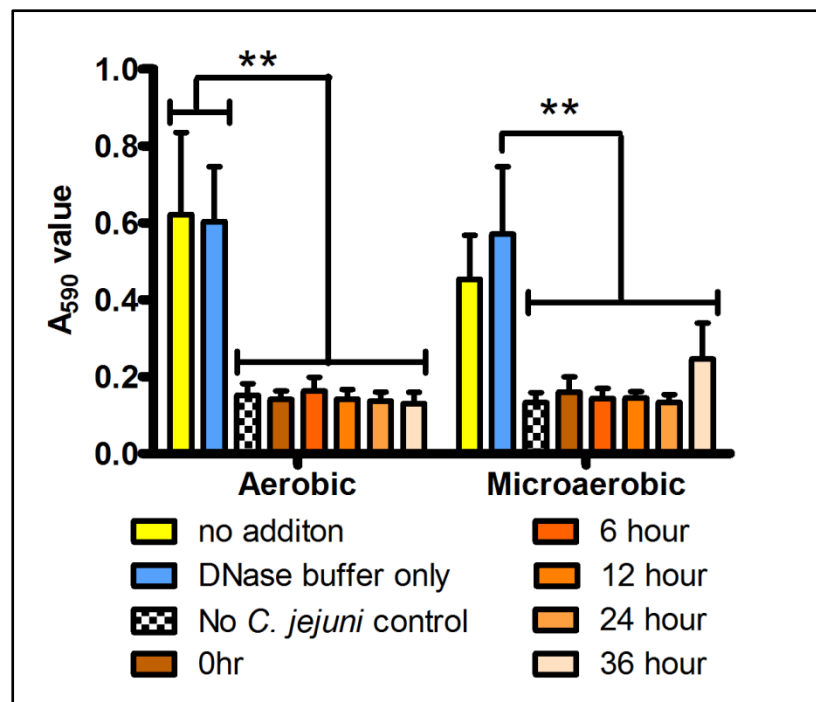


Figure 6-5 DNase I is able to degrade *C. jejuni* NCTC 11168 biofilms regardless of their maturity

DNase I was added at intervals to aerobically incubated NCTC 11168 cultures over a 48 hour static incubation and biofilm degradation assessed by crystal violet staining (A). At all time points a statistically significant reduction in biofilm biomass was observed, with DNase I treated samples being indistinguishable from a Brucella medium only control. Error bars show SE (N= 5), significance was measured using Bonferroni post-test following ANOVA analysis (** = P<0.01).

The rapidity of biofilm degradation was next assessed by treating mature (48 hour) biofilms with DNase I and then staining at intervals over a two hour period. Following only a five minute incubation with DNase I the majority of the biofilm was removed from the glass surface and showed no statistical difference in A_{590} value to a test tube containing only Brucella medium (Figure 6-6). No further degradation occurred at later time points, suggesting that a five minute treatment is able to achieve maximal biofilm degradation.

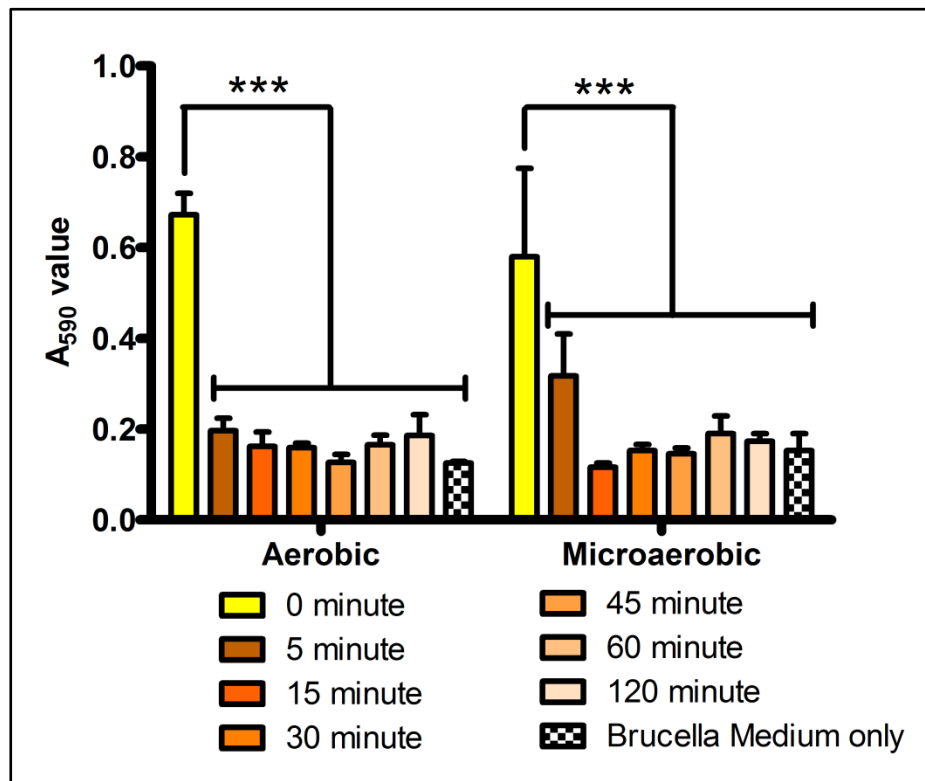


Figure 6-6 DNase I degradation of mature *C. jejuni* NCTC 11168 biofilms occurs with a 5 minute incubation

Following a 48 hour static incubation to allow biofilm formation, DNase I was added to biofilms for between 5 and 120 minutes before biofilm degradation was assessed. After a 5 minute incubation of the biofilm with DNase I there was a statistically significant decrease in biofilm biomass compared to an untreated control (yellow bar). No statistically significant further decrease in biofilm biomass, or regrowth of the biofilm was seen during a further two hour treatment. Error bars show SE (N = 5), significance was measured using Bonferroni post-test following ANOVA analysis (**=P<0.001).

Finally, the concentration of DNase I required to degrade the biofilm was also investigated. DNase I was diluted to concentrations, ranging from 5 to 0.01 U/ml, and added to biofilm cultures before incubating statically for 48 hours (Figure 4-8).

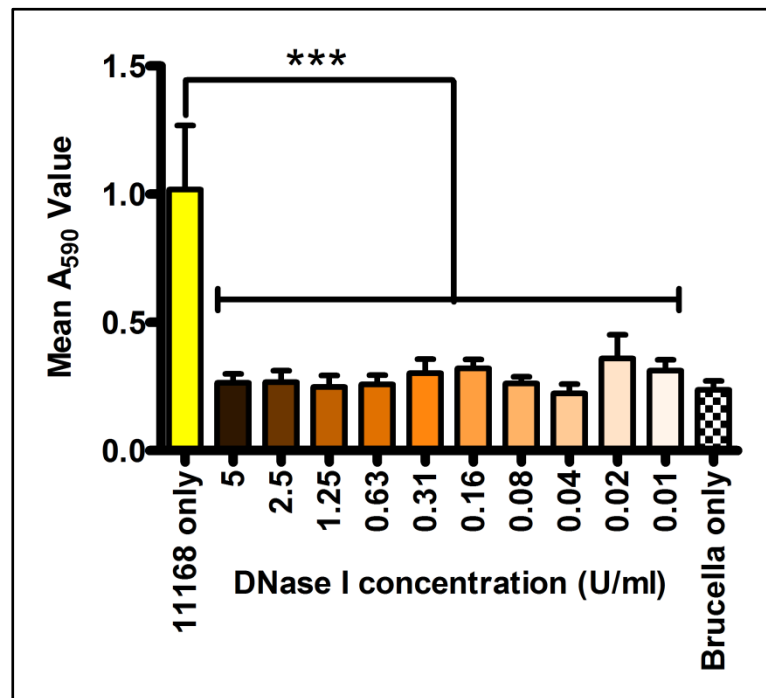


Figure 6-7 Concentrations of DNase I as low as 0.01 U/ml are able to achieve biofilm degradation

The concentration of DNase I required for biofilm reduction was assessed by adding various concentrations of DNase I to biofilm cultures before incubating statically for 48 hours and assessing biofilm biomass by crystal violet staining. The graph shows aerobic data only since it is industrially relevant, microaerobic data was comparable to the aerobic data shown. All DNase I concentrations tested were able to prevent measurable *C. jejuni* NCTC 11168 attachment and biofilm formation, with all treated samples being indistinguishable statistically from the Brucella only control (black and white checked bar). Error bars show SE (N = 5), significance was measured using Bonferroni post-test following ANOVA analysis (** = $P < 0.01$, *** = $P < 0.001$).

All the tested concentrations significantly reduced the level of *C. jejuni* NCTC 11168 biofilm. As previously there was no statistically significant difference between DNase I treated test tubes and the negative control tube containing Brucella medium only. It is interesting to note that DNase I treatment had little impact on MPN, simply degrading the biofilm and releasing the attached cells into suspension (Figure 6-7).

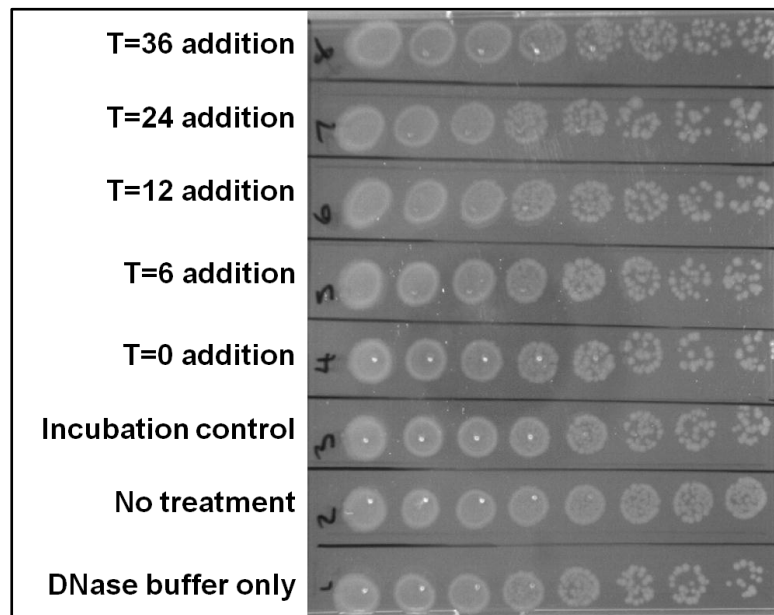


Figure 6-8 MPN of the *C. jejuni* NCTC 11168 planktonic phase is not effected by DNase I treatment.

Representative image (N = 5) of a spot plate containing serial 1:10 dilutions (highest dilution on the left of the image and lowest dilutions on the right) of *C. jejuni* NCTC 11168 cells incubated statically for 48 hours in aerobic conditions. The labels on the left of the image denote at what time during the incubation DNase I was added to the culture. There is little difference in MPN between those cultures incubated with DNase I (top four lines) and those without DNase I (bottom four lines), suggesting that although DNase I is able to effect the biofilm biomass, it does not significantly impact MPN. Control samples were as follows: DNase buffer only = biofilm suspension containing 4 μ l of manufacturers DNase buffer, No treatment = biofilm suspension not containing any buffers or enzyme but physically handled in the same manner as the treatment samples, Incubation control = sample was incubated continuously throughout the experiment, rather than being removed for incubation for manipulations.

Since biofilms formed in both aerobic and microaerobic conditions showed the same pattern of disruption (Figure 6-8), it is suggested that the effects observed were not a response to atmospheric condition, but rather DNase I treatment. DNase I which had been heat inactivated (by heating to 95 $^{\circ}$ C for 10 min) lost the ability to inhibit the formation of *C. jejuni* NCTC 11168 biofilms (Figure 6-9).

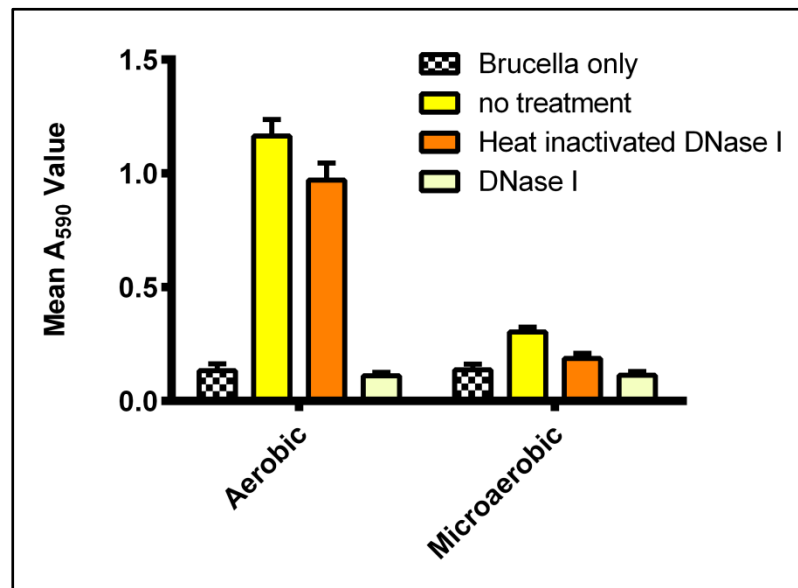


Figure 6-9 Heat inactivation of DNase I inhibits its ability to degrade *C. jejuni* biofilms

Biofilm formation by *C. jejuni* NCTC 11168 cultures treated with either DNase I (pale yellow bars), Heat treated DNase I (orange bars) or cultures with no DNase I added (yellow bars) were compared. DNase I enzyme which had been heated to 95 °C for 10 min was unable to statistically significantly inhibit *C. jejuni* NCTC 11168 biofilm formation during a 48 hour static incubation at 37°C, unlike its un-treated counterpart. Graph shows a representative replicate (with three internal, technical replicates). Error bars show SE of the three internal replicates.

1.1.1 Treatment of the biofilm with exogenous DNase I leads to irreversible biofilm disruption

DNase I treatment is able to both degrade pre-existing biofilms, and inhibit biofilm formation when added at the start of static incubations. Next, it was investigated whether DNase I treatment of abiotic surfaces can inhibit *de novo* biofilm formation. *C. jejuni* NCTC 11168 and 81116 cultures were incubated statically for 48 hours in borosilicate test tubes to allow biofilm formation. Following this primary incubation the biofilms were treated with 4 U/ml of DNase I for 15 min. Supernatant was then removed and the test tube washed twice to remove any residual cell suspension or DNase I. The following solutions were then added to the test tubes: either fresh *Brucella* medium or fresh *C. jejuni* NCTC 11168 cell suspension. The tubes were then incubated for a further 48 hours before crystal violet staining, to assess if any *de novo* biofilm formation had occurred following DNase I treatment. There appeared to be neither regrowth of DNase I treated biofilm or fresh biofilm formation (Figure 6-10). This suggests that DNase I treatment is not only a rapid method of degrading existing *C. jejuni* NCTC 11168 biofilms, but also provided a lasting impairment to biofilm re-growth. The same trend was observed for *C. jejuni* 81116 biofilms, although statistical significance was not reached in this instance. As in the previous experiments, microaerobic biofilms responded in a similar manner, showing no further biofilm formation following treatment.

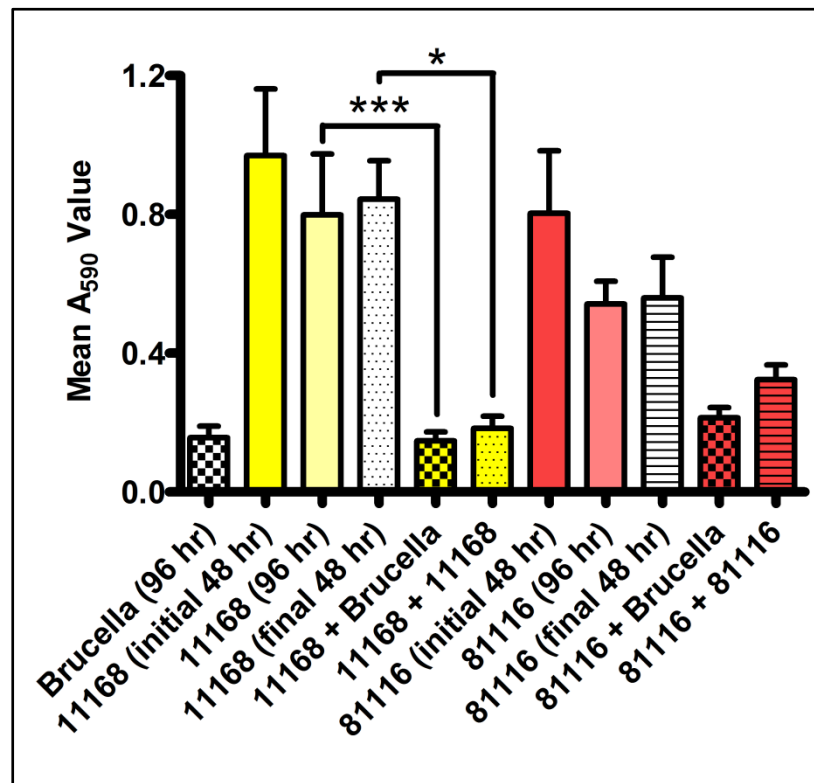


Figure 6-10 Treatment of abiotic, biofilm supporting, surfaces with DNase I leads to inhibition of biofilm regrowth.

Mature (48 hour) *C. jejuni* NCTC 11168 (yellow and spotted bars) and 81116 (red and striped bars) biofilms were allowed to form in sterile borosilicate glass test tubes before a 15 minute treatment with 4 U/ml DNase I. Following treatment the tubes were washed with PBS and then either fresh Brucella medium (hatched bars) or fresh NCTC 11168 suspension (striped or spotted bars) was added to the tubes. DNase I treated tubes showed no further biofilm growth. Error bars show SE (N = 5), significance was measured using Bonferroni post-test following ANOVA analysis (* = P<0.05, ***=P<0.001).

6.2.3 Restriction enzymes also have the ability to degrade *C. jejuni* NCTC 11168 biofilms

Earlier results showed that the eDNA found within the *C. jejuni* NCTC 11168 and 81116 biofilms is of a high molecular weight (Figure 6-1), and so it was speculated that high molecular weight genetic material, rather than simply the presence of any genetic material, was required for biofilm formation. Restriction enzymes are able to cut DNA at specific sequences. Depending on the rarity of the recognition sequences within the genome, DNA fragments of various lengths can be prepared. In order to assess the impact different sized DNA fragments may have on *C. jejuni* biofilm structure six restriction enzymes with different cutting profiles were selected. A variety of restriction enzymes with different recognition site frequencies were selected in order to give a variety of DNA degradation patterns (Figure 6-11). The recognition sites for the chosen enzymes can be found in Table 2-12.

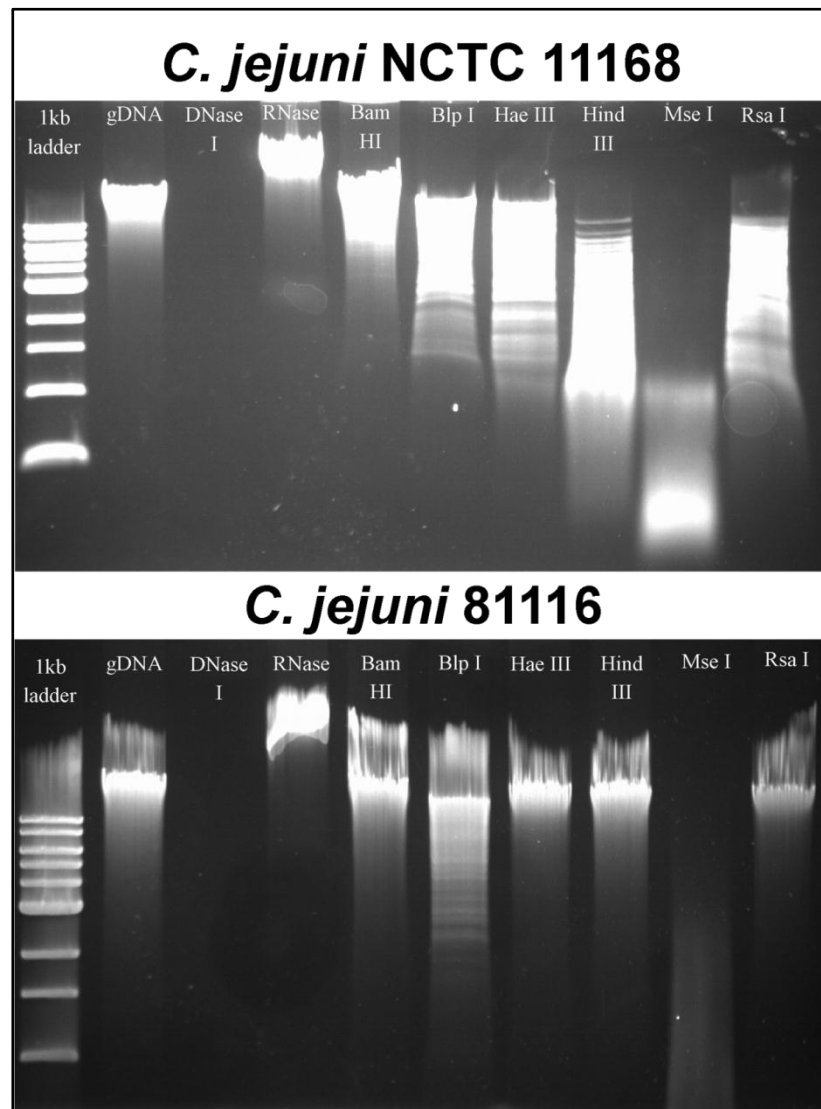


Figure 6-11 Cut patterns of the selected restriction enzymes on genomic *C. jejuni* DNA

Representative images of *C. jejuni* NCTC 11168 and 81116 purified genomic DNA following digestion for 60 minutes at 37 °C with the restriction enzymes *Bam*HI, *Bln*I, *Hae*III, *Hind*III, *Mse*I and *Rsa*I, RNase and DNase I.

C. jejuni NCTC 11168 showed a significant reduction in biofilm formation for all six restriction enzymes tested, with little variation between treated enzymes and a *Brucella* only control (Figure 6-12). Although the same trend was shown within the 81116 biofilms, statistical significance was not reached except in the case of DNase I treatment. This may be explained by the slightly different cutting patterns of the enzymes on the 81116 DNA, producing fragments of a higher molecular weight than those found following NCTC 11168 digest.

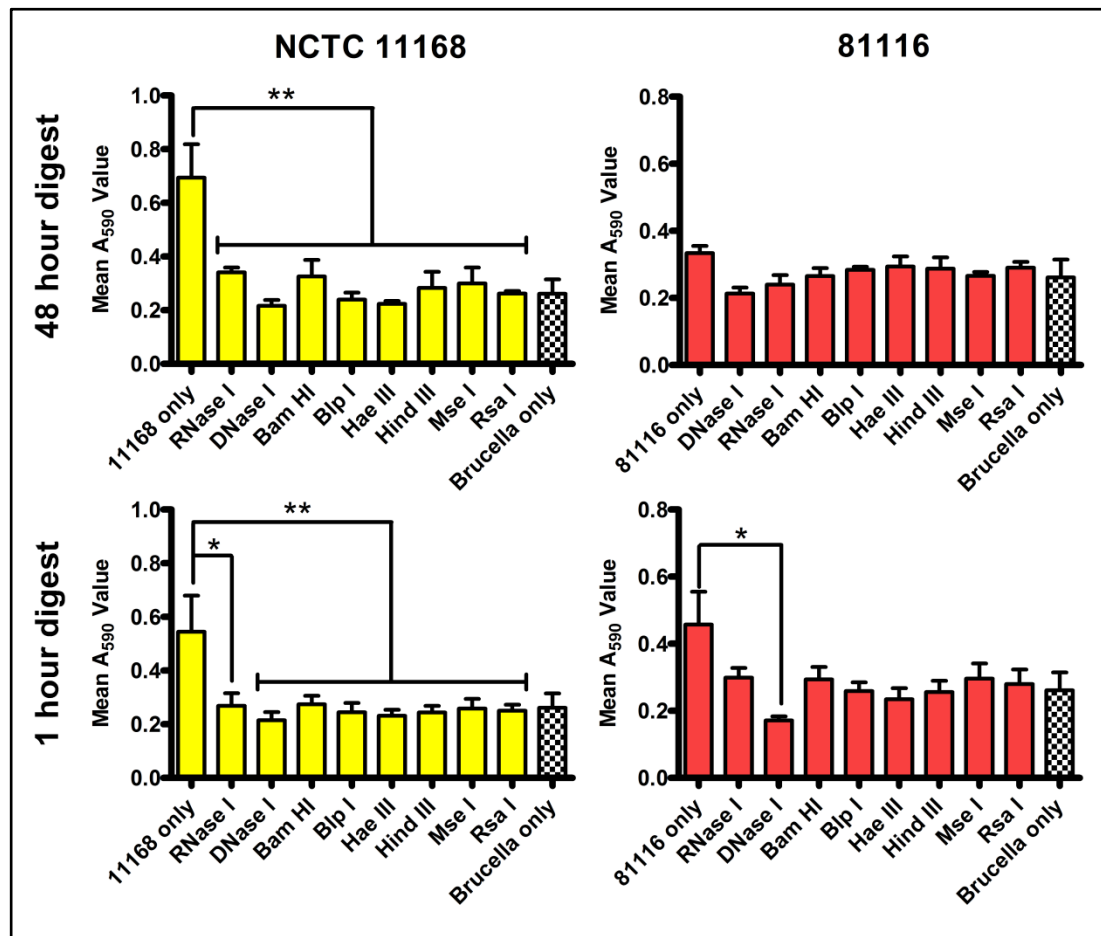


Figure 6-12 Restriction enzyme treatment of *C. jejuni* biofilms also reduces biofilm formation.

C. jejuni NCTC 11168 (yellow) and 81116 (red) were incubated statically for 48 hours at 37°C in aerobic conditions to allow biofilm formation. Prior to incubation either one restriction enzyme, RNase or DNase I were added to the tubes. Enzyme treatment of *C. jejuni* NCTC 11168 biofilms led to a significant reduction in biofilm formation ($P \leq 0.01$ for DNase I and restriction enzymes and $P \leq 0.05$ for RNase treatment). Although the reduction in *C. jejuni* 81116 biofilms did not reach statistical significance for any treatment, with the exception of DNase I treatment ($P \leq 0.05$), a clear trend of biofilm reduction could be observed. All error bars show SE ($N = 5$).

6.2.4 DNase I treatment is effective on food chain relevant surfaces

Since *C. jejuni* is a foodborne pathogen, frequently present throughout the food chain (Wassenaar, 2011), its ability to survive on food relevant surfaces such as stainless steel, rubber and plastics (Thormar and Hilmansson, 2010, Somers et al., 1994), or on soiled surfaces (Brown et al., 2014, De Cesare et al., 2003) is important.

C. jejuni NCTC 11168 biofilms formed on sterile stainless steel coupons showed a significant reduction following DNase I treatment (Figure 6-13). The coupons showed no observable biofilm formation following static aerobic incubation in the presence of DNase I, however significant levels of biofilm formation were observed when DNase I was not present.

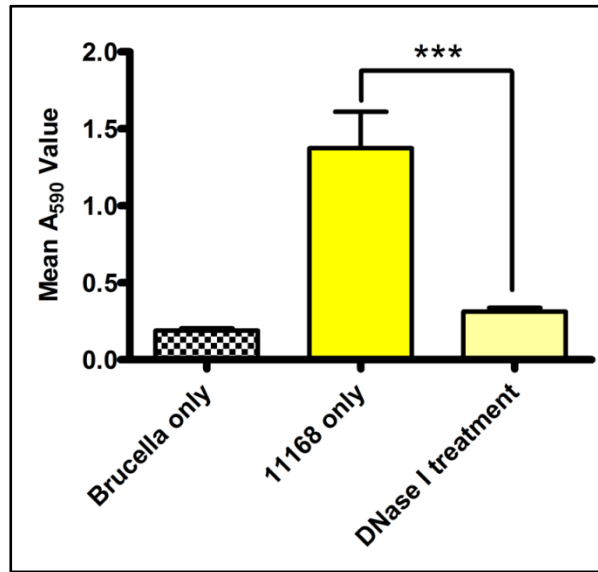


Figure 6-13 DNase I treatment is effective on biofilms formed on stainless steel surfaces.

The ability of DNase I to inhibit biofilm formation of *C. jejuni* NCTC 11168 on sterile, stainless steel coupons was assessed. Stainless steel coupons were incubated statically at 37°C in aerobic conditions with *C. jejuni* NCTC 11168. Following a 48 hour incubation biofilm biomass was assessed by crystal violet staining. A statistically significant difference in biofilm biomass can be observed between DNase I treated (pale yellow bars) and untreated (yellow bars) cultures, with treated cultures showing no statistically significant difference to the Brucella medium only control (hatched bars). Error bars show SE (N = 3) and significance was measured using Bonferroni post-tests following ANOVA analysis (***) ($P < 0.001$).

6.2.5 *C. jejuni* biofilms formed in heavily soiled areas are also susceptible to DNase I treatment

In order to mimic environments in which soiling occurs, *C. jejuni* NCTC 11168 cultures were allowed to form biofilms in Brucella medium containing 5% v/v chicken juice. Chicken juice is a complex, undefined meat exudate obtained from frozen chickens, and is known to have a high protein and lipid content (for further details of the chicken juice model see 4). It has also previously been shown to increase biofilm formation in several *C. jejuni* strains due to its ability to condition abiotic surfaces (Brown et al., 2014). These properties make it a suitable model to replicate soiled and conditioned surfaces, such as might be encountered within the food chain (Birk et al., 2004). DNase I treatment of biofilms formed in the presence of 5% v/v chicken juice did show a significant ($P = \leq 0.01$) reduction compared to untreated biofilms (Figure 6-14), but some biofilm was still detectable. This suggests that on conditioned surfaces DNase I treatment, although significantly decreasing biofilm formation, does not degrade the biofilm to the same extent as observed in culture medium only. As described in 4, chicken juice forms a conditioning layer on abiotic surfaces, into which *C. jejuni* integrates during the biofilm formation. This conditioning layer may inhibit penetration of the DNase I enzymes, leaving some cells attached to the surface.

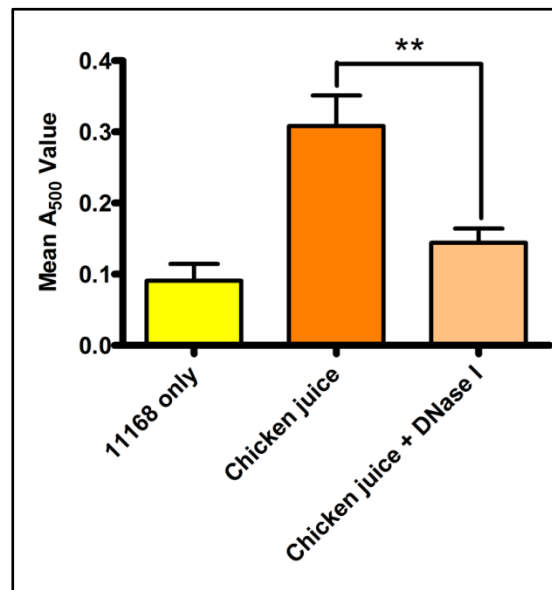


Figure 6-14 DNase I treatment is effective in the presence of surface conditioning

The ability of DNase I to inhibit biofilm formation by *C. jejuni* NCTC 11168 in the presence of chicken juice, mimicking heavy surface soiling, was assessed. Although there was a slight increase in biofilm formation in when cultures were incubated with chicken juice and DNase I (pale orange bars) compared to untreated cultures incubated in Brucella medium only (yellow bars), this was not statistically significant. As expected, a significant increase was observed when Brucella medium was supplemented with 5% chicken juice (orange bars). When chicken juice supplemented biofilms with and without DNase I treatment were compared there was a statistically significant reduction in biofilm biomass in DNase I treated cultures. Error bars show SE (N = 5), significance was measured using Bonferroni post-test following ANOVA analysis (** = P<0.01).

6.2.6 Natural transformation is able to occur within the *C. jejuni* biofilm

Given the presence and structural importance of the eDNA it was hypothesised that supplementation of biofilm cultures with additional DNA may further increase biofilm formation. This was tested by the addition of 2 µg of *C. jejuni* NCTC 11168 GFP⁺ gDNA at either the start of the biofilm incubation, or following 24 hours of incubation. Addition of gDNA did not lead to significant changes to the levels of crystal violet staining (Figure 6-15). This indicates that although eDNA is essential for biofilm formation and structural stability, in contrast to previous research in this field using *C. jejuni* 81-176 biofilms (Svensson et al., 2014, Svensson et al., 2009), the DNA produced by the *C. jejuni* NCTC 11168 and 81116 is adequate for biofilm formation to occur. Further DNA addition does not lead to increased biofilm formation.

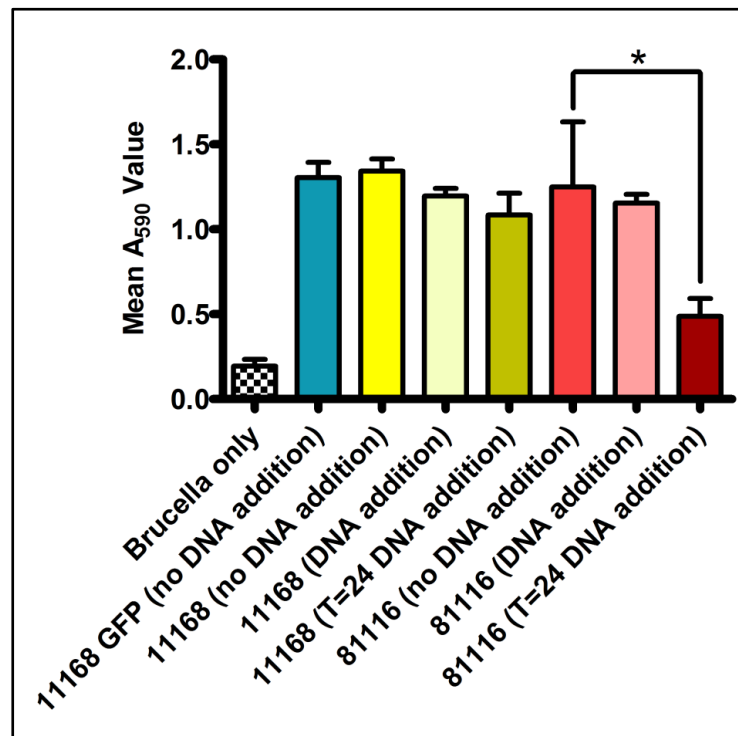


Figure 6-15 Addition of DNA to *C. jejuni* NCTC 11168 and 81116 biofilm cultures does not increase biofilm biomass.

C. jejuni NCTC 11168 and 81116 biofilms were supplemented with 2 µg gDNA isolated from *C. jejuni* NCTC 11168 GFP⁺ and incubated statically for 48 hours at 37 °C in aerobic conditions, before quantification of the biofilm biomass by crystal violet staining. There was no statistically significant difference in crystal violet staining between unsupplemented NCTC 11168 (yellow bar), 81116 (red bar) and NCTC 11168 GFP⁺ (blue bar) cultures. Neither was a statistically significant difference observed when NCTC 11168 cultures were supplemented with gDNA at either the start for the static incubation (pale yellow bars) or after 24 hours of static incubation (dark yellow bar). In contrast a significant reduction in biofilm biomass was observed when 81116 cultures were supplemented following 24 hours of incubation (dark red bar), but not when gDNA was added immediately before static incubation commenced (light red bar). Error bars show SE (N = 5) and significance was calculated using Bonferroni post-test following ANOVA analysis (* = P<0.05).

Although it was observed that gDNA was not able to increase biofilm formation, in its presence genetic exchange occurred in cells of both the planktonic and biofilm phase (Figure 6-16). *C. jejuni* NCTC 11168 GFP⁺ contains a cassette expressing both a GFP⁺ gene and a chloramphenicol resistance gene (*cat*). At the end of a 48 hours static incubation to allow biofilm maturation, the cell suspension was removed from the test tube and plated onto both Brucella agar and Brucella agar containing 10 µg/ml chloramphenicol. Any bacteria containing the *cat* gene from NCTC 11168 gDNA within its chromosome would be able to form colonies on antibiotic containing plates. This method was also repeated with bacteria released from biofilms using a sterile cotton wool swab. Enumeration and comparison of colonies on both agar types allowed calculation of the proportion of bacteria which had become chloramphenicol resistant. No resistance was observed in cultures not containing *C. jejuni* NCTC 11168 GFP⁺ gDNA, suggesting that neither planktonic or biofilm

cultures of strains NCTC 11168 or 81116 are naturally resistant to 10 µg/ml chloramphenicol, even following biofilm growth.

Where gDNA had been added to the suspensions at the start of the static incubation, some resistance was observed in both planktonic (Figure 6-16a) and biofilm (Figure 6-16b) cultures. In cultures where gDNA had been added at a later (24 hour) time point, less resistance was apparent. There appeared to be no resistance of 81116 biofilms to 10 µg/ml chloramphenicol following 24 hours of contact with the gDNA within the medium, although low numbers of resistant cells were present within the planktonic phase. These results suggest that *C. jejuni* NCTC 11168 and 81116 are able to utilise eDNA as a source of new genetic elements, incorporating eDNA found within biofilms and planktonic phase into the genomes via natural transformation.

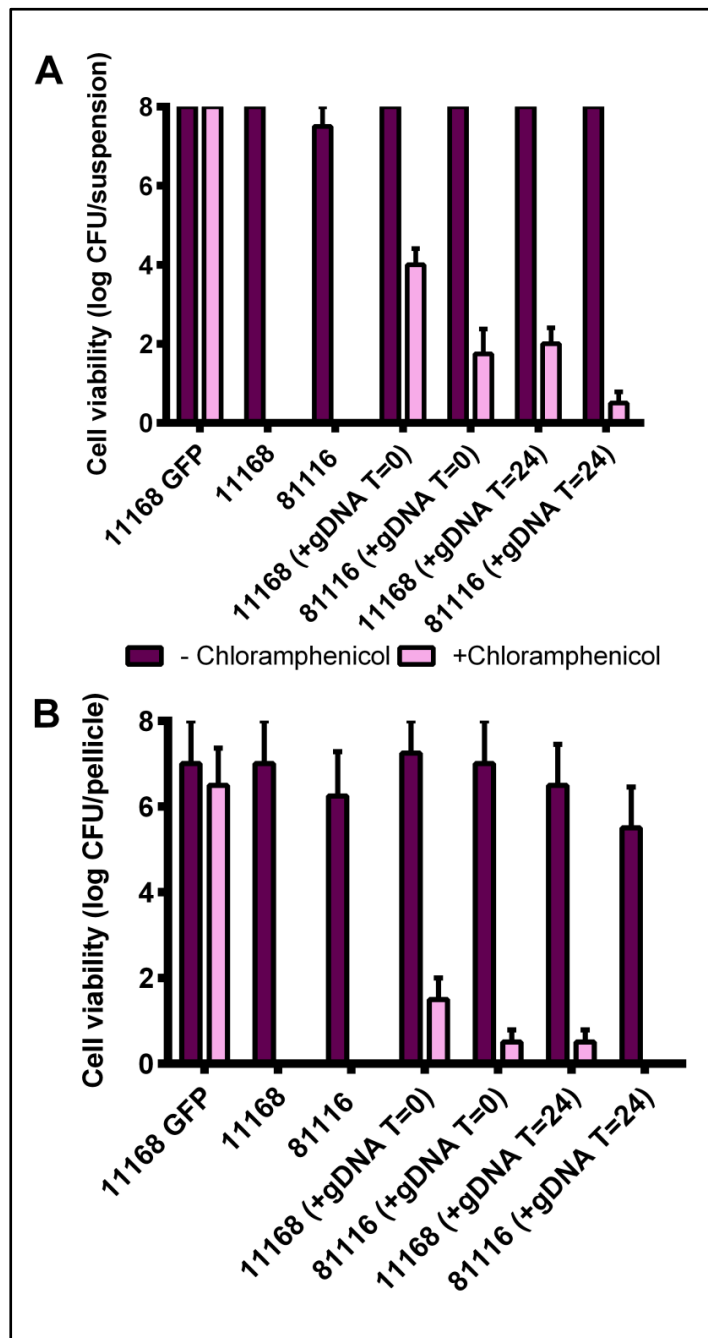


Figure 6-16 Natural transformation can be observed in *C. jejuni* NCTC 11168 and 81116 during static biofilm incubation

MPN assessment of planktonic (A) and biofilm (B) phase cultures showed that cultures supplemented with *C. jejuni* NCTC 11168 GFP⁺ gDNA were able to incorporate the *cat* gene into their genome and proliferate on Brucella agar containing 10 µg/ml chloramphenicol. Dark purple bars indicate the number of cell present on spot plates containing Brucella agar only (no antibiotic was added to these plates), light purple bars indicate the number of cells present when cultures were incubated on Brucella agar supplemented with 10 µg/ml chloramphenicol. Error bars show SE (N = 5).

6.3 Discussion

The importance of eDNA in bacterial biofilms is now well recognised (Svensson et al., 2014, Whitchurch et al., 2002), and has attracted attention as a target for enzymatic or chemical treatment for disinfection purposes. DNase I is effective in interfering with the biofilms of the foodborne pathogens *L. monocytogenes* (Harmsen et al., 2010) and of *E. coli* (Zhao et al., 2013), but also mixed species biofilms. Biofilms found in activated sludge flocs form close interactions with the DNA occurring from cell lysis (Dominiak et al., 2011), affecting microcolony formation within the biofilm. Similarly, mixed biofilms containing *S. epidermidis* and *C. albicans* are also affected by DNase treatment (Pammi et al., 2013), suggesting that DNase is able to modify both mixed species and mixed kingdom biofilms. Addition of exogenous DNase is effective in reducing biofilms of pathogenic bacteria, such *Neisseria gonorrhoeae* (Zweig et al., 2014), *Gardnerella vaginalis* (Hymes et al., 2013) and *L. monocytogenes* (Nguyen et al., 2014).

C. jejuni strain 81116 appear to be less susceptible to DNase I treatment than its counterpart NCTC 11168. It is important to note that not all bacterial species show reduction of biofilm formation following treatment with DNases. When the opportunistic pathogen *B. cenocepacia* was exposed to DNase, it produced significantly denser biofilms (Novotny et al., 2013). *H. pylori* biofilms also remain unaffected following treatment with DNase I (Grande et al., 2011). The presence of eDNA on a surface inhibits biofilm formation by *S. enterica* serovars Typhimurium and Typhi (Wang et al., 2014). These examples show that DNase treatment may not be effective in the case of all biofilms. However many naturally occurring biofilms, such as are found in processing plants, are comprised of multiple species and so DNase treatment should still be considered an effective mechanism of at least partially degrading biofilms and possibly allowing better penetration of antimicrobials.

Recently, research on food chain biofouling has benefited from a focus on not only antimicrobial treatment but methods of biofilm dispersal and prevention. Examples of biofilm control methods (reviewed in Section 1.1.6) include use of quorum sensing molecules (Kalia, 2013), nanoparticles (Chorianopoulos et al., 2011), bacteriophage treatments (Siringan et al., 2011), use of food additives to reduce bacterial attachment (Furukawa et al., 2010), and the development of antifouling surfaces (Carlson et al., 2008). It has also been shown that combination treatment including various enzymatic treatments, surfactants and chelating agents may provide a suitable alternative to the chemical treatments currently in use for biofilm removal within food processing areas (Lequette et al., 2010). The use of DNase I is an example of one such enzymatic treatment.

Previous research in *P. aeruginosa* biofilms has shown that eDNA can not only provide structural stability at early stages of biofilm formation (Whitchurch et al., 2002) but is also found to be localised to specific areas of the biofilm as it matures (Ma et al., 2009), again suggesting a structural role. Gloag et al. (2013) show that eDNA is also integral to *P. aeruginosa* biofilm organisation and expansion, with DNase I treatment of developing biofilms leading to significant decreases in biofilm expansion. The results presented here show that eDNA is an important component of the *C. jejuni* ECM at all stages of maturation. This is in contrast to species such as *P. aeruginosa*, which become less susceptible to DNase I treatment as the biofilm matures (Whitchurch et al., 2002). Some outer membrane and flagella proteins have been identified as been important in *C. jejuni* biofilm formation but

to date there has been little investigation of the ECM components themselves, or their genes. Genes such as *pel* and *psl*, the two major *P. aeruginosa* ECM polysaccharide genes (Colvin et al., 2012), are not present within the *C. jejuni* genomes, although the presence of a polysaccharide containing beta1-3 and/or beta 1-4 linkages, and reactive to calcofluor white, has previously been identified in *C. jejuni* biofilm (McLennan et al., 2008). It is possible that eDNA has greater importance in *C. jejuni* biofilms because it lacks many of the extracellular matrix genes found in other biofilm forming species. It would be of interest to extend this study by investigating how the eDNA interacts with other ECM components and the bacteria within the biofilm. It would also be interesting to understand what proportion of the ECM is comprised of eDNA, and if this proportion alters of the lifespan of the *C. jejuni* biofilm.

For naturally transformable species such as *C. jejuni*, the eDNA is a useful source of genetic material for incorporation in the genome. Genetic material can be transferred within the biofilm either by direct cell to cell transmission or uptake of exogenous DNA. Conjugation within biofilms is a well reported phenomenon, with examples reported in mixed species oral biofilm models (Hannan et al., 2010), drinking water systems (Lisle and Rose, 1995) and within bacterial populations colonizing the nasopharynx (Marks et al., 2012). Recent work has shown that *C. jejuni* strains NCTC 11168 and 81-176 in microaerobic cultures are able to transfer genetic material between bacterial cells both within biofilms and planktonic suspension (Bae et al., 2014, Svensson et al., 2014). The results presented here show that *C. jejuni* is also able to utilise the eDNA itself to obtain new genetic elements.

The eDNA within *C. jejuni* NCTC 11168 and 81116 biofilms is not only present in both aerobic and microaerobic conditions, but appears to be of structural importance. It is interesting to note that only high molecular weight DNA appears to provide structural support to the *C. jejuni* biofilm. Further analysis of the spatial positioning of the eDNA within the biofilm could indicate why the fragment size is of importance. It would also be interesting to investigate if other sources of eDNA, such as salmon sperm or DNA obtained from other bacterial species, is also incorporated into the *C. jejuni* biofilm and contributes to its structural integrity.

Treatment of biofilms with DNase I provided a rapid and lasting degradative effect, and could be successfully used on food chain relevant surfaces such as stainless steel, and in environments containing heavy soiling. Another problem frequently encountered within food processing environments is the presence of food product debris. This presence of this debris on surfaces can lead to surface conditioning and increased bacterial attachment. The attachment of *L. monocytogenes* to stainless steel surfaces is enhanced by surface pre-conditioning with fish and meat emulsions (Gram et al., 2007), and surface conditioning by chicken juice has been shown to enhance *C. jejuni* biofilm formation (Brown et al., 2014). Surface conditioning can also decrease the effectiveness of chemical cleaning products, leading to reduced killing or biofilm removal (Gram et al., 2007). In heavily soiled environments broad spectrum enzymatic treatments may provide a useful and effective addition to current cleaning regimes as they are able to degrade not only biofilm ECM, but potentially the conditioning layer. Our results show that DNase I treatment is able to significantly reduce *C. jejuni* biofilms formed on surfaces conditioned with chicken juice, suggesting that DNase I treatment could provide a useful addition to current treatment regimens.

It should be noted that DNase I treatment had no effect on MPN, only biofilm dispersal. This is as expected since DNase I is only in contact with the DNA of the ECM, reducing the structural integrity of the colonies forming the biofilm but not able to cause a loss of viability in bacterial cells with intact membranes. The implication of this finding is that although the DNase I treatment provides a rapid and effective method of biofilm dispersal, it would best be used in combination with antimicrobial treatments, ensuring effective biofilm removal and bacterial inactivation.

In conclusion, eDNA is an essential component of the *C. jejuni* biofilm and its removal leads to rapid biofilm disintegration. Treatment of abiotic surfaces with DNase I leads to both rapid and long lasting biofilm removal, releasing the cells into planktonic phase, potentially allowing more efficient antimicrobial treatment. DNase I treatment is effective on food chain relevant surfaces and could provide a useful addition to current food chain cleaning regimes.

7 *C. jejuni* strain RM1221 contains three extracellular DNases which modify its biofilm forming ability and degrade *C. jejuni* biofilms

This chapter is based on the paper: [Brown H L](#), Reuter M, Hanman K, Betts R P, van Vliet A H M (2014) Prevention of biofilm formation and removal of existing biofilms by extracellular DNases of *Campylobacter jejuni*, *PLoS One*. 10(3): e0121680.

7.1 Introduction

As shown in 6, and in previous work by other researchers (Svensson et al., 2009, Svensson et al., 2014), eDNA is present within the *C. jejuni* biofilm. This eDNA can be degraded by treatment of the biofilm with DNase I, leading to a release of the cells from abiotic surfaces, and inhibiting reattachment. It has also been reported that some bacteria are able to secrete their own DNase enzymes into the extracellular environment (hereafter these enzymes are referred to as eDNase). These eDNase proteins have diverse functions, reflecting the diversity of the bacterial species they have been found within. Examples of eDNase functions include immune evasion by *S. aureus* (Berends et al., 2010), biofilm modification by *P. aeruginosa* and *S. aureus* (Beenken et al., 2012, Seper et al., 2011), scavenging of carbon and phosphate sources by *E. coli* and *P. aeruginosa* (Finkel and Kolter, 2001, Mulcahy et al., 2010), efficient bacterial predation of prey bacterium by *Bdellovibrio bacteriovorus* (Lambert and Sockett, 2013), and inhibition of natural transformation within *C. jejuni* (Gaasbeek et al., 2010, Gaasbeek et al., 2009).

The species *C. jejuni* contains several mobile genetic elements, such as prophages, plasmids and insertion elements, all of which contribute to the diversity shown within the species (Dingle et al., 2001). Most *C. jejuni* isolates are naturally competent, and readily take up DNA from the environment. One of the consequences of this is that *C. jejuni* shows a high level of genetic diversity (Parker et al., 2006, Clark et al., 2014). *C. jejuni* strain RM1221 contains four insertion elements named *Campylobacter jejuni* Insertion Element (CJIE) 1 to 4 (Fouts et al., 2005a, Parker et al., 2006). Three of these, CJIE1, CJIE2 and CJIE4, each contain a gene encoding a DNase protein (*dns*, *cje0556* and *cje1441* respectively). These encoded proteins are predicted to be extracellular due to the presence of signal peptide cleavage sites (Gaasbeek et al., 2010). The genes have previously received attention as their expression prevents natural competence within strain RM1221 (Gaasbeek et al., 2010, Gaasbeek et al., 2009). The genes are widely distributed within *C. jejuni* and appear either individually or in combination within the genomes of many strains (Figure 7-1).

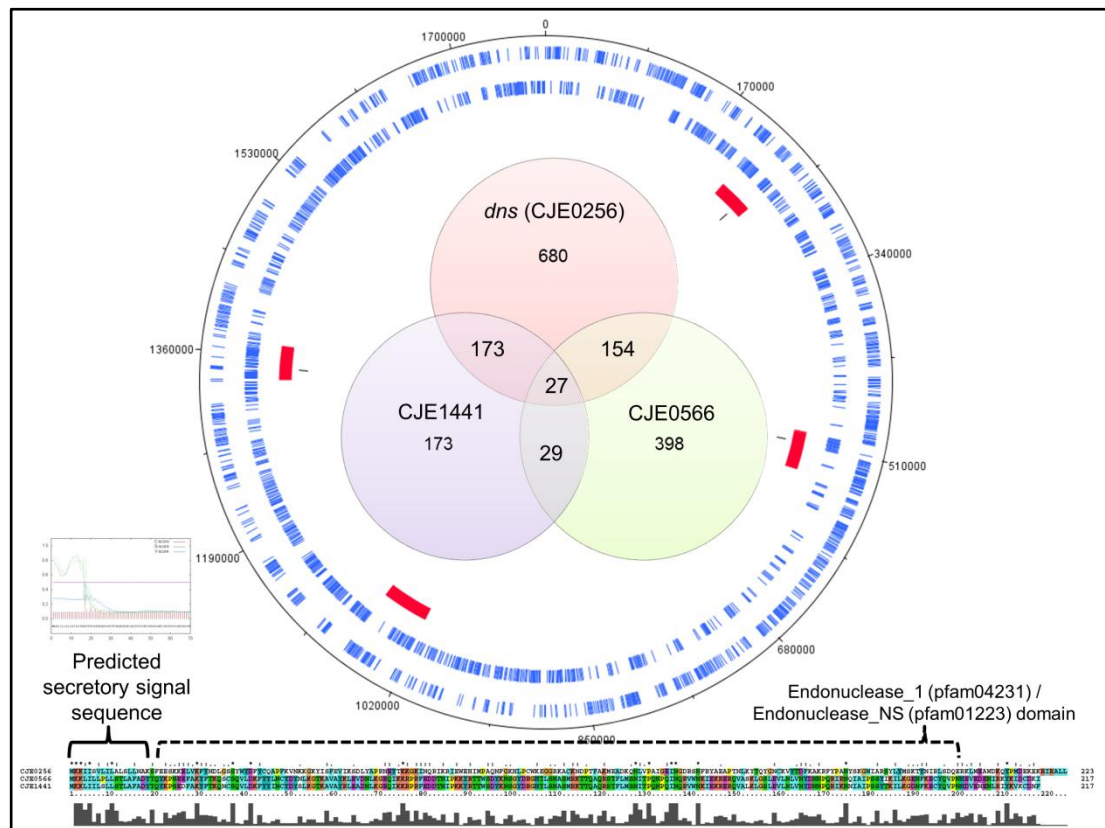


Figure 7-1 Distribution of eDNase genes across *C. jejuni* sequences.

Diagram from Brown et al. (2015) showing the distribution of eDNase genes within *C. jejuni* strains. The Venn diagram shows the distribution of the eDNase genes *dns* (*cje0256*), *cje0566* and *cje1441* in the genome sequences of 1621 eDNase gene-positive *C. jejuni* strains. The Venn diagram is encircled by the RM1221 chromosome showing open reading frames (blue), CJIE1-4 insertion elements (red), and the position of the three eDNase genes (black). Finally, the bottom part shows an amino acid sequence alignment of the Dns, CJE0566 and CJE1441 proteins, with the signal sequence and Pfam domains indicated. Signal sequences were predicted using PSORTb version 3.0.2.

The impact of eDNase expression on *C. jejuni* biofilm formation has not previously been investigated. In this chapter, phenotypic and genetic data demonstrates that eDNase activity by *C. jejuni* RM1221 not only inhibits biofilm formation by the strain RM1221, but can also effect biofilm formation in *C. jejuni* isolates lacking eDNase genes. Isolation of DNase proteins from strains such as RM1221 could provide a cheap and relatively easily obtainable source of DNase for use within novel biofilm eradication treatments.

7.2 Results

7.2.1 *C. jejuni* strain RM1221 is unable to form a biofilm during static incubation

In Section 4.2.3 (Figure 4-5) it was observed that *C. jejuni* strain RM1221 was unable to form a biofilm in Brucella medium alone. To confirm the previous observations a comparison of biofilm formation by *C. jejuni* strains NCTC 11168, 81116 and RM1221 was performed, using crystal violet staining of borosilicate glass test tubes. A clear difference between the levels of biofilm formation in strains NCTC 11168 and 81116 was observed compared to strain RM1221, with the latter showing very little difference to the negative control (Brucella medium only) (Figure 7-2a).

Swarming (Figure 7-2b) of RM1221 was significantly ($P = <0.001$) reduced compared to NCTC 11168. Motility has previously been shown to be important in *C. jejuni* biofilm formation (Reuter et al., 2010, Svensson et al., 2014). Microscopic observation of overnight RM1221 cultures indicated that strain RM1221 is able to swim in a comparable manner to NCTC 11168, and was significantly more motile than an 11168 $\Delta flaAB$ mutant. This indicates that although swarming was reduced, strain RM1221 was still able to swim in a comparable manner to NCTC 11168. Autoagglutination (Figure 7-2c) was also measured and compared to NCTC 11168 WT and the NCTC 11168 $\Delta flaAB$ mutant. Analysis showed that autoagglutination of RM1221 was not statistically different to the biofilm-forming NCTC 11168 strain. Taken together these results suggest that the lack of biofilm formation in RM1221 is not due to deficiencies in either motility or autoagglutination.

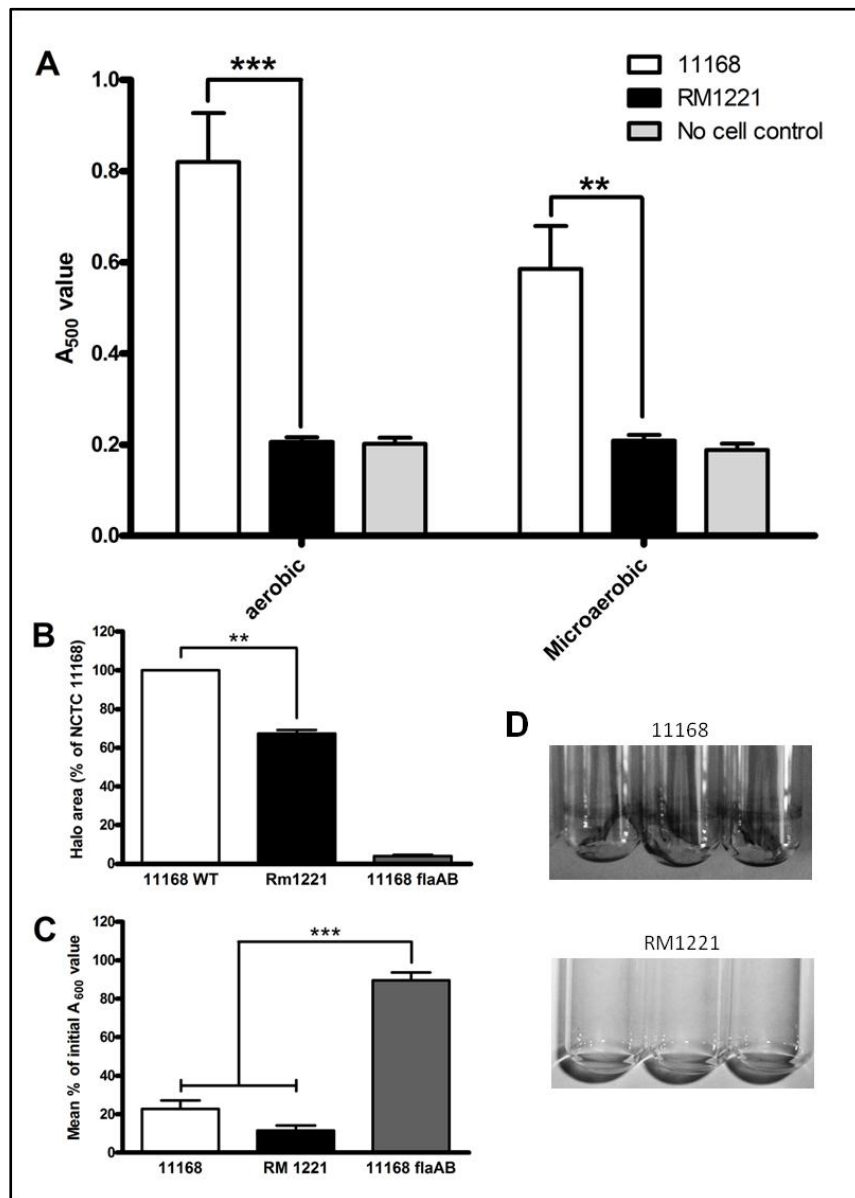


Figure 7-2 Strain RM1221's inability to form a mono-species biofilm cannot be attributed to either swarming or autoagglutination (AAG) deficiency.

Biofilm formation (A) of RM1221 (light grey bars) was measured by crystal violet staining and compared to NCTC 11168 (white bars) and a test tube containing only Brucella medium (black bar). Swarming ability (B) was calculated by measuring halo area on soft agar after 48 hours incubation in microaerobic conditions. Autoagglutination assessment (C) was carried out by observing the reduction in OD₆₀₀ measurement over a 24 hour period. Both B and C show data for 11168 (white bars), RM1221 (light grey bars) and 11168 $\Delta flaAB$ (dark grey bars). Error bars show SE (N = 5) and significance was measured using Bonferroni post-test (** = P<0.01, *** = P<0.001) following ANOVA analysis. Panel D shows representative images of crystal violet stained test tubes following a 48 hours static incubation of 11168 and RM1221 cultures.

7.2.2 Microscopy indicates that *C. jejuni* RM1221 cannot effectively attach to abiotic surfaces

Crystal violet staining has a high background level due to its non-specific nature. This means that low levels of cell attachment to abiotic surfaces cannot be distinguished using this technique. In order to address this issue and discover whether RM1221 is able to attach at low levels to abiotic surfaces, RM1221 and NCTC 11168 cultures were incubated statically for 48 hours in vessels containing sterile glass slides. Following static incubation the glass slides were stained with crystal violet to allow visualisation and light microscopic analysis.

Images showed that although RM1221 cells displayed some initial attachment to the slide, there was no progression towards the development of microcolonies or mature biofilm formation (Figure 7-3a). This was in contrast to strain NCTC 11168 which showed mature biofilm formation following 48 hours of static incubation (Figure 7-3b). Assessment of MPN showed comparable values between strains RM1221, NCTC 11168 and 81116 following static culture for 48 hours (Figure 7-3c). This suggests that RM1221 is able to survive for extended periods in both aerobic and microaerobic static cultures but, unlike strains NCTC 11168 or 81116, is unable to attach to the glass surface in sufficient quantities to allow biofilm initiation.

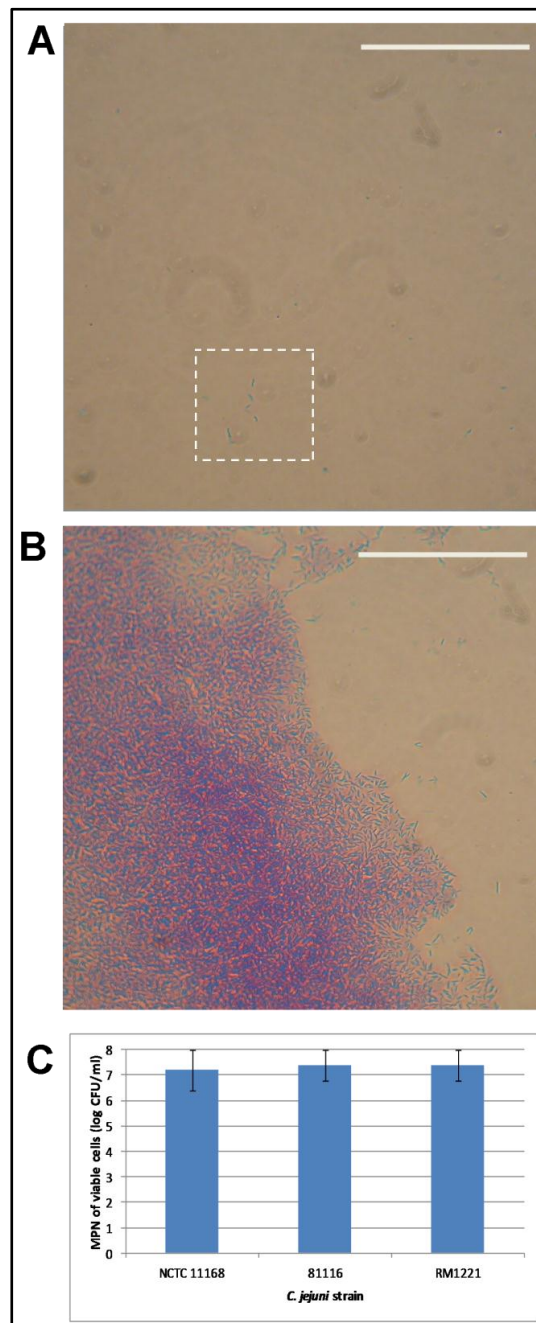


Figure 7-3 *C. jejuni* strain RM1221 is unable to form microcolonies or biofilms

Panels A and B show representative images of the air/liquid interface of a glass slide following 48 hours of static incubation at 37°C in aerobic conditions. Panel A shows a slide incubated with RM1221 cells and panel B shows a slide incubated with NCTC 11168 (right side of image is above the air liquid interface). The highlighted area in panel A shows attached RM1221 cells, although no progression to microcolony or mature biofilm formation was observed. Panel C shows a MPN assessment of strains 81116, NCTC 11168 and RM1221 following a 48 hour static incubation at 37°C in aerobic conditions. Scale bar indicates 100 µm and enlarged versions of the microscopy images can be found in Appendix 3.

7.2.3 Cell suspensions of *C. jejuni* RM1221 are able to degrade exogenous, genomic DNA

The DNase genes of *C. jejuni* strain RM1221 have previously been shown to have DNA degrading activity when expressed individually in *C. jejuni* C019168 (Gaasbeek et al., 2010). In order to confirm that the DNase proteins were also active in strain RM1221, cells from overnight shaking cultures were concentrated and spotted on to DNase agar plates before further incubation, allowing degradation of the DNA within the plates and subsequently halo production (Figure 7-4).

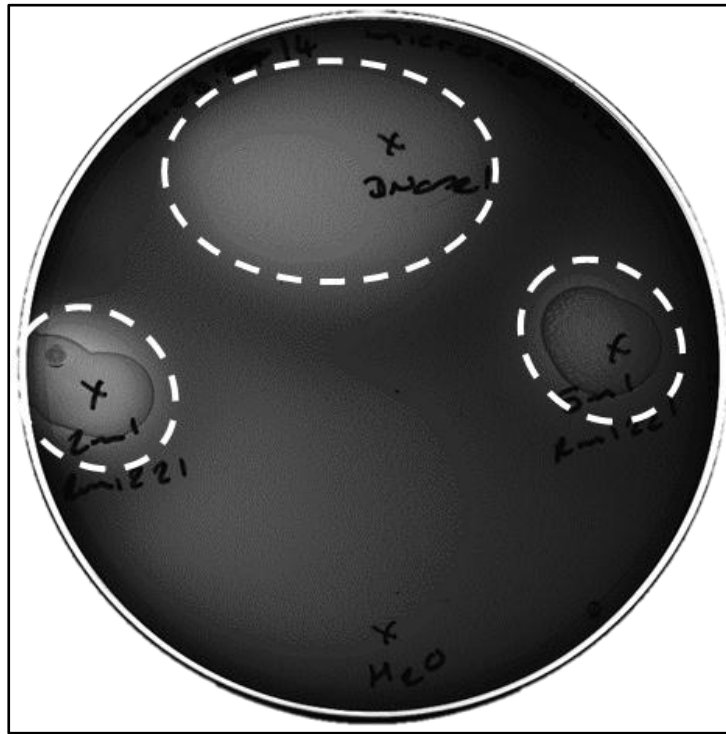


Figure 7-4 *C. jejuni* strain RM1221 is able to degrade exogenous DNA

Representative image of DNase agar plate (Oxoid) incubated with RM1221 cell cultures or a sterile H₂O negative control. 15 ml RM1221 cell suspensions were concentrated to a final volume of 2 ml (left halo) and 5 ml (right halo) and incubated for 48 hours to allow potential expression of eDNase proteins. DNase I was used as a positive control (top halo) and sterile water as a negative control (labelled H₂O at bottom of plate).

The presence of a halo surrounding the cell culture spots confirmed that RM1221 does express active DNase proteins, albeit at a low level. A positive control of 1 µl of DNase I in final volume of 10 µl water containing 1 x DNase I buffer was used to confirm that DNA present within the plates could be degraded in microaerobic conditions at 37°C (conditions which were chosen to allow optimal RM1221 viability and potential DNase protein expression). Detection of DNase activity in the cell free supernatant of RM1221 cultures was not possible. This is potentially due to the attachment of the DNase proteins to the RM1221 cells, or due to their low level of expression. Since 15 ml overnight cultures had to be concentrated to a 2 ml volume before spotting on to the agar in order to allow a easily

observable halo it is hypothesised that any expression of the proteins in supernatant could be too dilute to measure in a system with low sensitivity.

To further investigate the DNA-specific activity of the *C. jejuni* RM1221 eDNases, 2 µg of genomic DNA from *C. jejuni* strain NCTC 11168 was combined with washed RM1221 cells, and incubated at 37°C to allow digestion of the gDNA by the RM1221 DNases. A time-dependent degradation of *C. jejuni* genomic DNA was observed (Figure 7-5) by RM1221 cultures, however no DNA degradation was observed when *C. jejuni* strain NCTC 11168 was added to genomic DNA. This suggests that the DNase activity displayed by RM1221 is not found in a biofilm forming strain such as NCTC 11168.

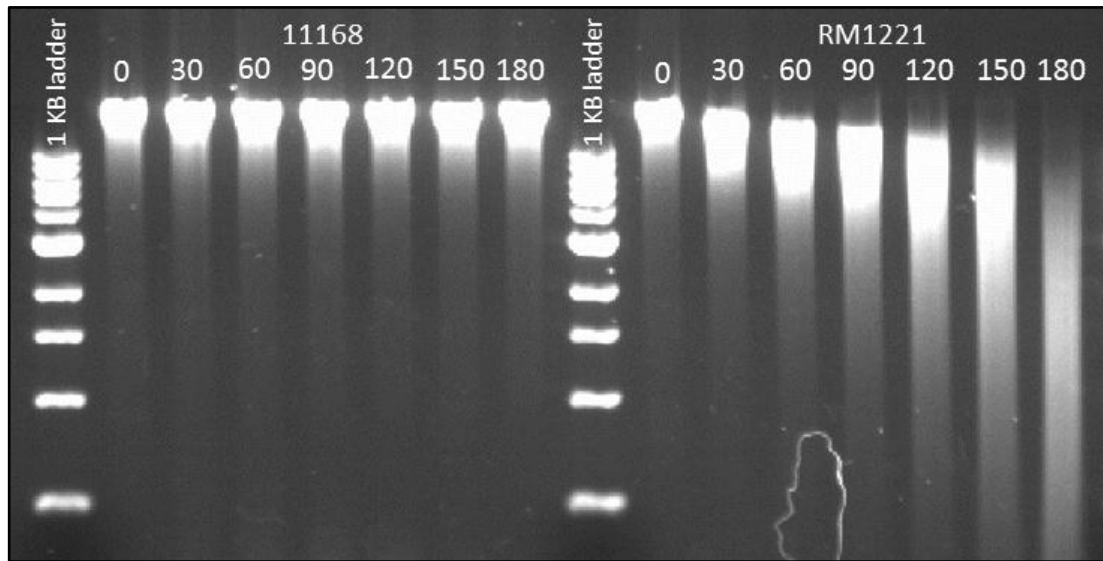


Figure 7-5 *C. jejuni* RM1221 is able to degrade NCTC 11168 genomic DNA over a three hour time period.

The ability of NCTC 11168 and RM1221 to degrade NCTC 11168 genomic DNA was assessed by incubation of cell suspensions (in log phase of growth) with genomic DNA at 37°C for three hours. The numbers at the top of the images indicate the incubation time (in minutes) and a 1KB ladder is included for size comparison. *C. jejuni* NCTC 11168 culture (left-hand image) is unable to degrade the genomic DNA, with a band of genomic DNA of >10 kb remaining for the duration of the assay, while incubation with RM1221 (right-hand image) results in degradation of genomic DNA, indicated by the 'smearing' of the DNA as the time course progresses.

7.2.4 eDNA cannot be detected in *C. jejuni* RM1221 cell suspensions

Since eDNA from *C. jejuni* strain NCTC 11168 is able to be degraded by RM1221 cell suspensions, it was hypothesised that RM1221 supernatants may be able to degrade their own eDNA. In order to assess this possibility, the supernatant from shaking NCTC 11168, 81116 and RM1221 cultures were collected and sterile filtered. They were treated for one hour with DNase I or RNase before gel electrophoresis to allow visualisation of the DNA. This allowed not only genetic material within the supernatant to be detected, but also information to be gathered on its composition, with respect to DNA and RNA content, and degradation status. Genetic material was detected in NCTC 11168 and 81116 supernatants, but not RM1221 supernatant (Figure 7-6). In both NCTC 11168 and 81116 RNase treatment

had not visible effect on the genetic material, whereas DNase I treatment led to loss of bands.

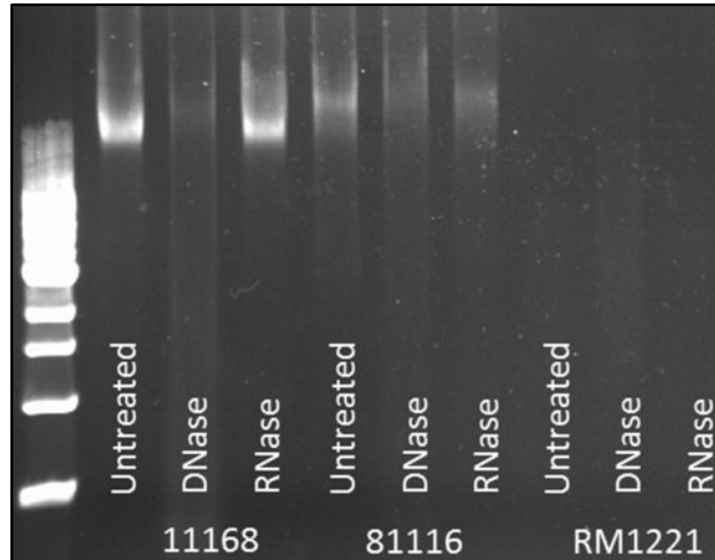


Figure 7-6 eDNA cannot be detected in RM1221 suspensions.

Representative images of gel electrophoresis of *C. jejuni* NCTC 11168, 81116 and RM1221 supernatants following overnight shaking growth. The image indicates that although NCTC 11168 and 81116 cultures contained genetic material, which could be degraded by DNase I but not RNase, no genetic material could be detected in RM1221 cultures, even prior to enzymatic treatment. Gels were run at 100V for 40 minutes, the ladder shown in the first lane is a NEB 1kb ladder.

In order to better gauge the time period over which this degradation took place shaking cell suspensions were cultured at 37°C in microaerobic conditions for 48 hours. At regular intervals cell concentrations (Figure 7-7a) and eDNA concentrations (Figure 7-7b) were measured. Throughout the 48 hour time course cell concentration of all three *C. jejuni* strains increased as expected. With regards to eDNA concentration all strains showed high levels of variability in eDNA concentrations over the first ten hours of growth, and an overall decrease in eDNA concentration throughout the 48 hour time course (Figure 7-7b). This trend was particularly clear within RM1221 cultures, where from six hours onwards a statistically significant reduction was seen, when RM1221 eDNA concentrations were compared to both NCTC 11168 ($P = \leq 0.05$) and 81116 ($P = \leq 0.01$) eDNA concentrations.

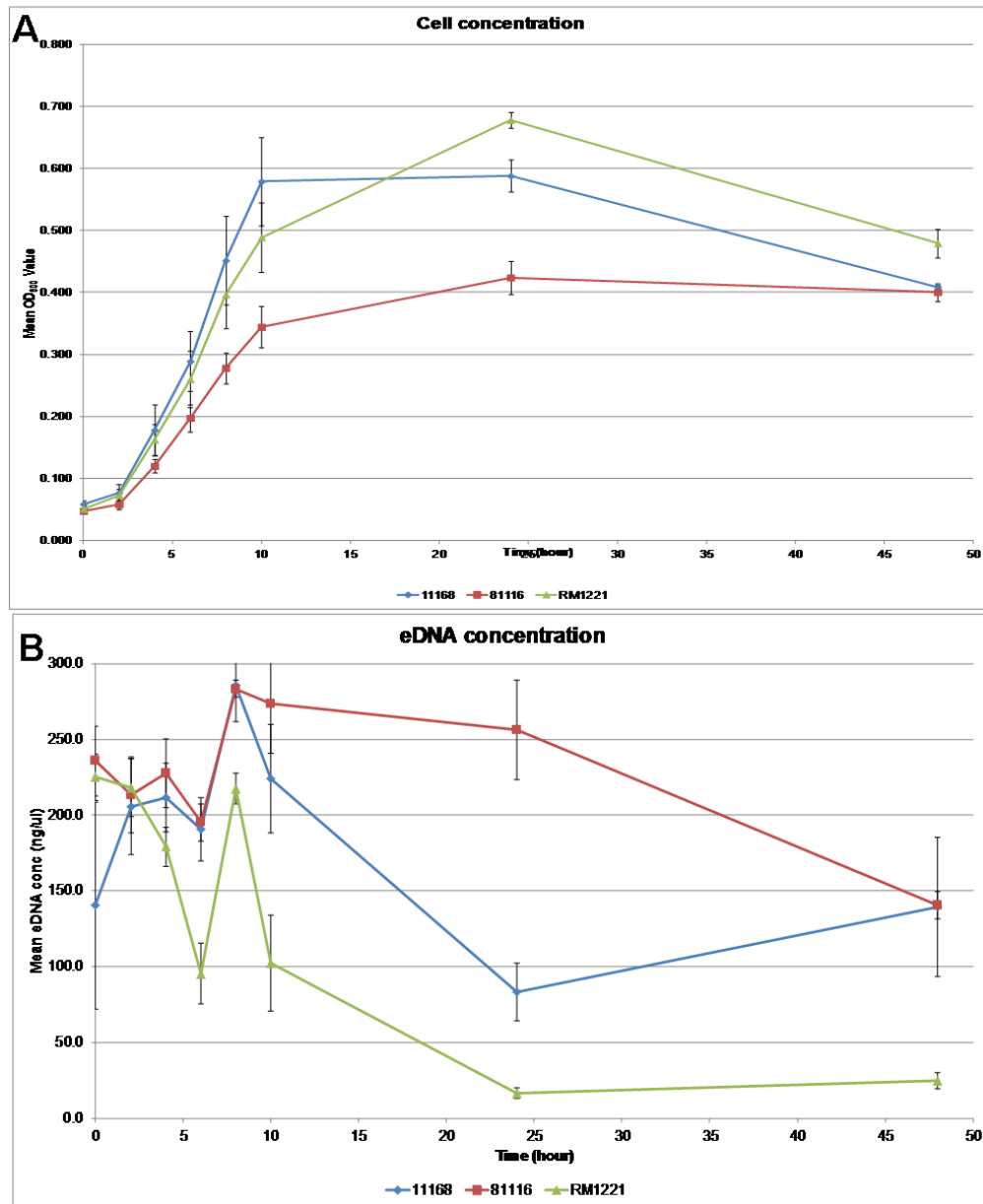


Figure 7-7 eDNA concentrations rapidly decrease in RM1221 cell suspensions.

Cell concentration (A), measured by optical density, and eDNA concentration (B), measured by Nano drop following isolation from supernatant of shaking microaerobic *C. jejuni* NCTC 11168 (blue bars/lines), 81116 (red bars/lines) and RM1221 (green bars/lines) cultures, were measured over a 48 hour period. For all strains the concentration of eDNA decreased over the 48 hour time period. This decrease was particularly apparent within RM1221 samples, which showed a statistically significant reduction ($P = \leq 0.05$) from the six hour measurement onwards. This reduction was not related to cell growth, which continued to increase as expected over the time course. Error bars show SE ($N = 3$) and statistical analysis was carried out using Student T Test.

Taken together, the results from 7.2.3 and 7.2.4 suggest that RM1221 does express active eDNase proteins, which are likely to be responsible for the degradation of DNA within RM1221 cultures. It was shown in 6 that eDNA is not only a component of the *C. jejuni* ECM, but its degradation by DNase I leads to biofilm disintegration. It is suggested that RM1221's eDNase genes are responsible for its lack adhesion to abiotic surfaces, and its inability to form biofilms.

7.2.5 Genetic manipulation of RM1221 eDNase genes is confounded by the strains DNase activity

In the *C. jejuni* NCTC 11168 background, genes can routinely be insertional inactivation and complementation to better understand their function. Inactivation of the three eDNase genes in RM1221 was therefore attempted by insertion of an antibiotic resistance cassette into each of the genes. Although manipulation of all three genes was tried, only *cje1441* was successfully inactivated. The *cje0566* gene was particularly hard to work with as its similarity to *cje1441* meant that attempts to amplify *cje0566* led to amplification of fragments from both the *cje0566* and *cje1441* genes (Figure 7-8).

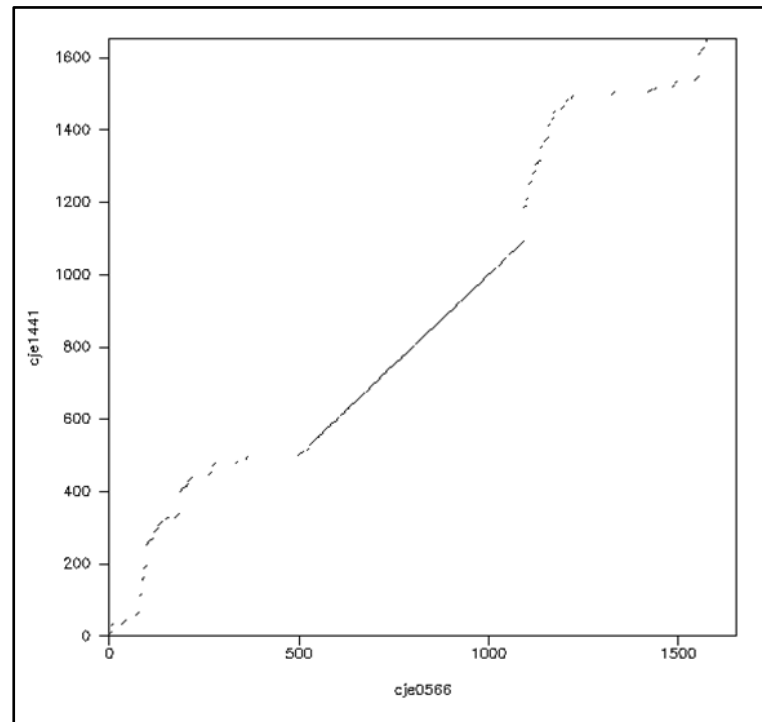


Figure 7-8 Dot plot comparison of *cje0566* and *cje1441* including the surrounding 500 base pair sequences.

A dot plot comparison of the sequences of the RM1221 genes *cje0566* and *cje1441* and the surrounding genomic sequence (500 bp. either side of the genes). Analysis showed that the two genes are almost identical in their genetic code and have very similar flanking regions. This similarity increases the complexity of primer design and in part may explain the difficulties experienced in the cloning of these genes.

This similarity confounded efforts to insert a fragment containing the *cje0566* gene into the pNEB193 plasmid. Creation of the *dns* inactivation plasmid (pHB010) was more successful, with a *dns* containing fragment been successfully inserted into the pNEB193 plasmid and inactivated by insertion of the kan cassette (for plasmid map see Section 10.2). Although this plasmid was confirmed to contain the correct genetic code by sequencing, despite multiple attempts to insert the plasmid into RM1221, a successful transformation was never achieved. This result confirms the proposed role of *dns* in preventing natural competence (Gaasbeek et al., 2010, Gaasbeek et al., 2009).

Although successful inactivation of the *cje1441* gene was achieved, its complementation was not possible. Constructs expressing the RM1221 eDNase genes from a constitutive *C. jejuni* promoter invariably accrued spontaneous promoter mutations (Figure 7-9), meaning it was not able to be transcribed.

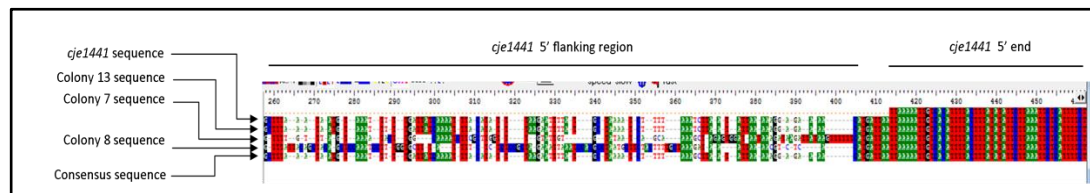


Figure 7-9 Constructs expressing *cje1441* from a constitutive promoter accumulate spontaneous point mutations in the promoter region.

Alignment of sequenced plasmid fragments was carried out using BioEdit software and compared to the *cje1441* sequence obtained from Artemis. This image is a representative example of three plasmid sequences (in this case from pHB002 – see Section 10.2 for plasmid map) in comparison to the RM1221 gene sequence. The lack of consensus in the gene flanking regions can be clearly observed, however the gene itself shows a high degree of preservation. Plasmid DNA isolated from three different colonies from the ligation transformation (colony 7, 8 and 13) all contained spontaneous but different point mutations in the promoter region, most likely resulting in no transcription of the open reading frame, suggesting that expression of the active gene is detrimental to the viability of the plasmid encoding *E. coli* cells.

This accumulation of spontaneous mutations was observed in plasmids pHB001 to pHB006. These plasmids were designed for insertion of *dns* and *cje1441* into the cj0046 pseudo-gene region of *C. jejuni* NCTC 11168 or RM1221 as part of the complementation strategy. The genes were under control of either an *fdxA* (pHB001 to pHB003) or *metK* (pHB004 to pHB006) promoter (hereafter referred to as *fdxA*^{Pr} and *metK*^{Pr}). Since it has previously been reported that *E. coli* is able to recognise *C. jejuni* promoters, such as *metK*^{Pr} (Wosten et al., 1998), it hypothesised that translation of the eDNase genes by *E. coli* was lethal, with only *E. coli* containing non-translational DNase genes being able to grow following transformation. Transformation of *E. coli* Top 10 cells with the pCporAGFP⁺ plasmid led to expression of GFP (Figure 7-10), confirming that *E. coli* is able to transcribe and translate genes under control of *C. jejuni* promoters.

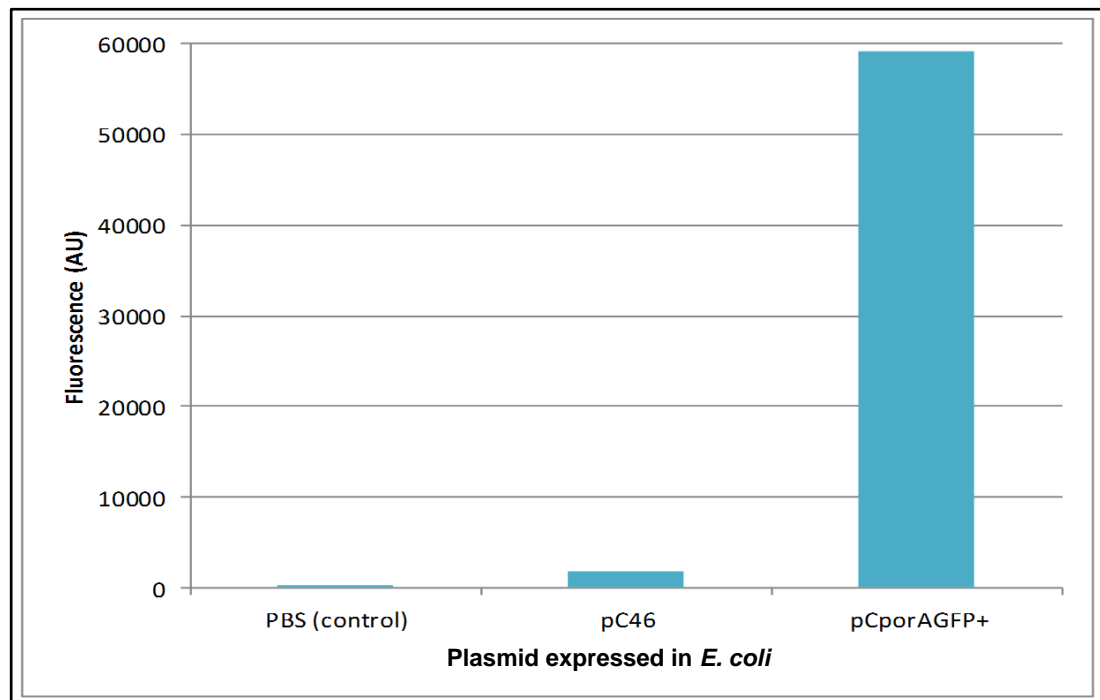


Figure 7-10 *E. coli* Top 10 cells are able to express proteins from a *C. jejuni* promoter.

E. coli Top 10 was transformed with pCporAGFP⁺ (expressing GFP⁺ under control of the *C. jejuni* porA promoter) and pC46 (a plasmid analogous to pCporAGFP⁺ but lacking the porA promoter and GFP⁺ gene). Following 24 hours of growth on LB plates the cells were resuspended, adjusted to an OD₆₀₀ of 4.5 and their fluorescence measured. The high fluorescence readings obtained for the pCporAGFP⁺ suspension confirms that *E. coli* is able to translate proteins expressed from *C. jejuni* promoters.

7.2.6 Transformation of *C. jejuni* RM1221

The major difference between *C. jejuni* strains NCTC 11168, 81116, and their counterpart *C. jejuni* strain RM1221 is the presence of the CJIE1-4 insertion elements, which have previously been reported to inhibit natural transformation (Bleumink-Pluym et al., 2013, Gaasbeek et al., 2010, Gaasbeek et al., 2009). It is hypothesised that the three DNase genes contained within these insertion elements also limit the ability to genetically manipulate RM1221 *in vitro*. In order to successfully manipulate RM1221, modifications to the standard transformation protocol were required, to inhibit DNase activity. To achieve this inhibition two methods of EDTA supplementation of bacterial suspensions were compared. Growth of RM1221 in EDTA supplemented Brucella medium and supplementation of CTB medium with EDTA were compared with respect to their ability to reduce DNase activity and maintain a high level of cell viability. The toxicity of EDTA towards both planktonic bacteria and biofilms is well reported (Al-Bakri et al., 2009) and so the effect of six EDTA concentrations, ranging from 1mM to 10mM was assessed (Figure 7-11).

Initially RM1221 growth in EDTA supplemented overnight cultures was considered as a potential solution, it was thought that this would allow a relatively high cell density to be achieved while also allowing the EDTA to act on eDNases produced at various stages of growth (since it is currently unknown if RM1221 eDNase expression is linked to a particular

growth phase). Incubation of RM1221 cultures with all concentrations of EDTA lead to significant inhibition of growth, and as the concentration of EDTA increased above 1 mM, the viability of the cultures dropped below the MPN limit of detection and viable cells could be recovered (Figure 7-11).

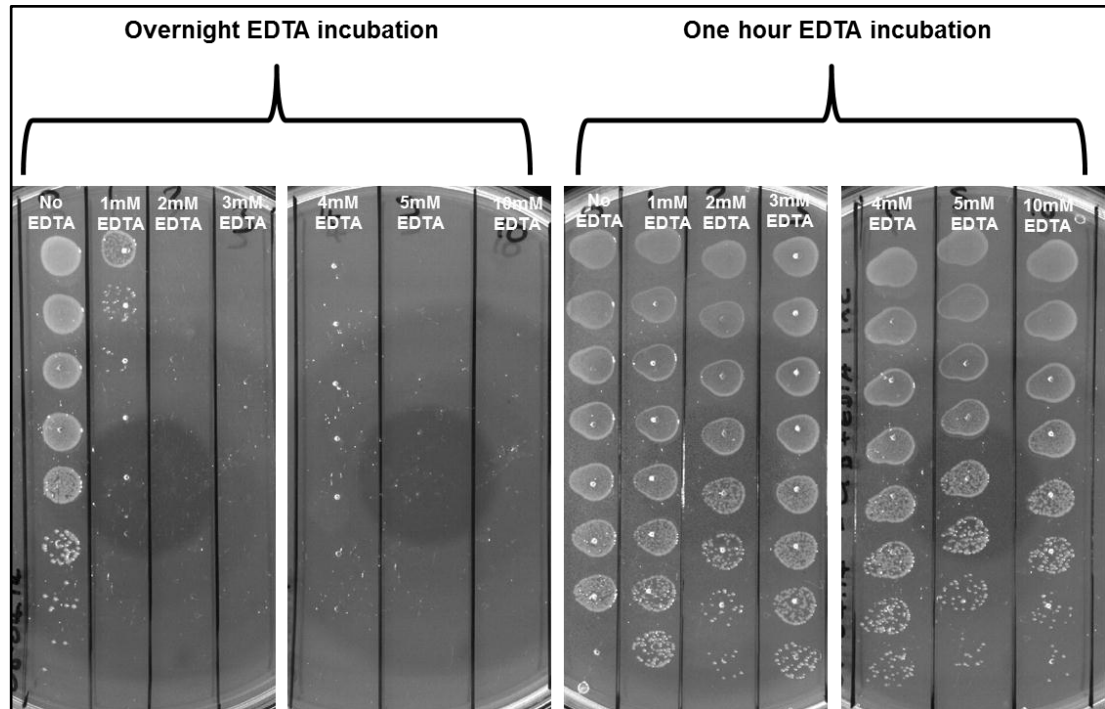


Figure 7-11 MPN assessment of *C. jejuni* RM1221 following exposure to EDTA containing medium.

Representative images of MPN assessment following either an overnight (two left-hand panels) or one hour (two right-hand panels) incubation of *C. jejuni* RM1221 cultures with several EDTA concentrations (the concentrations used are listed in white at the top of the images). While overnight incubation with EDTA led to a loss of viability in all EDTA containing medias, a one hour incubation showed good recovery of viable cells.

Since overnight growth of RM1221 in the presence of EDTA lead to cell death a second method of EDTA supplementation was trialed. Here CTB was supplemented with the same EDTA concentrations used for overnight growth. *C. jejuni* RM1221 cells from a Skirrow plate were resuspended within this buffer and incubated for one hour on ice. This method not only more closely reflects the current laboratory transformation protocol (Section 2.5.10.1), but limits the exposure time to EDTA, potentially increasing viability. It was also hoped that incubation of the cells on ice would naturally reduce enzymatic activity, as RM1221 shows DNase activity at 37°C (Section 7.2.3). Following one hour incubation both cell viability (by MPN estimation) and DNase activity were assessed. MPN was unaffected in all EDTA concentrations, being comparable to that of the no EDTA control (Figure 7-11). This suggests that both the presence of EDTA and ice incubation do not measurably affect MPN. DNase plates were used to assess the DNase activity of RM1221 following the treatments. A

concentration of 10 mM EDTA led to decreased DNase activity (Figure 7-12), and so was chosen for use in the RM1221 transformation protocol.

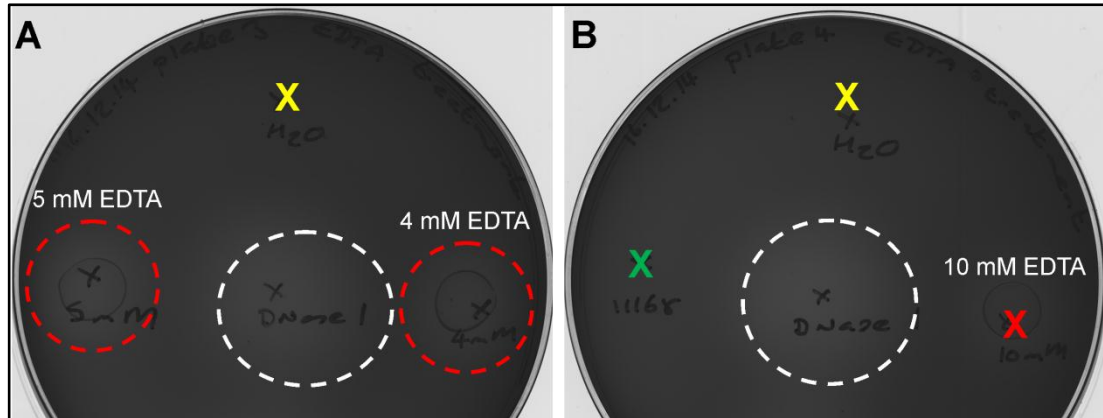


Figure 7-12 Incubation of RM1221 cells with EDTA leads to DNase activity inhibition

Representative image of DNase agar plates containing RM1221 cell suspensions (red circles/cross) following one hour incubation on ice with EDTA containing medium (concentration of EDTA is given above the halo/cross). Water (yellow cross) and *C. jejuni* NCTC 11168 (green cross) were used as positive controls. DNase I (white halo) was added as a positive control.

This new protocol (one hour incubation of RM1221 culture from a Skirrow plate, on ice with 10 mM EDTA supplemented CTB medium), along with methylation of the plasmids (Section 2.5.9) to reduce potential restriction enzyme activity, was trialed for the insertion of pHB012b (*cje1441* insertionally inactivated with *cat*) into RM1221. Following transformation, as described in Section 2.5.14, cultures were incubated on selective agar plates for 72 hours to allow growth of transformants. Analysis of the agar plates (Figure 7-13) shows that large numbers of cells are present on unsupplemented plates, again confirming that incubation of cells in 10 mM EDTA containing CTB did not adversely affect RM1221 MPN (Figure 7-13a and c). Agar plates containing chloramphenicol only showed multiple colony growth when cells had been transformed with pHB012b, suggesting that some colonies had taken up the suicide vector following electroporation (Figure 7-13b and d).

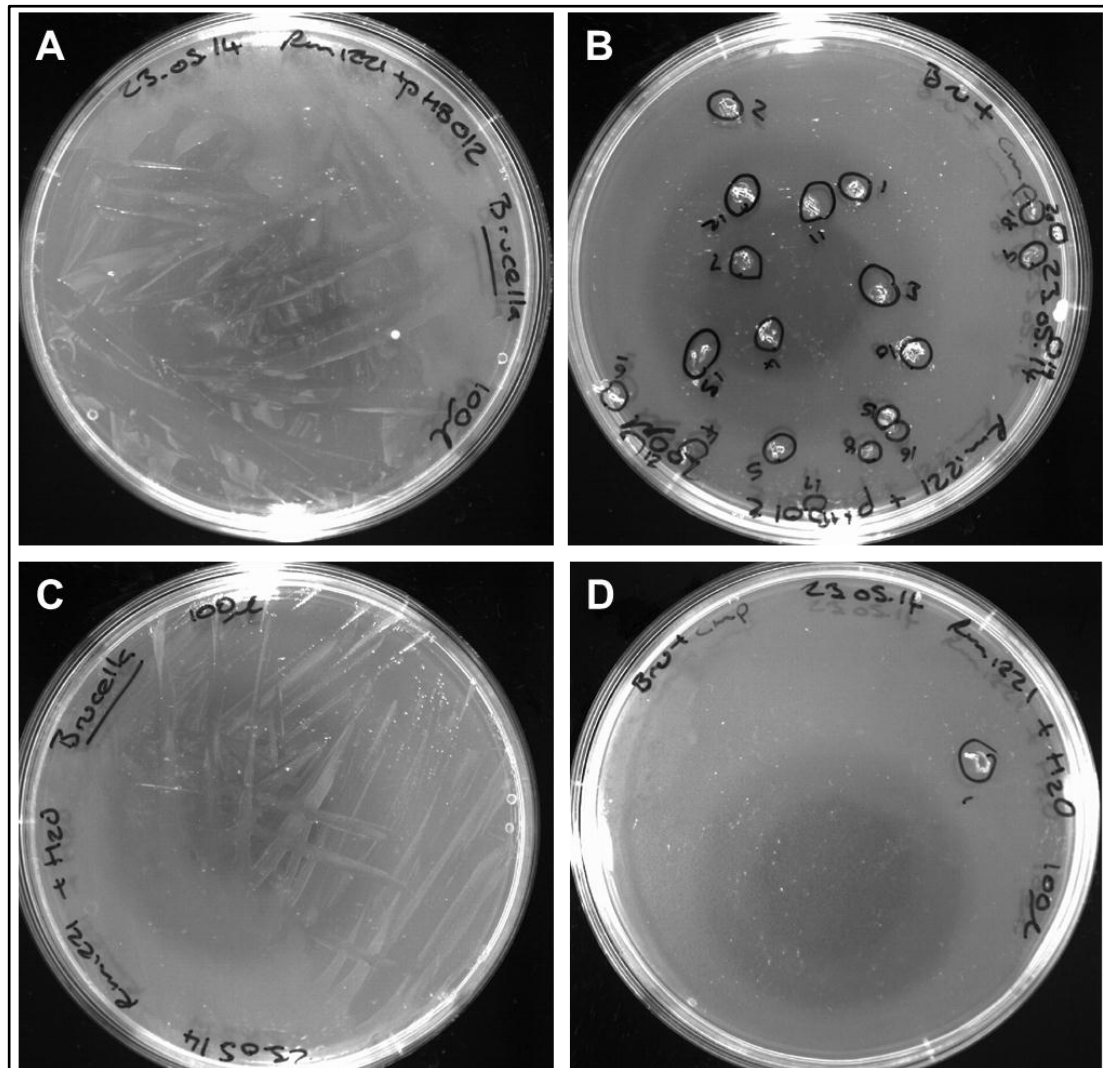


Figure 7-13 Images of agar plates following outgrowth of RM1221 pHB012b transformants

Representative images of *Brucella* (A and C) and *Brucella* Cm¹⁰ (B and D) agar plate following outgrowth of electroporated RM1221 cultures transformed with either pHB012b (A and B) or sterile H₂O (C and D). Both *Brucella* only plates show high levels of growth, indicating good survival of the cell cultures following the transformation procedure. Several resistant colonies can be seen on B but only one on D suggesting that the pHB012b plasmid has been taken up by some of the RM1221 cells, conferring chloramphenicol resistance, however electroporation in water has only resulted in the production of one chloramphenicol resistant colony. This indicates that the method has an acceptable rate of false positive results.

PCR amplification was used to confirmation that *cat* had successfully been inserted into *cje1441*. Primers were chosen which would allow amplification of both *cat* and the *cje1441* flanks, confirming that the whole construct had successfully been inserted into the RM1221 genome (Figure 7-14).

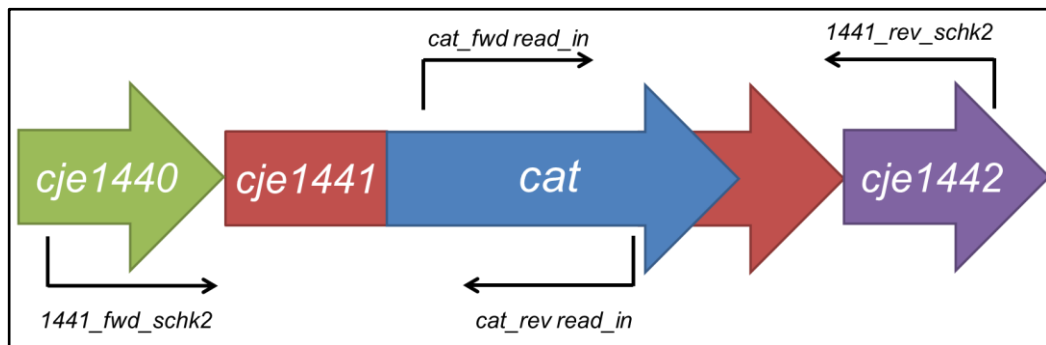


Figure 7-14 Diagram of pHB012b inactivation construct

Diagrammatic representation of the interrupted *cje1441* sequence contained within the pHB012b plasmid (for a full plasmid map of pHB012b see Section 10.2). The arrows indicate both the primer attachment sites and the direction of the read. Primers were designed to produce a fragment including both the *cat* gene and *cje1441* flanking region. This primer selection gave confidence that the *cat* gene had been inserted into *cje1441* and disrupted its production successfully.

Taken together these results suggest that the modified transformation protocol is able to be used successfully to insert a suicide vector into RM1221 cells. Eight colonies from the initial overnight growth plate (Figure 7-13b) were selected for further analysis. Genomic DNA was extracted from each colony and fragments generated using the primers shown above (Figure 7-14). Gel electrophoresis confirmed that in one of the colonies tested both fragments were present. This colony was renamed RM1221 $\Delta cje1441$ and used for all further analysis.

7.2.7 Disruption of *cje1441* restores robust biofilm formation and abolishes degradation of existing biofilms.

Phenotypic assessment of the RM1221 $\Delta cje1441$ mutant showed that growth and the ability to swarm and autoagglutinate were unaffected by inactivation of *cje1441* (Figure 7-15).

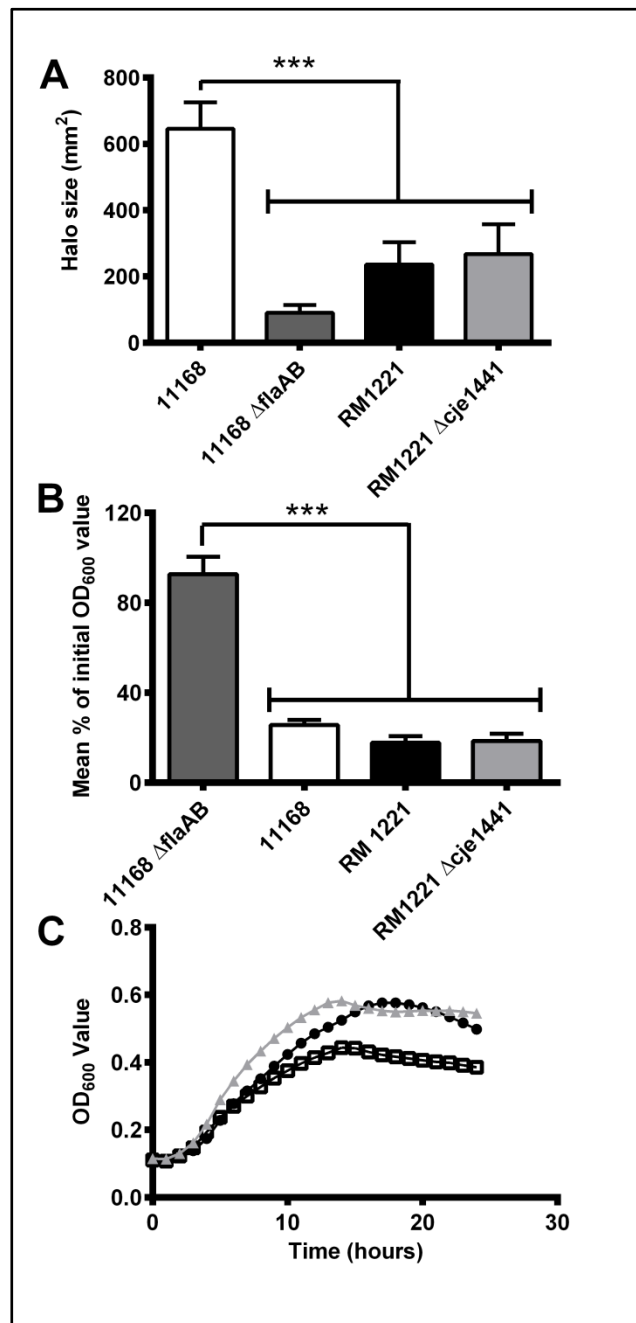


Figure 7-15 RM1221 Δ cje1441 and its parent strain show no significant difference in swarming, autoagglutination or growth.

Strains NCTC 11168 (white), its non-motile Δ flaAB mutant (dark grey), RM1221 (black bars) and the Δ cje1441 mutant (light grey) were compared for their ability to swarm (A) and autoagglutinate (B). In both tests no statistical difference was observed between Δ cje1441 and the wild-type. Panel C shows growth over a 24 hour period for Δ cje1441 (light grey triangles), RM1221 wild-type (black circles) and NCTC 11168 (white squares). Error bars show SE (N = 5) and significance was measured using Bonferroni post-test (***) = P<0.001, **** = P<0.0001) following ANOVA analysis.

Motility was also unaffected although cell morphology did appear altered, producing cells which were straighter in appearance than their WT counterparts and also slightly larger (Figure 7-16). Morphologically the RM1221 $\Delta cje1441$ mutant is more similar in appearance to *C. jejuni* NCTC 11168 than its parent strain.

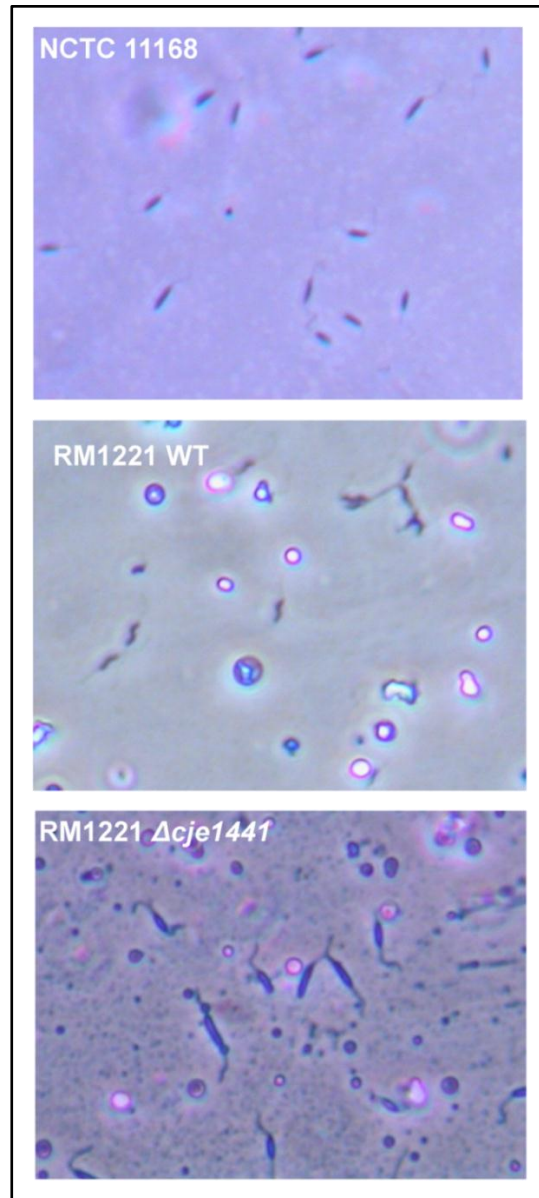


Figure 7-16 Ryu stained *C. jejuni* NCTC 11168, RM1221, and RM1221 $\Delta cje1441$

Representative microscopic images (1000x magnification) of *C. jejuni* NCTC 11168 (top panel), RM1221 (middle panel) and RM1221 $\Delta cje1441$ (bottom panel) showing alterations in morphology between RM1221 WT and mutant strains. The RM1221 WT has a spiral morphology, whereas the $\Delta cje1441$ mutant is non-spiral, like the NCTC 11168 WT. Ryu stain indicates that all strains have retained their flagella, and the mutant flagella appears to be of a similar length to both the NCTC 11168 and RM1221 strains.

Assessment of the ability to degrade DNA was undertaken in both DNase plates and in a sterile PBS solution containing 2 µg of NCTC 11168 gDNA. Analysis showed that inactivation of *cje1441* led to the loss of the eDNase activity that RM1221 displays.

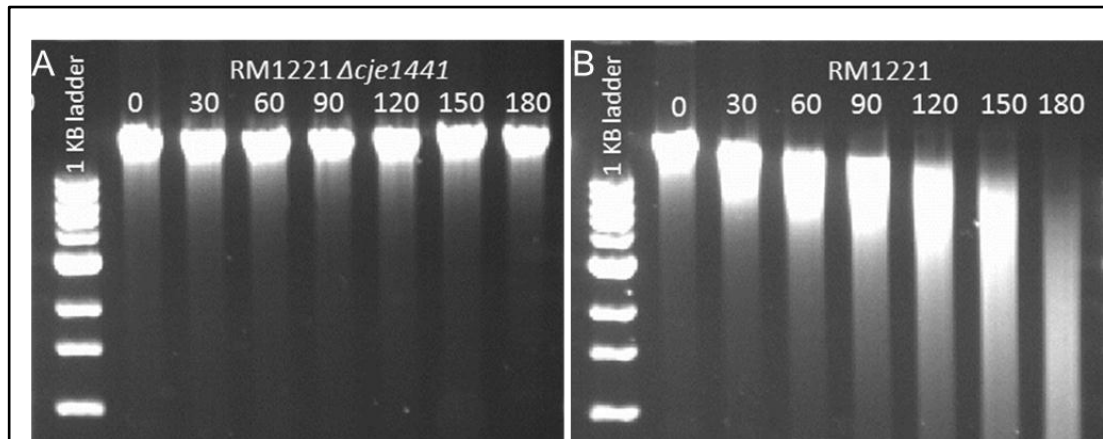


Figure 7-17 *C. jejuni* RM1221 is able to degrade exogenous DNA in both static and shaking suspensions.

The ability of RM1221 $\Delta cje1441$ mutant (A) and the RM1221 WT (B) to degrade NCTC 11168 genomic DNA was assessed by incubation of cell suspensions with genomic DNA at 37°C for three hours. Unlike the RM1221 WT strain, the $\Delta cje1441$ mutant was unable to degrade the genomic DNA, with a band of genomic DNA of >10 kb remaining for the duration of the assay. Numbers at the top of the images indicate the length of time (minutes) the incubation was carried out for and a NEB 1KB ladder has been included for size comparison.

Inactivation of the *cje1441* gene also resulted in a significant increase in biofilm formation, to levels similar to strain NCTC 11168 (Figure 7-18a). Biofilms formed by the $\Delta cje1441$ mutant were also sensitive to DNase I treatment (Figure 7-18b), once again highlighting the importance of eDNA as a component of the ECM.

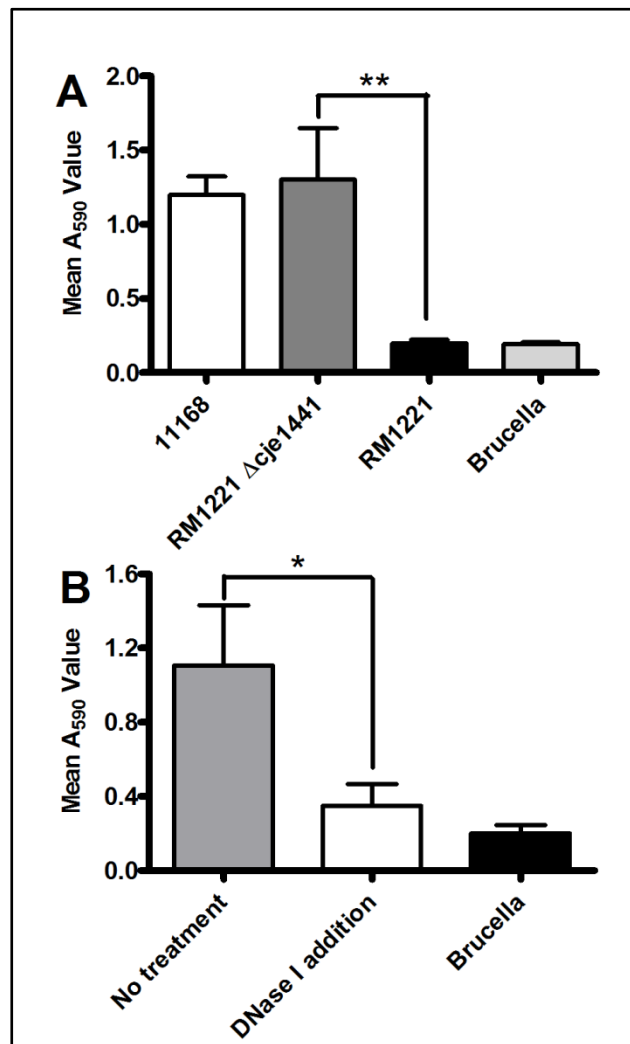


Figure 7-18 Inactivation of the *cje1441* eDNase gene restores biofilm formation by *C. jejuni* strain RM1221

(A) shows biofilm formation of NCTC 11168 (white bars), $\Delta cje1441$ (dark grey bars), RM1221 (light grey bars) and a Brucella medium only control (black bars). The $\Delta cje1441$ mutant shows similar levels of biofilm formation to NCTC 11168 and a significant increase in biofilm formation compared to the parent strain. (B) shows that the biofilm produced by the $\Delta cje1441$ mutant is susceptible to degradation by DNase I (white bar) and leads to levels of staining indistinguishable from the Brucella medium only control (black bars). Error bars show SE (N = 5) and significance was measured using Bonferroni post-test (* = P < 0.05 ** = P < 0.01).

7.2.8 Overexpression of RM1221 eDNase genes in *E. coli*

Since genetic manipulation of the RM1221 genome, or insertion of the eDNase genes into NCTC 11168, proved very difficult, it was decided that further investigation of the DNase genes should be carried out following their expression and purification in *E. coli*. In order to reduce the problems described in Section 7.2.5, the pET28 α expression system was used. This system allows tighter regulation of protein expression, as expression is suppressed in *E. coli* BL21 DE3 cells due to the presence of a T7 promoter which drives expression of the

heterologous protein. This promoter allows expression of the protein of interest to be controlled by addition of IPTG into the growth medium. It should however be noted that although expression is controlled and very much reduced in pET28 α plasmids compared to the pC46 system used previously, it is not completely repressed, and some 'leaky' expression is to be expected. The pET28 α system also allows a terminal six histidine tag (his-tag) to be added to the proteins, allowing easier purification using metal affinity columns.

The three eDNase genes from RM1221 were inserted separately into the pET28 α plasmid (plasmids containing N terminal his-tagged *dns*, *cje0566* and *cje1441* were called pHB007, 008 and 009 respectively). Correct insertion of the gene was confirmed by PCR amplification of the region between the T7 promoter and terminator, giving a fragment of approximately 850 bp. in length, when the gene had been ligated correctly. All PCR fragments of plasmids showing a band of the correct size were sequenced to ensure their accuracy before use.

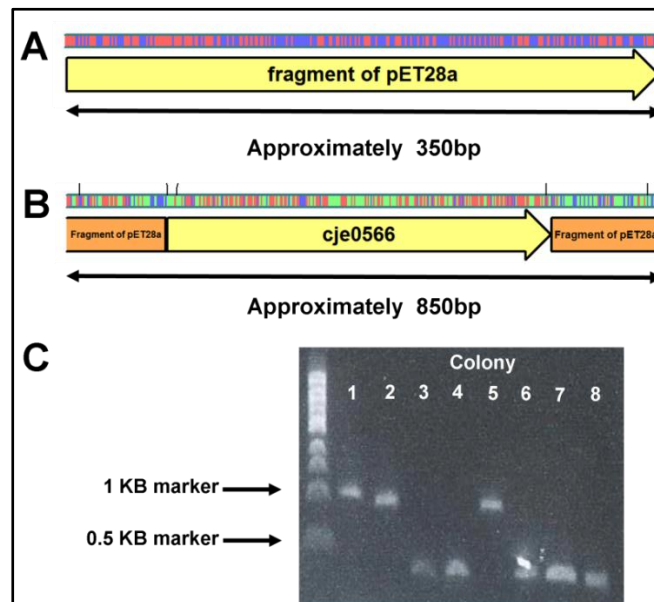


Figure 7-19 Selection of *E. coli* colonies containing pET28 α for sequencing was performed using colony PCR

Colony PCR of the T7 promoter region was performed on *E. coli* colonies following overnight growth on selective agar plates. All pET28 α plasmids without the insert gave a fragment of approximately 350 bp. (A), whereas the eDNase containing the correct regions gave a fraction of approximately 850 bp. (B) allowing distinction between colonies with and without the eDNase insert. The figure (C) shows an agarose gel with pHB008 (*cje0566*) colonies, however both pHB007 and pHB009 produced fragments of the correct size.

Following confirmation of the plasmids, *E. coli* BL21 (DE3) cells were transformed with pHB007, pHB008, pHB009 or pET28 α . Cells were grown aerobically, shaking, at 37°C for 120 minutes in LB containing 2% glucose and 30 μ g/ml Kanamycin. After 120 minutes of incubation 1 mM IPTG was added to induce expression of the DNase proteins contained within the plasmids. Cell growth was monitored throughout (Figure 7-20). Cells containing pHB007 and pHB008 showed very little growth throughout the whole time course, and

following IPTG addition the cell concentration of pHB009 containing cells also declined. Growth was unaffected in pET28 α expressing cells, suggesting that the reduced viability observed within the other cells lines was not due to the presence of the pET28 α plasmid, but rather the inclusion of the DNase genes within the plasmid.

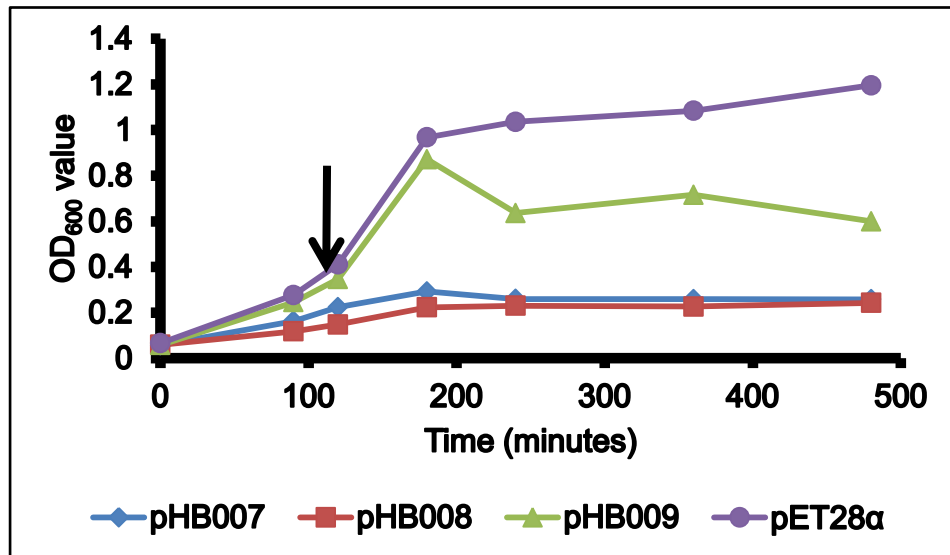


Figure 7-20 IPTG induction of *E. coli* BL21 (DE3) cell containing eDNase plasmids leads to severely retards growth

Representative example of growth curves of *E. coli* BL21 (DE3) cells containing either a plasmid expressing one of the eDNase genes (pHB007 to pHB009 expressing genes *dns*, *cje0566* and *cje1441* respectively) or plasmid with no insert (pET28 α). Following addition of IPTG at 120 minutes (indicated by the arrow), OD₆₀₀ values of pHB007 to pHB009 is consistently found to be lower than that of the cells containing pET28 α .

In order to increase cell viability during IPTG induction, cells were incubated at either 30°C or 15°C. It was hoped that this reduction in temperature would lead to reduced metabolism, protein expression and, potentially, reduced DNase activity. SDS-PAGE and Western blot analysis showed no visible DNase protein expression following the reduced temperature incubations. A pET28 α plasmid expressing *cj1388* (referred to as pCASO040) was used as a positive control and did show IPTG inducible expression (Figure 7-21). Although *cj1388* currently has no function assigned, it has previously been expressed using BL21 (DE3) cells, so is known to be non-toxic when over-expressed in *E. coli* (Reuter et al., 2015).



Figure 7-21 Western blot analysis of *E. coli* BL21 (DE3) cells shows no visible DNase expression following IPTG induction.

E. coli BL21 (DE3) cells containing either a plasmid expressing a non-toxic protein (pCASO040), or the eDNase expressing plasmids (pHB007 to 009) were incubated in either the presence or absence of IPTG at 37 °C, with 200 rpm shaking, for four hours before collection of cell pellets and western blot analysis. A representative image of a blot is shown and the labelling above the image indicates the sample run in each lane. Samples were probed using an anti-His antibody. Although a band appears in the positive control (pCASO040 +IPTG) there are no other bands on the gel. This indicates that the *E. coli* BL21 (DE3) cell line is not a suitable system for the expression of the RM1221 eDNase proteins, although expression of other *C. jejuni* proteins, such as that contained in pCASO040, is possible in this system.

In order to increase expression of the DNase proteins, the pET28 α plasmids were inserted into alternative *E. coli* strains. The *E. coli* C41 and C43 cell lines are derived from BL21 (DE3) but contain additional, un-characterised, mutations. These strains have previously been shown to have greater resistance to toxic recombinant proteins (Dumon-Seignovert et al., 2004), although a literature review shows that expression of DNase proteins has not previously been reported.

The manufacturers protocols were used for both transformation and IPTG induction. Antibiotic selection was used to confirm insertion of the plasmid before growth. Growth was monitored throughout the course of the induction procedure, in order to assess if there was a reduction in cell viability following IPTG induction. For initial toxicity assessment only pHB007 and pHB009 were used and compared to cells transformed with pET28 α or pCASO040. Growth curves for induced and un-induced cells were compared (Figure 7-22) and showed that IPTG induction did lead to reduced growth for approximately 100 minutes following IPTG addition, although cell concentrations did recover at later time points and were comparable to un-induced cells at the end of the time course.

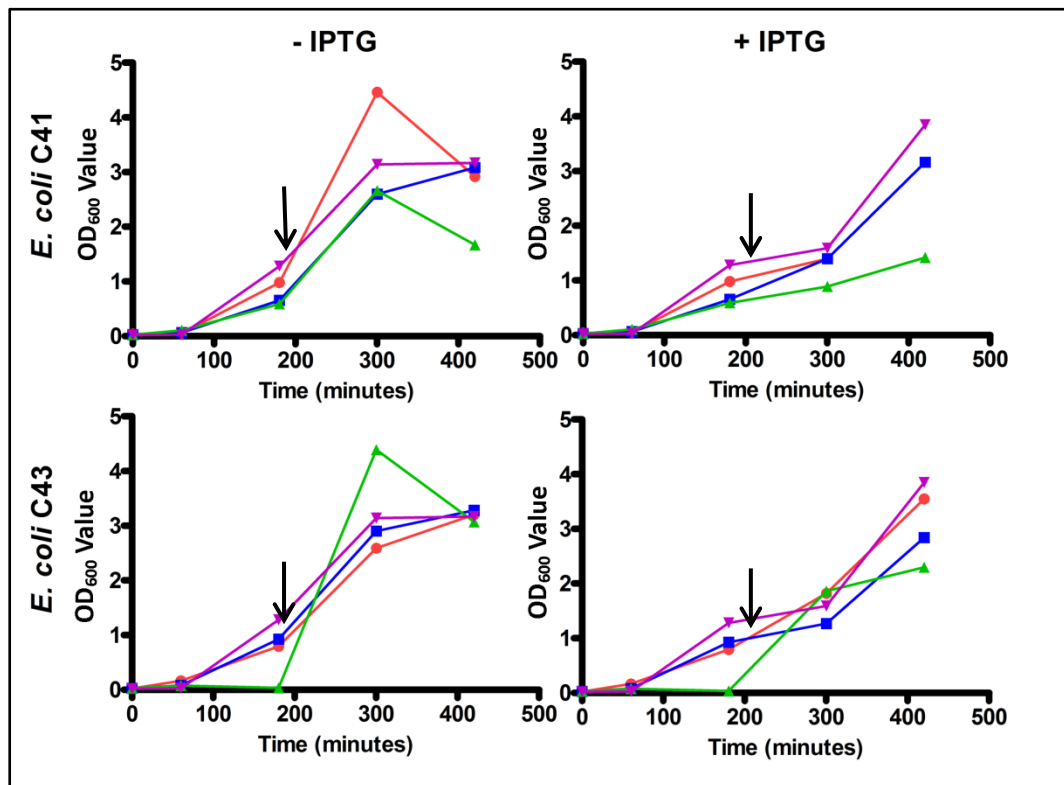


Figure 7-22 *E.coli* C41 and C43 cells have greater viability than *E. coli* BL21 (DE3) following IPTG induction

E.coli C41 and C43 cells transformed with pHB007 (blue), pHB009 (green) or pET28 (red) were grown for 180 minutes before addition of IPTG to allow expression of eDNase proteins (arrow indicated the point of IPTG addition). In order to assess the expansion of the population OD₆₀₀ readings were taken at regular intervals. As a comparison, BL21 (DE3) cells containing the positive control pCASO040 plasmid were also grown and induced with IPTG (purple). Graphs show that even following induction with IPTG (right hand column) cells expressing the eDNase containing genes were still able to proliferate. Each graph shows the growth course of a single experimental replicate.

As well as assessing growth, the *E. coli* C41 and C43 cells transformed with the three test plasmids (pHB007, pHB009, pET28 α and pCASO040) were also spotted onto DNase plates in order to assess DNA degradation activity (Figure 7-23). Following IPTG induction, halos were present around cells containing both pHB007 and pHB009, but not in the pET28 α or BL21 (DE3) cells containing pCASO040. This suggests that active DNase is expressed in detectable quantities by both C41 and C43 cells. It was also noted that C41 cells also showed un-regulated expression of pHB009 since a small halo was also observed surrounding the un-induced C41 pHB009 colony.

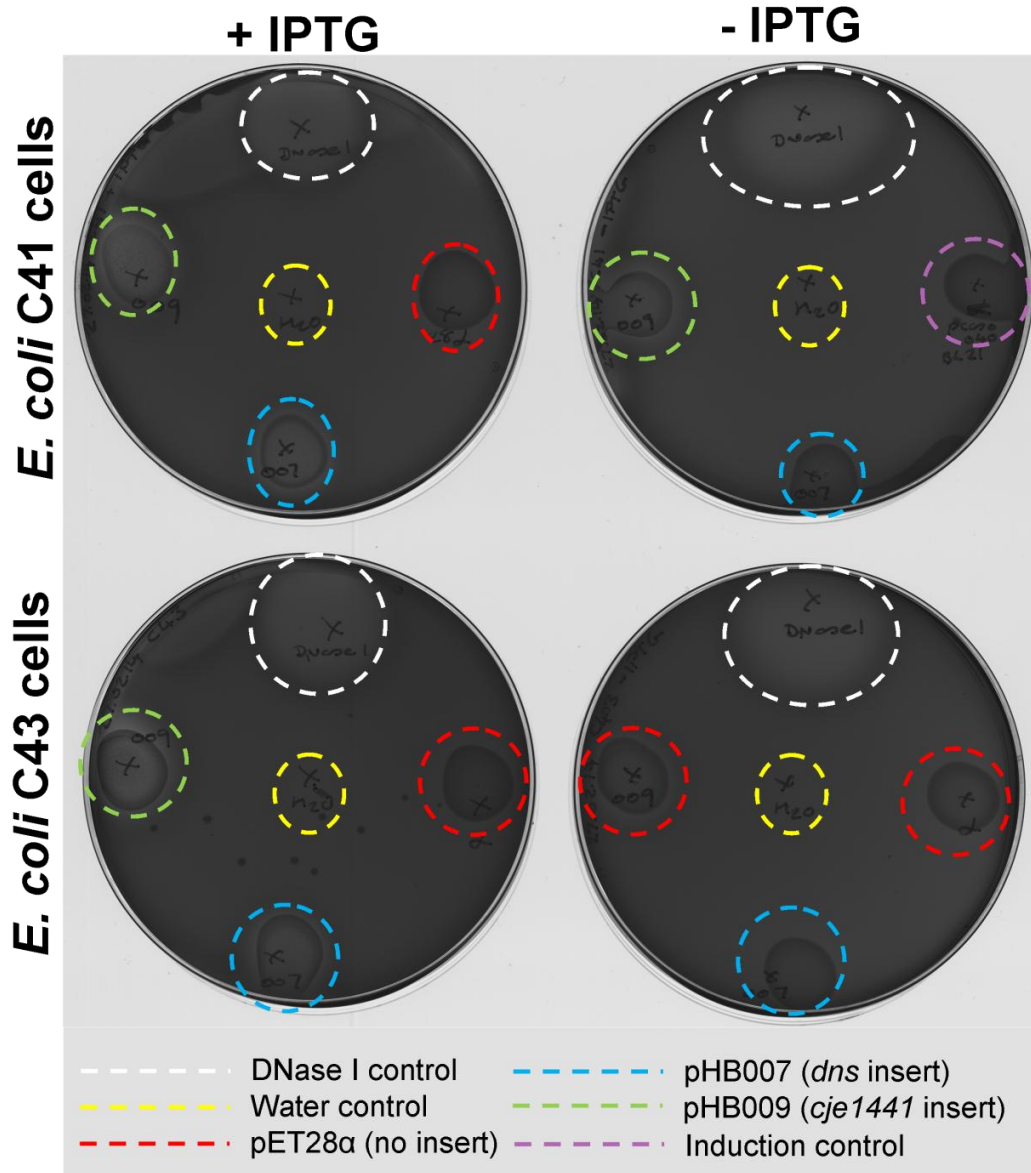


Figure 7-23 DNase activity is observed following IPTG induction of *E. coli* C41 and C43 cells.

Representative images of DNase plates spotted with *E. coli* C41 and C43 cultures containing either a pET28α plasmid with no insert (red dotted line), pHB007 (blue dotted line), pHB009 (green dotted line) or pCASO040 (purple dotted line). Cells were incubated in the presence or absence of IPTG at 37 °C with 200 rpm shaking before 5 μl was spotted onto the plates and incubated statically at 37 °C for a further 24 hours to allow halo development. As a positive control, 5 μl of DNase I was added to the plates ((white dotted line) and 5 μl of sterile water was included as a negative control (yellow dotted line). Halos can be observed surrounding the IPTG induced pHB007 (C43 cells only) and pHB009 cultures and around the positive controls but not around the other cultures.

Microscopic analysis of the C41 and C43 cells also appeared to show C43 cells were better suited to expression of the eDNase genes (Figure 7-24). *E. coli* C41 cells containing the

pHB009 plasmid appeared elongated prior to IPTG addition, suggesting an increased level of stress. They also had a reduced cell density compared to their C43 counterparts. Following IPTG induction the elongated phenotype of pHB009 containing C41 cells became more noticeable. *E. coli* C43 had a constant morphology regardless of the plasmid they contained or their induction with IPTG.

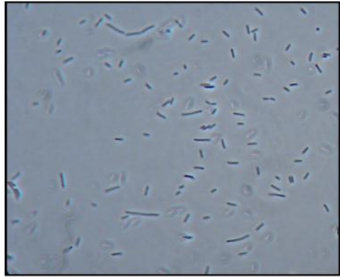
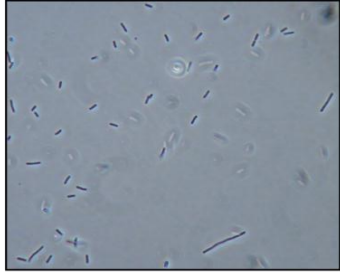
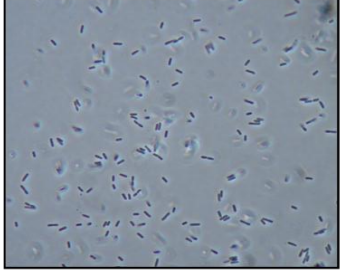
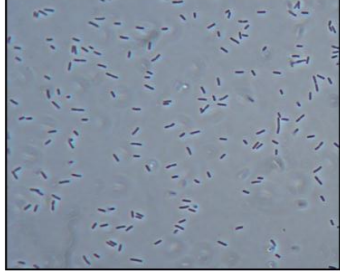
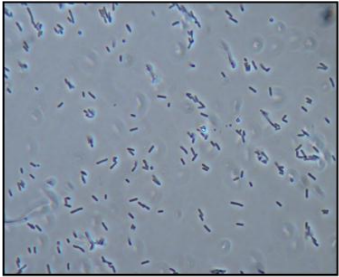
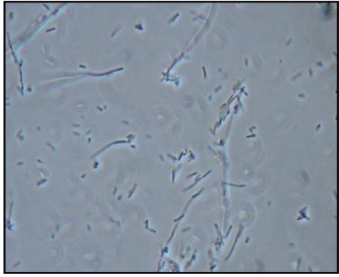
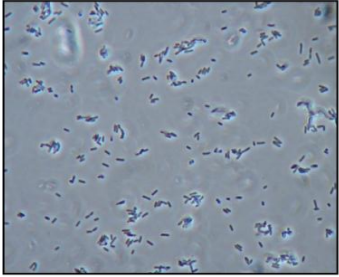
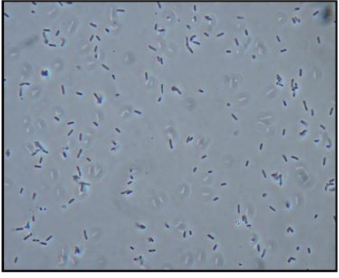
	<i>E. coli</i> C41 (pET28α)	<i>E. coli</i> C41 (pHB009)	<i>E. coli</i> C43 (pET28α)	<i>E. coli</i> C43 (pHB009)
Prior to IPTG addition				
Four hours post IPTG addition				

Figure 7-24 Cell morphology analysis suggests that *E. coli* C43 cells are more suitable for eDNase expression

Representative images of *E. coli* C43 and C41 cells containing either the empty pET28α plasmid or the eDNase containing plasmid pHB009, both before (top panels) and after (bottom panels) IPTG induction. The C41 cells containing the pHB009 plasmid have an elongated phenotype, indicating stress. There are also fewer cells in these images. This is not observed in the pHB009 containing C43 cells, indicating that they may be more suitable for use in expression of the eDNase proteins.

Although DNase activity could be observed in both *E.coli* C41 and C43 cells, protein expression was also confirmed using SDS-PAGE and western blotting (Figure 7-25). No DNase expression could be observed on SDS-PAGE gels, although the presence of Cj1388 was observed. Western blot analysis did show that DNase expression was detectable within C43 cells, but no detectable DNase expression was observed in C41 cells. Taken together these results suggest that C43 cells are more suitable for the expression of DNase than either BL21 (DE3) or C41 cells. All further induction was carried out using C43 cells only.

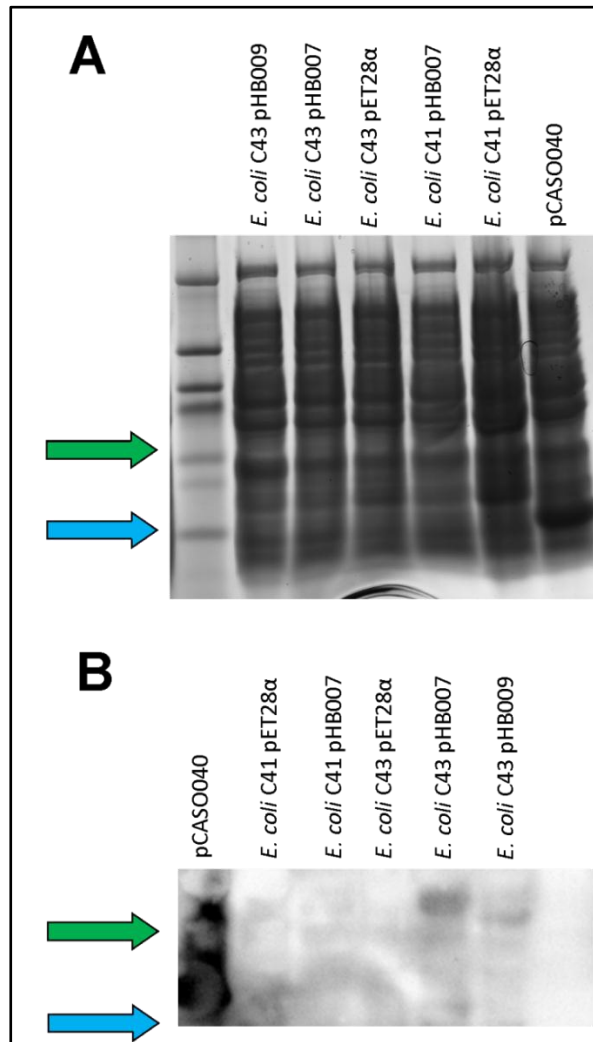


Figure 7-25 Expression of DNase proteins can be confirmed by SDS-PAGE and Western Blot analysis.

SDS-PAGE (A) and Western blot (B) analysis was carried out following IPTG induction of *E. coli* C41 and C43 cells. Expression of *cj1388* was confirmed by both SDS-PAGE and Western blot (blue arrow indicates the level on the gel of the *cj1388* band). DNase expression (size indicated by green arrow) was not observable by SDS-PAGE, however faint bands of the correct size were observed in C43 cells following Western blot analysis.

Since all the cell lysates showed DNase activity following IPTG induction it was thought that they might also impact *C. jejuni* NCTC 11168 biofilms. Cell lysates from IPTG induced C43 cells containing pHB007, pHB008, pHB009 or pET28 α were added to semi-mature *C. jejuni* NCTC 11168 biofilms using the co-culture method described in Section 2.8.11. All *E. coli* C43 cell lysates were able to degrade the *C. jejuni* NCTC 11168 biofilm, including those from cells not expressing the DNase proteins. This highlights that the degradative effect of DNase was not solely responsible for biofilm degradation in this experimental system. The presence of LB medium or its additives did not impact biofilm formation, since addition of 1ml of fresh LB medium with glucose, kanamycin or IPTG did not impact biofilm formation. This indicates that other components of the *E. coli* lysates are able to degrade *C. jejuni* biofilms, again highlighting the multi-factorial nature of biofilm formation and maintenance.

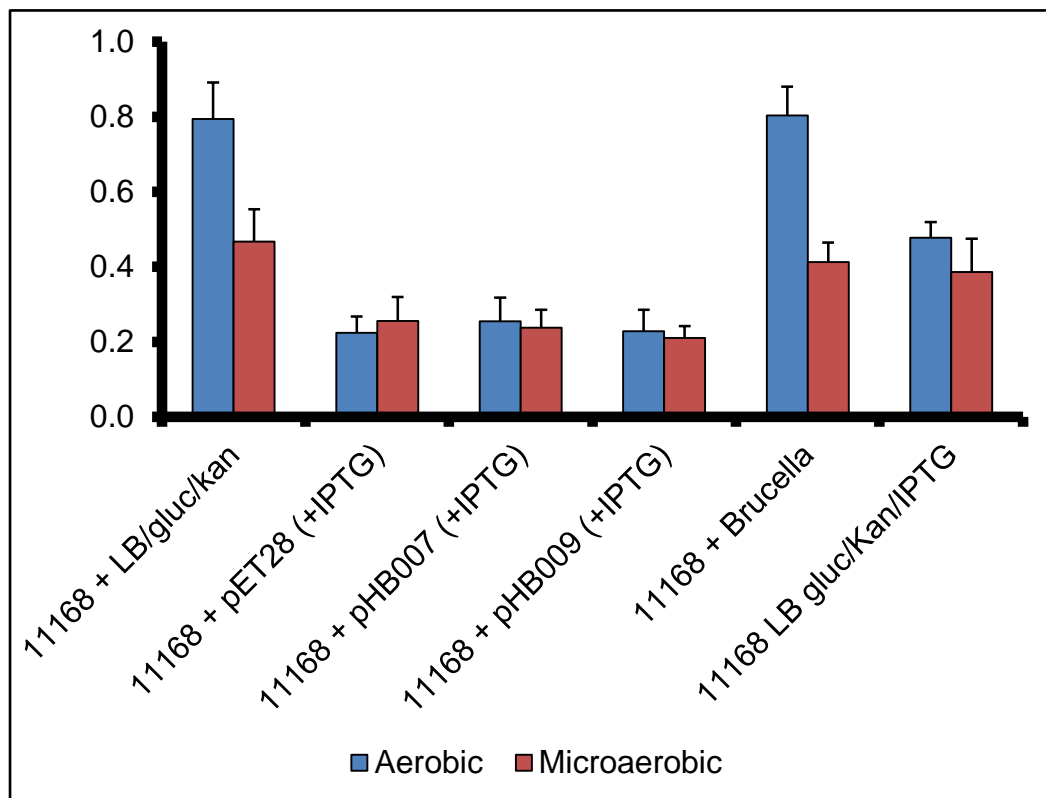


Figure 7-26 Addition of *E. coli* lysates to preformed *C. jejuni* NCTC 11168 biofilms degrades biofilm regardless of plasmid content

C. jejuni NCTC 11168 biofilms were allowed to form for 24 hours in static conditions at 37 °C in either aerobic or microaerobic conditions before addition of a second volume of sterile cell supernatant from either *E. coli* C43 cells expressing the eDNase proteins. In order to insure that the medium that the *E. coli* was grown in would affect biofilm formation samples of medium both with and without IPTG were also used assessed, along with a negative control sample of *E. coli* C43 cells containing the empty pET28 α plasmid. The addition of *E. coli* supernatant, regardless of the contents of its pET28 α plasmid led to a decrease in biofilm formation, suggesting that further optimisation is required before this assays can be used to determine if the pET28 α expressed plasmids are able to have an effect on *C. jejuni* biofilm formation. Error bars show SE (N = 3).

7.2.9 *C. jejuni* RM1221 is able to degrade pre-existing biofilms of other *C. jejuni* strains

Since expression and purification of RM1221 eDNases was technically challenging and required extensive optimisation, an alternative method of investigating RM1221 DNase activity against *C. jejuni* biofilms was sought. *C. jejuni* NCTC 11168 and 81116 biofilms were allowed to form for 24h, before incubation for a further 24 hours with fresh Brucella medium containing either biofilm forming strains (NCTC 11168 or 81116), or strain RM1221 (A schematic of the assay method is provided in Figure 2-1). Addition of fresh, un-supplemented Brucella medium provided a negative control. Results showed that replacement with either fresh medium, 81116, or NCTC 11168 cell suspensions had two consequences: biofilm formation at the first air-surface interface was enhanced, and a new biofilm formed at the newly formed, second, air-surface interface (Figure 7-27). Biofilm levels were significantly reduced in tubes containing *C. jejuni* RM1221 cells in the secondary culture, suggesting that not only is RM1221 a poor biofilm forming strain, but the presence of viable *C. jejuni* RM1221 can degrade a pre-existing *C. jejuni* biofilm.

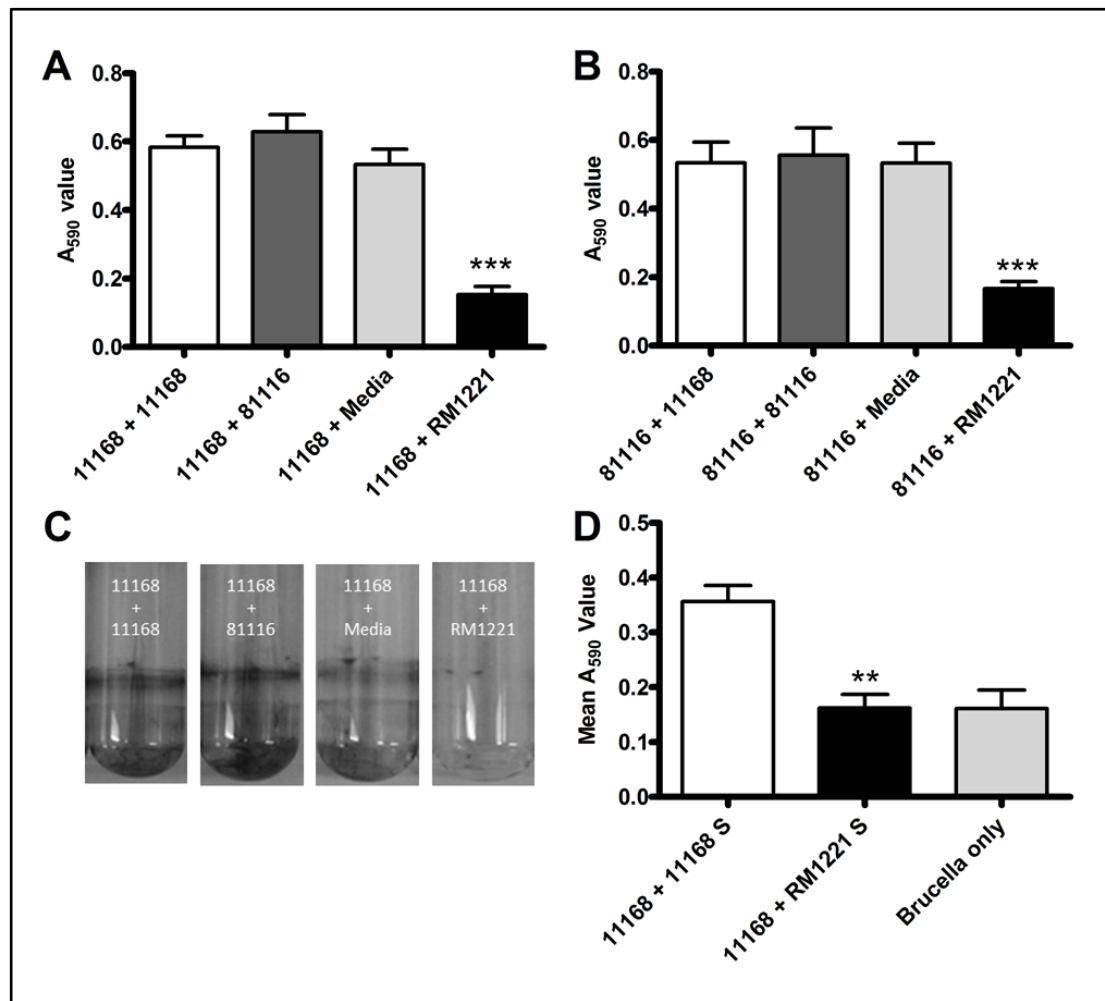


Figure 7-27 Co-incubation of preformed biofilms with RM1221 leads to biofilm degradation.

Biofilms of NCTC 11168 (A and D) and 81116 (B) were allowed to form in static aerobic conditions for 24 hours before a further 24 hour treatment with RM1221 cell culture (A and B), or the cell free spent medium of RM1221 (D). Graphs A, B and D show mean A_{590} values of each treatment. Error bars show SE (N =5) and images are representative of the CV staining observed throughout the experiments. Significance was measured using Tukey tests following ANOVA analysis (***) = $P < 0.001$, ** = $P < 0.01$).

Although previous work in this Section has shown that the DNase proteins are likely to be displayed extracellularly it is still not known if the eDNase proteins are attached to the cell surface or able to freely diffuse throughout the supernatant. To assess this, cell-free spent medium was prepared from *C. jejuni* RM1221 cultures grown overnight in microaerobic conditions at 37°C. Cell-free *C. jejuni* strain RM1221 supernatant was added to a 24 hour biofilm of *C. jejuni* NCTC 11168, resulting in degradation of the biofilm to levels comparable to the Brucella medium only control (Figure 7-27d). The same effect was not observed when cell-free supernatant from a *C. jejuni* NCTC 11168 culture was used to repeat the method (Figure 7-27d labelled '11168 + 11168 S'). This indicated that any factors disrupting biofilm

formation are produced solely by *C. jejuni* RM1221 and that the factors disrupting biofilm formation are soluble in spent medium.

Finally, use of the RM1221 $\Delta cje1441$ mutant in the co-culture assay showed that not only was $\Delta cje1441$ unable to degrade existing biofilms but it was able to be degraded by RM1221 when used as a primary culture (Figure 7-28). This strongly suggests that it is RM1221's ability to degrade eDNA which is solely responsible for its biofilm degrading capacity and biofilm formation deficiency.

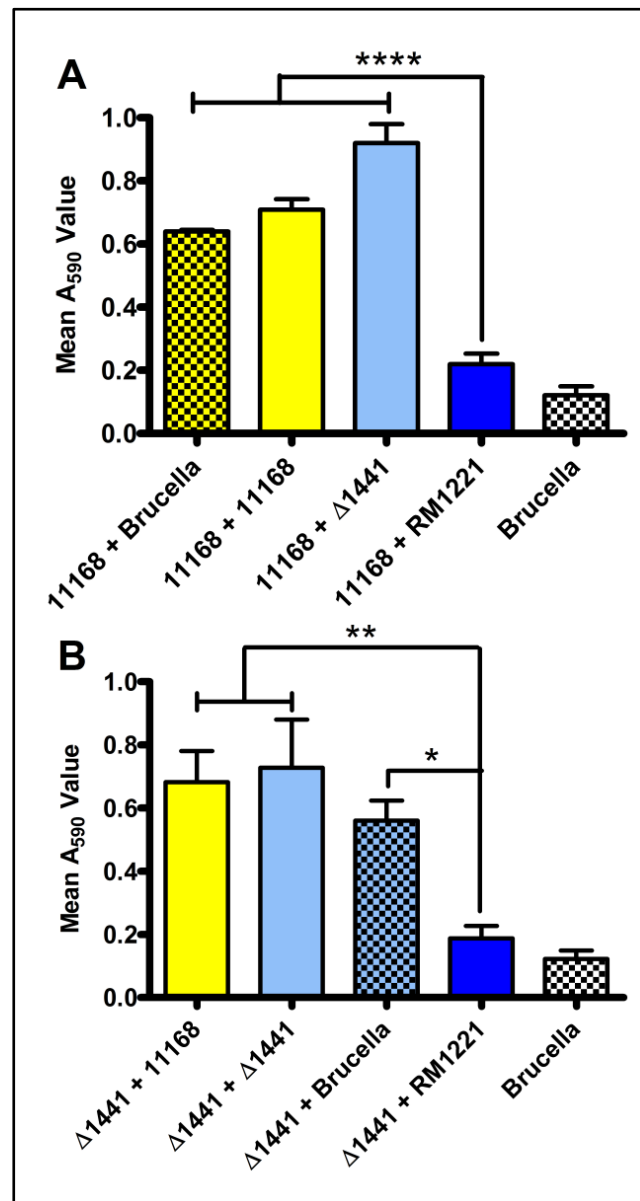


Figure 7-28 *C. jejuni* RM1221 $\Delta cje1441$ is unable to degrade preformed biofilms

The ability of RM1221 $\Delta cje1441$ to degrade the preformed biofilms of NCTC 11168 was assessed (A). When $\Delta cje1441$ was added to NCTC 11168 cultures (light blue bars) no significant difference in biofilm formation was observed compared to NCTC 11168 with Brucella medium added (yellow checked bar) or NCTC 11168 with more NCTC 11168 added (yellow bar). This is in contrast to the addition of RM1221 (dark blue bar), which led to a statistically significant reduction in biofilm staining. Similarly RM1221 $\Delta cje1441$ biofilms could only be degraded by the RM1221 WT (B). RM1221 $\Delta cje1441$ biofilms were allowed to form for 24 hours before addition of NCTC 11168 (yellow bar), a second volume of RM1221 $\Delta cje1441$ (light blue bar), Brucella medium (light blue checked bar) or RM1221 WT (dark blue bar). A statistically significant decrease in biofilm biomass was only observed in RM1221 WT containing cultures. Error bars show SE (N = 5), significance was measured using T-Tests (**** = $P < 0.0001$, ** = $P < 0.01$, * = $P < 0.05$).

7.2.10 The *C. jejuni* RM1221 eDNase proteins are small, heat stable molecules.

It has been reported by other investigators that several of the secreted DNase proteins found in other bacterial species, including *C. jejuni*, are heat stable and small (Erickson and Deibel, 1973, Ruiz et al., 2000, Lior and Patel, 1987). In order to elucidate more information about the eDNase proteins expressed by RM1221, its supernatant, shown to be able to degrade NCTC 11168 and 81116 biofilms (Section 7.2.9), was subjected to heat treatment and size fractionation. It was hypothesised that these treatments would be able to inactivate, or remove, the DNases within the supernatants, which in turn would lead to reduced anti-biofilm activity.

In order to separate the supernatant into solutions containing molecules of specific size fractions, RM1221 supernatant was filtered through 5, 10, 50 and 100 kDa filters and the flow through retained for use in assays. This produced a medium deficient in some of the content the original solution. In addition, heat treatment of the sterile RM1221 cell supernatant was performed by heating small aliquots of supernatant to 95°C for 10 minutes prior to use in the co-culture assay. This treatment has been shown to deactivate DNase I (Figure 6-9), and so was considered to be a suitable method of deactivation for other non-heat stable DNase enzymes.

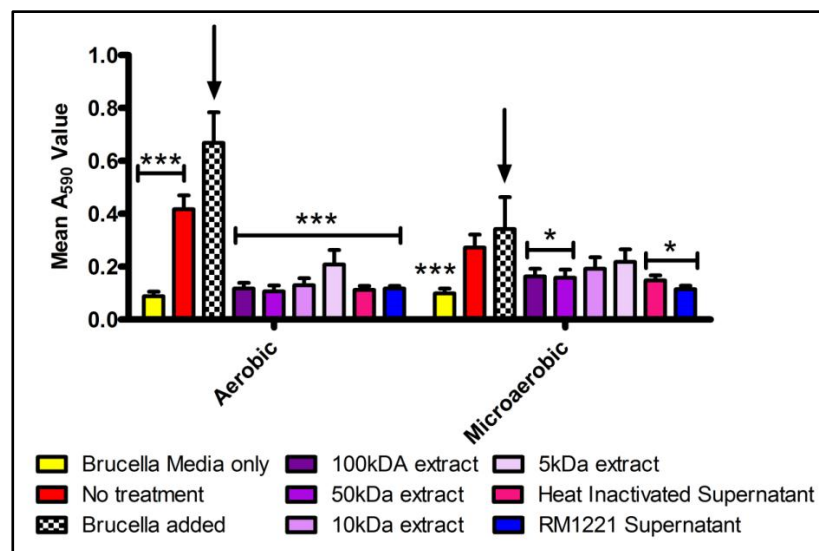


Figure 7-29 Size fractionation or heat inactivation of the supernatant has no effect on degradation activity.

Size fractionated (lilac and purple bars) or heat treated (pink bars) RM1221 culture supernatant was added to primary NCTC 11168 cultures in the co-culture assay and their ability to degrade biofilms assessed by crystal violet staining following a further 24 hour static incubation at 37 °C in either aerobic or microaerobic conditions. All fractions and heat treated supernatants were able to reduce biofilm biomass compared to cultures where only Brucella medium was added (black and white checked bars). This reduction was statistically significant for all fractions and heat treated samples in aerobic conditions, and for selected size fractions and the heat treated sample where the incubation took place in microaerobic conditions. Error bars show SE (N = 5), significance was measured using Bonferroni post-test following ANOVA analysis (* = P<0.05 *** = P<0.001). The arrow indicates the data used for comparison during statistical analysis.

Neither the heat inactivation of size fractionation treatments gave a significant difference in activity when compared to untreated RM1221 supernatant (Figure 7-29). In size fractionated samples there was a trend towards decreased activity as contents of the suspension reduced in size, however this did not reach statistical significance when compared to untreated RM1221 cell supernatants. It should be noted however that in microaerobic conditions both the 5 and 10 kDa fraction were also not significantly reduced compared to the 'Brucella added' sample, again highlighting their reduced capacity to effectively degrade preformed biofilms. Heat treatment did not appear to have an effect on activity, suggesting that the DNase protein(s) responsible for the biofilm degrading activity are relatively heat stable.

7.3 Discussion

In Figure 4-5 it was shown that there is variation between different strains of *C. jejuni* in their ability to form a biofilm. It has also been shown that in strains which form biofilms, such as *C. jejuni* NCTC 11168 and 81116, eDNA is not only present within the biofilm but appears to have a role in biofilm structural stability. In this chapter it was shown that the presence of three eDNase genes within the strain RM1221 are not only responsible for its inability to form *de novo* biofilms, but also its capacity to degrade biofilms of other *C. jejuni* strains. This work highlights how naturally-occurring eDNase activity may be able to weaken or destroy natural biofilms, e.g. in food processing environments.

In *C. jejuni* RM1221 the eDNase activity, and lack of natural competence, has so far precluded robust genetic manipulation (other than conjugation via tri-parental mating (Miller et al., 2000)), and the successful inactivation of *cje1441* is, to our knowledge, the first reported manipulation of strain RM1221 chromosome. The eDNase genes pose technical problems for genetic manipulation and cloning, as their intracellular expression can lead to cytoplasmic DNase activity and cell death, thus hampering cloning and expression in *E. coli*. This has also been reported for eDNase proteins of other bacteria, such as the eDNase proteins from the predatory bacterium *Bdellovibrio bacteriovorus*, where expression was found to be lethal in *E. coli* (Lambert and Sockett, 2013). Similarly, expression of active DNase I by *E. coli* could only be achieved by the use of the very tightly controlled expression plasmid pDOC55 (Worrall and Connolly, 1990). Such plasmids are not available for *C. jejuni*, and hence genetic manipulation of the eDNase genes in *C. jejuni* is technically challenging. It is interesting to note that (John et al., 2011) reported that the presence of hydrogen gas during microaerobic incubation led to decreased expression of genes associated with natural transformation inhibition. The microaerobic gas mixture used throughout this work did not contain any hydrogen, which may have further contributed to the poor transformation efficiency of RM1221.

It would be of interest to further investigate how *C. jejuni* RM1221 overcomes the toxicity of the DNase genes. The genes *cje0566* and *cje1441* are closely surrounded both several other genes, which may function as chaperone proteins, inactivating the DNase protein until it is exported. Future work may include expression of *cje0566* and *cje1441* with their neighbours and assessment of cell toxicity or DNase activity. The gene *dns* do not appear to be associated with any other genes, and so its association with neighbouring chaperone proteins is less likely. It is possible that *dns* associates with other proteins, not in close proximity to *dns* on the genome, during its translation, folding and export. It is also possible that the genes are transported to the surface in a non-active formation, therefore reducing their activity within RM1221 cells.

Many species which form biofilms are also able to produce and export extracellular DNase proteins, and eDNase proteins appear to have multiple functions. Although the biological function of the eDNase activity in *C. jejuni* is yet to be elucidated, it is possible that it protects isolates with the insertion element against allelic exchange containing insertion element-negative flanking sequences, as this incurs the risk of losing the insertion element, which offer some evolutionary advantage. The *P. aeruginosa* eDNase PA3909 is involved in DNA degradation, using DNA as a nutrient source, and its expression is induced in phosphate

limiting conditions (Mulcahy et al., 2010), whereas in *Shewanella oneidensis*, expression of the nucleases ExeM and ExeS is strongly induced if DNA is the sole nutrient source, and deletion of the ExeM gene leads to a significantly reduced growth rate (Godeke et al., 2011). Finally, the eDNase genes of *Staphylococcus aureus* are involved in immune evasion, and their expression during host infection aids the escape of *S. aureus* from the DNA 'nets' which are secreted by neutrophils (Berends et al., 2010). In the case of *C. jejuni* (Gaasbeek et al., 2010, Gaasbeek et al., 2009), and other bacteria such as *Vibrio cholerae* (Focareta and Manning, 1991), the eDNase proteins restrict natural transformation.

Although not the case in *C. jejuni* RM1221, many bacteria which produce eDNase enzymes are still able to form biofilms, appearing to utilise the enzymes in order to modify their biofilm structures. Two well-studied examples of eDNase-positive bacterial species that can form biofilms are *V. cholerae* (Seper et al., 2011) and *S. aureus* (Kiedrowski et al., 2011). Since the eDNase genes of RM1221 are classified as non-specific DNA/RNA endonucleases, they are not expected to have stringent specificity regarding the source, methylation or sequence of the DNA targeted for digestion. Indeed this was confirmed by absence of eDNA in RM1221 cultures (Figure 7-6 and Figure 7-5 respectively) alongside the ability of RM1221 cultures to degrade purified NCTC 11168 gDNA and the ability of the RM1221 eDNase enzymes to produce halos on DNA agar plates (Figure 7-4).

It is possible that it is the lack of controlled expression of the three eDNase proteins in RM1221 which leads to its ability to degrade DNA and inhibit biofilm formation. To date no analysis of transcription levels or the growth phase(s) transcription takes place in has been carried out. Reverse transcriptase PCR (RT-PCR) could be used to rapidly answer these questions. It is also interesting to note that both *C. jejuni* NCTC 11168 and 81116 contain genes similar to *cje1441*, and it is possible that their effective regulation allows these two strains to form biofilms while RM1221 cannot.

It is also important to note that some *C. jejuni* strains and their prophages are able to enter a 'carrier life cycle state' in which the majority of a population maintains a stable prophage element, while a small subset of the population produce lytic phages (Siringan et al., 2014). *C. jejuni* strains R14 and R20 both contained prophage elements similar to those found in strain RM1221, and were both able to produce infectious Mu like phage particles (Scott et al., 2007). Since phage treatment has previously been reported to reduce biofilm formation by *C. jejuni* (Siringan et al., 2011) it is possible that phage particles such as these also contribute to the reduction of biofilms by strain RM1221, and it would be of interest for future investigations to consider this possibility.

DNase enzymes are becoming increasingly common in the treatment of some biofilm infections and chronic conditions such as cystic fibrosis, but DNase production is costly. Although this is not considered problematic within the medical industry, high production costs severely limit potential DNase use in the food chain. Within the food industry, the use of naturally produced bacterial eDNases could be a suitable alternative to DNase I use. Bacteria such as *Aeromonas* sp. produce several secreted DNase enzymes (Pemberton et al., 1997) and in species such as *Streptococcus agalactiae*, some of these eDNase proteins are heat stable (Derre-Bobillot et al., 2013) as was found in RM1221. Many DNase-positive bacteria have low complexity growth requirements and do not have the ethical or legal issues surrounding their growth, which may preclude or limit the use of DNase obtained from

animals, or recombinant products from genetically modified organisms. The cell-free extracts of *C. jejuni* RM1221 retain their DNase activity, and are able to degrade *C. jejuni* biofilms even after a ten minute heat treatment (Figure 7-29). This suggests that the eDNase enzymes of RM1221 are relatively heat stable and could potentially be a source of easily obtainable DNase proteins for use during food chain cleaning. Although their use in such applications requires further consideration and investigation to ensure that any supernatant derived products are safe for food chain use.

In conclusion, eDNase activity inhibits biofilm formation by *C. jejuni* RM1221, and this eDNase activity can be utilised to degrade biofilms formed by other *C. jejuni* strains, using either live RM1221 cells or cell-free supernatant. Since DNase treatment has been proved to be so effective against both bacterial and fungal biofilms, extraction of eDNase enzymes from *C. jejuni* strains such as RM1221 could, in future, provide a cost effective alternative source of DNase enzymes, and assist in improving food safety by prevention of biofilm-assisted transmission of foodborne pathogens such as *C. jejuni*.

8 General Discussion

The aim of this work was to gain a better understanding of how biofilm formation might contribute to the survival of *C. jejuni* within the food chain. In order to achieve this aim, investigation was carried out in three complementary areas: the development of food chain relevant models, further investigation into the four stages of biofilm formation, and study of the role that eDNA may play in maintaining the structure of *C. jejuni* biofilms, and eDNase enzymes in their degradation.

As part of this work, a novel *C. jejuni* biofilm staining technique was developed, allowing visualisation of metabolically active *C. jejuni* biofilms in protein and lipid rich medium (3). The development of this staining method allowed biofilms formed in the presence of chicken juice to be studied. Following this development, *C. jejuni* biofilm formation in the presence of chicken juice was studied (4). Results showed that the juice was able to condition abiotic surfaces such as glass and stainless steel, increasing *C. jejuni* attachment and subsequent biofilm formation. The observed increase was due to the ability of chicken juice to alter the physic-chemical properties of the surface. This allowed *C. jejuni* to form attachments to the surface more easily, as indicated by the increased biofilm formation of the aflagellate *C. jejuni* NCTC 11168 Δ *flaAB* mutant.

Analysis of the biofilm as it matured indicated that in aerobic conditions biofilm formation is accelerated. Microaerobic biofilm formation could also be accelerated by the presence of particulates or conditioning layers on the abiotic surface. Biofilm formation also allowed *C. jejuni* cells to significantly increase the time they were metabolically active in microaerobic conditions, while also allowing survival for up to two weeks in temperatures below 20 °C (5).

Finally, eDNA was isolated from both planktonic and biofilm cultures and shown to be an essential component of the *C. jejuni* ECM. The degradation of eDNA, for example by DNase I enzymes, was identified as a potential biofilm removal tool suitable for use in the food chain (6). It was also discovered that a *C. jejuni* strain expressing extracellular DNase proteins, *C. jejuni* RM1221, was unable to form biofilm. RM1221 cultures and cell free supernatants were also shown to be able to degrade *C. jejuni* biofilms. The production of eDNase enzymes by bacteria could provide a cost effective source of biofilm degrading enzymes for use within the food chain (7).

Several important questions still remain unanswered with respect to *C. jejuni* biofilm formation. Firstly, although both eDNA and polysaccharide have been identified within the ECM of the *C. jejuni* biofilm, to date there has been little investigation of how the ECM is released into the extracellular milieu. Previous work by Svensson et al. (2014) indicated that eDNA release was due to cell lysis. This could be explored further by inhibiting or promoting cells lysis and measuring the effect this may have on biofilm formation. Cell lysis inhibition in *S. aureus* biofilms has previously been achieved by the use of polyanethole sulfonate (Mann et al., 2009). Polyanethole sulfonate is typically included as a medium component during clinical isolation of bacteria from whole blood, since it is able to inhibit complement-mediated bacterial killing (Palarasah et al., 2010). It is also able to inhibit the activity of bacterial autolysins (Wecke et al., 1986), leading to reduced cell lysis within bacterial populations. In addition, the *C. jejuni* flagella apparatus has been shown to be essential for the secretion of proteins such as virulence factors, and inactivation of key proteins within the apparatus lead to decreased secretion of *Campylobacter* invasion antigens (Konkel et al., 2004). If ECM factors are also secreted, inactivation of the flagella is also likely to impair their release,

although due consideration should also be given to how flagella protein manipulation may impact *C. jejuni* motility and, by extension, motility related biofilm reduction. By using techniques such as these, in combination with stains such as DAPI, calcofluor white and Congo red, it would be possible to investigate ECM release in more detail.

It is also important to consider what factors other than aerobic stress, may contribute to biofilm formation. The work presented here has focused on the impact of aerobic conditions on biofilm formation, but many other stresses are encountered during *C. jejuni* transit through the food chain. Nutrient deficiency, osmotic stress and temperature fluctuations are all encountered within poultry processing plants. Although some study has previously been carried out on how each of these affect *C. jejuni* biofilm formation (Buswell et al., 1998, Dykes et al., 2003, Reeser et al., 2007, Tatchou-Nyamsi-Konig et al., 2008, Hanning and Slavik, 2009, Duffy and Dykes, 2009), there has been little investigation of how a combination of factors may impact on *C. jejuni* biofilm formation or survival within biofilms. High throughput testing, allowing analysis of multiple conditions, and techniques such as transcriptomics, metabolomics and proteomics are becoming increasingly accessible to researchers in the biofilm field and will be useful to better understand how the *C. jejuni* biofilm is able to respond to the food chain environment. To date only two studies into *C. jejuni* biofilms have used 'omics technologies extensively (Kalmokoff et al., 2006, Sampathkumar et al., 2006), and the scope of these studies did not include food chain relevant conditions. Several studies in other bacterial species have shown that combination of 'omics technologies can provide useful information about the dynamics of biofilm populations in environmentally relevant conditions (Beale et al., 2013). Increasingly this technology is also being applied to multispecies biofilms, which *C. jejuni* is known to populate (Sanders et al., 2007, Zimmer et al., 2003, Balamurugan et al., 2011).

One limitation of this study is the reliance on measurement of bacterial growth and concentration of cell cultures by assessment of OD₆₀₀ values and MPN. It was previously shown by Wright et al. (2009) that there was a poor correlation between OD₆₀₀ values and viable cell numbers once the bacterial population had entered stationary phase. This was due to the high volume of cell debris and non-viable cells at later time points, which contribute to light scattering when measuring optical density. This effect can also be compounded by the alterations in cell morphology which are observed when *C. jejuni* cells encounter stress conditions (Hwang et al., 2011, Oh et al., 2015) or progress beyond log phase growth (Thomas et al., 1999). Throughout this study optical density measurement were used to both measure growth of planktonic populations and the bacterial concentration (to allow accurate dilution of the *C. jejuni* cultures before beginning static culture to allow biofilm formation). Wright et al. (2009) do state that in activity growing (log phase) cultures, such as those used to prepare the biofilm cultures, OD₆₀₀ can be used to predict the viable population of cultures. This was also shown in Section 3.2.5, where good correlation was observed between TTC conversion and OD₆₀₀ value, up to a value of OD₆₀₀ 0.8. However where OD₆₀₀ was used for measurement of populations in stationary and decline phases populations were overestimated. In order to correctly estimate viable cells in biofilms, where the bacterial population is not growing logarithmically, or in stationary phase populations total viable cell (TVC) counts should be used alongside other measures. Although throughout this study a method of estimating MPN was used, it should not be considered to be comparable to TVC counting since it had a high limit of detection and could not be used

to estimate populations with more than 8 log of growth. Alternative TVC calculation methods, such as whole plate counts or Miles-Misra, should be considered instead of the currently used technique, since they offer a better resolution than the currently used MPN technique.

It would be of interest to better understand how *C. jejuni* is able to interact with other food chain relevant biofilms in mixed species populations. *C. jejuni* is an obligate microaerophile (Stern and Meinersmann, 1989), but is able to transit the food chain, an environment where aerobic conditions predominate. Biofilms have been shown to contain 'micro-environments' where bacterial species are able to find environmental conditions more suited to their metabolic needs (Hidalgo et al., 2009). Inter-species collaboration within biofilms has been shown to enhance the function of the bacteria within the biofilm, increasing their resistance to stresses such as antimicrobial treatment (Burmolle et al., 2014). Previous research has shown that *C. jejuni* surface attachment is enhanced by the presence of species such as *P. aeruginosa* (Trachoo et al., 2002) and *Enterobacter sp.* (Sanders et al., 2007), and *C. jejuni* survival at low temperatures is directly influenced by the other species within the biofilm (Hanning et al., 2008). *C. jejuni* survival in aerobic conditions is also enhanced by the presence of the protozoan *Acanthamoeba castellanii*. The increase in *C. jejuni* survival was attributed to the consumption of oxygen by *A. castellanii*, producing atmospheric conditions more suited to *C. jejuni* survival (Bui et al., 2012). These studies provide an interesting insight into how *C. jejuni* biofilm formation is affected by the presence of other bacterial species. Further research, particularly into how *C. jejuni* senses and communicates with other species in the biofilm, is still required though.

How *C. jejuni* cells are released from the biofilm is of particular interest when considering the impact of biofilm formation on the transit of *C. jejuni* within the food chain. Passive release and biofilm turnover could be studied using flow cell models to simulate the shear forces encountered within pipes and areas of high liquid flow, such as scald tanks. Active dispersal will be more challenging to study and potentially require the identification of *C. jejuni* signaling systems and secretion of enzymes for ECM degradation.

A recent review by Teh et al. (2014) hypothesized that *C. jejuni* was more likely to form simple attachments to surfaces, or integrate into pre-existing biofilms, than form *de novo* biofilms within the food chain. The work presented here demonstrates that complex, structured *C. jejuni* biofilms do form on plastic, glass and stainless steel surfaces in aerobic conditions. It is also shown that biofilms are more likely to form in heavily soiled areas. The presence of eDNA, reported to be a component of the ECM in many bacteria (Seper et al., 2011, Allesen-Holm et al., 2006, Whitchurch et al., 2002, Gloag et al., 2013, Bockelmann et al., 2006, Pammi et al., 2013, Nguyen and Burrows, 2014, Hymes et al., 2013, Zhao et al., 2013) including *C. jejuni* (Svensson et al., 2014), suggests that true biofilms, rather than non-biofilm attached populations, are being formed by *C. jejuni* in food chain relevant conditions.

Although the results presented in this work appear to contradict the conclusions made by Teh et al. (2014), their conclusions should not be entirely discounted. It is well established that the majority of biofilms in environments, including the food chain, are comprised of multiple bacterial species (Jahid and Ha, 2014). These multispecies biofilms allow 'partnerships' to develop between genetically diverse species of bacteria, benefiting both species. These interactions are termed 'co-aggregation' (for reviews see Rickard et al.

(2003) and Katharios-Lanwermeier et al. (2014)), and although initially described in oral biofilms they have been discovered in other biofilm communities, including those present within food chains and drinking water communities (Palmer et al., 2007, Vornhagen et al., 2013, Simoes et al., 2008a, Sasahara and Zottola, 1993). *C. jejuni* has also been shown to benefit from co-aggregation, with *C. jejuni* showing increased biofilm formation and heat resistance in the presence of *Enterococcus faecium* biofilms (Trachoo and Brooks, 2005), and increased survival at 32°C in the presence of mixed species food chain biofilms (Hanning et al., 2008). Other foodborne pathogens, such as *L. monocytogenes*, also utilise co-aggregation as part of their food chain survival mechanisms. *L. monocytogenes* biofilm formation on stainless steel increases significantly in the presence of *Pseudomonas fragi*. This increase was attributed to the production of large volumes of ECM by *P. fragi*, allowing *L. monocytogenes* to attach to the ECM, rather than directly to the surface and therefore overcome the surfaces repellent physio-chemical properties (Sasahara and Zottola, 1993).

It is therefore entirely possible that *C. jejuni*, as Teh et al. (2014) suggests, does integrate into existing, multi species, biofilms throughout the food chain. This is the mode of existence favoured by the majority of food chain related bacterial species and so it would be unwise to assume that *C. jejuni* would be any different. It is easy to assume that because *C. jejuni* typically lives in mixed species communities it is a poor biofilm former; however the results presented here suggest that this is not the case. Following 48 hours of incubation in aerobic conditions *C. jejuni* is able to form dense, structured biofilms which allow survival of viable *C. jejuni* for several weeks at temperatures between 5 and 42°C. These biofilms are able to form on stainless steel, a food chain relevant material, and are increased by the presence of chicken exudates, a material ubiquitous within the poultry processing plant. Viable *C. jejuni* cells are shed from these mature biofilms, while a permanent population is maintained at the original site, allowing continual contamination of other areas of the food chain.

In conclusion, the results presented within this work suggest that although *C. jejuni* may take advantage of preformed biofilms in the majority of cases, it is able to establish a biofilm in areas without prior biofilm populations. In aerobic conditions structured, biofilms are identifiable within 24 hours and in areas of heavy soiling, where surfaces may be conditioned, this maturation may be further accelerated. Although these biofilms appear to have little resistance to enzymatic treatments, constant vigilance is required to ensure that *C. jejuni* biofilm populations are not able to establish themselves and contribute to contamination of food stuffs.

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10 Appendices

10.1 List of outcomes

Below are four tables detailing the outcomes of this PhD project:

Peer reviewed papers		
Date	Details	Type
June 2015	<u>Brown H L</u> , Reuter M, Hanman K, Betts R P, van Vliet A H M (2015) <i>Campylobacter jejuni</i> biofilms contain extracellular DNA and are sensitive to DNase I treatment. <i>Frontiers in Microbiology</i> . 6, article 699	Paper
June 2015	Reuter M, Periago P M, Mulholland F, <u>Brown H L</u> , van Vliet A H M (2015) A PAS Domain-Containing Regulator Controls Flagella-Flagella Interactions in <i>Campylobacter jejuni</i> . <i>Frontiers in Microbiology</i> . 6, article 770	Paper
January 2015	<u>Brown H L</u> , Reuter M, Hanman K, Betts R P, van Vliet A H M (2015) Prevention of biofilm formation and removal of existing biofilms by extracellular DNases of <i>Campylobacter jejuni</i> . <i>PLoS One</i> . 10(3): e0121680.	Paper
November 2014	<u>Brown H L</u> , Reuter M, Salt L J, Cross K L, Betts R P, van Vliet A H M (2014) Chicken juice enhances surface attachment and biofilm formation of <i>Campylobacter jejuni</i> . <i>Applied Environmental Microbiology</i> , 80, (22) 7053 - 7060	Paper
December 2013	Marlow V L, MacLean T, <u>Brown H L</u> , Kiley T B, Stanley-Wall N R (2013) Blast-a-biofilm: a hands on activity for school children and members of the public. <i>Journal of Microbiology and Biology Education</i> , 14, p252-4	Paper
July 2013	<u>Brown H L</u> , van Vliet A H M, Betts R P, Reuter M (2015) Tetrazolium reduction allows quantification of biofilm formation by <i>Campylobacter jejuni</i> in a food matrix model. <i>Journal of Applied Microbiology</i> , 115, (5), 1212-21	Paper

Table 10-1 List of PhD outcomes (peer reviewed papers)

Technical reports		
Date	Details	Type
July 2014	<u>Brown H L</u> , Walshaw J, Barker G C, van Vliet A H M (2014) Confidential literature review on the use of 'omics technologies in biofilm study	Technical Report

Table 10-2 List of PhD outcomes (technical reports)

Oral presentations		

Date	Details	Type
March 2015	<u>Brown H L</u> , Reuter M, Hanman K, Betts R P, van Vliet A H M (2015) DNA is an essential component of the <i>Campylobacter jejuni</i> biofilm extracellular matrix. SGM Annual Conference, Birmingham	Accepted oral Presentation
December 2014	<u>Brown H L</u> , Reuter M, Hanman K, Betts R P, van Vliet A H M (2014) Prevention of biofilm formation by extracellular DNases of <i>Campylobacter jejuni</i> . Norwich Students Microbiology Symposium, Norwich	Accepted oral Presentation
September 2014	<u>Brown H L</u> , Reuter M, Hanman K, Betts R P, van Vliet A H M (2014) Prevention of biofilm formation by extracellular DNases of <i>Campylobacter jejuni</i> . CampyUK Conference, Liverpool	Accepted oral Presentation
September 2014	<u>Brown H L</u> , Reuter M, Betts R P, van Vliet A H M (2014) Surface conditioning allows efficient attachment of <i>Campylobacter jejuni</i> biofilms to abiotic surfaces. SGM Young Microbiologist Award Nomination, London	Invited oral presentation
March 2014	<u>Brown H L</u> , Reuter M, Hanman K, Betts R P, van Vliet A H M (2014) DNA is an essential component of the <i>Campylobacter jejuni</i> biofilm extracellular matrix. SGM Annual Conference, Liverpool	Accepted oral Presentation
September 2013	<u>Brown H L</u> (2013) A rough guide to outreach. SGM autumn conference, Brighton	Invited oral Presentation
May 2013	<u>Brown H L</u> , Reuter M, Betts R P, van Vliet A H M (2013) The role of extracellular DNA in biofilm formation by <i>Campylobacter jejuni</i> . IFR student showcase, Norwich	Accepted oral Presentation
May 2012	<u>Brown H L</u> (2012) Kitchen Nightmares: the dark side of dinner. Night at the museum event, Sainsbury Centre, Norwich	Accepted oral Presentation

Table 10-3 List of PhD outcomes (oral presentations)

Poster presentations		
Date	Details	Type
September 2014	<u>Brown H L</u> , Reuter M, Betts R P, van Vliet A H M (2014) DNA is an essential component of the <i>Campylobacter jejuni</i> biofilm extracellular matrix. CampyUK Conference, Liverpool	Poster Presentation
June 2014	<u>Brown H L</u> , Reuter M, Betts R P, van Vliet A H M (2014) DNA is an essential component of the <i>Campylobacter jejuni</i> biofilm extracellular matrix. IFR Student showcase, Norwich	Poster Presentation

Poster presentations		
Date	Details	Type
May 2014	<u>Brown H L</u> , Reuter M, Betts R P, van Vliet A H M (2014) Surface conditioning allows efficient attachment of <i>Campylobacter jejuni</i> biofilms to abiotic surfaces. Biofilm 6, Vienna	Poster Presentation
May 2014	<u>Brown H L</u> , Reuter M, Betts R P, van Vliet A H M (2014) DNA is an essential component of the <i>Campylobacter jejuni</i> biofilm extracellular matrix. Biofilm 6, Vienna	Poster Presentation
March 2014	<u>Brown H L</u> , Reuter M, Betts R P, van Vliet A H M (2014) Surface conditioning allows efficient attachment of <i>Campylobacter jejuni</i> biofilms to abiotic surfaces. SGM Annual Conference, Liverpool	Poster Presentation
September 2013	<u>Brown H L</u> , Reuter M, Betts R P, van Vliet A H M (2013) Chicken juice is an essential component of the <i>Campylobacter jejuni</i> extracellular matrix. CHRO, Aberdeen	Poster Presentation
September 2013	<u>Brown H L</u> , Reuter M, Betts R P, van Vliet A H M (2013) DNA is an essential component of the <i>Campylobacter jejuni</i> biofilm extracellular matrix. CHRO, Aberdeen	Poster Presentation
September 2013	<u>Brown H L</u> , Reuter M, Betts R P, van Vliet A H M (2013) Use of a tetrazolium dye for monitoring growth, viability and biofilm formation of <i>Campylobacter jejuni</i> in food matrices. CHRO, Aberdeen	Poster Presentation
September 2013	<u>Brown H L</u> , Reuter M, Betts R P, van Vliet A H M (2013) Chicken juice is an essential component of the <i>Campylobacter jejuni</i> extracellular matrix. SGM autumn conference, Brighton	Poster Presentation
September 2013	<u>Brown H L</u> , Reuter M, Betts R P, van Vliet A H M (2013) DNA is an essential component of the <i>Campylobacter jejuni</i> biofilm extracellular matrix. SGM autumn conference, Brighton	Poster Presentation
May 2013	<u>Brown H L</u> , Reuter M, Betts R P, van Vliet A H M (2013) The role of biofilms in persistence and transmission of <i>Campylobacter jejuni</i> in the food chain. IFR student showcase, Norwich	Poster Presentation
March 2013	<u>Brown H L</u> , Reuter M, Betts R P, van Vliet A H M (2013) Chicken juice contributes to increased growth and biofilm formation of <i>Campylobacter jejuni</i> . SGM Spring conference, Dublin	Poster Presentation

Poster presentations		
Date	Details	Type
January 2013	<u>Brown H L</u> , Reuter M, Betts R P, van Vliet A H M (2013) Chicken juice contributes to increased growth and biofilm formation of <i>Campylobacter jejuni</i> . CampyUK, London	Poster Presentation
September 2012	<u>Brown H L</u> , Reuter M, Betts R P, van Vliet A H M (2012) Biofilms assist in the survival of <i>Campylobacter jejuni</i> in food-chain relevant conditions. International Biodeterioration and Biodegradation Society Conference, Winchester	Poster Presentation
June 2012	<u>Brown H L</u> , Reuter M, Betts R P, van Vliet A H M (2012) Biofilms assist in the survival of <i>Campylobacter jejuni</i> in food-chain relevant conditions. IFR student showcase, Norwich	Poster Presentation
July 2011	<u>Brown H L</u> , Reuter M, Betts R P, van Vliet A H M (2011) Chicken juice contributes to increased growth and biofilm formation of <i>Campylobacter jejuni</i> . IFR student showcase, Norwich	Poster Presentation
October 2011	<u>Brown H L</u> , Reuter M, Betts R P, van Vliet A H M (2011) Chicken juice contributes to increased growth and biofilm formation of <i>Campylobacter jejuni</i> . CampyGERM, Gottingen	Poster Presentation

Table 10-4 List of PhD outcomes (poster presentations)

10.2 Plasmid maps

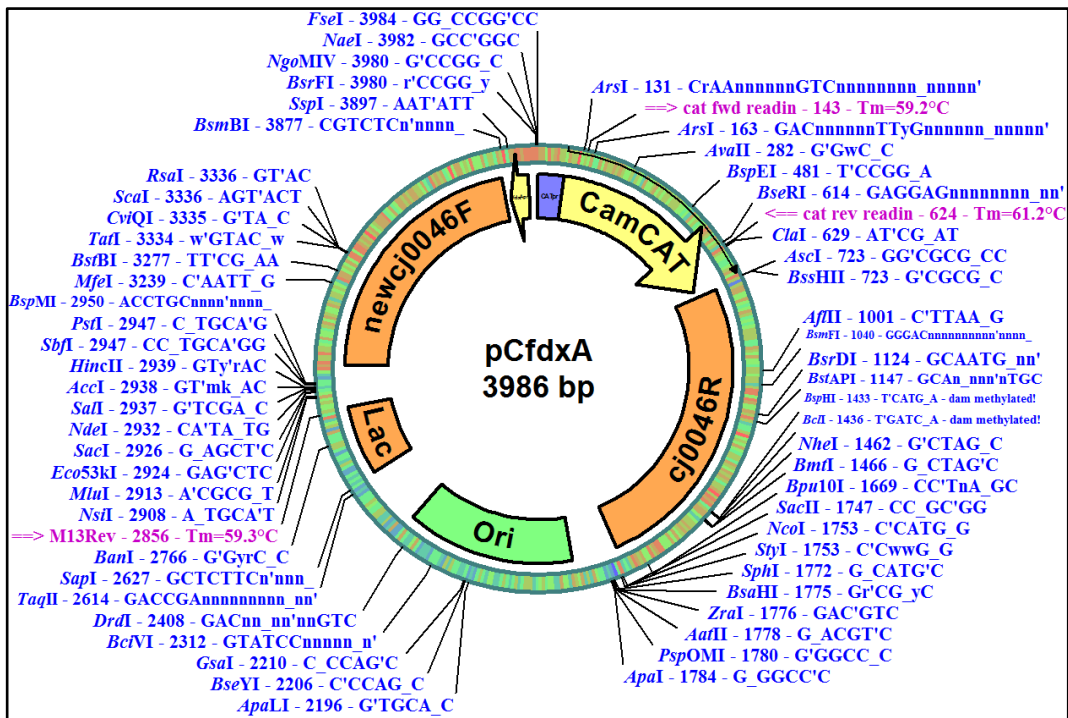


Figure 10-1 Plasmid map of pCfdxA

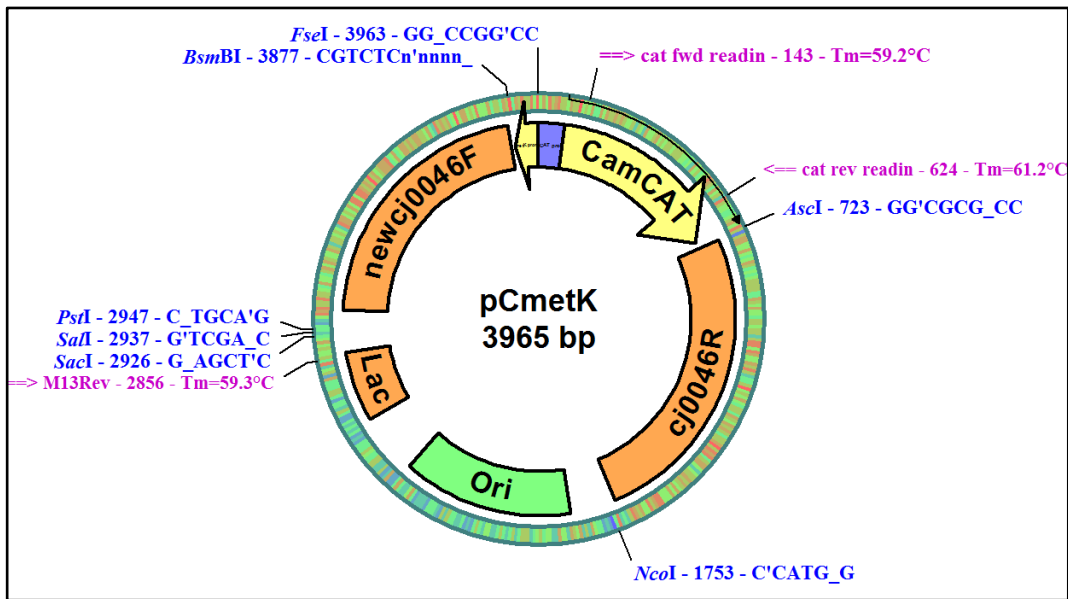


Figure 10-2 Plasmid map of pCmetK

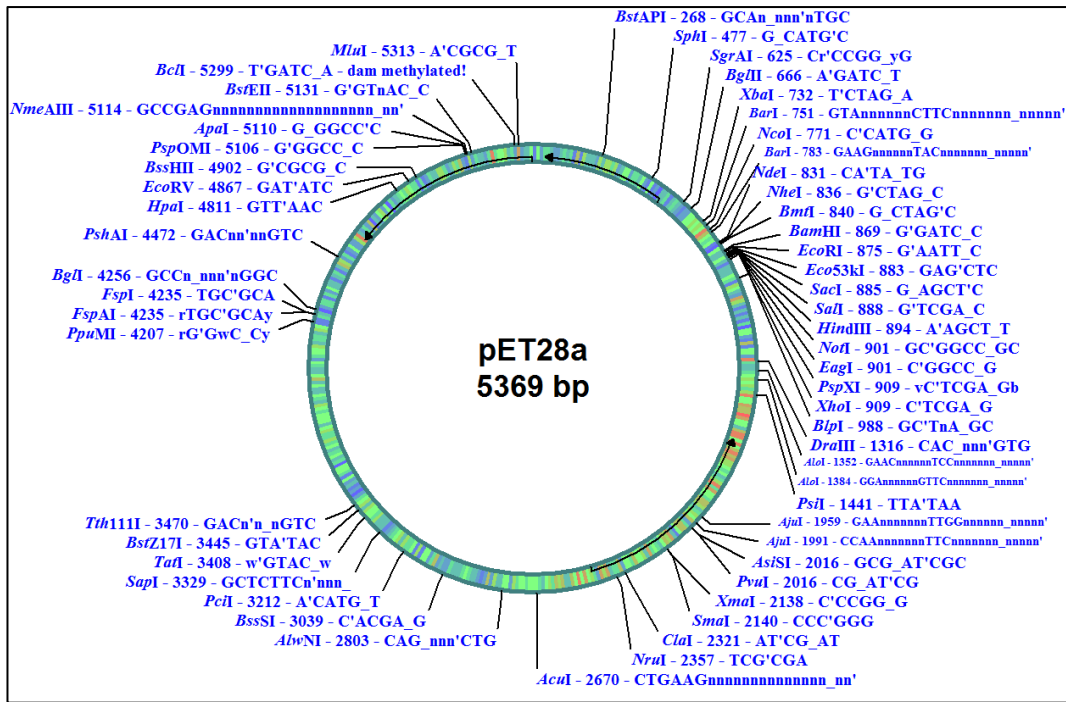


Figure 10-3 Plasmid map of pET28a

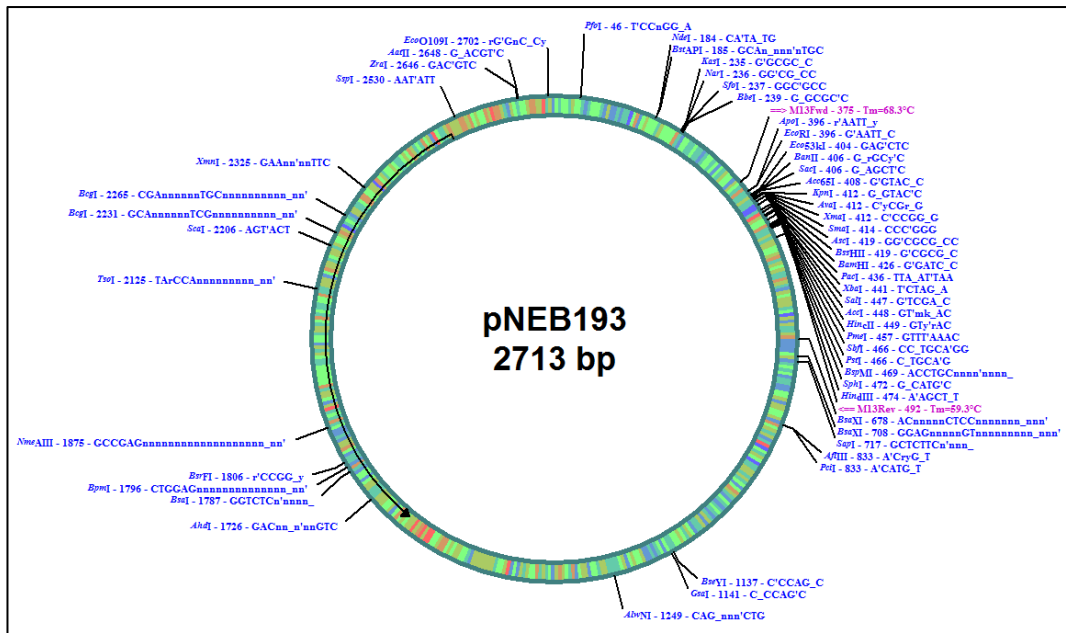


Figure 10-4 Plasmid map of pNEB193

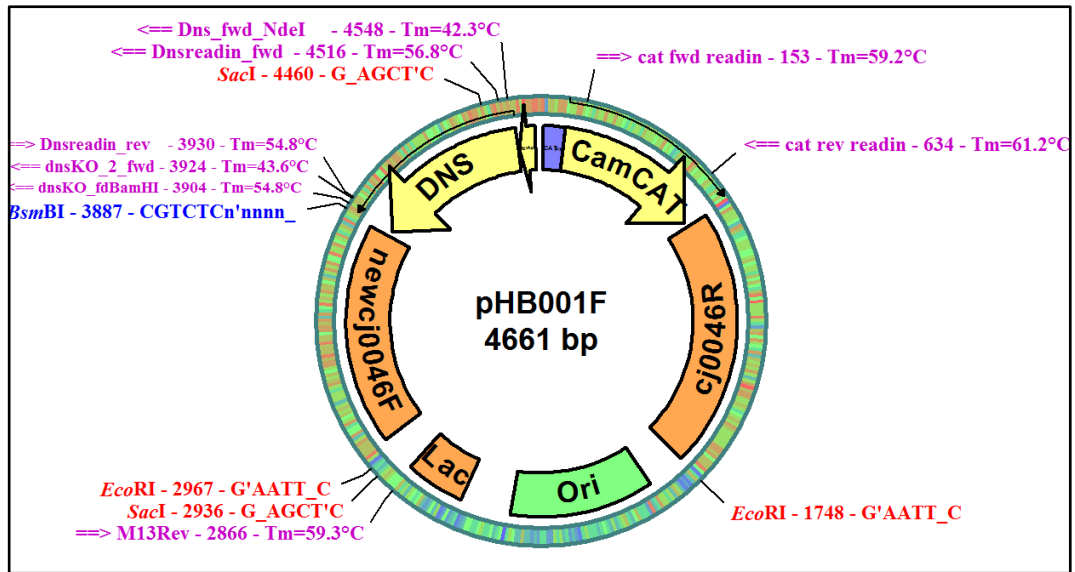


Figure 10-7 Plasmid map of pHB001

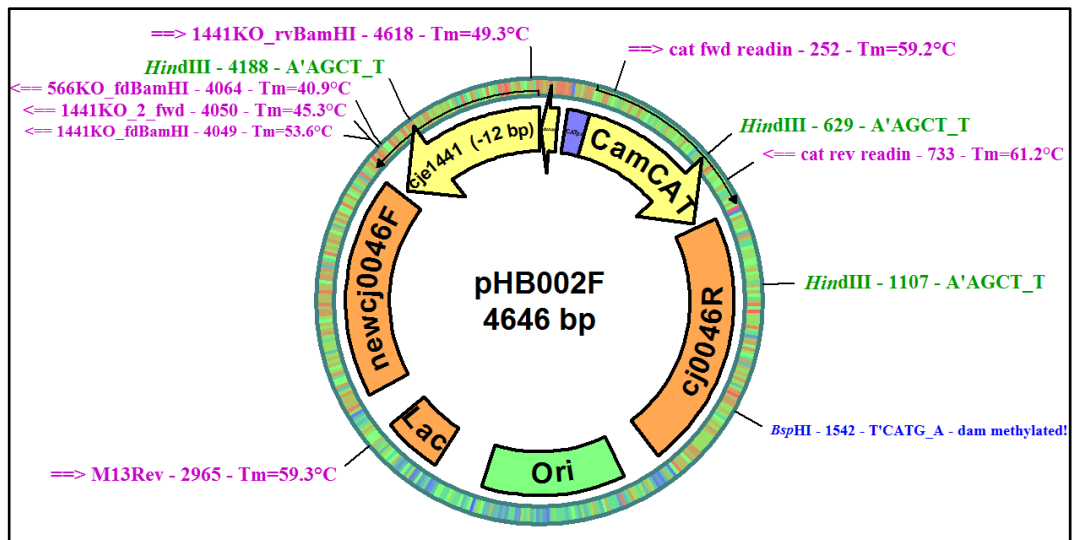


Figure 10-8 Plasmid map of pHB002

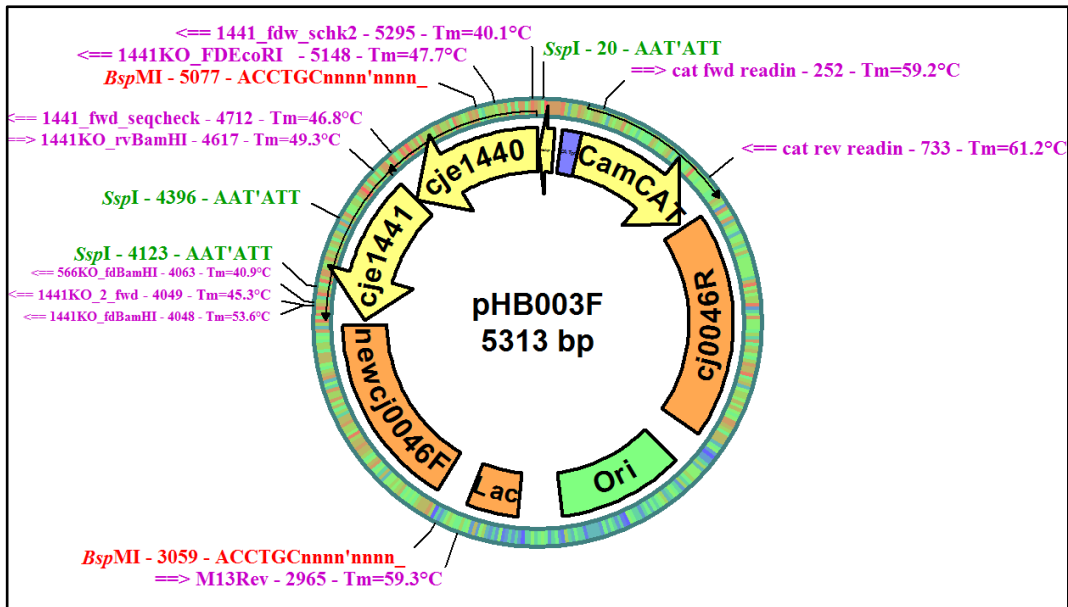


Figure 10-9 plasmid map of pHB003

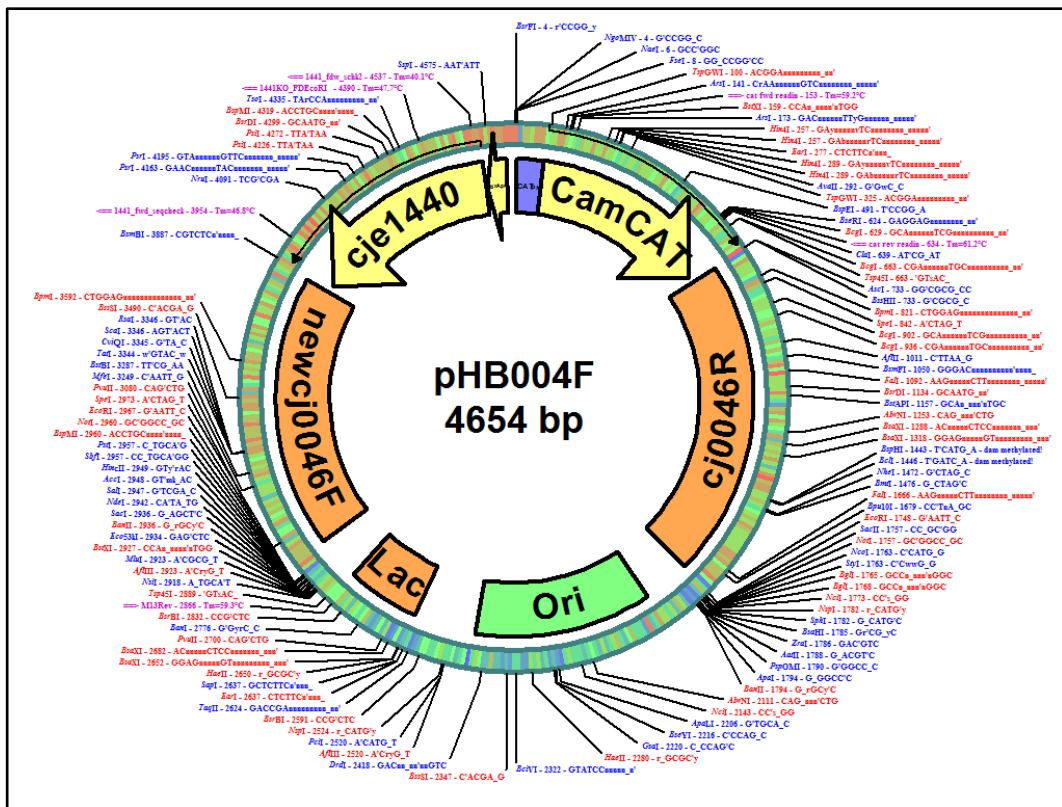


Figure 10-10 Plasmid map of pHB004 (correct orientation)

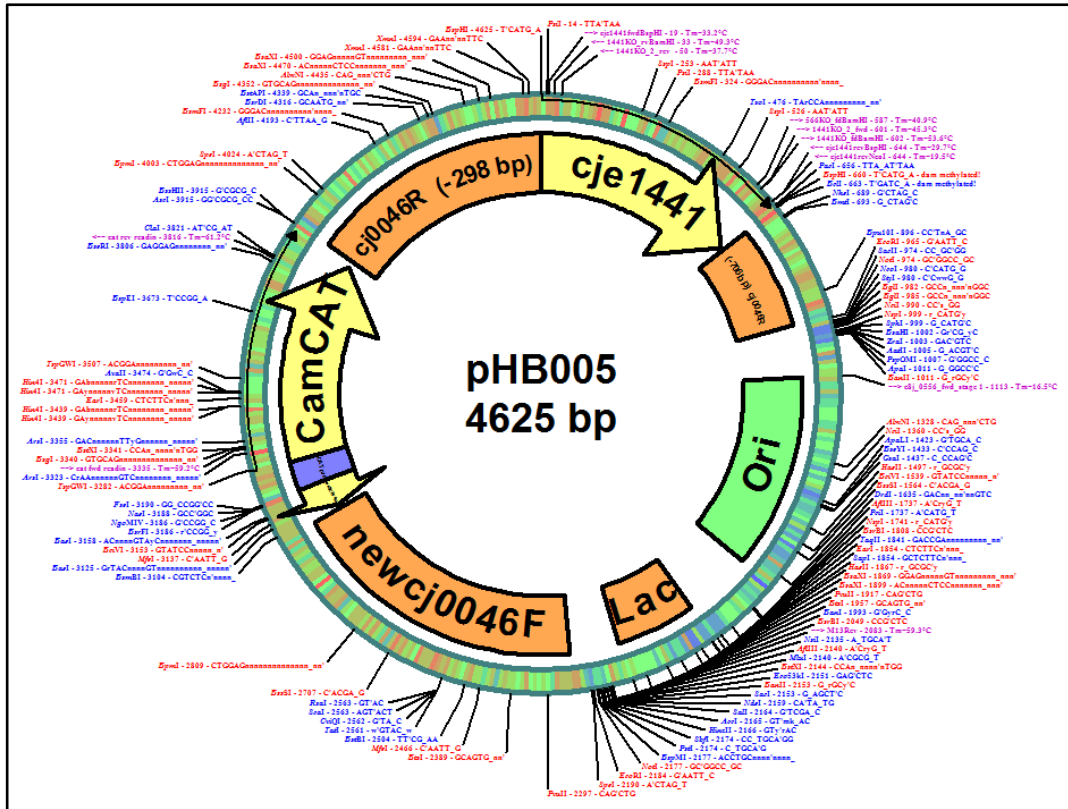


Figure 10-11 Plasmid map of pHB005

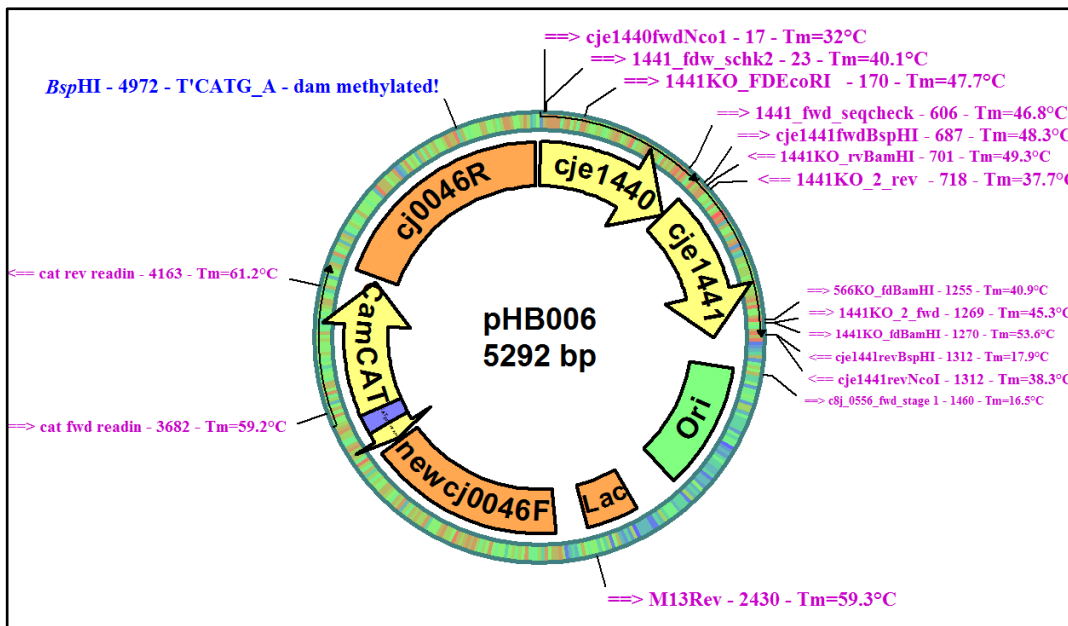


Figure 10-12 Plasmid map of pHB006

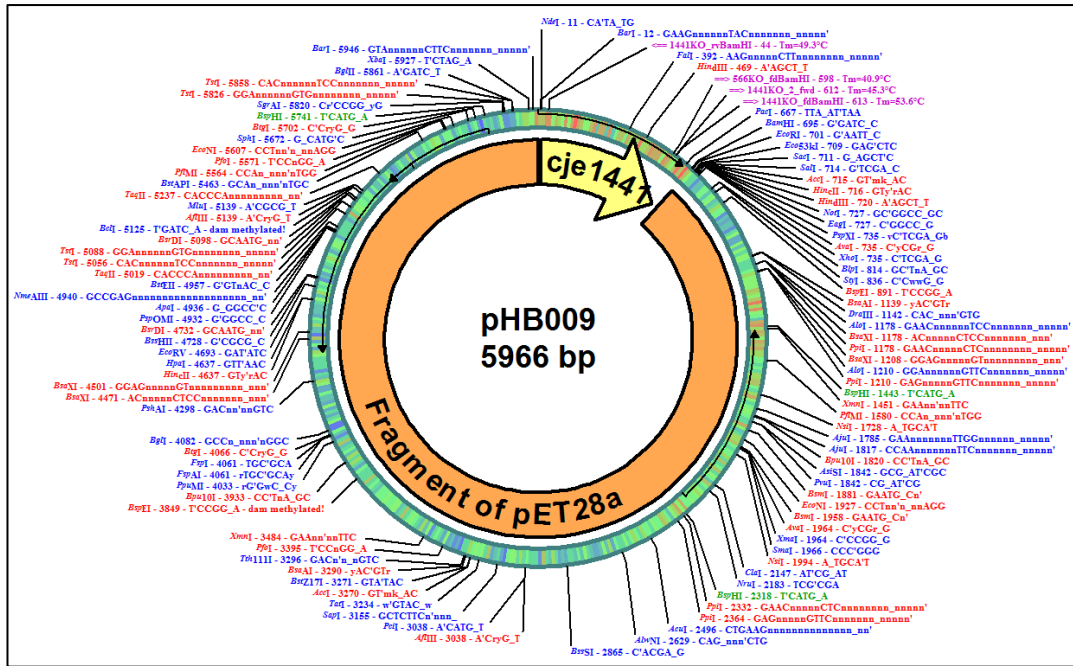


Figure 10-15 Plasmid map of pHB009

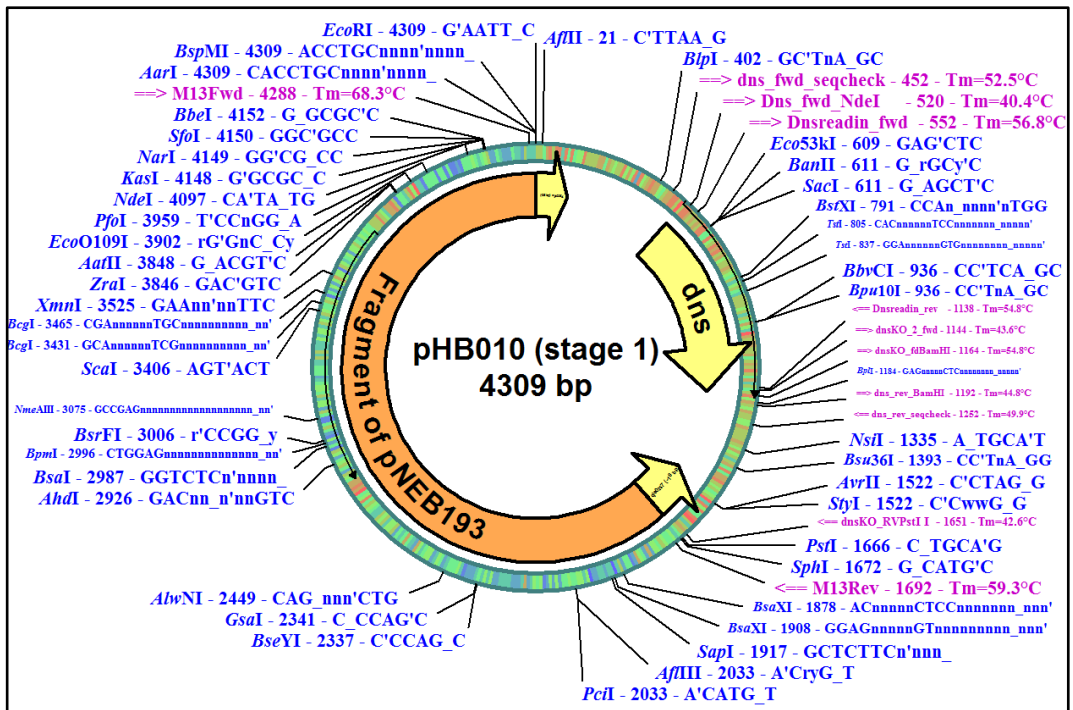


Figure 10-16 Plasmid map of pHB010a

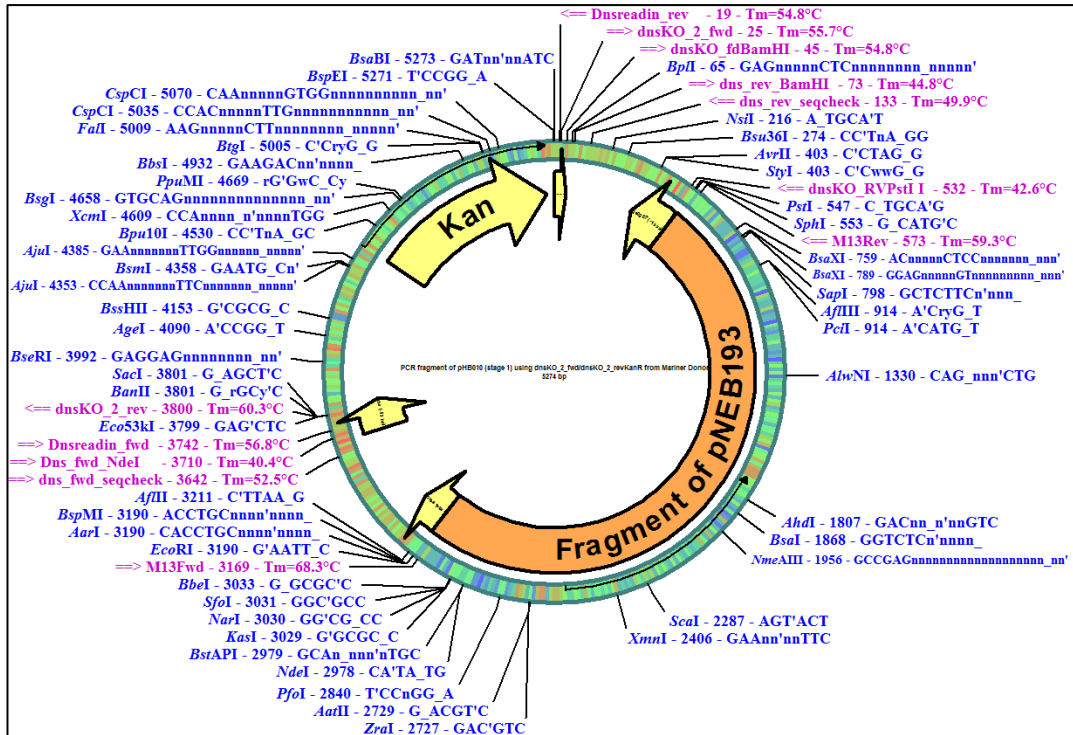


Figure 10-17 plasmid map of pHB010b

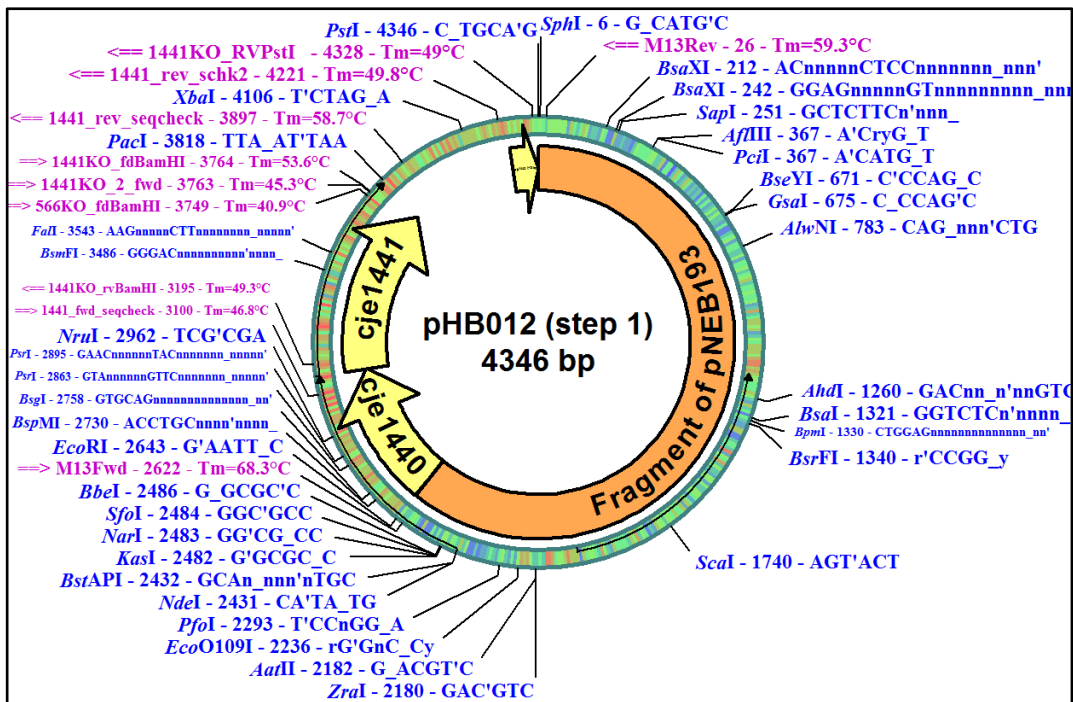


Figure 10-18 Plasmid map of pHB012a

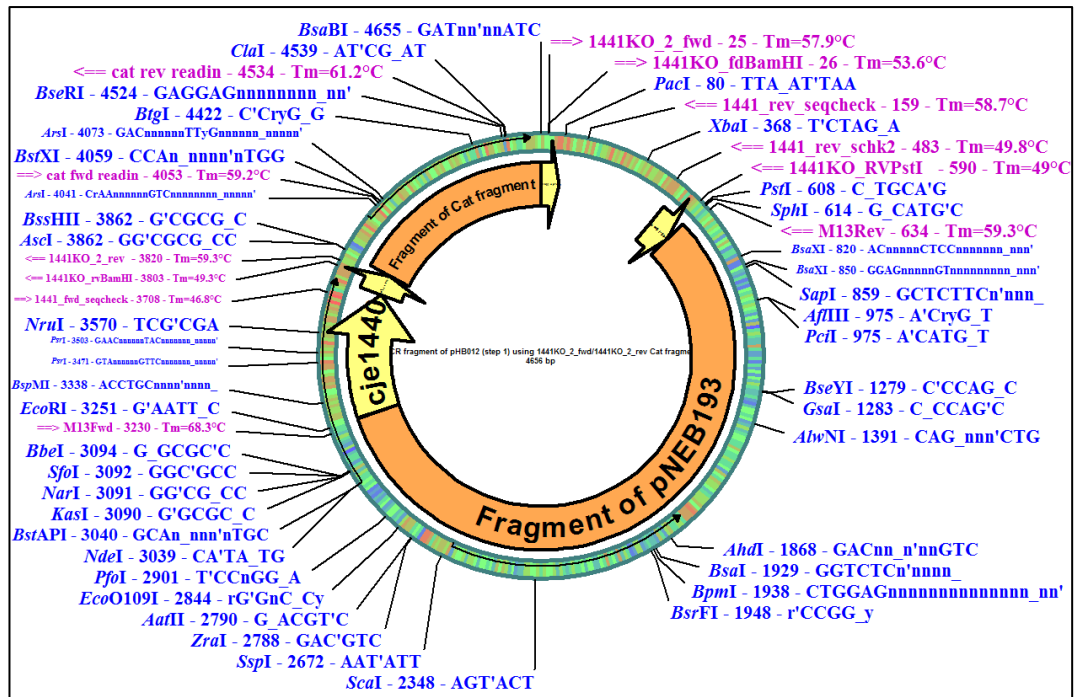


Figure 10-19 Plasmid map of pHB012

10.3 Large microscopy images.

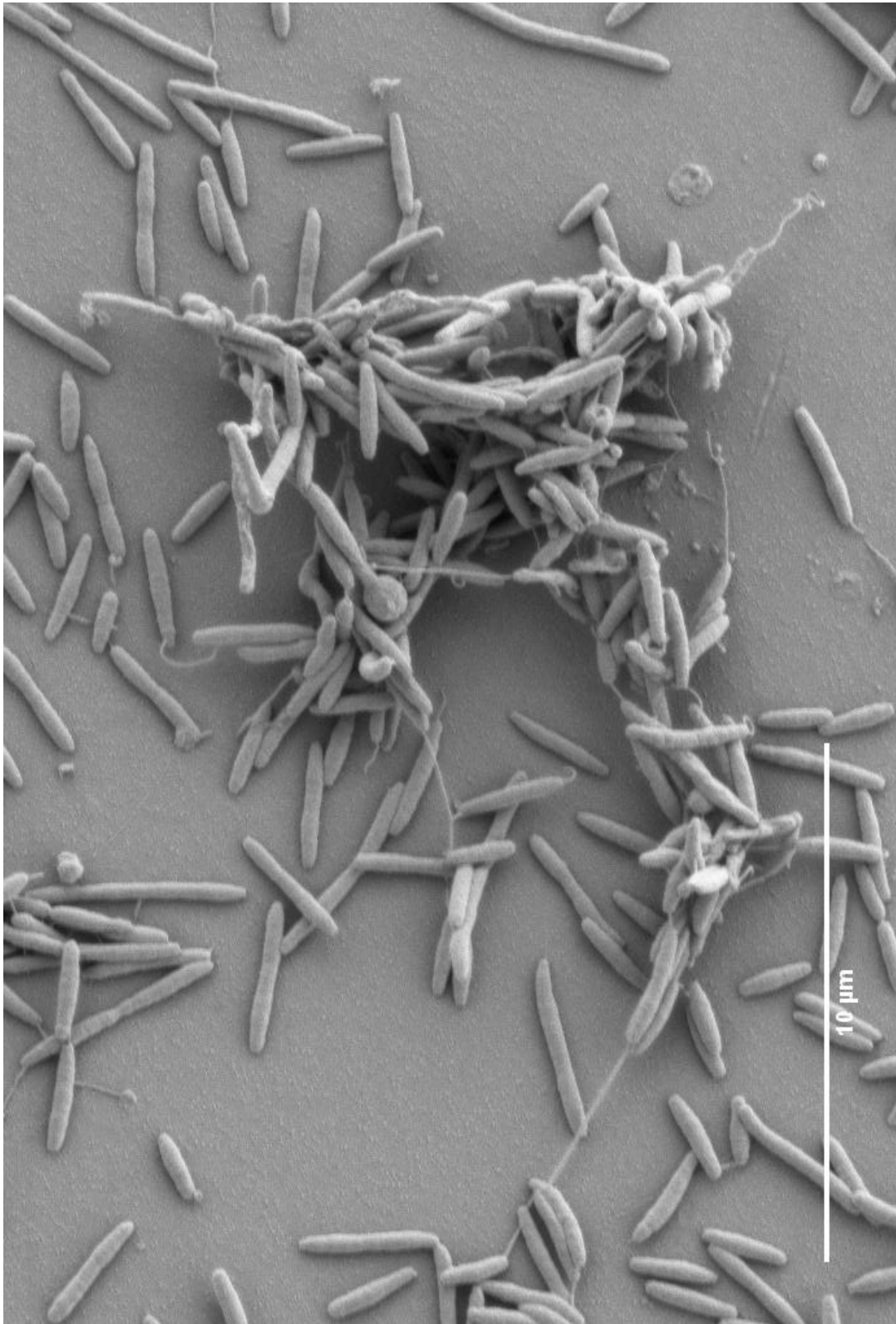


Figure 10-20 Enlarged 0% chicken juice image (original in Figure 4-11)

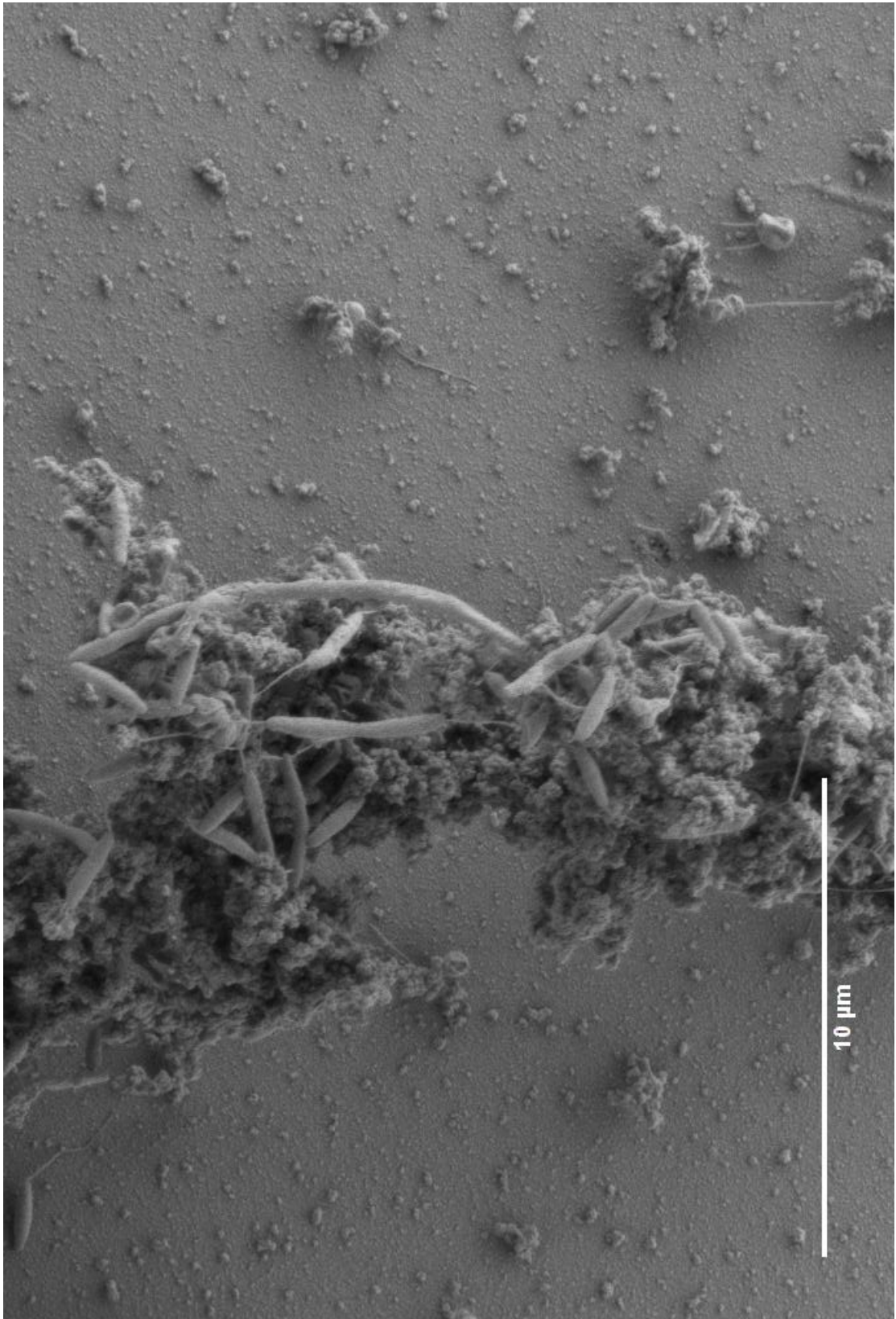


Figure 10-21 Enlarged 5% chicken juice image (original in Figure 4-11)

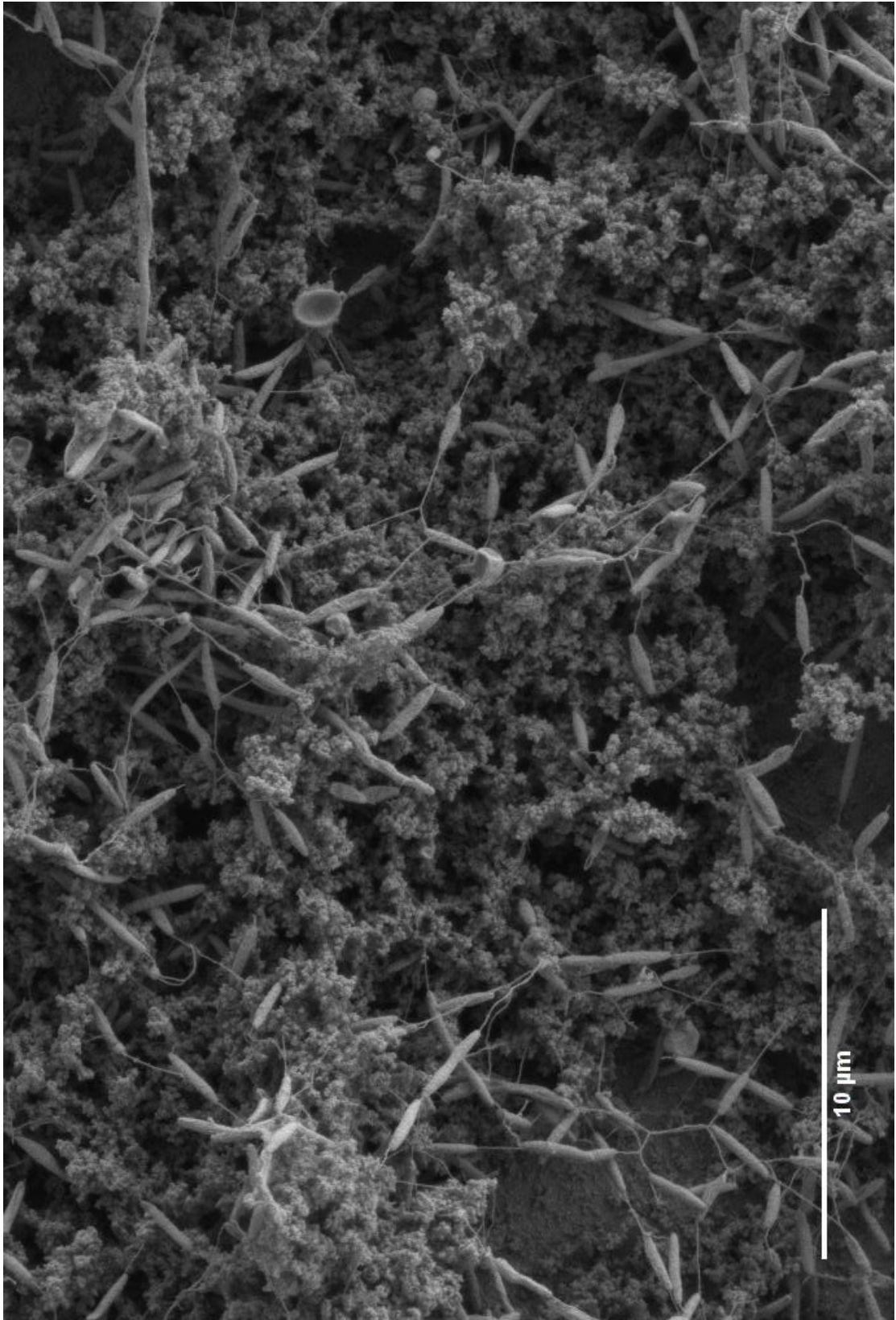


Figure 10-22 Enlarged 100% chicken juice image (original in Figure 4-11)

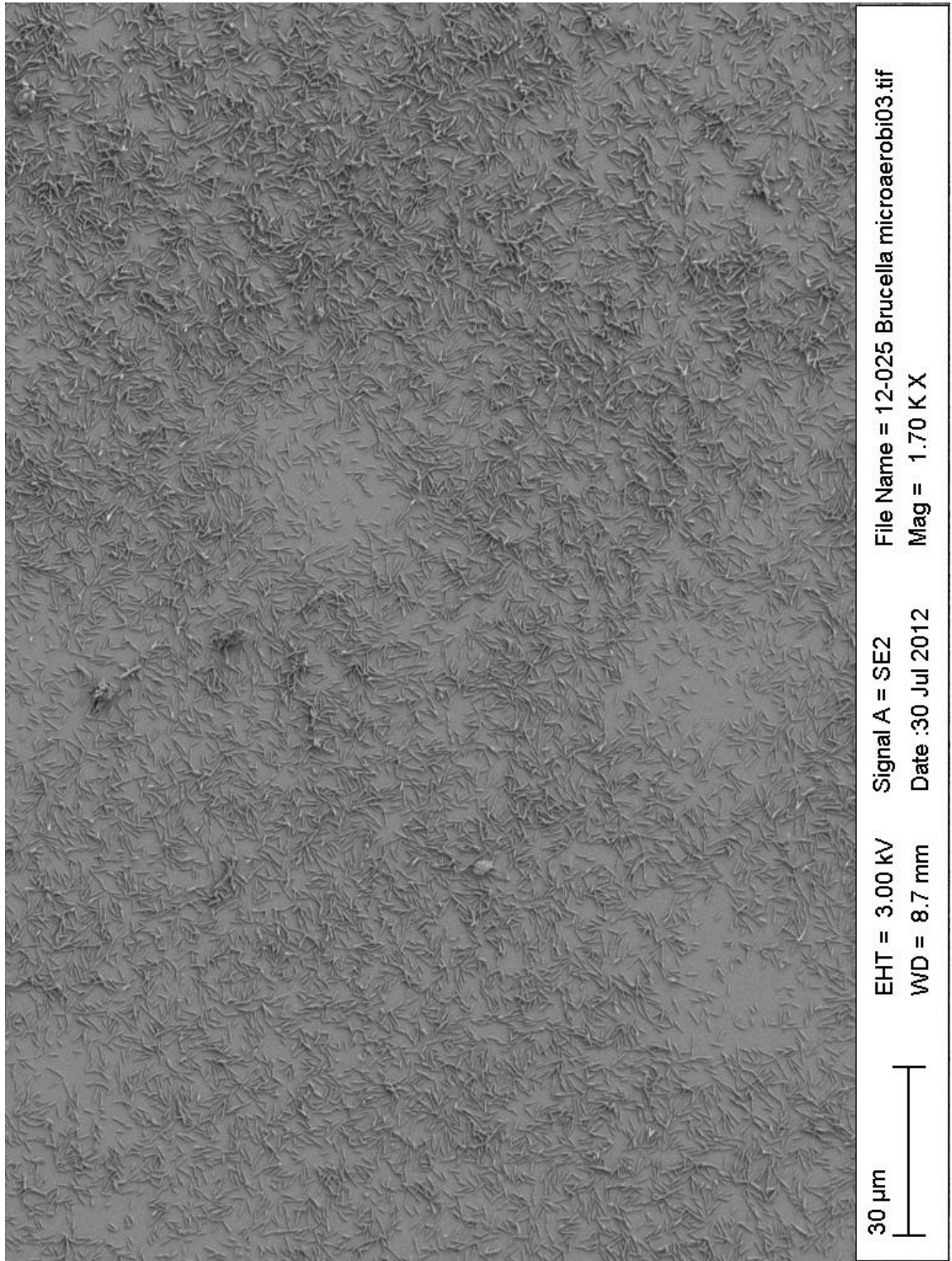


Figure 10-23 Enlarged microaerobic biofilm image (original in Figure 5-4a)

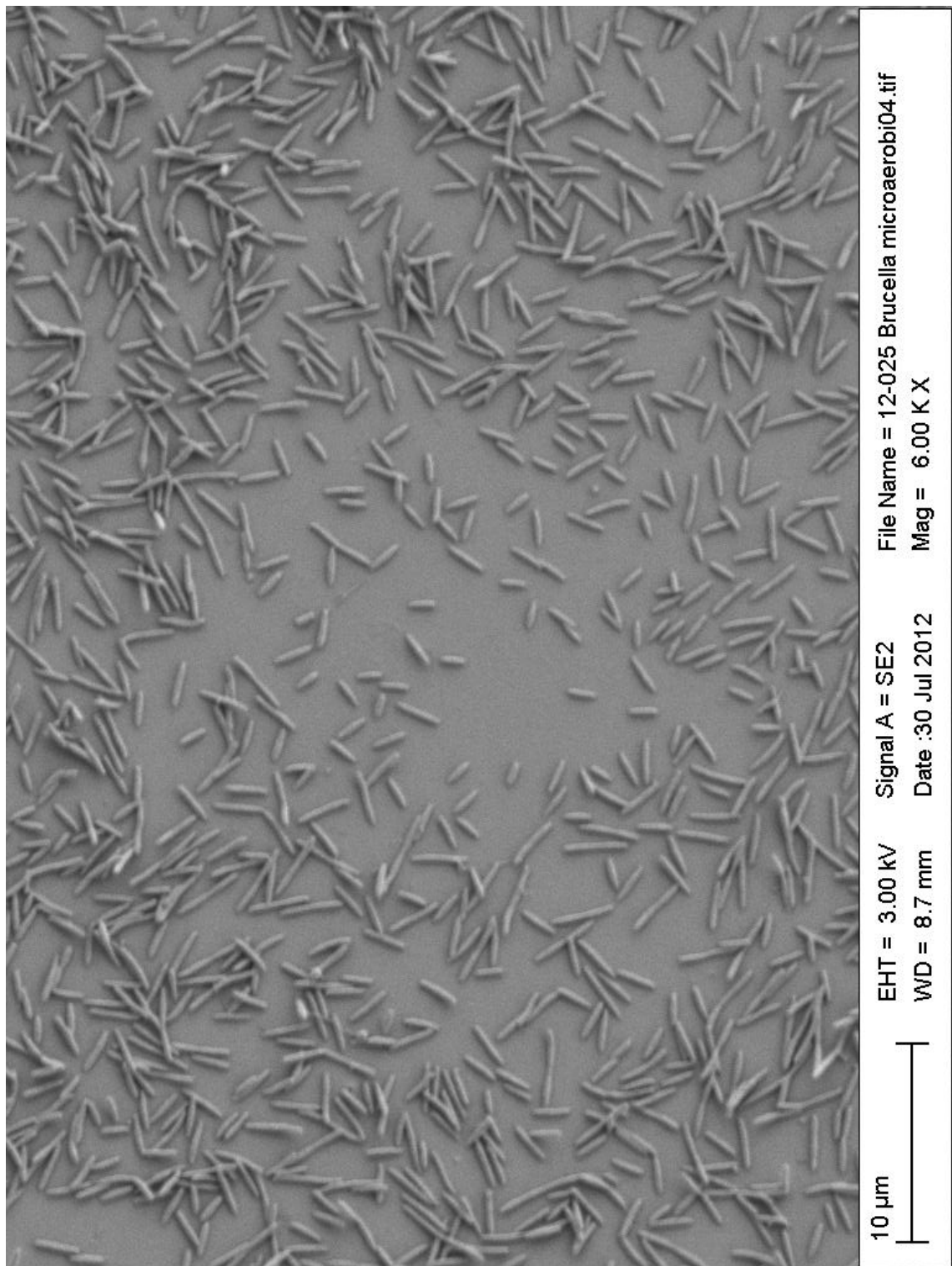


Figure 10-24 Enlarged microaerobic biofilm image (original in Figure 5-4b)

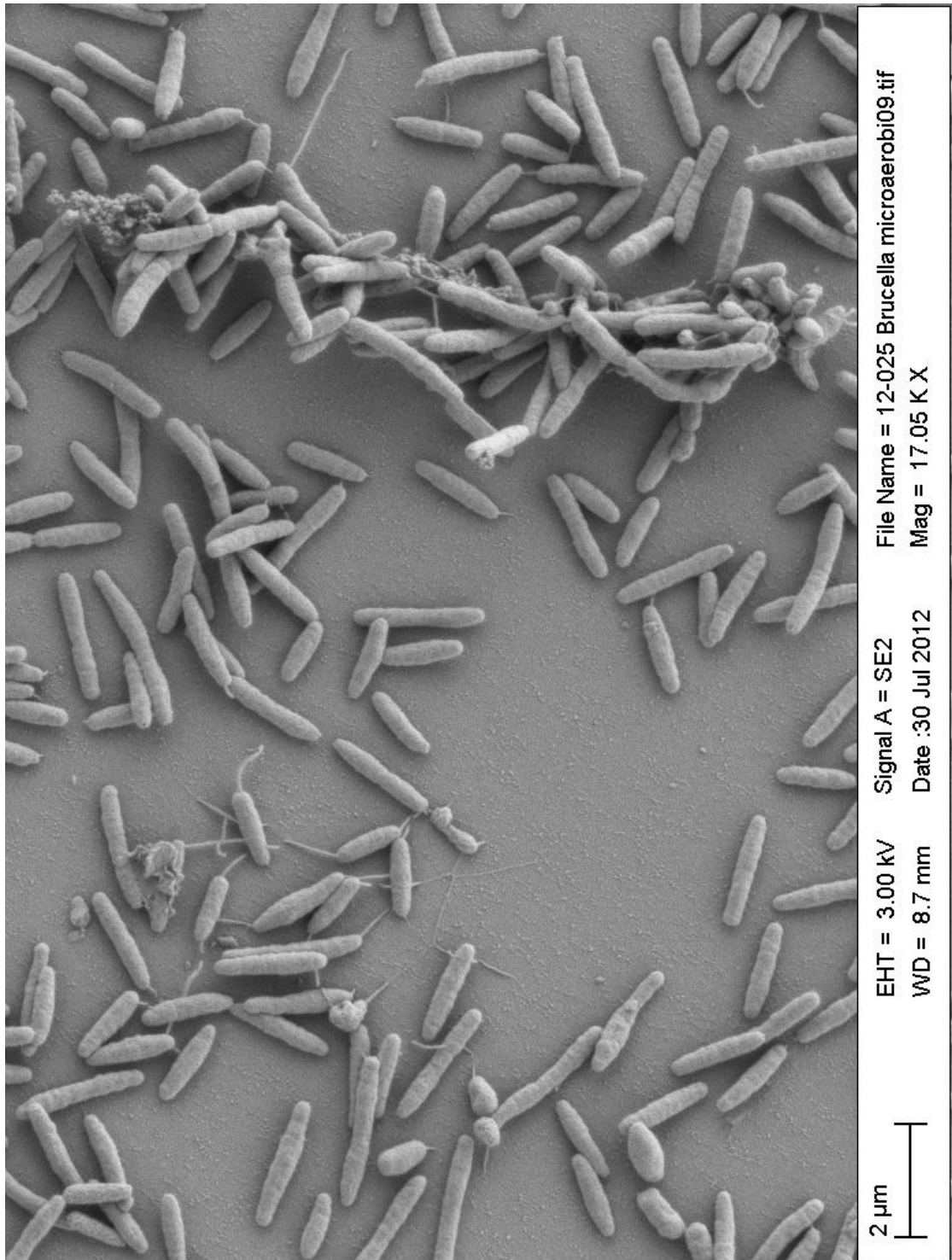


Figure 10-25 Enlarged microaerobic biofilm image (original in Figure 5-4c)

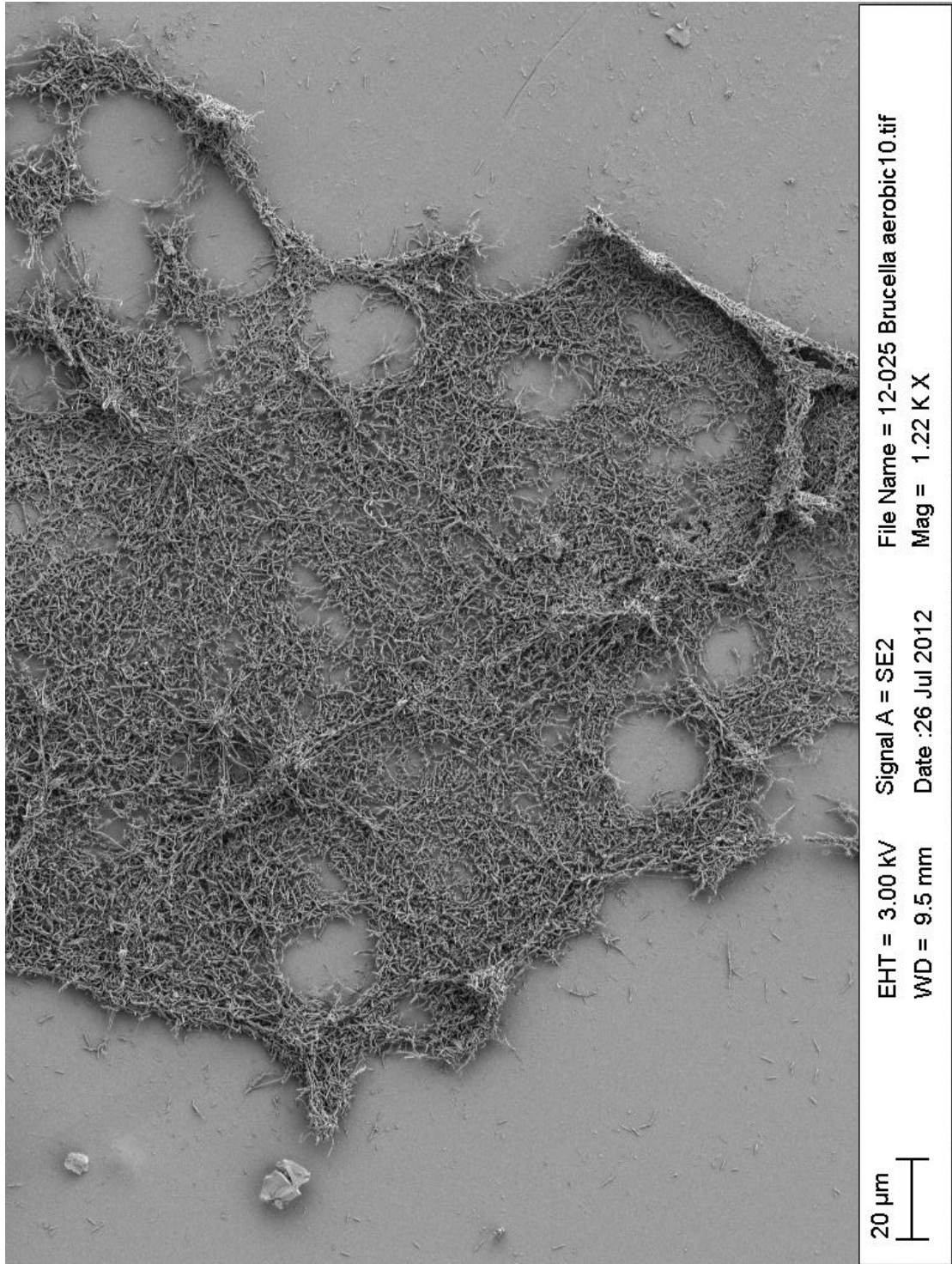


Figure 10-26 Enlarged microaerobic biofilm image (original in Figure 5-4d)

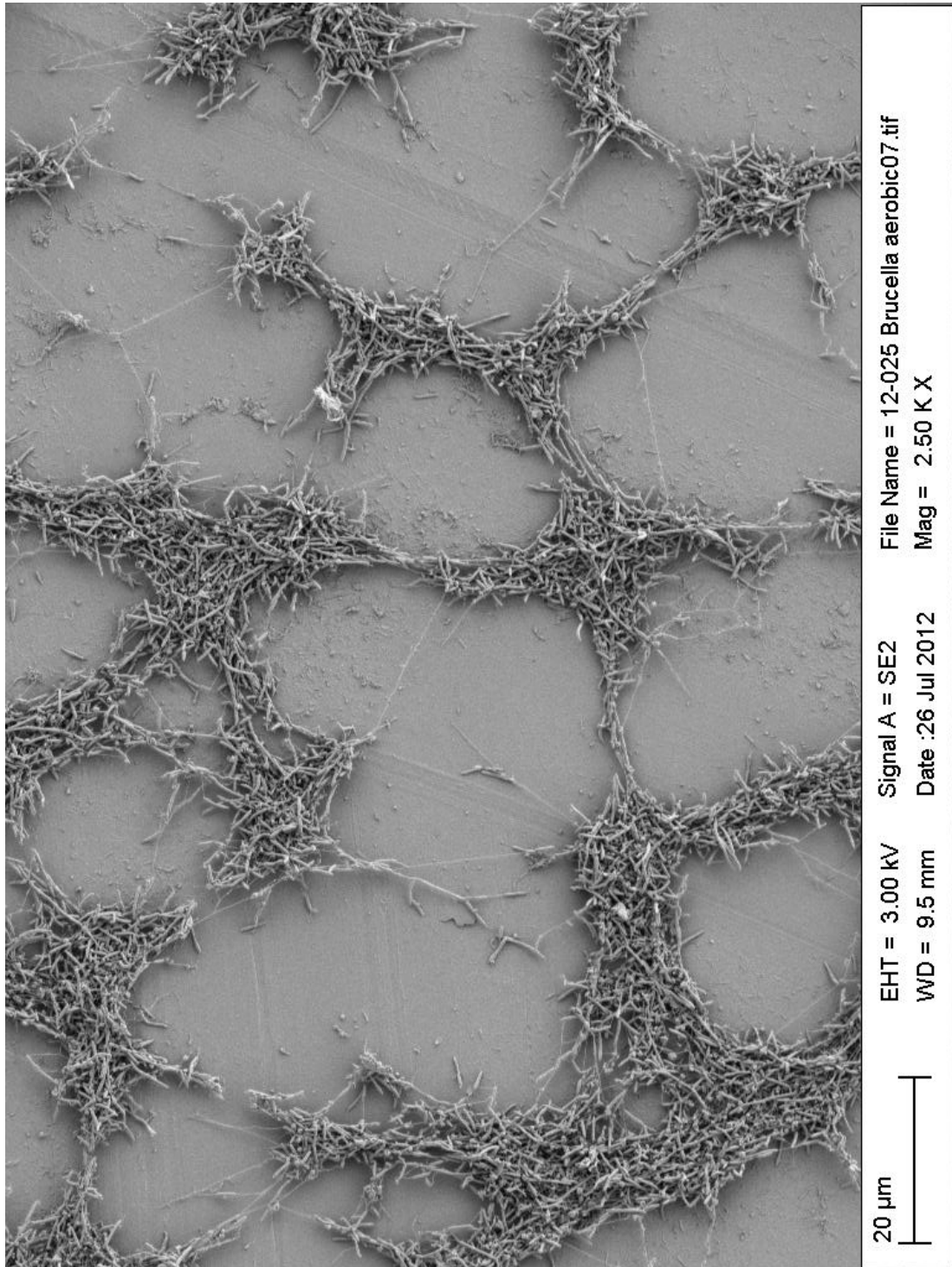


Figure 10-27 Enlarged microaerobic biofilm image (original in Figure 5-4e)

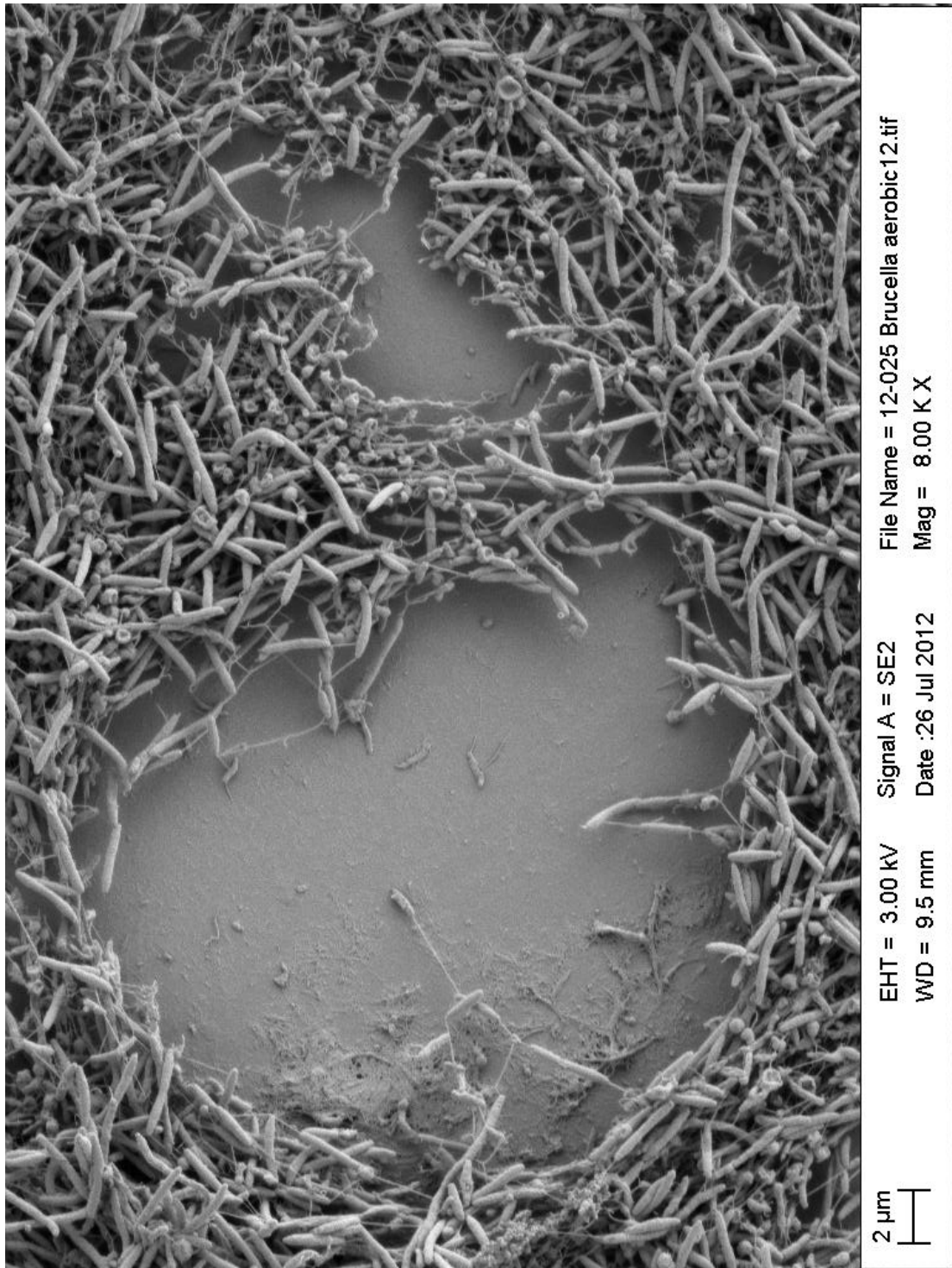


Figure 10-28 Enlarged microaerobic biofilm image (original in Figure 5-4f)

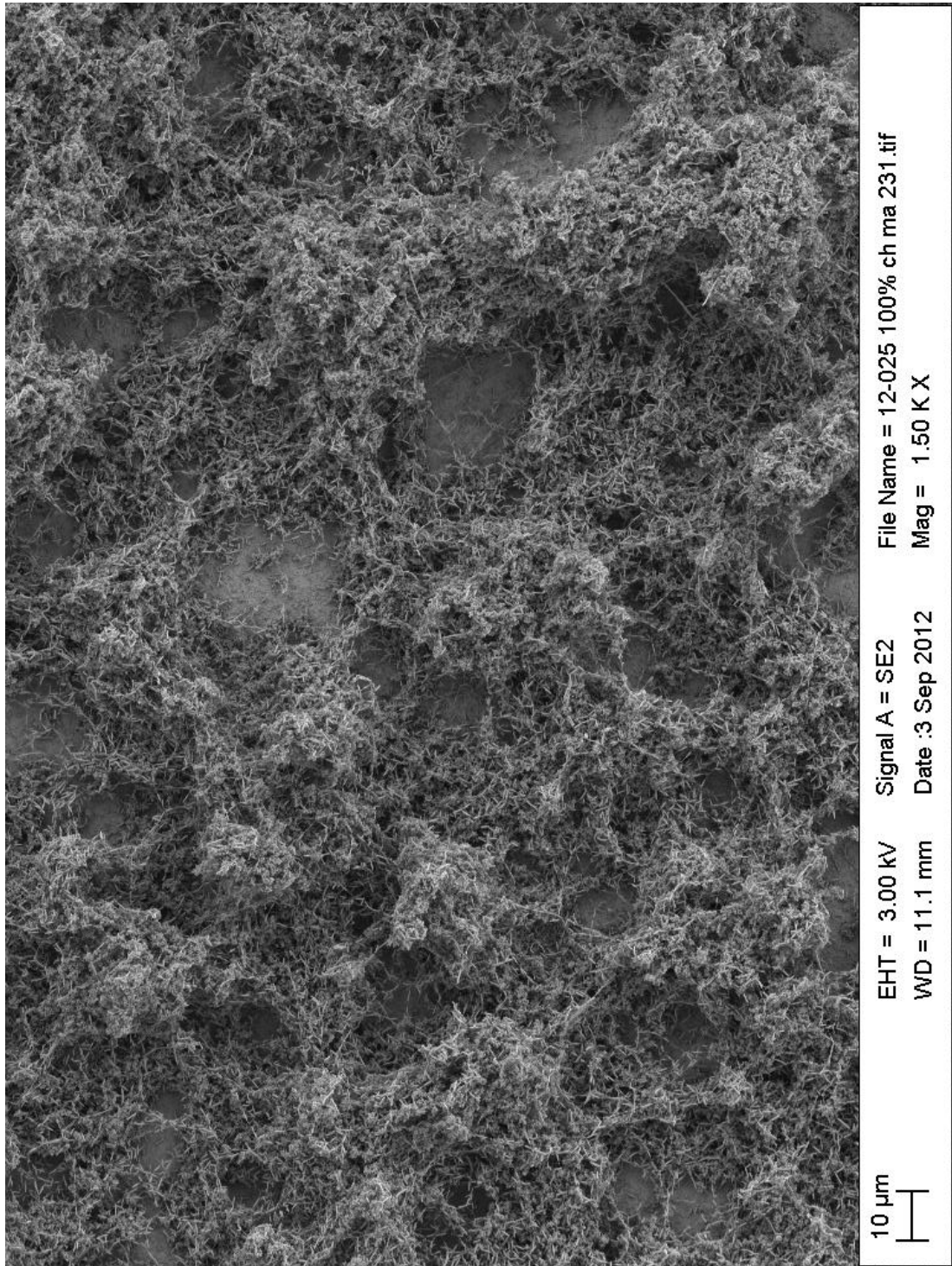


Figure 10-29 Enlarged microaerobic biofilm image (original in Figure 5-5a)

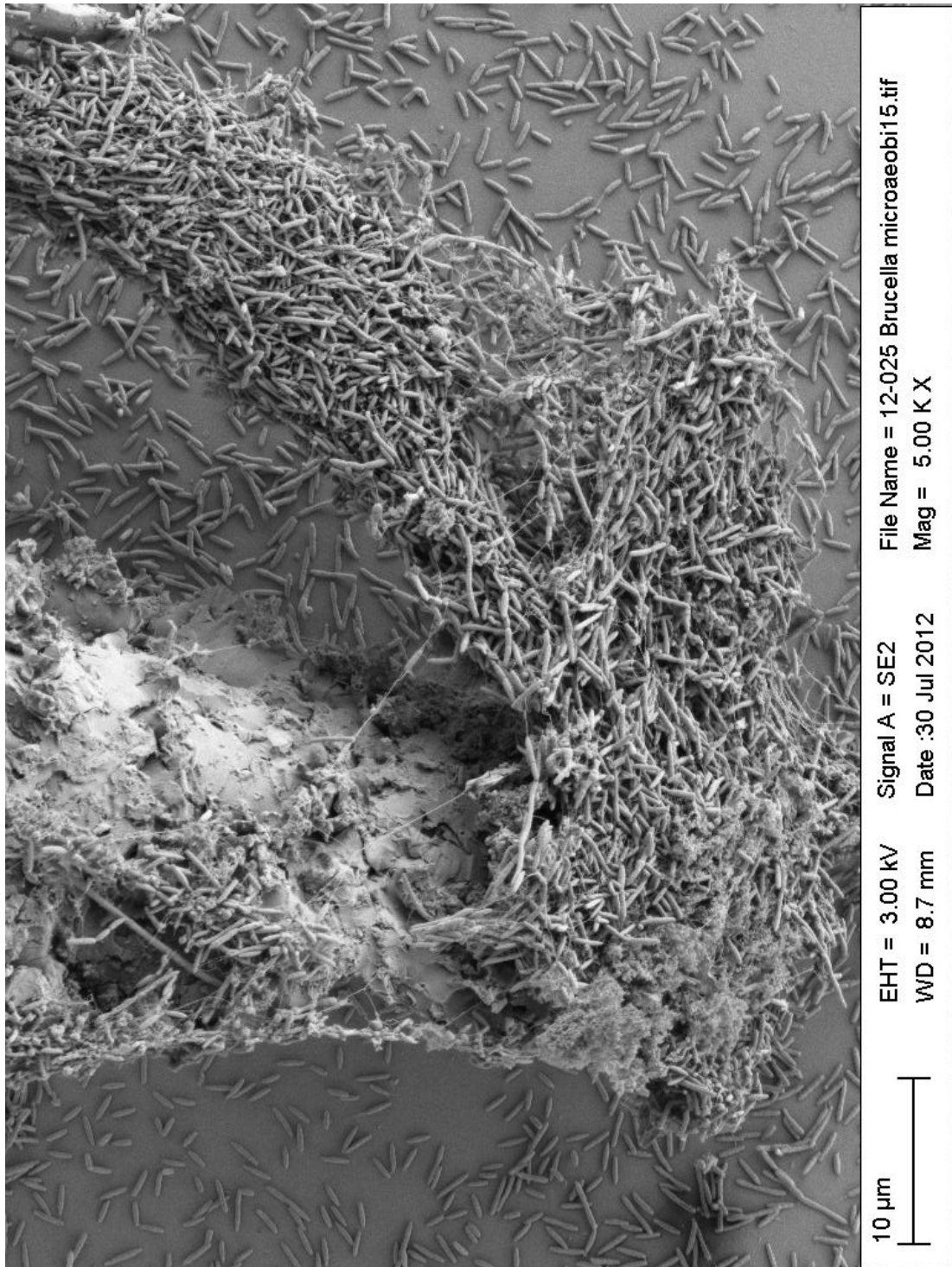


Figure 10-30 Enlarged microaerobic biofilm image (original in Figure 5-5b)

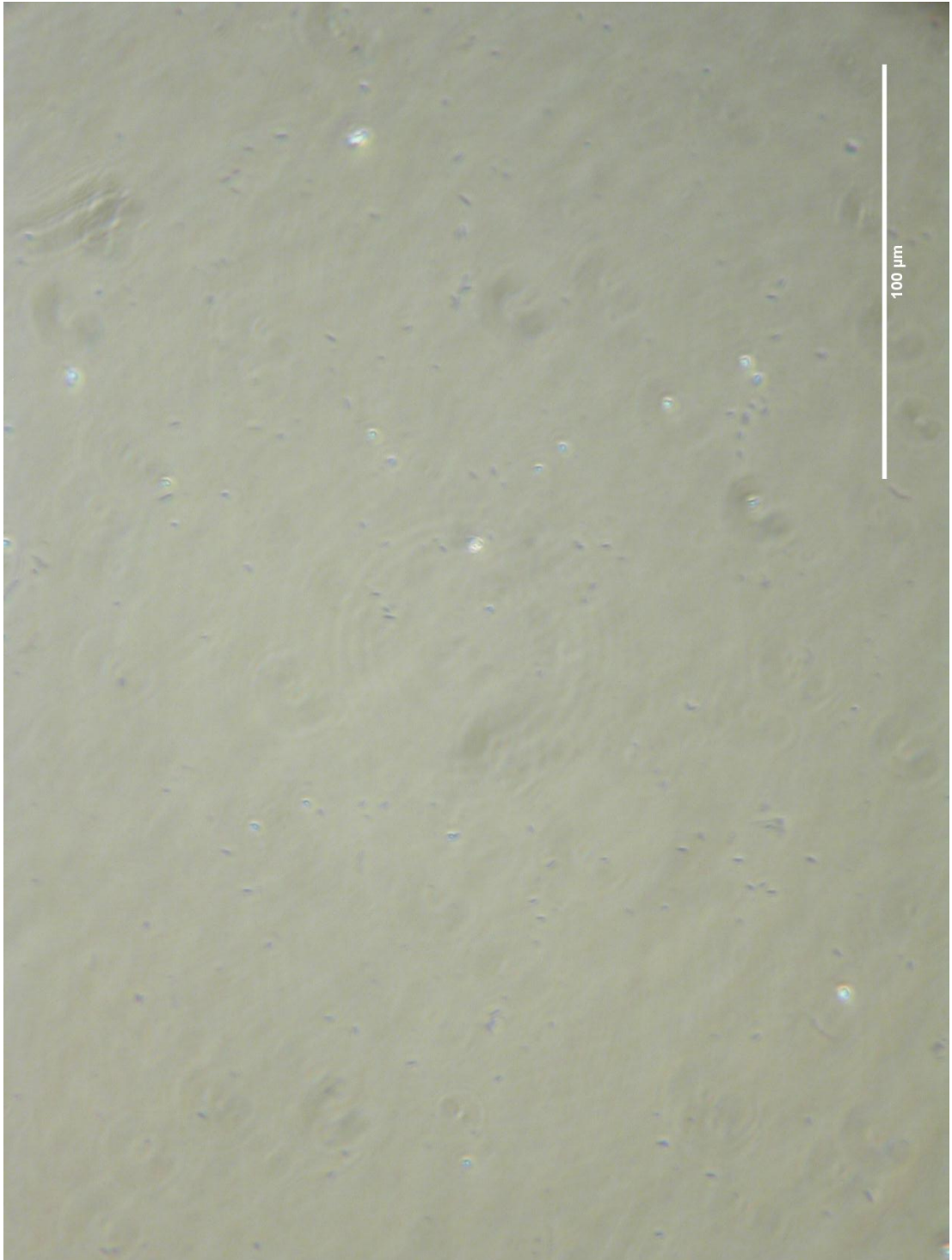


Figure 10-31 Enlarged microaerobic 12 hour biofilm image (original in Figure 5-9)

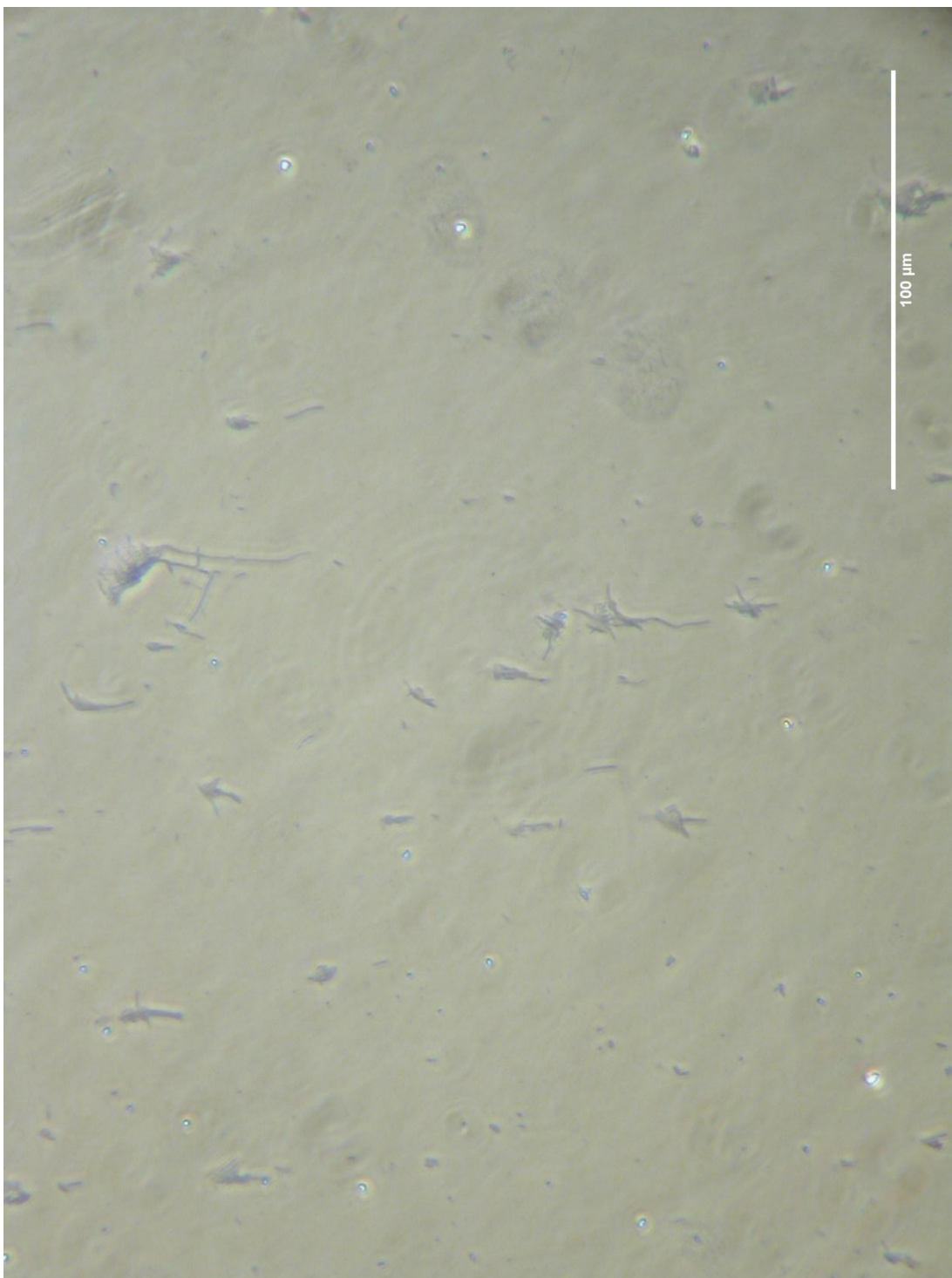


Figure 10-32 Enlarged aerobic 12 hour biofilm image (original in Figure 5-9)

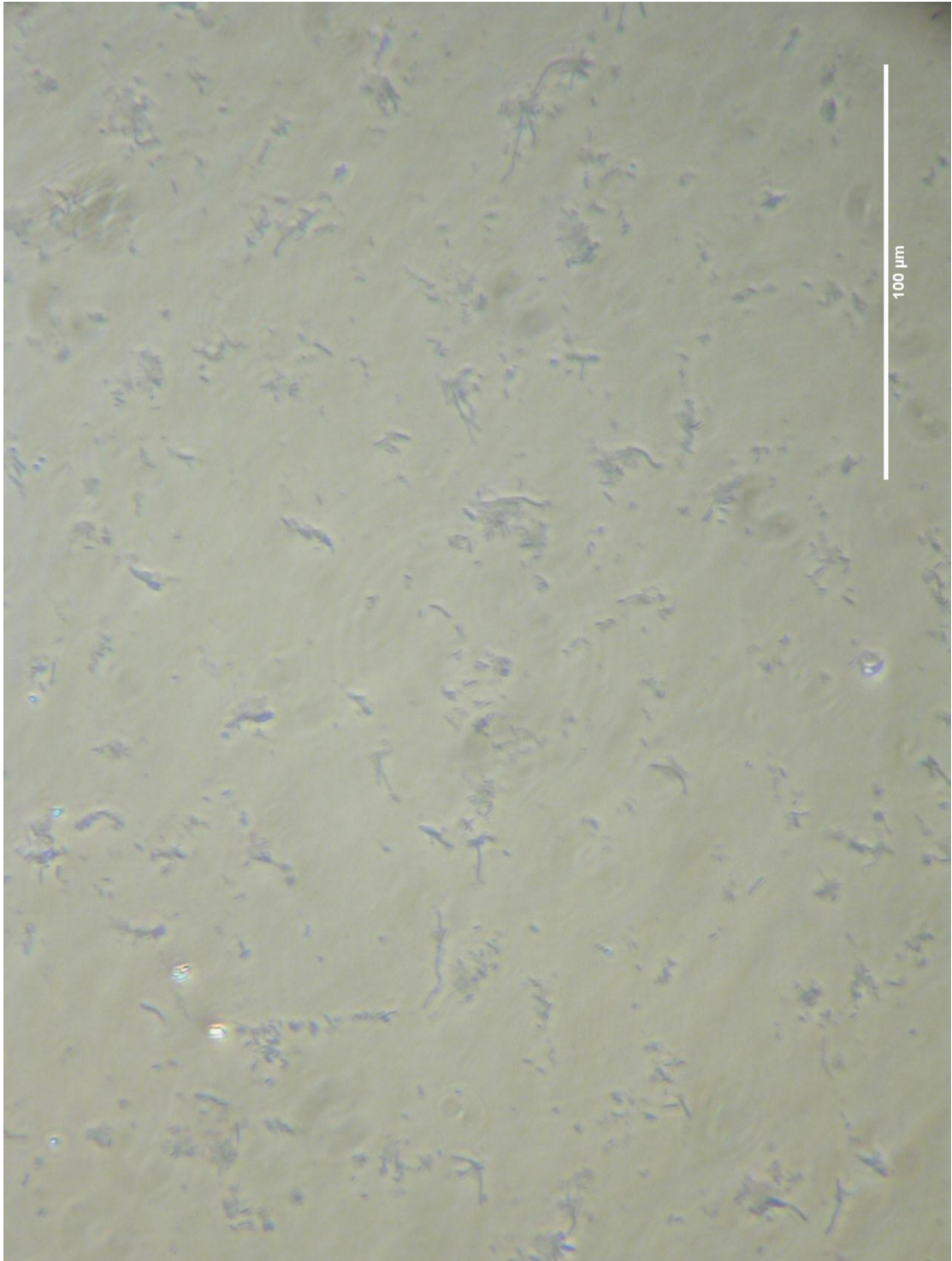


Figure 10-33 Enlarged microaerobic 24 hour biofilm image (original in Figure 5-9)

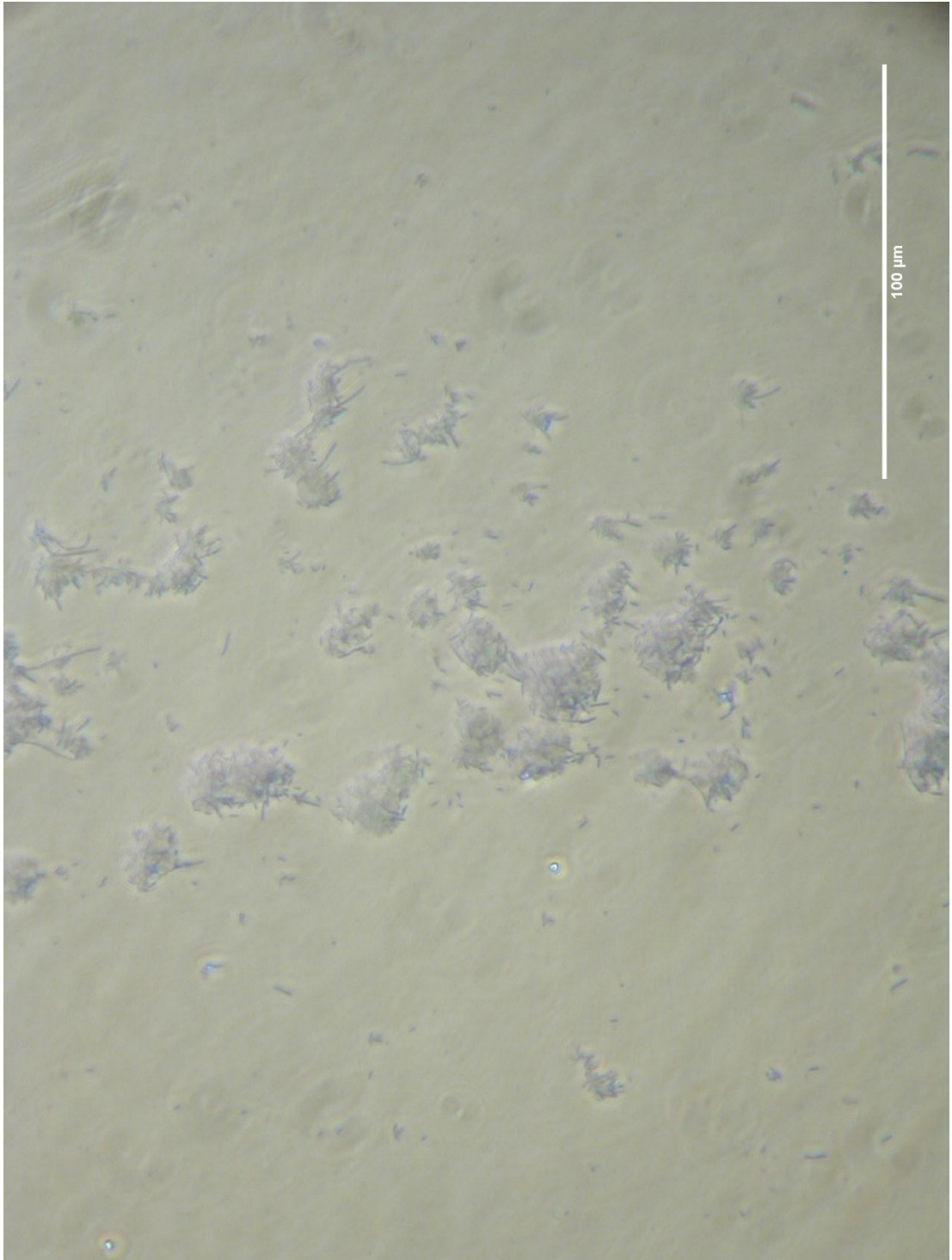


Figure 10-34 Enlarged aerobic 24 hour biofilm image (original in Figure 5-9)



Figure 10-35 Enlarged microaerobic 48 hour biofilm image (original in Figure 5-9 and Figure 5-12)

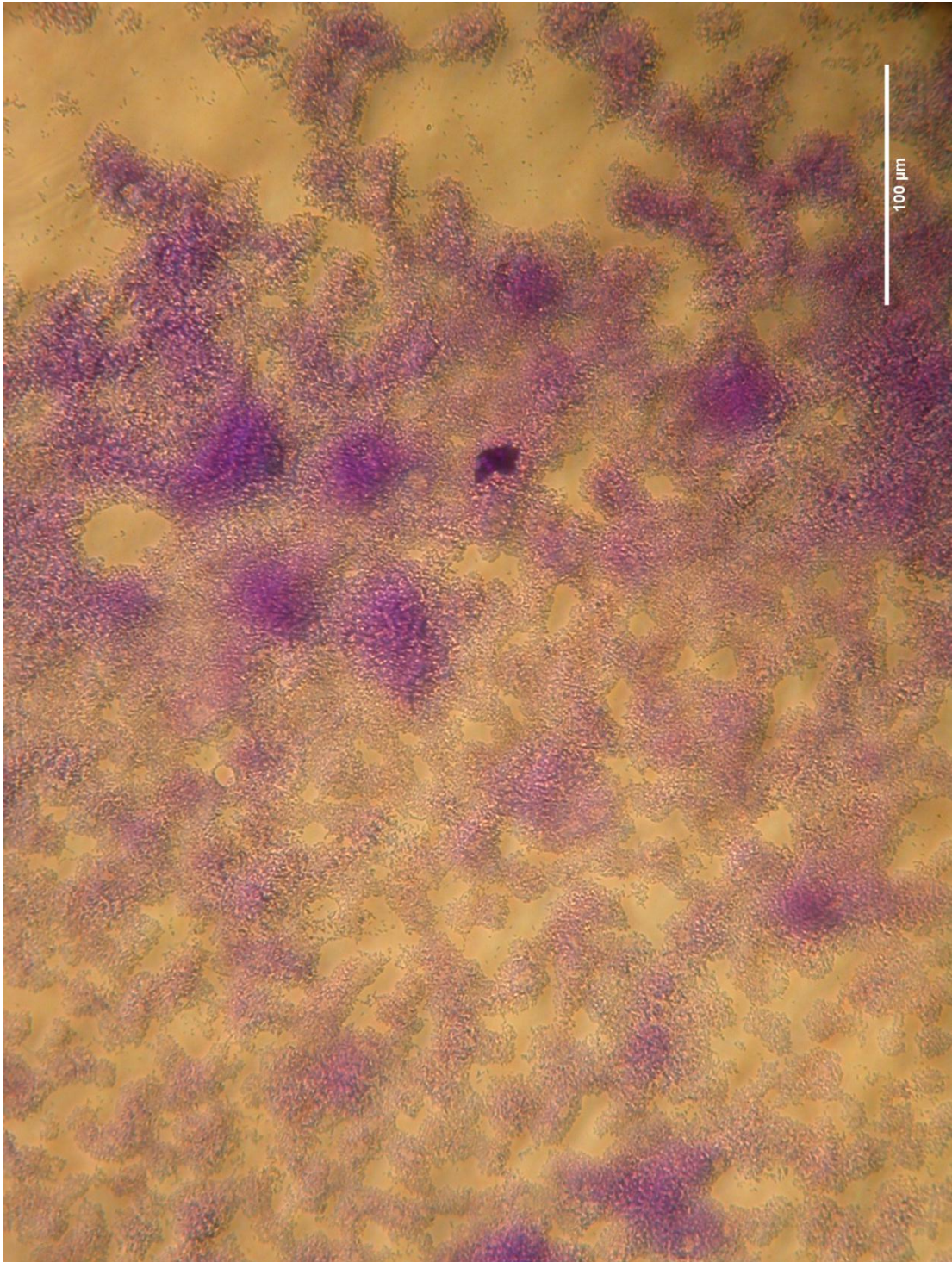


Figure 10-36 Enlarged microaerobic 48 hour biofilm image (original in Figure 5-9 and Figure 5-12)

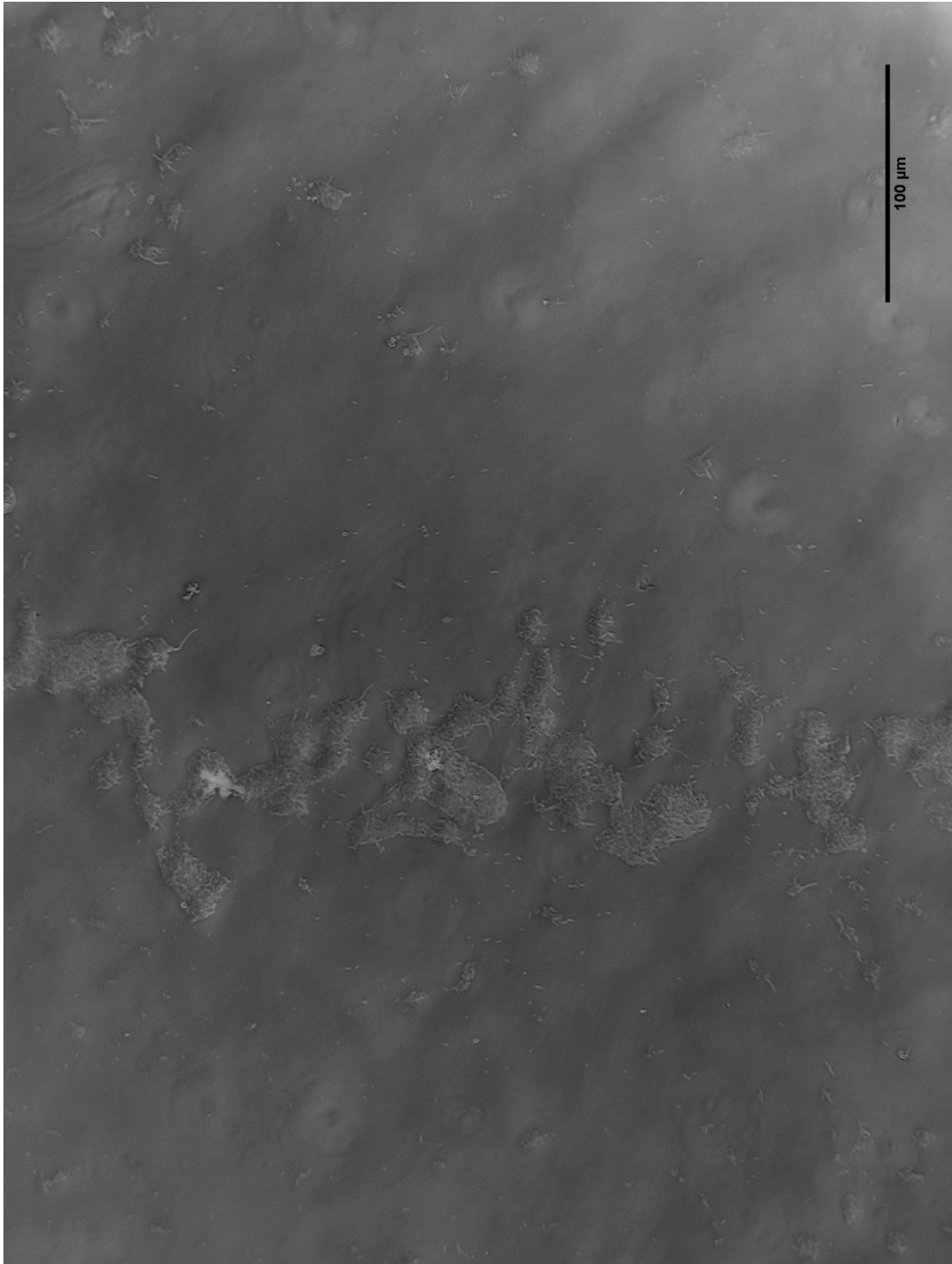


Figure 10-37 Enlarged aerobic 24 hour biofilm image in negative (original in Figure 5-11)

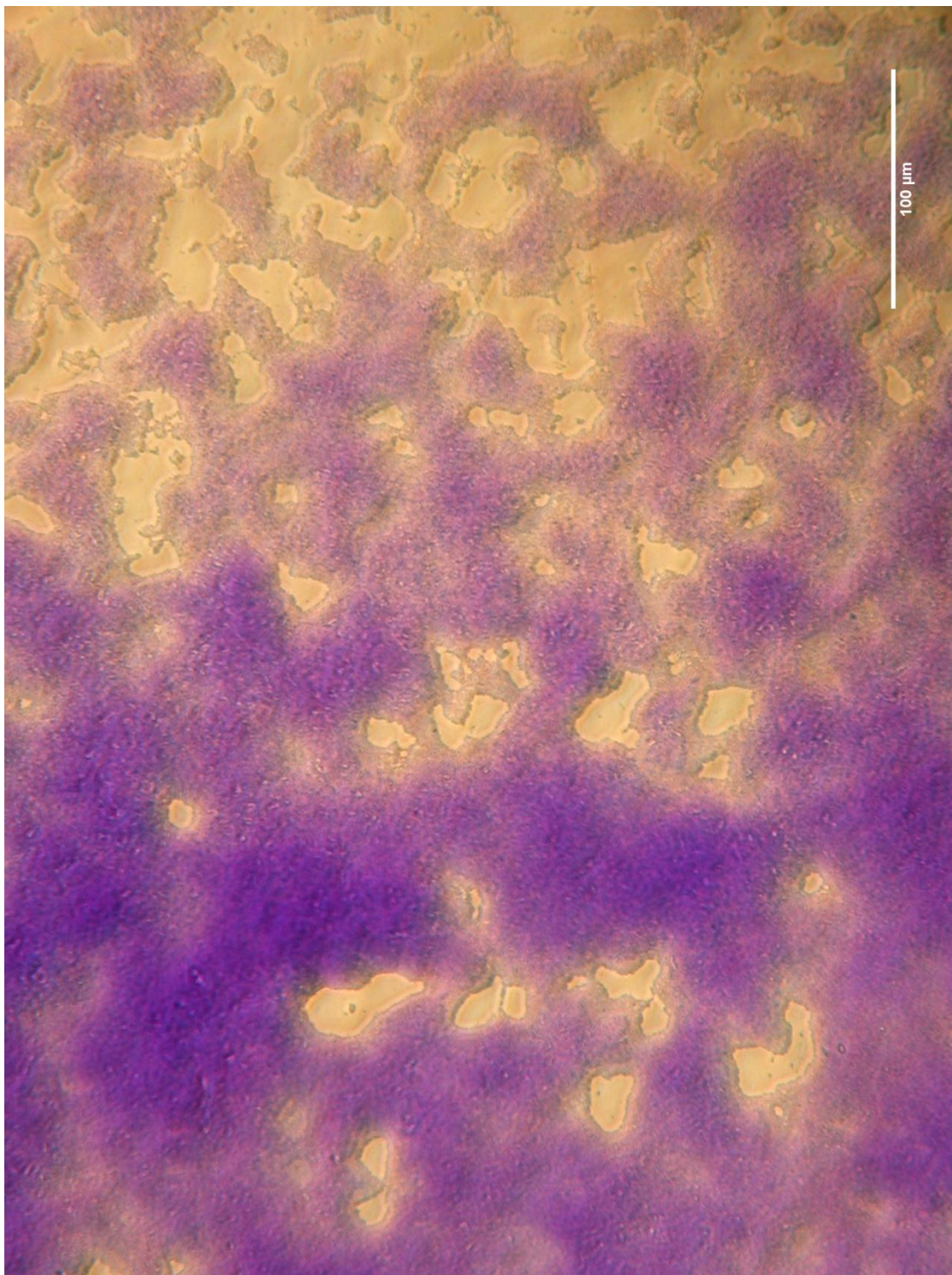


Figure 10-38 Enlarged microaerobic 72 hour biofilm image (original in Figure 5-12)

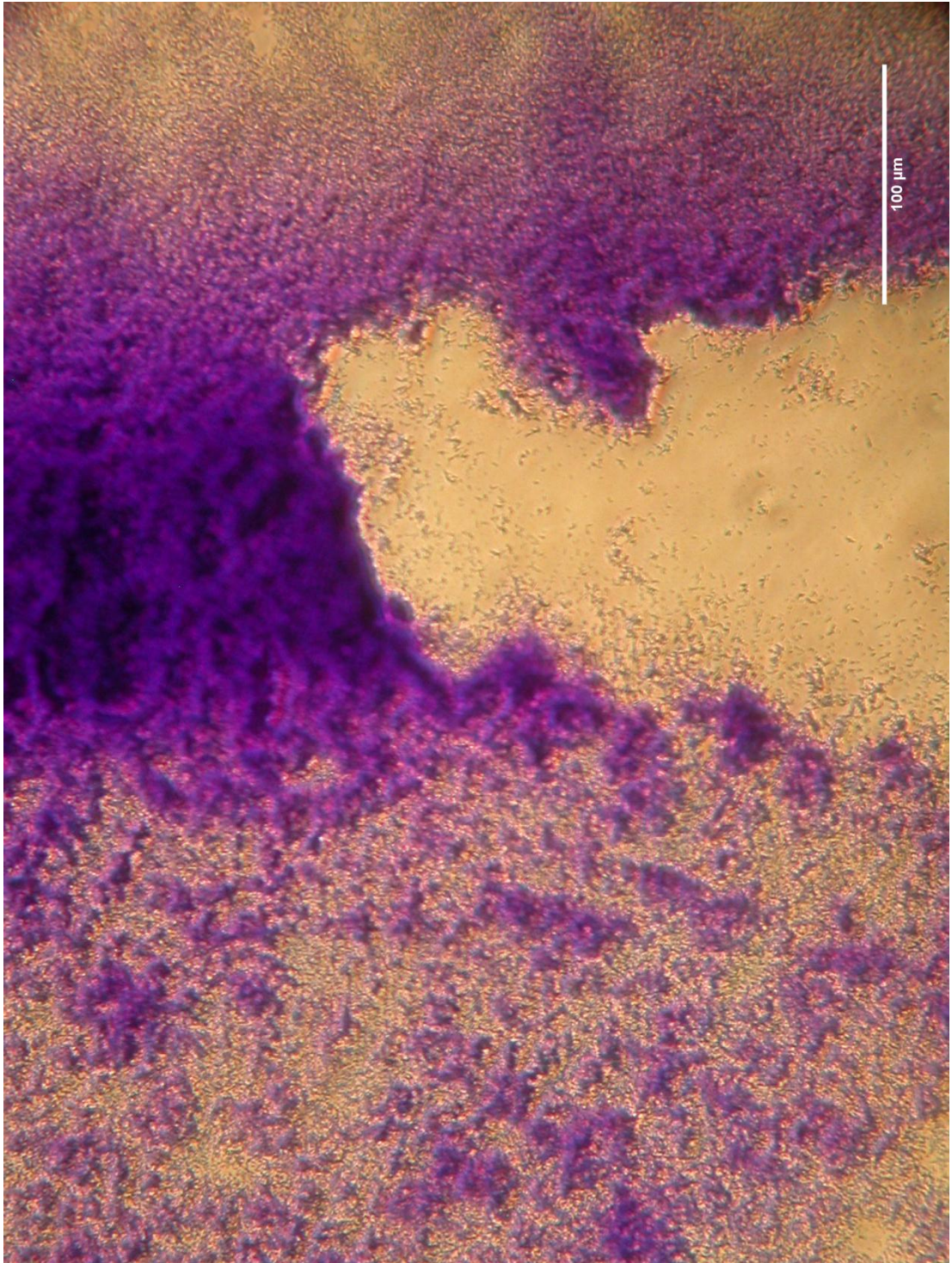


Figure 10-39 Enlarged aerobic 72 hour biofilm image (original in Figure 5-12)

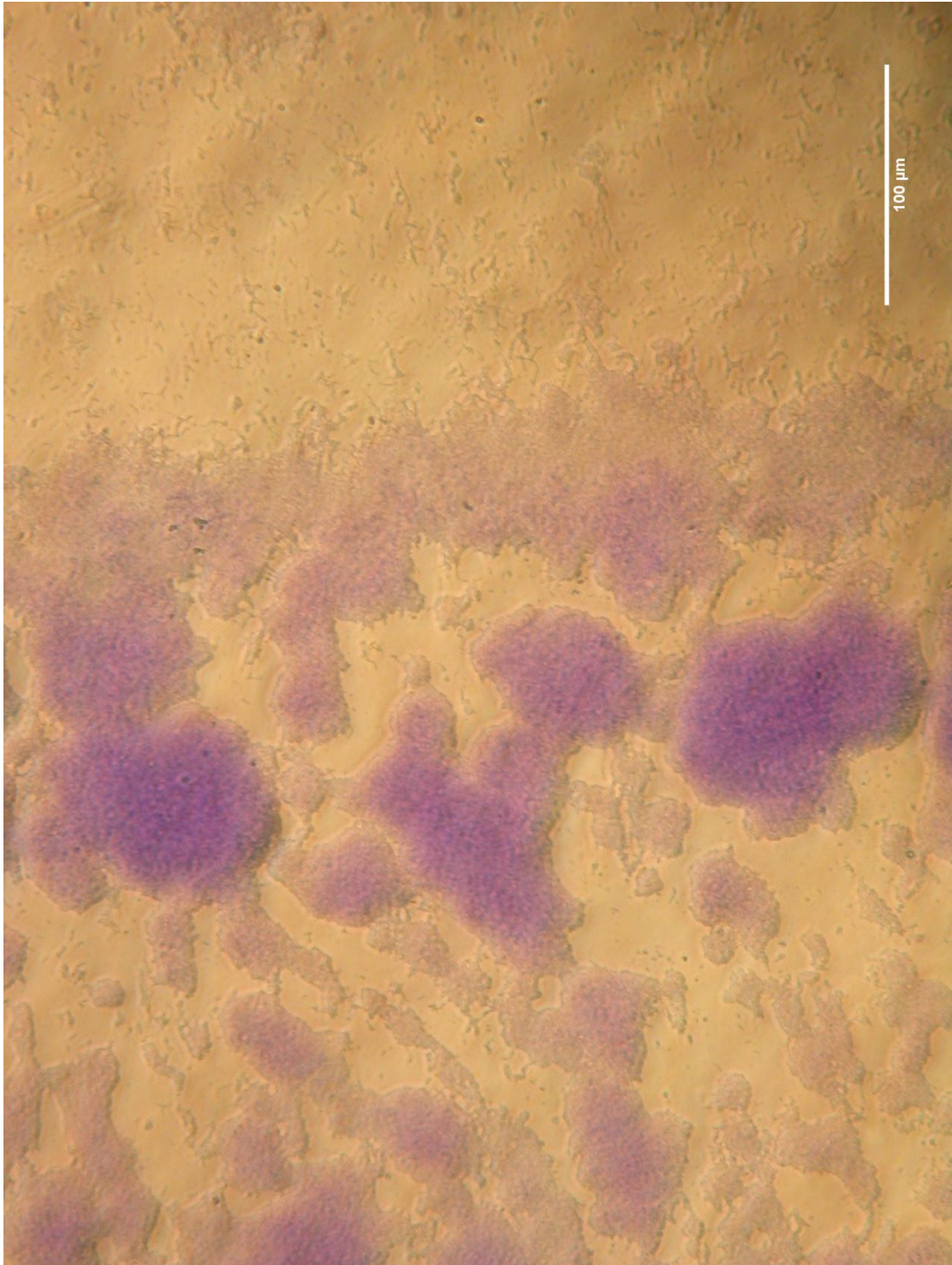


Figure 10-40 Enlarged microaerobic 96 hour biofilm image (original in Figure 5-12)

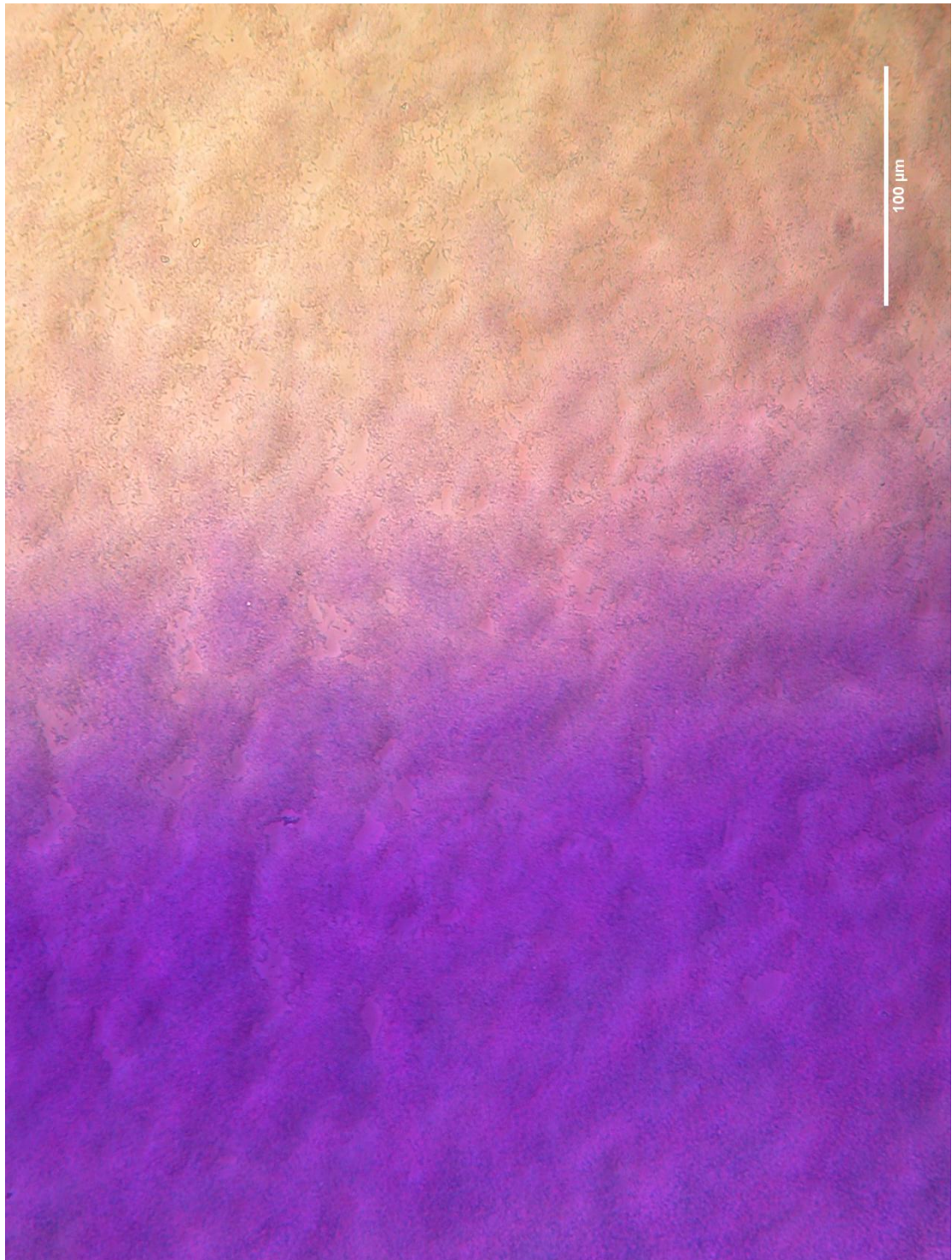


Figure 10-41 Enlarged aerobic 96 hour biofilm image (original in Figure 5-12)



Figure 10-42 Enlarged image of below the air-liquid interface in a 72 hour aerobic biofilm (original in Figure 5-13)

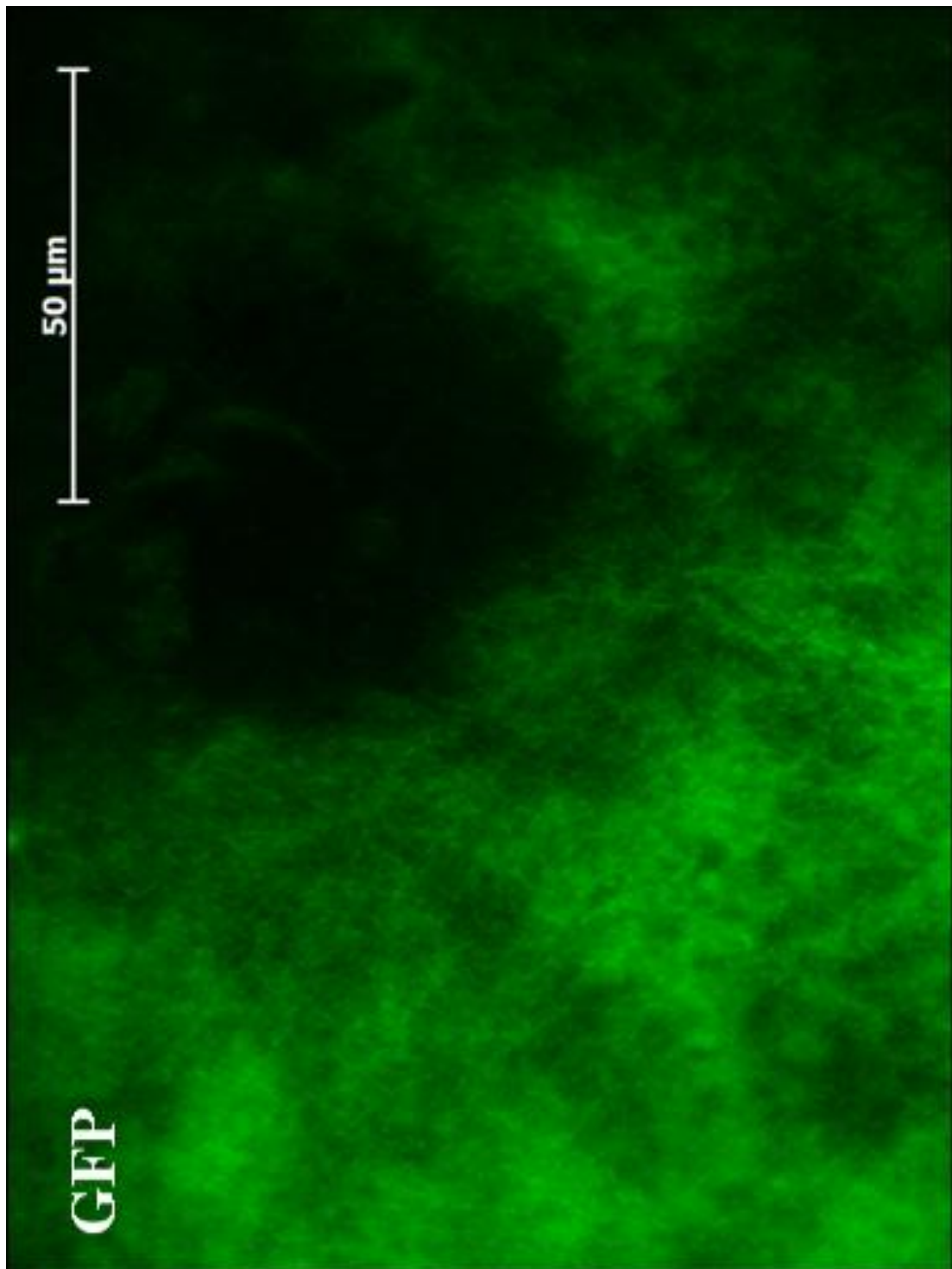


Figure 10-43 Enlarged aerobic 48 hour biofilm of GFP expressing *C. jejuni* NCTC 11168 image (original in Figure 6-3)

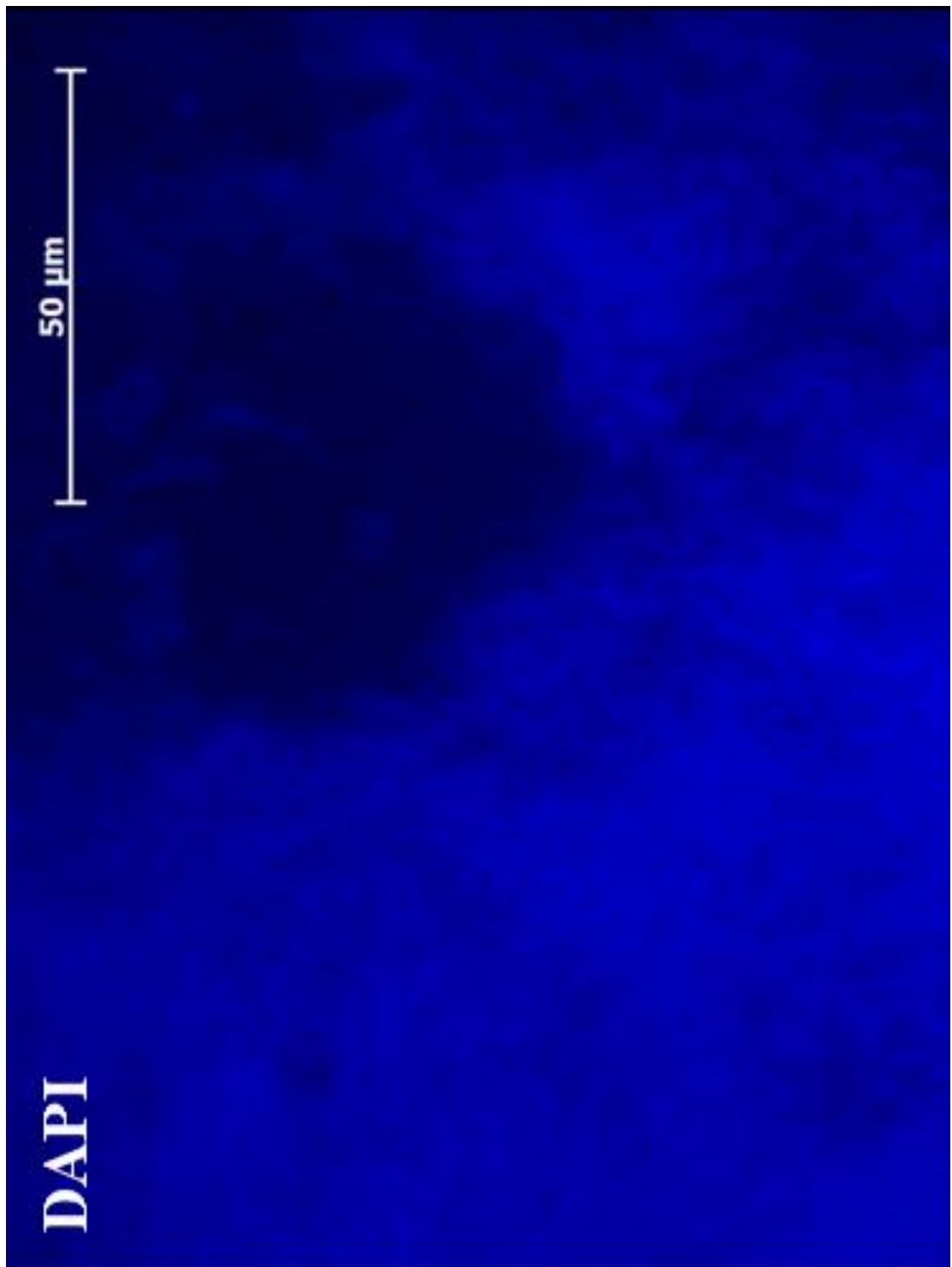


Figure 10-44 Enlarged aerobic 48 hour biofilm of GFP expressing *C. jejuni* NCTC 11168. Image counterstained with DAPI (original in Figure 6-3)

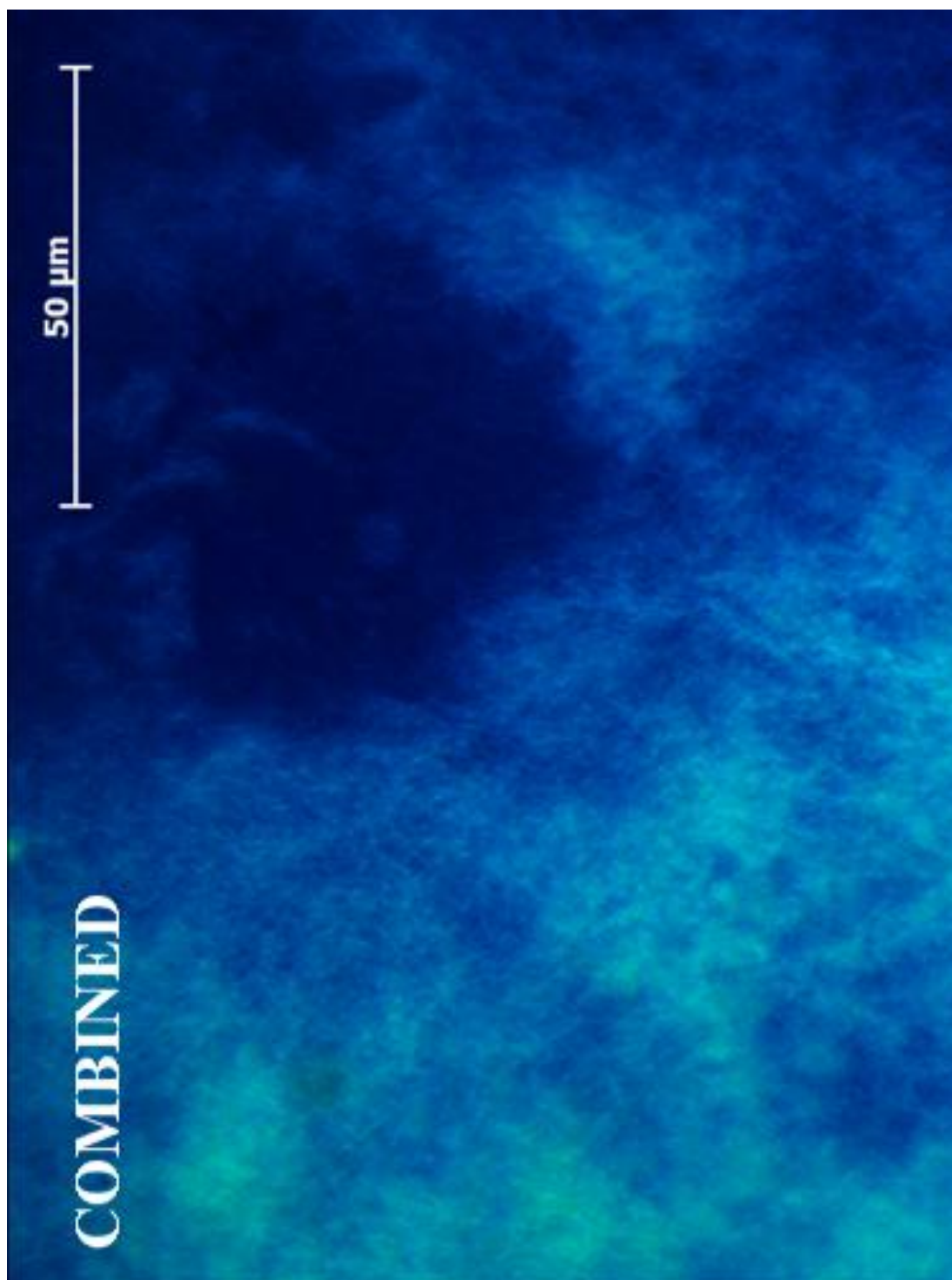


Figure 10-45 Enlarged aerobic 48 hour biofilm of GFP expressing *C. jejuni* NCTC 11168 counterstained with DAPI to visualise eDNA (original in Figure 6-3)

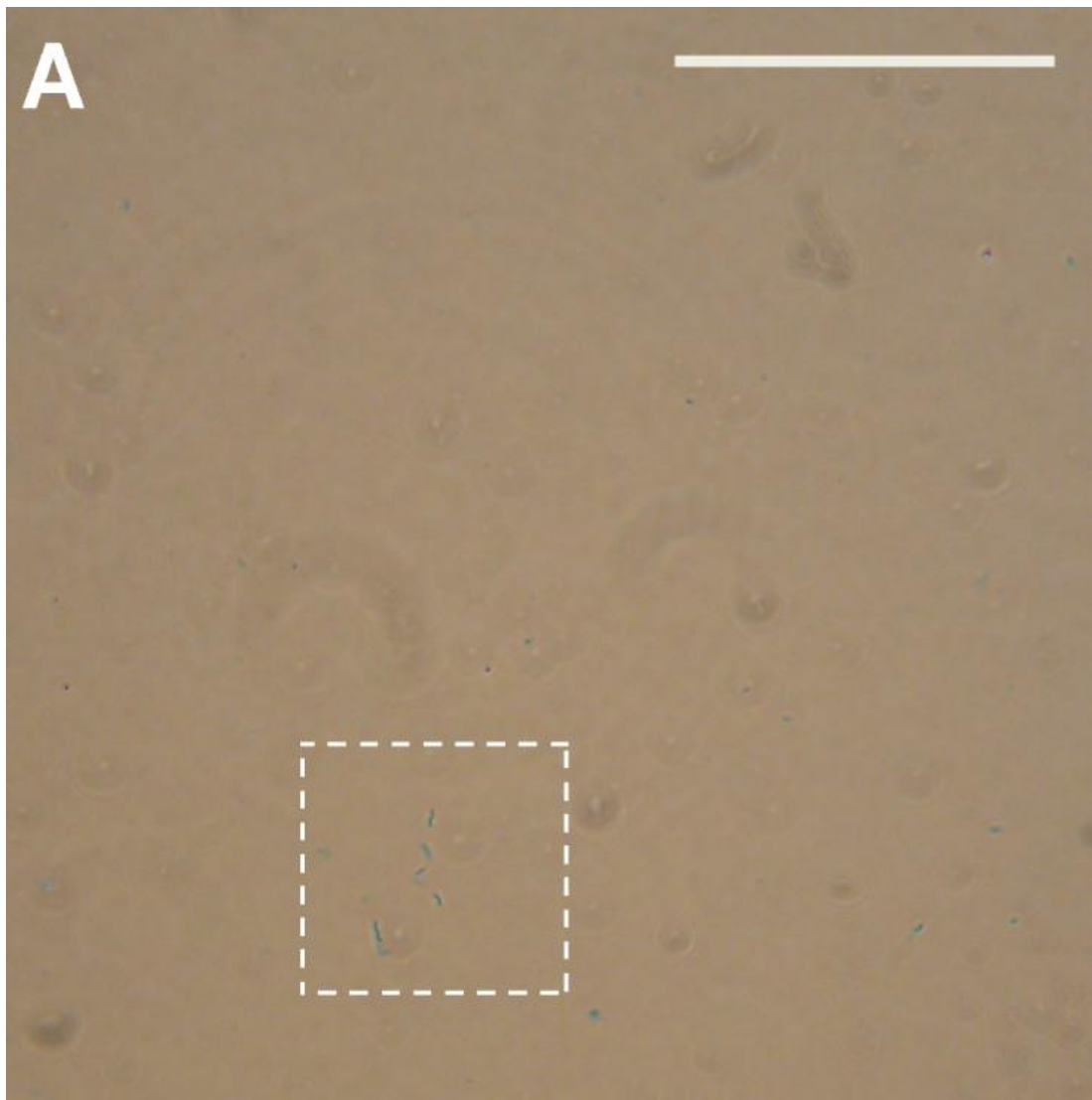


Figure 10-46 Enlarged image of surface attached *C. jejuni* RM1221 cells (Originally in Figure 7-3)

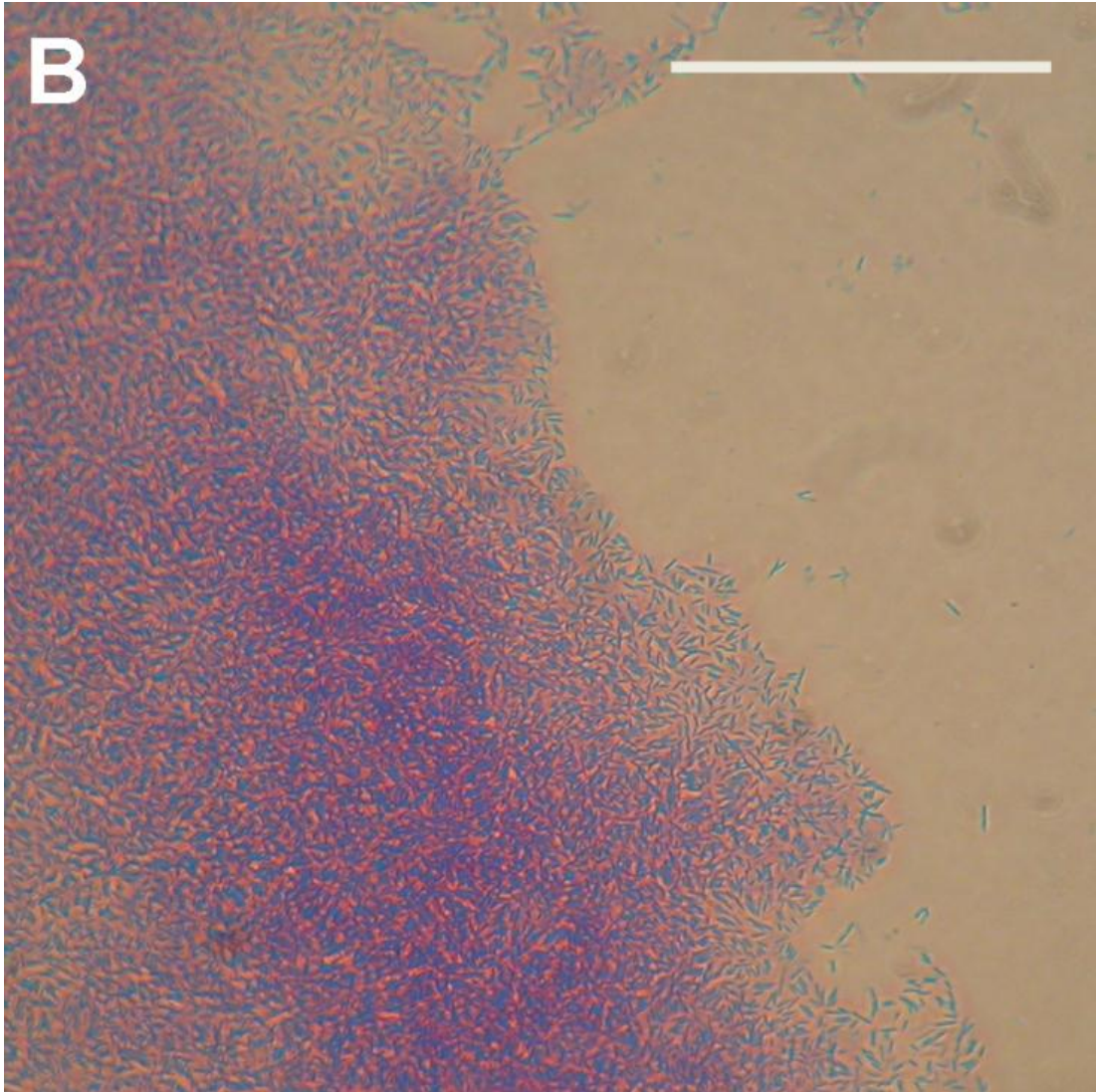


Figure 10-47 Enlarged image of surface attached *C. jejuni* NCTC 11168 cells (Originally in Figure 7-3)