Signalling Molecule Production by *Escherichia coli* and *Campylobacter jejuni*

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Abstract

Quorum sensing (QS) is a density-dependent gene regulation signalling mechanism utilized by bacteria to enable the simultaneous expression of bacterial phenotypes in a given population. Several QS mechanisms and different classes of signalling molecules, including acyl-homoserine lactones (AHLs), have now been identified in numerous bacterial species. AHL production by clinical and laboratory isolates of *Escherichia coli* and *Campylobacter jejuni* has been investigated in this study. Laboratory, blood and urine *E. coli* isolates were analysed via three reporter strain bioassays for putative AHL production. The initial results indicated that the *E. coli* blood isolates produced a compound(s) capable of activating one of the bioassays. Subsequent analysis by thin layer chromatography and mass spectroscopy suggested that this active compound may have been a cyclic dipeptide, although the apparent inability to isolate subsequent samples of this active compound has prevented confirmation of this finding. All of the *C. jejuni* isolates tested induced activity in the *Agrobacterium* liquid culture bioassay, in a growth-dependant manner, indicating the possible presence of an exogenous AHL. Comparative analysis of the genome sequenced *C. jejuni* strain, NCTC 11168-GS and its clinical counterpart, *C. jejuni* NCTC 11168-O, has indicated that these variants respond differently to changes in the levels of dissolved oxygen and toxic oxygen derivatives and as a consequence produce different levels of the active compound. HPLC separation and HPLC-mass spectroscopy has indicated that this active compound may be *N*-hexadecanoyl-homoserine lactone, providing preliminary evidence of a previously unidentified AHL-mediated QS mechanism within the Epsilon *Proteobacteria* class.

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List of Abbreviations

Acyl carrier protein	ACP
Acyl homoserine lactone	AHL
Agrobacterium tumefaciens maintenance medium	ATMM
Amended-MOPS	A-MOPS
Amplified fragment length polymorphism	AFLP
Antibiotic minimal medium	ABMM
Attaching and effacing lesions	AE lesions
Autoinducer(s)	AI(s)
Autoinducer-1	AI-1
Autoinducer-2	AI-2
Autoinducer-3	AI-3
Autoinducing peptide(s)	AIP(s)
Base pairs	bp
Blackburn Royal Infirmary	BRI
Bolton broth	BB
5'-bromo-4-chloro-3-indoyl-β-D-galactopyranoside	X-GAL
Cerebral spinal fluid	CSF
Cholera toxin-like enterotoxin	CTLT
Coenzyme A	CoA
Cystic fibrosis	CF
Cytolethal distending toxin	CDT
Diffusely adherent *Escherichia coli*	DAEC
Diketopiperazine(s)	DKP(s)
Distilled deionised water	ddH_2O

Electron impact mass spectrometry	EI-MS
Electrospray ionisation	ESI
Enteroaggregative *Escherichia coli*	EAEC
Enterohaemorrhagic *Escherichia coli*	EHEC
Enteroinvasive *Escherichia coli*	EIEC
Enteropathogenic *Escherichia coli*	EPEC
Enterotoxigenic *Escherichia coli*	ETEC
Extraintestinal pathogenic *Escherichia coli*	ExPEC
Flagellin gene restriction fragment length polymorphism	*fla* Typing
Gas chromatography	GC
Gastrointestinal	GI
Guillain-Barré syndrome	GBS
Haemolytic uremic syndrome	HUS
Heat-labile	HL
Heat-labile toxin(s)	LT(s)
Heat-stable	HS
Heat-stable toxin(s)	ST(s)
High performance liquid chromatography	HPLC
High performance liquid chromatography time of flight-mass spectroscopy	LC/TOF-MS
Homoserine lactone	HSL
High performance liquid chromatography-mass spectroscopy	LC-MS
Infrared spectroscopy	IR
Lancashire Polytechnic	LP
Locus of enterocyte effacement	LEE

Megabases	Mb
Multilocus sequence typing	MLST
N-butanoyl-L-homoserine lactone	BHL
N-hexadecanoyl-L-homoserine lactone	HDHL
N-hexanoyl-L-homoserine lactone	HHL
N-(3-oxo-dodecanoyl)-L-homoserine lactone	OdDHL
N-(3-oxo-octanoyl)-L-homoserine lactone	OOHL
Neonatal meningitis associated *Escherichia coli*	NEMEC
Nuclear magnetic resonance spectroscopy	NMR
o-nitrophenyl-β-D-galactopyranoside	ONPG
Paraoxonase(s)	PON(s)
Phosphate buffered saline	PBS
Public Health Laboratory Service	PHLS
Pulse field gel electrophoresis	PFGE
Quorum sensing	QS
Retention factor	R*f*
Rhomboid protein	RHO
S-adenosylmethionine	SAM
Sepsis associated *Escherichia coli*	SEPEC
Selective ion monitoring	SIM
Shiga toxins	Stx
Sodium dodecyl sulphate	SDS
Thin layer chromatography	TLC
Tumour-inducing	Ti
University of Central Lancashire	UL

Urinary tract infection(s)	UTI(s)
Uropathogenic *Escherichia coli*	UPEC
Viable but non-culturable	VBNC

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Dedicated to those who have gone before and paved the way for us to tread

For Luke and Isabel

With Love

"Science is a human endeavour, not a magical method

for finding the truth"

Alom Shaha

Pincock, S. (2005) The most important thing in Science is...*The Scientist* **19**: 12

CHAPTER 1

General

Introduction

1.1 Quorum sensing

Quorum sensing (QS) is a cell density-dependent signalling mechanism that enables bacterial cells to regulate the expression of specific genes in response to changes in the local cell population density. This mechanism allows the bacteria to perceive and respond to the presence of neighbouring microbial populations, therefore enabling the bacteria within a confined environment to act in a multi-cellular fashion, expressing the same genes simultaneously (Miller and Bassler, 2001).

This phenomenon was first observed by scientists studying the control mechanism for the production of bioluminescence (*lux* gene expression), in the marine bacterium, *Vibrio fischeri* (Nealson *et al.*, 1970). This primary example has become the general model for explaining the QS mechanism, which relies upon the synthesis, accumulation and subsequent sensing of small, low molecular weight, diffusible signalling molecules (sometimes called pheromones but more frequently autoinducers [AI]). These self-generated AIs play a crucial role in QS in that they are produced at a basal level and diffuse freely between the cellular and external environment, accumulating in the environment local to the cell (Winzer *et al.*, 2003). The AI concentration increases, as a function of cell number, until a minimum threshold level indicative of a critical population size - the quorum, is attained (Fuqua *et al.*, 1994). Then the AI binds to the inactive transcriptional activator initiating a signal transduction cascade, which leads to the positive regulation of an AI synthase resulting in the synthesis of additional AI (hence the term autoinducer) and expression of the regulated phenotype.

1.1.1 Signalling molecules

The term 'cell-to-cell signal molecule' which is synonymous with AI, was re-defined by Winzer *et al.* (2002*a*) via four key criteria:

- The production of the signalling molecule occurs during specific growth stages, under certain conditions or in response to environmental change.
- The signalling molecule accumulates extracellularly and is recognised by a specific receptor.
- Accumulation of the signalling molecule generates a concerted response, once a critical threshold concentration has been reached.
- The cellular response to signalling molecule accumulation extends beyond physiological changes required to metabolise or detoxify the signalling molecule (Winzer *et al.*, 2002*a*).

This final criterion defines cell-to-cell signalling as a phenomenon that is greater than the presence and degradation of toxic or nutritional molecules which may indirectly influence the expression of genes of other metabolic pathways and for this reason the first three criteria on their own are insufficient to define an AI (Winzer *et al.*, 2002*a*).

Several distinct classes of QS AIs, including but not limited to acyl homoserine lactones (AHL), furanones (AI-2), cyclic peptides, 4-quinolones, and oligopeptides, have now been identified (Visick and Fuqua, 2005; Williams, 2006). The isolation and characterisation of these molecules which are utilised by numerous bacterial species has furthered the understanding of QS and has led many researchers to the belief that this phenomenon is ubiquitous rather than limited to a few specific bacterial species.

1.1.2 Autoinducer-1 mediated QS

The first observed signalling molecule, (autoinducer-1), was *N*-(3-oxo-hexanoyl)-L-homoserine lactone, which is utilised by the symbiotic marine bacterium *V. fischeri* in bioluminescence control. Therefore, initial QS research has focused on the identification of bacteria that can produce and respond to AHLs (Figure 1.1.1) and the genes that these AHL-mediated QS mechanisms regulate (Watson *et al.*, 2002). Importantly AHLs from different bacterial species all share a common homoserine lactone ring but differ in the degree of saturation of the acyl chain (presence or absence of a double bond), the nature of the groups attached to carbon 3 of the acyl side chain (no substituent, keto or hydroxyl) and the length of their acyl side chain moieties (generally from four to 16 carbon atoms long, usually increasing in increments of two carbon units, C4, C6, C8 etc.) (Fuqua and Greenberg, 2002; Watson *et al.*, 2002; Anand and Griffiths, 2003).

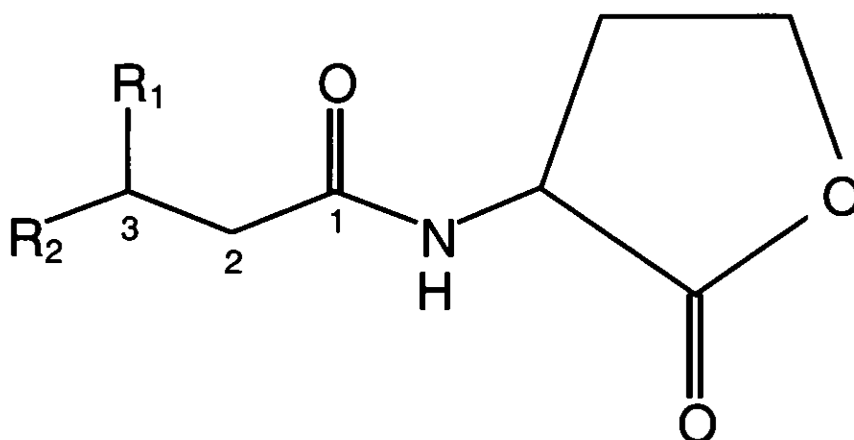


Figure 1.1.1: Generalised AHL structure

The R_1 group can be –OH, =O, or –H. The R_2 group is an acyl chain varying in the number of carbons, and the chain may be saturated or unsaturated.

1.1.2.1 AHL biosynthesis

AHLs are derived from two synthetic pathways. The homoserine lactone moiety is derived from S-adenosylmethionine (SAM), an essential metabolite that is used as a methyl donor for DNA, RNA, and proteins. The acyl chain, to which the homoserine moiety is linked, is usually donated by an acylated acyl carrier protein and synthesised via the common fatty acid biosynthesis pathway but may also be provided by the fatty acid degradation pathway in the form of acyl-coenzymeA (Figure 1.1.2) (Moré *et al.*, 1996; Schaefer *et al.*, 1996*a*; Schaefer *et al.*, 1996*b*; Fuqua and Eberhard, 1999). All AHL-mediated QS systems appear to comprise of two major components: an AHL synthase enzyme that catalyses the formation of AHLs and the cytoplasmic receptor protein that binds the AHL signal molecule and functions as a DNA binding transcriptional activator.

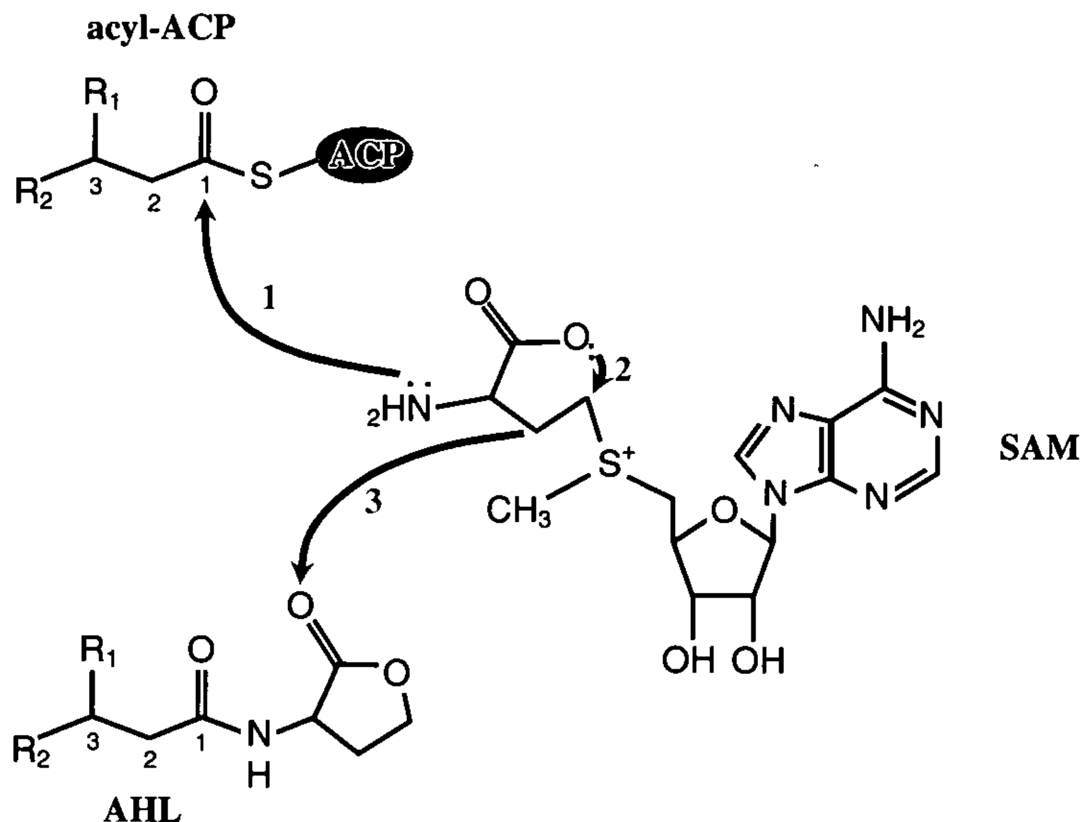


Figure 1.1.2: Generalised AHL biosynthesis from *S*-adenosylmethionine and acyl-acyl carrier proteins by a LuxI-like enzyme

The R_1 group can be –OH, =O, or –H. The R_2 group is an acyl chain varying in the number of carbons, and the chain may be saturated or unsaturated. ACP, acyl carrier protein; AHL, acyl homoserine lactone. **(1)** The LuxI like enzyme promotes the formation of an amide bond between the *S*-adenosylmethionine (SAM) and the acyl side chain from acyl-acyl carrier protein (acyl-ACP) provided by the fatty acid synthesis pathway. **(2)** This intermediate subsequently undergoes lactonization, releasing methylthioadenosine as a side product and **(3)** an acyl homoserine lactone. Figure adapted from Miller and Bassler, (2001).

There are several important proteins and enzymes involved in AHL biosynthesis and at least three different classes of AHL synthase, the most common of which is the LuxI-type transcriptional factor, although others include the LuxM-type and HdtS-type synthases (Visick and Fuqua, 2005). These LuxI-type enzymes catalyse the production of AHL by coupling the acyl-side chain of a specific ACP from fatty acid biosynthesis to the homocysteine moiety of *S*-adenosylmethionine. The ligated intermediate lactonizes to form AHL and methylthioadenosine is released (Schauder *et al.*, 2001; Federle and Bassler, 2003). LuxI-type proteins exhibit a preference for acyl chains of a certain length and oxidation state, only recognising the acyl carrier protein containing a specific acyl chain moiety ensuring that each LuxI-type protein generates only the correct AHL signal molecule (Taga and Bassler, 2003; Visick and Fuqua, 2005).

1.1.2.2 AHL receptor proteins

The AHL receptor proteins (mainly LuxR homologues) are required for the activation of QS-dependent production of AHLs. These cytoplasmic receptor proteins are dimeric, with each monomer consisting of two functional domains: the amino-terminal domain, which binds AHL in an enclosed cavity and a carboxy-terminal domain, which binds DNA via a helix-turn-helix motif (Vannini *et al.*, 2002). When bound to the receptor, specific interactions are made between the conserved lactone ring of the AHL ligand and the binding pocket, with the pocket's shape mediating specificity, enabling each LuxR-type receptor protein to bind and be activated only by its cognate signal (Vannini *et al.*, 2002; Zhang *et al.*, 2002*a*; Daniels *et al.*, 2004).

1.1.2.3 AHL screening

The most widely used method of screening for AHL production is via bioassays that are dependent upon the induction of a phenotypic response, such as β-galactosidase activity or violacin production, within a bacterial reporter strain. Such strains usually contain an AHL-activated promoter fused to a reporter gene(s) such as *lacZ*, together with a receptor protein (LuxR homologue) but lack the AHL synthases so that the reporter strain is incapable of AHL production. Therefore activation of the reporter fusion requires the presence of exogenous AHL.

However, each receptor protein is relatively ligand specific, limiting most bioassay sensors to a narrow range of AHL detection and several bacterial strains have now been shown to produce more than one AHL (Ravn *et al.*, 2001). Consequently, the simultaneous use of several different bioassay strains having different AHL specificities, although labour intensive, is usually advisable when screening for AHL production.

Diketopiperazines (DKPs) are small diffusible cyclic peptides produced by *Pseudomonas aeruginosa*, and other Gram-negative bacteria (Holden *et al.*, 1999; Degrassi *et al.*, 2002). They can also be generated via the non-enzymatic cyclisation of linear dipeptides at extremes of temperature and pH (Skwierczynski and Connors, 1993). Although structurally distinct from AHLs, at high concentrations DKPs can cross-activate several different LuxR homologue-based bioassays, previously considered to be specifically for AHLs (Holden *et al.*, 1999). Therefore activation of a LuxR homologue-based bioassay alone cannot be relied upon for identification of AHL production, so further analytical and structural analysis is required. The role of these cyclic dipeptides and whether they act as signalling molecules or have other

biological functions, have yet to be established, (Holden *et al.*, 1999; Degrassi *et al.*, 2002).

AHL-mediated QS mechanisms have now been identified in over 50 different Gram-negative bacterial species leading to speculation that this phenomenon is widespread. However, known examples of AHL-mediated QS mechanisms have to date only been identified in three out of the five classes (α, β and γ classes) of *Proteobacteria* (Manefield and Turner, 2002; Manefield *et al.*, 2004). Several different QS signalling mechanisms, utilising a wide range of AI signalling molecules have been identified in numerous bacterial species and new AHL-mediated QS mechanisms are being discovered with some regularity (Manefield *et al.*, 2004). However, to date, no AHL-mediated QS system has been identified in any Gram-positive bacterial species.

1.1.3 Autoinducer-2 (AI-2) mediated QS

LuxS, the synthase required for AI-2 production is highly conserved in both Gram-positive and Gram-negative bacteria, including *E. coli*, *Helicobacter pylori* and *Bacillus* spp. (Surrette *et al.*, 1999; Jones and Blaser, 2003), prompting speculation that AI-2-mediated QS is a universal signalling mechanism for communication amongst and between species. However, there is as yet little evidence to support this theory which remains purely speculative.

Chen *et al.* (2002), identified the structure of *Vibrio harveyi* AI-2 when associated with its receptor protein, LuxP. They concluded that this bound ligand was a furanosyl borate diester, and that it bore no resemblance to any previously characterised autoinducers. Further studies of different bacterial species are yet to reveal whether AI-2 represents a family of related molecules or is, in contrast to AHL, a single chemical entity that is identical for differing species. These studies may also

establish whether boron, which is widely available in the biosphere and has long been known to be an essential element in many organisms, is required in every case of AI-2 dependent signalling (Coulthurst *et al.*, 2002; Federle and Bassler, 2003).

1.1.3.1 AI-2 biosynthesis

Like AHL, the signalling molecule AI-2 is derived from SAM. In *V. harveyi* this occurs in three enzymatic steps during which the enzyme, LuxS, catalyses the conversion of *S*-ribosylhomocysteine to homocysteine and 4,5-dihydroxy-2,3-pentanedione, which is believed to spontaneously cyclize into a furanone ring. Borate is then added to this furanone product, forming the AI-2 signalling molecule, furanosyl borate diester (Elvers and Park, 2002; Schauder and Bassler, 2001; Schauder *et al.*, 2001).

1.1.3.2 AI-2 receptor proteins

In *V. harveyi* AI-2 detection requires two proteins: LuxP and LuxQ. LuxP, a soluble periplasmic protein is the primary receptor that binds AI-2. The LuxP-AI-2 complex then interacts with the second detection protein LuxQ, a membrane-bound sensor histidine kinase to transduce the autoinducer signal (Bassler *et al.*, 1994; Chen *et al.*, 2002). This interaction initiates a switch in the sensors from "kinase mode" to "phosphatase mode" leading to the derepression and transcription of the *luxCDABE* operon which encodes the enzymes necessary for light production (Martin *et al.*, 1989; Showalter *et al.*, 1990; Swartzman *et al.*, 1992).

1.1.3.3 The role of LuxS and AI-2 activity

Although the role of LuxS in quorum sensing in *V. fischeri* has been clearly established, the primary role of LuxS in different bacteria remains ambiguous. LuxS plays an important role in bacterial metabolism, contributing to the recycling of SAM which would otherwise have toxic effects on the cell. Therefore many researchers question whether LuxS/AI-2 really constitutes a signalling system in all the organisms in which it is found. In support of this Winzer *et al.* (2002*b*) found that *P. aeruginosa* was capable of stopping AI-2 activity and suggested that AI-2 in many bacteria acted as a nutrient, a metabolite which was released early and then metabolised in the later stages of growth. The assumption that the presence of a LuxS homologue and AI-2 activity automatically equates to a LuxS-dependent QS mechanism is counterfactual, especially considering that although many bacterial genera harbour the *luxS* gene and appear to produce AI-2, Lux P homologues have so far only been found in *Vibrio* spp. (Schauder *et al.*, 2001; Walters and Sperandio, 2006).

Many diverse roles have previously been attributed to AI-2 activity in bacteria by comparing *luxS* mutants with wild-type strains, and complementing these mutants with spent supernatants. However, mutation of the *luxS* gene will also interrupt the activated methyl pathway, in which *luxS* functions as an enzyme. This disruption will result in differential gene expression, the accumulation of *S*-ribosyl-homocysteine within the cell and in changes in amino acid synthetic and catabolic pathways (Walters and Sperandio, 2006). These changes in bacterial metabolism may, in many bacteria, actually be responsible for the diverse roles previously attributed to AI-2 activity through *luxS* mutant comparison. Therefore the only genes so far shown to be regulated by AI-2 in species other than *Vibrio* spp., encode for an ABC transporter,

which is responsible for AI-2 uptake, in *Salmonella typhimurium* (Taga *et al.*, 2001; Hardie *et al.*, 2003).

1.1.4 Putative autoinducer-3 mediated QS

Although originally attributed to AI-2 activity, induction of the locus of enterocyte effacement (LEE) - encoded type III secretion system and flagellum expression in enterohaemorrhagic *Escherichia coli* (EHEC) have subsequently been linked to a third, previously undescribed autoinducer (AI-3), whose synthesis may also be dependent upon LuxS (Sperandio *et al.*, 2003). Further structural analysis of AI-3 has indicated that this AI is an aromatic compound, and unlike AI-2 does not contain a sugar skeleton (Walters and Sperandio, 2006). Additionally Sperandio *et al.* (2003) postulated that EHEC, which has a very low infective dose, uses this novel AI to determine when it is within the intestine and it regulates its virulence genes accordingly, activating those essential for intestinal colonisation and initiating the disease process.

1.1.5 QS network architecture

Multiple QS-mechanisms, responding to a wide range of AIs have now been identified in many bacteria. In *V. harveyi* three separate QS systems consisting of three AIs (an AHL, a furanosyl borate diester and a third, as yet, unidentified molecule) and three cognate receptors functioning in parallel, have been reported (Cao and Meighen, 1989). *P. aeruginosa* produces several AIs, the QS regulatory systems for which are arranged in series, resulting in a temporally ordered sequence of gene expression that many believe is critical for the ordering of infection related events (Whiteley *et al.*, 1999; Schuster *et al.*, 2003). Competitive QS circuits enable

bacteria to commit to one of two mutually exclusive events, as in *B. subtilis* which has two autoinducing peptides that allow it to adopt a specific lifestyle, either taking up exogenous DNA or sporulating (Waters and Bassler, 2005).

1.1.6 Gene regulation/phenotypes

QS is thought to regulate a wide variety of phenotypes including bioluminescence, virulence factors, antibiotic production, biofilm formation and conjugation (Greenberg, 1999).

1.1.6.1 Bioluminescence

In *V. harveyi* and *V. fischeri* luminescence via the synthesis of luciferase, the light-emitting enzyme, are strongly regulated by QS (Cao and Meighen, 1989). *V. fischeri* colonises the light organs of certain marine fish and cephalopods, such as *Euprymna scolopes*, a small, nocturnal surface feeding squid, which is endemic to the Hawaiian Islands (DeLoney *et al.*, 2002). When freely suspended in seawater the bacterial cell density is low, but once bacteria are concentrated within the light organ, a quorum is reached and luminescence is expressed (Whitehead *et al.*, 2001; DeLoney *et al.*, 2002). The cephalopods counter-illuminate themselves by emitting light downwards, matching the intensity of the moon- and starlight above to prevent a shadow from being cast which would alert predators to their presence (Fuqua and Greenberg, 2002). Current theory suggests that this is a symbiotic relationship in which the bacterium gains nutrients from the cephalopod, which is in return camouflaged by the bioluminescence.

1.1.6.2 Virulence factors

The common soil dwelling organism *P. aeruginosa* is also an opportunistic human pathogen that infects immunocompromised individuals and is most notorious for its devastating effects on cystic fibrosis (CF) patients (Eberl and Tümmler, 2004). A major contributor to the pathogenesis of *P. aeruginosa* is its ability to secrete numerous virulence compounds and degradative enzymes, including exoprotease, siderophore, exotoxin and lipase production (for reviews see Pesci and Iglewski, 1997; Fuqua and Greenberg, 1998; van Delden and Iglewski, 1998). The production of many of these virulence factors are controlled by QS, as are adhesion and biofilm formation, all of which are essential for chronic *P. aeruginosa* respiratory infection, enabling persistence in the lung and disease progression (Davies *et al.*, 1998; Smith and Iglewski, 2003). Studies using *P. aeruginosa* mutants with defects in the QS mechanisms indicate that the mutation does not markedly influence their growth under normal laboratory conditions. However, it does affect colonisation cell density levels, and results in severe virulence defects in various mouse, invertebrate and plant model systems, rendering the mutant incapable of causing disease (Tang *et al.*, 1996; Parsek and Greenberg, 2000).

1.1.6.3 Enzyme and antibiotic production

Erwinia carotovora is a Gram-negative, pathogenic bacterium that causes soft-rot in a variety of plants including the potato, through the production and activity of various plant cell wall-degrading exoenzymes including pectate lyases, polygalacturonase, cellulase and several proteases (Barras *et al.*, 1994). Production of these macerating enzymes is regulated via QS, enabling this phytopathogen to amass sufficient

numbers of bacteria for successful tissue destruction and evasion of the plant phytodefence mechanism (Kievit and Iglewski, 2000).

Additionally the biosynthesis of the broad-spectrum antibiotic, carbapenem, by this bacterium is also controlled by QS, albeit by a second mechanism (Bainton *et al.*, 1992; Williams *et al.*, 1992). Antibiotic and exoenzyme production occurs simultaneously, with both QS systems responding to the same signalling molecule, coupling the timing of the individual phenotypic responses (Andersson *et al.*, 2000). It is hypothesised that as the exoenzymes macerate the plant cell wall, the antibiotic functions to inhibit other bacterial species and microflora, which will compete for the nutrients released by cell wall degradation and attempt to infect the plant via the wound produced by the *E. carotovora* exoenzymes (Miller and Bassler, 2001).

1.1.6.4 Biofilm formation

Microbial colonisation of the major airways of CF patients by *P. aeruginosa* leads to a destructive lung disease which results in a need for aggressive treatment with antibacterial drugs and is a major cause of morbidity and mortality (Govan and Deretic, 1996; Welsh *et al.*, 2001). *P. aeruginosa* is intrinsically resistant to numerous antimicrobial agents, including antibiotics, organic solvents and detergents. Bacteria living in biofilms can be up to 1000 times more resistant to antibacterial compounds than planktonic bacteria (Nickel *et al.*, 1985). Therefore the formation of *P. aeruginosa* biofilms within the lungs of people with CF is of extreme clinical consequence resulting in a requirement for frequent physiotherapy to encourage the physical removal of mucus and the bacterial biofilm (Kievit and Iglewski, 2000).

Davies *et al.* (1998) demonstrated that in *P. aeruginosa* QS is required for the differentiation of individual cells into complex mature biofilm structures, with mutants forming thin, unstructured biofilms. More specifically *P. aeruginosa lasI* mutants terminate biofilm formation at the micro-colony stage resulting in biofilms that are more sensitive to environmental stresses and the detergent biocide sodium dodecyl sulphate (SDS, 0.2 %) (Davies *et al.*, 1998).

1.1.6.5 Conjugation

Agrobacterium tumefaciens causes crown gall tumours in a wide range of dicotyledonous plants. The genes that enable this organism to exhibit virulence are encoded on a large Tumour-inducing (Ti) plasmid which is transferred from the bacterium to the host cell nucleus. Tumour formation is dependent upon the conjugal transfer of this oncogenic plasmid between the bacterium and the host cell, which in *A. tumefaciens* is controlled by QS (Zhang and Kerr, 1991; Piper *et al.*, 1993). The core components of this system are TraI, which synthesises the AI, *N*-(3-oxo-octanoyl)-L-homoserine lactone (OOHL); TraR, an OOHL-dependent transcriptional activator of the plasmid's conjugal transfer genes and OOHL, the inducing ligand (Piper *et al.*, 1993; Zhang *et al.*, 1993; Hwang *et al.*, 1994; Piper *et al.*, 1999).

1.1.6.6 Regulation responses

According to Greenberg (1999), the majority of the QS-mediated regulatory responses identified so far appear to be related to secondary metabolism. The author proposed that a general theme was emerging in that bacteria that exhibit QS-mediated gene regulation, experience a plant or animal host association as part of their lifestyle. Although the photosynthetic bacterium *Rhodobacter sphaeroides* may be an

exception to this, Greenberg felt that the possibility of an involvement of this bacterium with a eukaryotic host that had not yet been reported, could not be ignored. However, Greenberg's observation does not imply that this is fact. There is a 'selective pressure' applied to the scientific community, with regards to funding, that focuses research in the direction of organisms that are of medical and commercial importance. There is currently no financial impetus to research QS in bacteria that do not form a pathogenic or symbiotic relationship with either plant or animal cells, resulting in a research bias that would have a direct influence on Greenberg's observation.

1.1.7 Cross-species communication

In natural habitats bacteria usually exist in mixed populations potentially containing a wide variety of bacterial species. The ability to recognise and respond to multiple AI signals from different origins would enable any given species to monitor not only its own cell-population density but also the population density of other bacteria in the immediate environment. This would therefore enable each bacterial species to gauge when it constitutes a majority or minority of the total population.

Although AHL-mediated QS is generally considered to be highly specific with regards to the binding of the receptor protein and its cognate ligand, many bacteria actually produce and respond to the same AHL. The presence of multiple species producing the same AHL within any given environment would therefore result in cross-activation of receptors, although whether this is by design or is a fortuitous coincidence that has been adapted to augment bacterial survival within any specific environment, remains to be elucidated.

As previously stated, *luxS*, which encodes the synthase required for AI-2 production, is highly conserved in both Gram-positive and Gram-negative bacteria (1.1.3). This, combined with verification of AI-2 production by numerous species, has prompted great speculation that AI-2-mediated QS is a universal signalling mechanism for communication amongst and between species. However, LuxS functions in a major bacterial metabolic pathway and separating this function from QS is proving complex. To date the only genes shown to be regulated by AI-2 in species other than *Vibrio* spp., encode for an ABC transporter, which is responsible for AI-2 uptake, in *S. typhimurium* (Hardie *et al.*, 2003; Taga *et al.*, 2001). Therefore there is, as yet, little evidence to support the 'universal language' theory which currently remains purely speculative.

1.1.8 Cross-kingdom communication

Recent research has highlighted the possibility that some bacterial and eukaryotic signalling mechanisms have a common evolutionary origin. The inner membrane protein AarA of *Providencia stuartii*, which is required for the release of a currently structurally unidentified QS signalling molecule, has homology to the *Drosophila melanogaster* RHO (rhomboid protein) (Rather *et al.*, 1999; Gallio *et al.*, 2002). RHO is essential for many development processes in *D. melanogaster* including wing vein development (Schweitzer and Shilo, 1997). Gallio *et al.* (2002) demonstrated that the expression of *D. melanogaster rho* in a *P. stuartii aarA* mutant complemented the QS signal defect and that expression of *aarA* in a *D. melanogaster rho* mutant rescued vein development, indicating a common signalling function. The discovery that homologues of RHO/AarA are nearly ubiquitous in all three kingdoms: bacteria, archaea and eukaryotes, combined with this possible common cell-to-cell

communication system, establishes the potential for cross-kingdom communication, although its actual occurrence has as yet to be demonstrated (Koonin *et al.*, 2003; Waters and Bassler, 2005).

Several recent studies have demonstrated that AHLs with several different structures can enter eukaryotic cells and also retain their functionality (Neddermann *et al.*, 2003; Williams *et al.*, 2004; Shiner *et al.*, 2004). Evidence that bacterial AHLs can affect gene expression in host cells is increasing especially with regard to the multiple AHLs produced by *P. aeruginosa* and the effect that these AHLs appear to have on gene expression in a CF mouse model (Massion *et al.*, 1994; DiMango *et al.*, 1995). The subsequent isolation of these AHLs from the sputum of CF patients has provided further evidence in support of the clinical importance of these findings (Charlton *et al.*, 2000; Singh *et al.*, 2000; Erickson *et al.*, 2002; Middleton *et al.*, 2002 Chambers *et al.*, 2005). However the mechanisms involved in this inter-kingdom signalling and the implications of this research for other bacteria-host relationships are not yet fully understood (Shiner *et al.*, 2004).

1.1.9 Quorum quenching

As previously stated, the majority of bacteria for which QS systems have been identified experience a plant or animal host association as part of their lifestyle (1.1.6.6). Therefore it is highly likely that many of the eukaryotes susceptible to these bacteria have evolved methods designed to interfere with and/or manipulate bacterial QS to impede bacterial invasion/colonisation. AHL mediated QS is now recognised as a major mechanism in the regulation of virulence factors in many pathogenic bacteria. The potential for quenching QS mechanisms and therefore preventing the expression of virulence factors has been postulated for many years. However the transfer of idea

to reality has only recently become tangible with the discovery of several natural quorum quenching mechanisms.

There are three main targets for interfering with any QS mechanism; the AI synthase, the AI receptor and the AI itself. Although all three strategies are viable targets, to date very little research regarding the blockage of AI production has been undertaken (Rasmussen and Givskov, 2006*a*).

1.1.9.1 Target 1: the AI synthase

Although an obvious strategy for QS quenching, interference with AI synthases resulting in reduced AHL production, which in turn will prevent the population from 'sensing' when a quorum has been reached, has so far been relatively neglected, (Rasmussen and Givskov, 2006*a*; Rasmussen and Givskov, 2006*b*). Many of the few SAM and acyl-ACP (AHL substrate) analogues that have so far been identified, have proved to be potent inhibitors of the *P. aeruginosa* AHL synthase, RhlI, blocking AHL production *in vitro* (Parsek *et al.*, 1999). Parsek *et al.* (1999) found that the most effective inhibitor tested was L-*S*-adenosylcysteine, which lowered the activity of RhlI by 97 %. However, these tests were only performed *in vitro* and concerns have been expressed as to how such analogues of SAM and acyl-ACP, which are also essential metabolites in central amino acid and fatty acid catabolism, would affect other cellular functions (Rasmussen and Givskov, 2006*a*).

The widely used biocide Triclosan kills susceptible bacteria by interfering with fatty acid biosynthesis, which is essential for bacterial growth. Triclosan is a powerful inhibitor of enoyl-ACP reductase which is not only involved in fatty acid biosynthesis but also in the synthesis of acyl-ACP, which as previously stated, is an essential substrate for AHL biosynthesis (1.1.2.1; Hoang and Schweizer, 1999). Therefore

Triclosan reduces the amount of substrate available for AHL biosynthesis and it has been demonstrated to reduce AHL production *in vitro* (Hoang and Schweizer, 1999). However, as with many antibiotics and biocides, several bacterial pathogens have already developed resistance to this chemical, limiting its usefulness both as a biocide and in quorum quenching (Chuanchuen *et al.*, 2001; Schweizer, 2001).

1.1.9.2 Target 2: the AI receptor

The traditional pharmacological approach of "receptor antagonism" is to block the receptor site with small molecules that bind to but do not activate it. This strategy for QS quenching has been intensively investigated resulting in the identification of many AHL analogues. Most compounds investigated so far have either a substitution, such as a keto-oxygen or an extra carbon atom in the AHL ring or the acyl side chain (Schaefer *et al.*, 1996*a*; Rasmussen and Givskov, 2006*b*). Although many AHL analogues have now been generated, the majority are, as Schaefer *et al.* (1996*a*) experienced, actually competitive agonists rather than true inhibitors in that although they bind to the LuxR protein, and displace the AHL, they also activate the receptor and are therefore of limited application (Rasmussen and Givskov, 2006*b*). It may therefore prove prudent to look to natural examples of receptor inhibition, especially considering that the microorganisms that produce these analogues have had a selective pressure and hundreds of generations to perfect these strategies.

The macroalga *Delisea pulchra* produces and excretes halogenated furanones which are closely related in structure to AHL. The furanones, having been internalised by the bacteria, can therefore interfere with the QS system by binding to LuxR-type proteins, displacing the AHL. Furthermore, this interaction of the furanone with the LuxR-like receptor protein is believed to act to destabilise the furanone-LuxR

complex, encouraging its rapid degradation and therefore decreasing the number of LuxR-like receptor proteins available (Manefield *et al.*, 1999; Manefield *et al.*, 2002). Although the mode of action of this decrease remains to be characterised, the process has been shown to inhibit AHL-mediated gene expression, preventing the bacterial colonisation of the plant leaves, by inhibiting QS-controlled biofilm formation, essentially rendering the bacterium avirulent (Manefield *et al.*, 2002). Crucially Manefield *et al.* (1999) also demonstrated that ecologically realistic concentrations of these furanones show inhibitory activity, demonstrating the possibility of an *in vivo* application.

1.1.9.3 Target 3: the AI signal

AHL-degrading enzymes have been isolated from numerous bacterial sources prompting speculation that their production is a useful strategy for bacteria when competing with AHL producing strains in the environment. The structure of AHL suggests four possible enzymatic cleavage sites (Figure 1.1.3) and therefore the potential for at least four different degradation enzymes. However, to date, only two of these groups of enzymes have been demonstrated to use AHLs as substrates (Dong and Zhang, 2005).

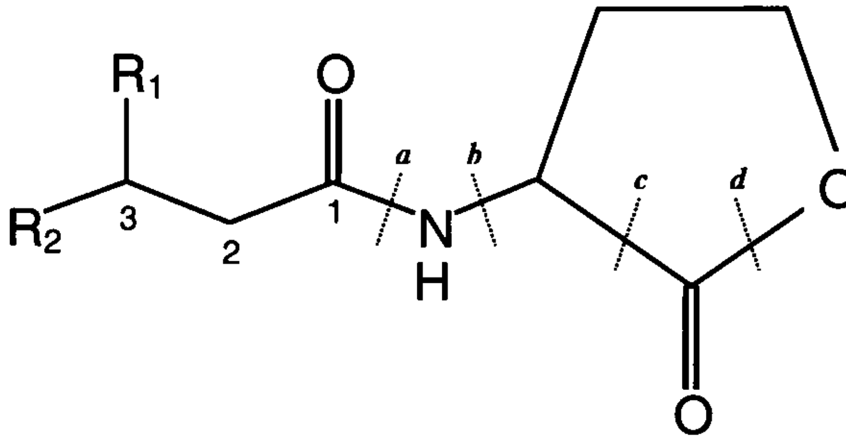


Figure 1.1.3: The general structure of AHL signalling molecules and their enzymatic degradation points

An acylase and deaminase could separate the homoserine moiety and acyl side chain at sites marked ***a*** and ***b***. Alternatively a lactonase and a decarboxylase could break the lactone ring at the positions marked ***c*** and ***d*** respectively.

1.1.9.3.1 AHL acylase

AHL acylase degrades AHL by hydrolysing the amide linkage between the acyl chain and the homoserine lactone moiety of AHL molecules, yielding fatty acids and homoserine lactone (Figure 1.1.3). Leadbetter and Greenberg (2000) demonstrated that a soil isolate of *Variovorax paradoxus* was capable of growth with AHLs as its sole energy and nitrogen sources. In this AHL acylase-mediated degradation, homoserine lactone is released into the medium, whilst the fatty acid is metabolised generating energy (Leadbetter and Greenberg, 2000).

AHL acylase activity has also been demonstrated for a *Ralstonia* spp. isolated from a mixed species biofilm (Lin *et al.*, 2003). Cloning of the signal inactivation gene, *aiiD*, from this isolate, has provided valuable information with regard to the molecular

details underlying this degradation mechanism. Sequence homology searches have indicated that AHL acylase is conserved in many different bacterial species. Further analysis has suggested that this *aiiD* gene produces a novel and potent AHL acylase, acting at point ***a*** on Figure 1.1.3, with the AHL degradation products apparently exhibiting no activity when tested with QS bioassays (Lin *et al.*, 2003).

1.1.9.3.2 AHL lactonase

Initially identified in the *Bacillus* isolate 240B1, the *aiiA* gene encodes an AHL-inactivating enzyme (Dong *et al.*, 2000). Further analysis of the gene product has indicated that it is a lactonase that hydrolyses the homoserine lactone ring of AHL to produce the corresponding acyl homoserines, which show much reduced biological activity. AiiA homologues have subsequently been identified in many subspecies of *Bacillus thuringiensis* and closely related *Bacillus* species (Dong *et al.*, 2002; Reimmann *et al.*, 2002; Lee *et al.*, 2002).

As previously stated, *A. tumefaciens* conjugation is controlled by QS, with the AI, OOHL, positively regulating the genes required for Ti plasmid transfer (1.1.6.5). Interestingly *A. tumefaciens* also produces an AHL lactonase, encoded by the the *aiiA* gene homologue *attM*, which is capable of degrading OOHL (Zhang *et al.*, 2002*b*). The expression of this AHL lactonase is growth-phase dependent, with enhanced expression occurring when the cells enter stationary phase, resulting in a sudden decrease in OOHL concentration and the termination of conjugation related QS (Zhang *et al.*, 2002*b*). Therefore *A. tumefaciens* can control both the initiation of conjugation via QS and its termination via quorum quenching maximising the efficiency of Ti plasmid transfer.

1.1.9.3.3 Paraoxonase

Sera of several mammalian species, including humans and horses, are known to have AHL inactivation activity due to lactonase-like enzyme(s), reminiscent of paraoxonases (PONs), hydrolysing the lactone ring to produce acyl homoserine (Yang *et al.*, 2005). There are three different members of the PON gene family in humans- PON1, PON2 and PON3. Although the physiological role(s) and natural substrates of the PON enzymes are currently uncertain, Dragonov *et al.* (2005) proposed that degradation of AHLs could represent a major physiological role for the PONs, especially for human PON2 and therefore could be important for host defence.

1.1.9.3.4 Chemical lactonolysis

Yates *et al.* (2002) demonstrated that AHLs are unstable in alkaline conditions. At pH levels above 7 the AHL undergoes lactonolysis diminishing its biological activity. Plants which are infected with the tissue-macerating pathogen *E. carotovora* (1.1.6.3) increase the pH at the infected site. This alkalinization leads to inhibition of AHL-mediated QS, reducing the extent of the bacterial attack (Byers *et al.*, 2002).

1.1.9.3.5 Human airway epithelia

P. aeruginosa produces two AHLs which are hierarchically regulated, in that the threshold levels of *N*-(3-oxododecanoyl)-L-homoserine lactone (OdDHL), the key AI, are required to activate the production of the secondary AI, *N*-butanoyl-homoserine lactone (BHL) (1.1.5; Pearson *et al.*, 1994; Pearson *et al.*, 1995; Latifi *et al.*, 1996; Pesci and Iglewski, 1997). Recent findings indicate that differentiated human airway epithelial cells contain a membrane-associated enzyme-like mechanism that inactivates OdDHL (Chun *et al.*, 2004). Despite this AHL-inactivating activity,

significant levels of 3OC12-HSL have been measured in the sputum of *P. aeruginosa*-infected CF patients (Erickson *et al.*, 2002). Chun *et al.* (2004) proposed that inadequacy or failure of AHL inactivation might contribute to the persistence of AHL and biofilm production in chronic lung infections and that augmenting the innate mechanisms or supplying new ones could be of value in treating chronic infections.

Interestingly OdDHL - degrading activity has been demonstrated for several other mammalian cells, with cells derived from human epithelia that have been exposed to pathogens, showing the highest extent of OdDHL inactivation (Chun *et al.*, 2004). Potentially, this AHL degrading activity could result in the inhibition of the regulation of hundreds of genes, production of extracellular virulence factors, biofilm development and most importantly OdDHL autoinduction and the hierarchically regulated production of BHL (Davies *et al.*, 1998; Schuster *et al.*, 2003; Smith and Iglewski, 2003; Wagner *et al.*, 2003). However, the full implications of these recent findings, especially with regard to *P. aeruginosa* biofilm formation in the lungs of people with CF (1.1.6.4) and how this opportunistic pathogen copes with this inactivation, remains to be seen (Zhang and Dong, 2004).

1.1.9.4 Quorum quenching application

Numerous examples of quorum quenching in the natural environment have now been identified, suggesting that signal interference has significant implications amongst microorganisms competing for nutrients and ecological niches. More importantly, such signal interference could be manipulated by scientists to control bacterial growth and gene expression in numerous environmental and clinical settings (Zhang and Dong, 2004). As quorum quenching does not kill or inhibit the growth of bacteria, a

repeat of the intense selective pressure applied by antibiotics for the development of resistant mutants is unlikely to occur, making the manipulation of quorum quenching for medicinal purposes, highly desirable (Hentzer and Givskov, 2003; Williams, 2002).

Although the production of AHL degrading enzymes may be a practical quorum quenching solution for plants and bacteria in the natural environment, Rasmussen and Givskov (2006*b*) have suggested that the medicinal application of these enzymes in a commercial setting will initially, at least, be limited to topical use due to the significant problems encountered in delivering proteinaceous agents systemically. It is also important to remember that both the chemical and enzymatic lactonolysis reactions are reversible and therefore once the lactonolysis catalyst (be it enzymatic or chemical) is depleted, the opened AHL ring will reform, reinstating the biological activity of the AHL (Càmara *et al.*, 2002). In addition to this, halogenated furanones (1.1.9.2) are unstable and several of the other quorum quenching compounds that have been identified, are toxic or naturally exist in low concentrations rendering them unsuitable for human use (Rasmussen and Givskov, 2006*b*).

However, given the numerous examples of quorum quenching in the natural environment, its potential for controlling bacterial growth and infection is unquestionable. Thus additional research, focusing on the identification and development of quorum quenching molecules for *in vivo* testing, is required.

1.1.10 QS in the gastrointestinal tract

The gastrointestinal (GI) tract is the largest and most complex environment in the mammalian host, with a large variety of ecological niches for aerobic, facultative anaerobic and anaerobic organisms. An estimated 500-1000 different bacterial species reside in the intestine, the majority of which have not yet been cultured *in vitro* (Xu *et al.*, 2003; Kaper and Sperandio, 2005). Kaper and Sperandio (2005) suggested that due to the extreme bacterial density, there is no environment in the human body with greater potential for cell-to-cell signalling than the GI tract.

Despite the identification of QS mechanisms for numerous pathogenic and commensal bacteria of the GI tract, there is, as yet, little evidence of QS *in vivo,* although AI-2 and AI-3 activity has been detected in faecal specimens from healthy individuals (Sperandio *et al.*, 2003). Additionally, concentrations of AI-2 activity were found to vary among subjects by as much as 10 fold, raising the possibility that this variation in AI-2 activity levels in different individuals' intestines, may result in different courses of disease (Sperandio *et al.*, 2003). AI-3 activity was also detected in a stimulated intestinal environment inoculated with a faecal specimen from a healthy individual (Sperandio *et al.*, 2003). As *E. coli* is one of the most common bacterial species in the GI tract, the detection of AI-3, which has been linked to induction of the type III secretion system and flagellum expression in EHEC, is of particular relevance (Farthing, 2004).

1.1.11 QS and human food

AI activity has been demonstrated for many foodborne pathogens *in vitro* and several studies have reported the isolation of AHLs from foods including bean sprouts, various fish and meat products (Gram *et al.*, 1999; Cloak *et al.*, 2002; Gram *et al.*, 2002; Buch *et al.*, 2003; Bruhn *et al.*, 2004; Rasch *et al.*, 2005). Rasch *et al.* (2005) demonstrated that the bacterial spoilage of some food products (more specifically soft rot in bean sprouts) is influenced by QS-regulated phenotypes, providing preliminary evidence that AI production may be exploited to control bacterial growth and survival in foods. However, as yet, little is known about the roles of QS in the growth and survival of bacteria in food environments *in vivo*, and its significance to the food industry with regard to food spoilage and food toxicity (Cloak *et al.*, 2002).

Theoretically environmental conditions, including the type of food, incubation temperatures, and the atmospheric conditions may have an effect on both the amount and type(s) of AI produced. However, a recent study of AHL and AI-2 production by food-derived *Enterobacteriaceae*, found that changes in environmental parameters including carbon source, temperature, salt concentration and co-existing lactic acid microflora had little effect on the specific AHL concentration and did not affect the types of AHL produced (Flodgaard *et al.*, 2003).

The contribution of QS and AHL production to food spoilage remains unclear, as does the consequences of the consumption of food containing AHL and contaminated with AHL producing bacteria. As all of the bacteria present in the GI tract enter through the mouth either with food and drink or by direct person-to-person contact, AI producing bacteria and AI activity in food may not only affect the speed and extent of microbial food spoilage, but may also affect the levels of AI and AI producing

bacteria within the GI tract and therefore, potentially affect the course of disease in individuals (Farthing, 2004).

The advancement of knowledge pertaining to QS, from its initial discovery in *V. fischeri* to the elucidation of the AI structures and the genes that they regulate is unquestionable. However, additional research with specific regard to the medical and industrial implications of this fascinating phenomenon is still required.

1.1.12 Aim of study

QS-quenching is now emerging as a potent potential method of infection control that avoids the usual problems associated with conventional antibiotic-based methods such as resistance and eradication of the natural commensal bioflora necessary for healthy GI tract function. Therefore a full understanding of the QS networks, AIs produced and the genes they regulate with particular regard to virulence factors, is vital. This study is intended to further the current understanding of AHL-mediated QS in the foodborne pathogens *E. coli* and *Campylobacter jejuni*.

CHAPTER 2

Escherichia coli

2.1 INTRODUCTION

2.1.1 Morphology and metabolism

E. coli is a Gram-negative, non-spore forming, facultative anaerobe that ferments a wide range of sugars, including lactose, producing acid and gas. *E. coli* is oxidase, Voges-Proskauer and citrate negative, but produces indole and is methyl red positive (Feng, 2001). Its morphology is typically a straight, round-ended rod, approximately 0.5-1.0 x 1-6 μm in size. They usually occur singly or in pairs and most strains are motile and commonly have fimbriae and/or pili. Its cells may have a polysaccharide capsule or microcapsule and a typical Gram-negative-type cell wall. It forms colonies on nutrient agar (37°C, 24 h) which are usually 1-3 mm in diameter and may be smooth, low-convex and greyish-translucent. Mucoid and slime-producing strains may also occur (Singleton and Sainsbury, 1999).

2.1.2 History

During a series of studies of the intestinal flora of infants in the late 19th century, Theodor Escherich first described *Bacterium coli*, a normal microbial inhabitant of healthy individuals. This bacterium was later renamed as *E. coli* in his honour (Kaper, 2005). *E. coli* is the most abundant facultative anaerobe present in the intestine of humans and many other warm-blooded species (Donnenberg, 2002). It has become a model organism, playing a critical role in the development of molecular biology, enabling the study of gene regulation and manipulation (Kaper, 2005).

2.1.3 Reservoirs and potential infection sources

E. coli is one of the most common bacterial species in the GI tract flora of warm-blooded animals (including birds and mammals) and is necessary for the normal development and function of the GI tract (Farthing, 2004). Under normal conditions,

commensal *E. coli* variants confer benefits to their human and animal hosts by suppressing the growth of harmful bacteria within the GI tract and by synthesising appreciable amounts of vitamins (Feng, 2001). Initially considered as a human/animal GI tract commensal with low virulence potential, *E. coli* is now known to be capable of causing serious disease and even death.

Unsurprisingly, as a natural inhabitant of the GI tract of many animals, *E. coli* is widely distributed throughout the environment and therefore both food and water supplies are susceptible to contamination. As previously stated all bacteria present in the GI tract enter through the mouth either with food and drink or by direct person-to-person contact (1.1.11). *E. coli* is frequently present in animal products such as meat and milk, and on crops treated with animal manure fertilisers and/or irrigated with water contaminated with faeces. This wide-scale distribution provides ample opportunity for the transmission of *E. coli* to the GI tract, especially when food is improperly prepared and/or prepared by unsanitary food handlers.

2.1.4 Infection

Russo and Johnson (2003) broadly categorised the *E. coli* strains of biological significance, from a genetic and clinical perspective, into three groups:- commensal strains, intestinal pathogenic strains (enteric or diarrhoeagenic) and extraintestinal pathogenic strains.

2.1.4.1 Commensal *E. coli*

Commensal *E. coli* variants, which as previously stated confer benefits to the host enabling the normal development and function of the GI tract, are generally considered to lack the pathogenic features and virulence factors that enable intestinal

and extraintestinal strains to cause disease (2.1.3). However, they can become problematic in specific situations such as when a foreign body (e.g. a urinary catheter) is present (Russo and Johnson, 2003).

2.1.4.2 Extraintestinal pathogenic *E. coli* (ExPEC)

ExPEC have been linked to infections of the urinary tract (2.1.3.2.1), cerebral spinal fluid (CSF) (2.1.3.2.2), blood stream (2.1.3.2.3), respiratory tract and peritoneum (spontaneous bacterial peritonitis) (Johnson *et al.*, 2002; Johnson, 2003). The majority of the strains involved in these infections belong to the same phylogenetic group and harbour genes encoding specialised virulence traits resulting in a highly virulent profile (Johnson *et al.*, 1994; Maslow *et al.*, 1995; Picard *et al.*, 1999; Johnson and Stell, 2000; Johnson *et al.*, 2002; Johnson, 2003). Most ExPEC strains are opportunistic and possess a wide range of virulence factors that enable survival, colonisation and infection of a wide variety of tissue, blood and urine based environments. ExPEC constitute an increasing problem for human medicine, especially in patients that are immunocompromised due to disease, chemotherapy or old age (Ron, 2006).

ExPEC strains have also been associated with abdominal and pelvic infections, pneumonia, surgical site infections, ulcers, cellulitis, burn wound infections and can occasionally be associated with sinusitis and endocarditis (Russo and Johnson, 2003).

2.1.4.2.1 Uropathogenic *E. coli* (UPEC)

The urinary tract is one of the most common sites of bacterial infections in women, with *E. coli* being the most common infectious agent accounting for as many as 90 % of all urinary tract infections (UTIs), seen among ambulatory populations (Kaper *et*

al., 2004; Marrs *et al.*, 2005). UTIs are defined as "a significant number of pathogenic organisms in the urinary system". If symptoms, such as painful or infrequent urination or blood in the urine, are present, as few as 100 colony forming units/ml urine may be considered significant (Stamm, 1982; Karram and Mallipeddi, 1999; Marrs *et al.*, 2005). Symptomatic UTIs may be acute, recurrent or chronic, but the most common form of UTI is asymptomatic bacteruria, in which despite the presence of large numbers of bacteria in the urine (possibly more than 10^5 colony forming units/ml urine) for months or even years, the mucosa remains inert, and no symptoms/sequelae develop (Lindberg *et al.*, 1978; Bergsten *et al.*, 2005). In some extreme cases *E. coli* from UTIs can ascend from the bladder through the ureters to the kidneys, and then potentially enter the bloodstream resulting in bacteraemia (Kaper *et al.*, 2004). These differences in disease severity result from the propensity of the host to respond to infection and the virulence potential of the infecting strain, which arises from the possession of type 1 fimbriae, the ability to acquire iron and grow in urine as well as the production of other virulence factors (Connell *et al.*, 1996; Bergsten *et al.*, 2005).

2.1.4.2.2 Neonatal meningitis associated *E. coli* (NEMEC)

E. coli is the most common cause of Gram-negative neonatal meningitis, with an overall mortality rate of 15 - 29 % that rises to nearly 40 % in premature infants (Mulder *et al.*, 1984; de Louvois *et al.*, 1991; Franco *et al.*, 1992; Unhanand *et al.*, 1993). Severe neurological sequelae affect as many as half (33 - 50 %) of the survivors, who frequently require extensive supportive and rehabilitative care, even after the infection is cured (Franco *et al.*, 1992; Unhananand *et al.*, 1993; de Louvois, 1994; Dawson *et al.*, 1999). NEMEC pathogenesis is a complex process of multiple steps including colonization in the GI tract, intestinal translocation, bacteraemia, and

passage across the blood-CSF barrier (Xie *et al.*, 2004). Once bacteria enter the central nervous system, they multiply and induce the release of proinflammatory and toxic compounds, which leads to increased permeability of the blood-CSF barrier and migration of white blood cells across the blood-CSF barrier (pleocytosis), resulting in meningitis (Xie *et al.*, 2004). A greater understanding of these pathogenic processes and the virulence factors involved is required to reduce the incidence and improve the treatment of this disease.

2.1.4.2.3 Sepsis associated *E. coli* (SEPEC)

Bacteraemia can result from any primary ExPEC invasive infection, including meningitis and UTIs (Russo and Johnson, 2003; Ron, 2006). Therefore, as all diseases caused by ExPEC potentially involve or have the potential to lead to septicaemia, Ron (2006) suggested that all ExPEC strains should be considered as septicaemia. ExPEC is the leading cause of blood stream infections in nursing homes, hospitals and in the young, especially children (Kim, 2002; Mylotte *et al.*, 2002; Siegman-Igra *et al.*, 2002). SEPEC strains have a high incidence of drug resistance, which is often transmissible by plasmids and the recent increase in the frequency of strains with extended spectrum β-lactamases, has severely limited the treatment option available (Girardeau *et al.*, 2003; Johnson *et al.*, 2004; Maslow *et al.*, 2004; Blomberg *et al.*, 2005; Branger *et al.*, 2005; Jackson *et al.*, 2005; Johnson *et al.*, 2005).

2.1.4.3 Intestinal pathogenic *E. coli*

There are several pathotypes of enterovirulent *E. coli* that cause gastroenteritis in humans. These pathogenic sub-types possess additional genetic material that encodes for specific virulence factors that directly determine the nature of the disease. The six most important pathotypes currently recognised are: enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC).

2.1.4.3.1 EHEC

The predominant illness caused by EHEC is haemorrhagic colitis, which is characterised by acute abdominal cramps and watery diarrhoea progressing to vomiting and bloody diarrhoea in about 90 % of culture confirmed cases (Karch *et al.*, 2005). Fever is rare when patients are being evaluated, although approximately 50 % of patients report fever early on in illness, prior to seeking medical advice (Karch *et al.*, 2005). Haemolytic uremic syndrome (HUS) develops in approximately 15 % of EHEC O157:H7 patients under 10 years of age. This is a profound disorder that requires immediate supportive treatment in specialised centres with particular regard to fluid and electrolyte management (Tarr and Neill, 2001; Andreoli *et al.*, 2002). Although most patients recover, some survivors suffer from chronic renal sequelae (Garg *et al.*, 2003). Other potential sequelae include diabetes mellitus, neurological disorders, hypertension, colonic strictures, cholelithiasis and urinary abnormalities of uncertain clinical significance (Siegler, 1995; Brandt *et al.*, 1998).

As the bovine intestinal tract is the main EHEC reservoir (other potential reservoirs include sheep, goats, horses, pigs, dogs, poultry and deer), initial outbreaks were

associated with consumption of undercooked hamburgers (Riley *et al.*, 1983; Kudva *et al.*, 1996; Bielaszewska *et al.*, 1997; Keene *et al.*, 1997; Heuvelink *et al.*, 1999). Subsequent foodborne disease associations include sausages, unpasteurised milk, lettuce, cantaloupe melon, apple juice and sprouts. EHEC has also been linked to numerous outbreaks associated with recreational and municipal drinking water, person-to-person transmission and from petting zoo/farm animals (Kaper *et al.*, 2004).

EHEC has an extremely low infectious dose (estimated to be < 100 cells), with an incubation period that generally ranges from 3 to 8 days but can be as short as 1 to 2 days (Bell *et al.*, 1994; Kaper *et al.*, 2004; Smith and Fratamico, 2005). The most important EHEC virulence factor is the production of large quantities of one or more related shiga toxins (Stx), (also known as verotoxins) that cause severe damage to the lining of the intestine, resulting in haemorrhagic colitis. Stx contribute to the inflammatory responses and the pathology of EHEC by modulating the expression of chemokines and cytokines by human epithelial and endothelial cells (Cherla *et al.*, 2003; Matussek *et al.*, 2003). For EHEC strains the main genes involved in virulence expression including *sep*, *esc*, *eae*, *espABD* are encoded on the LEE pathogenicity island, expression of which results in the formation of cup-like pedestals called attaching and effacing (AE) lesions on the microvilli of the intestinal cell membrane, and a (Frankel *et al.*, 1998; Donnenberg and Whittam, 2001).

The genes *sep* and *esc* encode a type III secretion system and the *espABD* encode proteins secreted by this system that form a filamentous-like structure that translocates bacterial proteins directly into the host cell. The *eae* gene encodes for an adhesion called intimin that is responsible for the intimate attachment of the bacteria to the epithelial cells whereas the *tir* gene encodes the intimin receptor and the *ler*

gene (LEE-encoded regulator) encodes a positive regulator of the LEE genes. *E. coli* O157:H7 is the most publicised serotype of this group accounting for the greatest proportion of disease cases, causing an estimated 73,000 cases of illness and 61 deaths each year in the United States of America (Mead *et al.*, 1999). Infections in the UK are considered uncommon compared with the United States of America, with provisional data indicating 699 verocytotoxin-producing *E. coli* O157 isolations from humans examined by the Laboratory of Enteric Pathogens England and Wales in 2004 (www.hpa.org.uk). However, *E. coli* O157:H7 infections are considered to be of increasing importance within the UK, especially with respect to the high hospitalisation rates (> 50 %) (Neill *et al.*, 2001).

2.1.4.3.2 ETEC

ETEC is the most frequent cause of bacterial diarrhoea in high risk populations, with high morbidity and mortality in infants, young children and the elderly, in developing countries (Kaper *et al.*, 2004). Despite being the main cause of diarrhoea in travellers to developing countries, ETEC-induced diarrhoea in developed countries is uncommon and therefore it is not considered to be a serious foodborne disease hazard in these countries (Nataro and Kaper, 1998). The main source of infection is food/water contaminated with human sewage, or foods such as salads, dipping sauces or ready-to-eat items such as hot dogs, that are contaminated by infected food handlers or by general poor standards of hygiene (Black *et al.*, 1981; Tilden *et al.*, 1996). ETEC causes watery diarrhoea, which can range from a mild, self-limiting disease to severe purging disease and when prolonged in children, it can lead to severe dehydration and malnutrition. Other symptoms included nausea and mild-to-moderate abdominal cramping without fever.

ETEC adheres to and colonises the surface of the small bowel mucosa and produces enterotoxins, which induce intestinal secretion and diarrhoeal illness by promoting chloride ion secretion from the epithelial cells of the small intestine (Farthing, 2004). ETEC secretory enterotoxins are either heat-labile toxins (LTs) or heat-stable toxins (STs), with each ETEC expressing either a LT or a ST, or both. The major adherence factors required for attachment of the ETEC strains to the host cells are the plasmid encoded, colonisation factor antigens which may or may not be encoded by the same plasmid as the ETEC secretory enterotoxins (Cohen and Giannella, 1995; Mol and Oudega, 1996).

2.1.4.3.3 EPEC

EPEC was the first *E. coli* pathotype identified when described in association with large outbreaks of diarrhoea in the UK (Bray, 1945; Bray and Beaven, 1948). Although usually transmitted via the faecal-oral route, transmission via contaminated hands and food also occurs. The incidence of infection is decreasing in many countries due to improved hygiene and sanitation, but it is still an important cause of diarrhoea in infants; children less than 2 years of age are at risk and infants less than 6 months old are particularly susceptible (Smith and Fratamico, 2005). Outbreaks in developed countries are rare although EPEC has been associated with outbreaks of infantile enteritis in nurseries and pediatric wards (Vallance and Finlay, 2000). Although usually mild and self-limiting, symptoms of severe cases may include fever, malaise, vomiting, food intolerance, weight loss/wasting and a failure to thrive (Fagundes-Neto and Scaletsky, 2000). Occasionally, acute EPEC-induced diarrhoea in infants is prolonged and can become chronic, leading to dehydration, electrolyte imbalance and death with a mortality rate approaching 50 % (Vallance and Finlay,

2000; Blank *et al.*, 2002; Clarke *et al.*, 2002). Treatment usually consists of oral hydration although in severe cases parenteral rehydration is necessary.

The natural reservoir for EPEC is the human GI tract (human serotypes are not found in other animals) and the site of infection is the small bowel epithelium, where the organism binds loosely (localised adherence) to the epithelial cells and injects virulence factors into the cells (Vallance *et al.*, 2002). The virulence factors interact with the host cell components, altering the host cell signalling pathways resulting in disease (Vallance and Finlay, 2000). Following localised adherence, which requires the EPEC adherence factor plasmid, proteins are secreted, resulting in intimate bacterial attachment and the formation of the characteristic AE lesions as with EHEC (2.1.3.3.1) (Farthing, 2004).

2.1.4.3.4. EAEC

EAEC are defined as *E. coli* that do not secrete LT, ST or Stx enterotoxin and that adhere to Hep-2 cells in a pattern known as "autoaggregative", in which bacteria adhere to each other in a 'stacked-brick' configuration (Nataro *et al.*, 1995; Law and Chart, 1998). Although EAEC are increasingly recognised as a cause of diarrhoea in children and adults in both developing and developed countries, not all EAEC strains are pathogenic. EAEC infection may be asymptomatic, but when symptoms do occur they often consist of protracted watery diarrhoea, abdominal pain, borborygmus (rumbling due to gas), low-grade fever and vomiting (Smith and Fratamico, 2005).

A three-stage model of EAEC pathogenesis, consisting of adherence, mucus production and elaboration of cytotoxins resulting in intestinal secretion and mild but significant damage to the mucosa, has been proposed (Hicks *et al.*, 1996; Nataro *et al.*, 1998; Onkeke and Nataro, 2001). Although several types of fimbriae are known to

be involved with aggregative attachment and several toxins have been described, no single virulence factor has been irrefutably associated with EAEC virulence indicating a 'package' of plasmid-borne and chromosomal virulence factors as in other enteric pathogens (Kaper *et al.*, 2004; Smith and Fratamico, 2005).

2.1.4.3.5 EIEC

EIEC strains are genetically, biochemically and pathogenically closely related to *Shigella* spp., possessing the same virulence genes and inducing similar disease symptoms that can result in bacillary dysentery. Infection induces watery diarrhoea that is indistinguishable from that due to other *E. coli* infections, and which can be voluminous and may contain blood and mucus (Nataro and Kaper, 1998). Diarrhoea may also be accompanied by abdominal pain, fever, and vomiting (Neill *et al.*, 2001).

Food- and water-borne outbreaks of EIEC are rare in developed countries, primarily due to the high standards of sanitation and personal hygiene. Although all age groups are affected by this infection, it is highly likely that EIEC infections are of more importance in young children in developing countries due to the poor standards of sanitation. Foods connected to outbreaks include French soft cheeses, potato salad and guacamole, and raw foods contaminated by infected workers, cooked foods that are not reheated after being handled by infected personnel, or food/water contaminated with faeces from an infected individual (Gordillo *et al.*, 1992; Hale *et al.*, 1997; Willshaw *et al.*, 2000). Direct person-to-person transmission can occur but is generally thought to be uncommon, possibly due to the relatively high infective dose required to induce disease when compared with *Shigella* infections (Harris *et al.*, 1985; Hale *et al.*, 1997).

EIEC possesses several surface proteins that permit attachment to and direct invasion of the host intestinal mucosal epithelial cells with enterocyte penetration occurring via endocytosis (Farthing, 2004). Penetration is followed by intracellular multiplication of the bacterial cells, lysis of the endocytic vacuole releasing the bacterial cells and directional movement through the cytoplasm, which enables the extension of the infection into adjacent epithelial cells (Nataro and Kaper, 1998). Interestingly the EIEC plasmid-borne genes required for invasion, multiplication and survival within the host intestinal mucosal epithelial cells, are identical to those found on the *Shigella* invasion plasmid and therefore as previously stated, induce similar disease symptoms (Hale *et al.*, 1997; Nataro and Kaper, 1998; Lan *et al.*, 2001).

2.1.4.3.6 DAEC

Currently considered to constitute a putative sixth group of diarrhoeagenic *E. coli*, DAEC are generally associated with watery diarrhoea that can become persistent in young children (Nataro and Kaper, 1998; Le Bouguénec, 1999; Russo and Johnson, 2000; Kaper *et al.*, 2004; Servin, 2005; Le Bouguénec and Servin, 2006). Other symptoms include fever and vomiting (Smith and Fratamico, 2005). However, not all strains of this heterogeneous group of *E. coli* induce diarrhoea and some isolates have been linked to UTIs (Servin, 2005; Smith and Fratamico, 2005). DAEC strains exhibit diffuse adherence to epithelial cells in a laboratory assay of their adherence to Hep-2 or HeLa cells, due to the production of adhesins encoded by a family of related operons (Cravioto *et al.*, 1991; Le Bouguénec and Servin, 2006). No secretion system-encoding genes have as yet been found associated with pathogenic DAEC, although Peiffer *et al.* (2000; 2001) observed adhesion-independent cellular lesions in DAEC-infected cells, suggesting the presence of additional virulence factors (Blanc-

Potard *et al.*, 2002). Therefore further research focusing on the virulence factors and the pathogenic mechanisms of this highly variable pathovar and their role in causing diarrhoeic syndromes, is required (Smith and Fratamico, 2005).

2.1.5 Therapy

As the routes of transmission and epidemiological features of these *E. coli* vary considerably between the different pathotypes and their geographical locations, treatment must be type specific.

The majority of *E. coli*-induced gastroenteritis cases are treated symptomatically with adequate hydration. For more severe or prolonged cases or for disease in elderly or debilitated patients, specific chemotherapy is required. However, widespread antibiotic resistance has now been recognised in many *E. coli* isolates from human, animal and environmental sources. Although the prevalence rates for resistance vary for different populations and environments, resistance levels are usually high for broad-spectrum penicillins and trimethoprim, and low for third generation cephalosporins and nitrofurantoin (Jones *et al.*, 2004; von Baum and Marre, 2005). Currently, the most common resistance found in bovine and human *E. coli* O157:H7 isolates is to tetracycline, followed by resistance to streptomycin and ampicillin (Wilkerson and van Kirk, 2004).

The treatment of *E. coli* O157:H7 and other EHEC strains with antibiotics is controversial as it may increase the expression of Stx, and/or cause bacterial injury resulting in an increased release of preformed toxins, thereby increasing the risk of HUS in both children and adults (Wong *et al.*, 2000; Dundas *et al.*, 2001; Mølbak *et al.*, 2002). Although there is currently no established treatment regime for *E. coli* O157:H7, there are several lines of investigation such as vaccines for use in both

cattle and humans that decrease shedding and the levels of the pathogen in faeces. Other possible treatment protocols include Stx receptor mimics, Stx-neutralising monoclonal antibodies and EHEC-haemolysin (Huppertz *et al.*, 1999; Paton *et al.*, 2001; Yamagami *et al.*, 2001).

As the incidence of serious *E. coli* induced extraintestinal infection increases with age, the ever increasing proportion of elderly people in the UK and other developed countries will result in a steady increase in the number of extraintestinal *E. coli* infections (Angus *et al.*, 2001; McBean and Rajamani, 2001; Russo and Johnson, 2003). This increase, combined with increasing antimicrobial resistance, will make the future treatment of such infections more challenging and costly. Therefore new treatments and preventative strategies, such as removal of contamination at source, improved food hygiene procedures and the enforcement of strict standard hygiene procedures in the healthcare setting, will be required. These measures, combined with the implementation of strategies designed to minimise the increase in antimicrobial resistance are necessary to tackle this growing problem (Russo and Johnson, 2003).

2.1.6 *E. coli* genome

Genome sequences are now available for both pathogenic (EHEC O157:H7 EDL933 and UPEC CFT073) and non-pathogenic (*E. coli* K12 MG1655) *E. coli* strains (Blattner *et al.*, 1997; Hayashi *et al.*, 2001; Perna *et al.*, 2001; Welch *et al.*, 2002).

The 4,639,221-basepair (bp) chromosomal sequence of *E. coli* K-12 MG1655 was first published by Blattner *et al.* (1997). The original K-12 strain was isolated by Lederberg in 1922 from a convalescent patient suffering from diphtheria and is possibly one of the most studied bacteria in science (Lederberg, 1951; Bachmann,

1996; Lederberg, 2004). The *E. coli* strain K-12 MG1655 was chosen as it has been maintained as a laboratory strain with minimal genetic manipulation and it is therefore the strain most similar to the original (Bachmann, 1996; Blattner *et al.*, 1997).

EHEC O157:H7 strain EDL933 is an isolate from Michigan ground beef and was originally linked to a multistate USA outbreak in 1982, in which *E. coli* O157:H7 was first associated with human disease (Riley *et al.*, 1983). The 5,528,445-bp chromosomal sequence of this strain which was first published by Perna *et al.* (2001), contains 1,387 genes that are not present in *E. coli* K-12. Although EHEC O157:H7 (EDL933) and *E. coli* K-12 appear to share a common backbone of 4.1 megabases (Mb), the similarity between the chromosomes is discontinued in EDL933 by 177 so-called O islands, some of which have been linked to pathogenicity and Stx (Perna *et al.*, 2001)

UPEC CFT073 was originally isolated from the blood of a woman with acute pyelonephritis at the University of Maryland Hospital (Mobley *et al.*, 1990). The 5,231,428-bp genome sequence of UPEC CFT073 contains no plasmids, is 590,209 bp longer than the *E. coli* K-12 MG1655 genome sequence and similar in size to the EHEC O157:H7 EDL933 strain (Welch *et al.*, 2002).

A three-way comparison of these strains by Welch *et al.* (2002) indicated that only 39.2 % of their combined (non-redundant) set of proteins are actually common to all three strains and that the pathogen genomes are as different from each other, as each pathogen is from the non-pathogenic strain. The results of this study also highlighted the differences in disease potential between the two pathogenic strains, in that the

genes encoding the type III secretion system and phage/plasmid encoded toxins are absent in the UPEC CFT073 strain. Additionally, in comparison to the other genomes the UPEC CFT073 genome is particularly rich in genes that encode potential fimbrial adhesins, autotransporters and iron-sequestration systems, confirming that the potential pathotype of a particular *E. coli* strain depends on the repertoire of specific virulence genes present within its genome (Donnenberg, 2002; Welch *et al.*, 2002).

2.1.7 QS in *E. coli*

Although many members of the *Enterobacteriaceae* produce AHLs, no strains of *E. coli* have, as yet, been found to produce these AIs (Williams, 2006). However, *E. coli* does possess a LuxR homologue termed SdiA (suppressor of division inhibition) which binds specific AHL AI molecules when a threshold concentration is attained. Therefore, *E. coli* is capable of responding to exogenous sources of AHL, possibly activating transcription of the *ftsQAZ* gene cluster, which encodes functions required for cell division in *E. coli* (Sitnikov *et al.*, 1996). This has stimulated speculation that *E. coli* may respond to exogenous AHLs produced by other species in mixed communities as found in the GI tract or in biofilms.

The *rpoS* gene encodes the alternative stationary phase sigma factor, σ^s (σ^{38}), which regulates the expression of several genes involved in starvation survival. Expression of the *rpoS* gene is stimulated by the presence of a factor in conditioned medium (Baca-DeLancey *et al.*, 1999). Although, this factor has not been identified and there is, as yet, no definitive evidence of endogenous AHL production in *E. coli* (Withers *et al.*, 2001), it has been suggested that a HSL derivative maybe the factor responsible for the regulation of *rpoS* expression (Anand and Griffiths, 2003).

Current research suggest that *E. coli* employs at least two LuxS-dependent QS systems, one of which operates at intermediate cell densities, but the structure of the LuxS-synthesised signalling molecules that regulate these QS systems have not yet been elucidated (Baca-DeLancey *et al.*, 1999). Equally the genes regulated by these QS systems have not yet been identified.

2.1.7.1 QS and pathogenicity

EHEC colonises the large intestine, where it causes AE lesions on intestinal epithelial cells and produces the potent Stx. As previously stated the genes that are involved in the formation of the AE lesion are on the LEE pathogenicity island which encodes a type III secretion system, the intestinal colonisation factor, and the intimin receptor protein that is translocated from the bacterium to the host epithelial cells (1.2.3.3.1; Frankel *et al.*, 1998; Donnenberg and Whittam, 2001).

The synthesis of both AI-2 and AI-3 depends on LuxS, therefore EHEC may potentially produce both of these AIs. Although the role of AI-2 signalling in EHEC remains to be established, research suggests that several EHEC virulence-associated genes, including the LEE genes, the *stx* genes and the flagellum regulon (assembly of flagella, motility and chemotaxis) are regulated by AI-3 mediated QS (Sperandio *et al.*, 2003; Kaper and Sperandio, 2005; 2.1.8.2). As previously stated, EHEC require a markedly low infectious dose which would appear inconsistent with the density dependent regulation of these genes (1.2.3.3.1; Tilden *et al.*, 1996; Sperandio *et al.*, 1999; Sperandio *et al.*, 2002). This observation has led to the proposal that intestinal colonisation by EHEC and LEE gene expression is induced by an AI produced from non-pathogenic *E. coli* and other commensal intestinal microflora that possess a *luxS*

homologue (Sperandio *et al.*, 1999; Gruenheid and Finlay, 2000; Anand and Griffiths, 2003).

2.1.7.2 QS and motility

Recent research has indicated that *E. coli* cells in nutrient-depleted environments produce chemoattractants, such as amino acids, that can result in the accumulation of cells, depending upon the geometry of their surroundings (Park *et al.*, 2003). Park *et al.* (2003) noted that assuming a sufficiently enclosed space is provided, then this chemotaxis-mediated self-attraction could readily produce local cell densities that exceed the threshold required for QS. This research also indicated that further association of *E. coli* via the formation of dense granular aggregates may be LuxS-dependent, suggesting a potential role for QS in *E. coli* biofilm formation (Park *et al.*, 2003). Additional studies have also suggested that transcription of the *mot* operon (encoding motility genes), and *flhDC* (encoding the master regulator of the flagellum regulon) which also regulates bacterial cell division and several metabolic processes, are controlled by a LuxS-dependent QS mechanism (Surrette and Bassler, 1998; Sperandio *et al.*, 2001; Sperandio *et al.*, 2002; Sperandio *et al.*, 2003; Taga and Bassler, 2003).

2.1.7.3 EHEC-host communication

Sperandio *et al.* (2003) demonstrated that in addition to sensing AI-3 when activating its virulence genes, EHEC also senses the hormones epinephrine/norepinephrine, which are both produced by the host and are present in the GI tract. These results indicate that cross-communication may occur between the EHEC LuxS-dependent QS system and the epinephrine/norepinephrine host signalling system. According to

Kaper and Sperandio (2005), this sensing of multiple signals of both bacterial and host origin, by sensor kinases in the membrane of EHEC, would allow the fine tuning of EHEC gene expression enabling the different genes sets (the LEE genes and the flagellum regulon) to be expressed at slightly different times.

The results from these research studies, suggests that the LEE genes and the flagellum regulon are activated by a complex regulatory cascade, the AI-3/epinephrine/norepinephrine cross-signalling mechanism, and that this bacterial-host signalling mechanism may also be present in several other bacterial species (Sperandio *et al.*, 1999; Sperandio *et al.*, 2001; Sperandio *et al.*, 2003; Kaper and Sperandio, 2005; Walters and Sperandio, 2006).

Theories pertaining to QS by pathogenic *E. coli* are abundant, ranging from the economics of virulence gene expression to pathogenesis regulation enabling coordinated stealth attacks and the overwhelming deployment of virulence factors (Parsek and Greenberg, 2000). Evidence in support of this theory is mounting, with numerous studies indicating that EHEC, more specifically *E. coli* O157:H7, uses QS to control biofilm formation, intestinal colonisation and virulence by influencing the transcription of genes in the LEE pathogenicity island (i.e. AE lesion formation and Stx production), the *mot* operon (flagellum assembly, motility and chemotaxis), and potentially the *ftsQAZ* gene cluster (cell division) (Sitnikov *et al.*, 1996; Surrette and Bassler, 1998; Sperandio *et al.*, 2001; Sperandio *et al.*, 2002; Park *et al.*, 2003; Sperandio *et al.*, 2003; Taga and Bassler, 2003; Kaper and Sperandio, 2005). However, the role of QS in non-pathogenic *E. coli*, beyond enhancing AI levels for pathogenic *E. coli* detection, is less clear, but may for example enable population density regulation (Sitnikov *et al.*, 1996).

2.1.8 Aim of the research

Current estimates suggest that approximately two-thirds of the 4,400 or so *E. coli* K12 genes have been identified, genetically and physically (Schaechter *et al.*, 2004). According to Schaechter *et al.* (2004), many of the remaining unidentified genes may be linked to survival in changeable natural environments, such as river water, soil, dung heaps, foods and healthy and diseased hosts. Although QS is emerging as an integral component of bacterial global gene regulatory networks, responsible for facilitating bacterial adaptation to environmental stress, the full extent of QS in *E. coli* and the role of QS in commensal strains is still unclear. The isolation and identification of AI molecules produced by *E. coli* and the study of the QS systems they regulate, is required to elucidate how bacteria cope with environmental changes by using intercellular interactions and thus further the understanding in this field (Withers *et al.*, 2001).

Despite being one of the most studied bacterial species, there is a general under-appreciation of the significant genetic differences amongst different *E. coli* strains. These differences determine whether the strains are commensal or pathogenic and what site of infection they will favour (Russo and Johnson, 2003).

Therefore, due to the extreme differences between *E. coli* strains, the work in this study employed several *E. coli* laboratory strains and two different types of clinical *E. coli* isolates. The primary isolate used in this study, UL51, has been used in several pilot studies at the University of Central Lancashire, Preston UK (unpublished data), which have all suggested that this isolate is positive for putative AHL production.

The aim of this research is to assess putative AHL production by both clinical and laboratory isolates of *E. coli*. Three bioassay reporter strains were exposed to extracts

of isolate-conditioned medium, enabling screening for both short and medium-long chain AHLs.

2.2 METHODS

2.2.1 Reagents

All chemicals were purchased from Sigma-Aldrich Company Ltd (Poole, Dorset, UK) or VWR International (Lutterworth, Leicestershire, UK) unless otherwise stated.

2.2.2 Bacterial strains/isolates

The bacterial strains/isolates used in this study are described in the following tables (2.2.1, 2.2.2, 2.2.3 and 2.2.4). Unless otherwise stated all strains/isolates used in this study were freeze dried and stored at 4°C as part of the University of Central Lancashire culture collection. Each culture in the collection is assigned a unique identification code with the designation UL (for University of Central Lancashire) or LP (for Lancashire Polytechnic) followed by the next sequential number.

Table 2.2.1: ***A. tumefaciens*** **strains used in this study**

Strain	Reference	Features	Source
A. tumefaciens NT1	UL32	Lacks the Ti plasmid so it cannot produce *Agrobacterium* AHL AI detected by UL34.	S. K. Farrand, (University of Illinois)
A. tumefaciens NT1(pTiC58ΔaccR)	UL33	Contains a *tra*^{c} Ti plasmid and synthesises *Agrobacterium* AI detected by UL34.	S. K. Farrand, (University of Illinois)
A. tumefaciens NTL4(pZLR4)	UL34	Reporter strain-clone containing inserts from pTiC58 that encode for *traR* and a *traG::lacZ* fusion and confers resistance to gentamicin (30 µg/ml) and carbenicillin (100 µg/ml). It cannot synthesise *Agrobacterium* AHL AI (OOHL). The *lacZ* fusion product is only expressed when exogenous AHL AI is present.	S. K. Farrand, (University of Illinois)
A. tumefaciens R10(pCF218)	UL224	Contains a *tra*^{c} Ti plasmid and synthesises *Agrobacterium* OOHL AI detected by UL225. Requires tetracycline (1 µg/ml) and spectinomycin (100 µg/ml) to prevent plasmid loss.	Prof. Stephen Winans, (Cornell University, Ithaca)
A. tumefaciens KYC55 (pJZ372)(pJZ384) (pJZ410)	UL225	Reporter strain-contains 3 plasmids that confer resistance to gentamicin and spectinomycin (100 µg/ml) and tetracycline (2 µg/ml). It cannot synthesise the *Agrobacterium* OOHL. The *lacZ* fusion product, β-galactosidase, is only expressed when exogenous AHL AI is present.	Prof. Stephen Winans, (Cornell University, Ithaca)

Table 2.2.2: ***E. coli*** **laboratory strains used in this study**

Strain	**Reference**	**Features**	**Source**
E. coli CGSC 4969	UL27	*met*K blocked in the production of *S*-adenosylmethionine and acyl-homoserine lactone from methionine.	*E. coli* Genetic stock centre (Yale University, New Haven, United States of America)
E. coli CF1648	LP50	Standard laboratory isolate Wild-type MG1655; relA^{+} spoT^{+}.	Dr. M. Cashel NIH-Bethesda USA
E. coli W3110	UL52	W3110 (ATCC 27325) Derivative of K12.	The American Type Culture Collection, Manassas, USA
E. coli SD1648Δlac	UL56	Derivative of CF1648 (LP50) created at the University of Central Lancashire, Preston. Δ*lac*Z58.	Dr. S. G. Danby (University of Central Lancashire)

Table 2.2.3: *E. coli* clinical strains used in this study

Strain	Reference	History	Source
E. coli blood isolate	UL47	Isolated from a human blood sample.	PHLS, Royal Preston Hospital
E. coli blood isolate	UL48	Isolated from a human blood sample.	PHLS, Royal Preston Hospital
E. coli blood isolate	UL49	Isolated from a human blood sample.	PHLS, Royal Preston Hospital
E. coli blood isolate	UL51	Isolated from a human blood sample.	PHLS, Royal Preston Hospital
E. coli urine isolate	BRI 7	Isolated from a mid-stream urine sample from a female patient (D.O.B. 23/5/12).	Blackburn Royal Infirmary
E. coli urine isolate	BRI 8	Isolated from a mid-stream urine sample from a female patient (D.O.B. 11/12/68).	Blackburn Royal Infirmary
E. coli urine isolate	BRI 9	Isolated from a mid-stream urine sample from a male patient (D.O.B. 01/10/36).	Blackburn Royal Infirmary
E. coli urine isolate	BRI 10	Isolated from a mid-stream urine sample from a female patient (D.O.B. 31/8/16).	Blackburn Royal Infirmary

All urine isolates were isolated in the 1st week of April 2003.

Table 2.2.4: Other strains used in this study

Strain	Reference	History	Source
Chromobacterium violaceum CV026	LP41	Violacein-negative. Mini *Tn5* mutant. Exogenous AI stimulates pigment production. Requires kanamycin (50 μg/ml).	Professor P. Williams (University of Nottingham)

2.2.3 Culture conditions

A. tumefaciens strains UL32, UL33 and UL34 (Table 2.2.1) were grown at 25°C, (agitated at 180 rpm in a Gallenkamp orbital incubator) in 9 ml aliquots of antibiotic minimal medium (ABMM) (Appendix 1, Table A1.2) (Chilton *et al.*, 1974; Cha *et al.*, 1998; Luo *et al.*, 2003). The reporter strain, UL34, required the addition of gentamicin (5 µg/ml) to prevent plasmid loss. Both UL32 and UL33 were stored on ABMM slopes at 4°C. Strain UL34 was stored on ABMM slopes containing gentamicin (5 µg/ml) at 4°C. All strains were renewed every 28 days and resuscitated via a single 16 h passage in the appropriate conditions as stated above.

A. tumefaciens strains UL224 and UL225 (Table 2.2.1) were grown at 25°C (agitated at 180 rpm) in 1 ml aliquots of A. tumefaciens maintenance medium (ATMM) (Appendix 1, Table A1.3) (Fuqua and Winans, 1994). Strain UL225 required the addition of gentamicin and spectinomycin (both at 100 µg/ml) and tetracycline (2 µg/ml). Strain UL224 required tetracycline (1 µg/ml) and spectinomycin (100 µg/ml) to prevent plasmid loss (Zhu *et al.*, 1998; Zhu *et al.*, 2003). Both UL224 and UL225 were stored on ATMM slopes containing the appropriate antibiotics for each strain at 4°C and refreshed every 28 days. Resuscitation of both strains consisted of one 16 h passage in the appropriate conditions as stated above.

The *C. violaceum* reporter stain, LP41, (Table 2.2.4) was grown at 30°C, and agitated at 180 rpm in 9 ml aliquots of nutrient broth (Oxoid), with the addition of kanamycin (to a final concentration of 50 µg/ml). LP41 stocks were stored in 1 ml volumes of nutrient broth containing glycerol (20 % v/v) at -80°C. Resuscitation consisted of a single 16 h passage in the growth conditions stated above.

All *E. coli* laboratory strains (Table 2.2.2) and blood isolates (Table 2.2.3) used in this study were freeze dried and stored at 4°C as part of the University of Central Lancashire culture collection. The *E. coli* urine isolates used in this study were all isolated in the Blackburn Royal Infirmary and assigned a unique identification code with the designation BRI (Blackburn Royal Infirmary) followed by the next sequential number. All *E. coli* isolates were stored in 1 ml amended-MOPS (A-MOPS) growth medium (Appendix 1, Table A4) containing glycerol (20 % v/v) at -80°C. Resuscitation for all *E. coli* strains consisted of a single 6 h passage in A-MOPS growth medium (9 ml).

E. coli strains were grown at 37°C in 50 ml aliquots of A-MOPS growth medium with shaking (180 rpm) for 24 h. Culture supernatants were prepared, by centrifuging culture samples at 6000 x g, 4°C for 15 min (MISTRAL 3000i). The supernatant was stored at -80°C for future extraction.

2.2.4 Extraction methods

QS signalling molecules are readily extracted using organic solvents which can then be removed by evaporation. Two extraction techniques using two different solvents were employed during this study.

2.2.4.1 Ethyl acetate extraction

Ethyl acetate can be used to efficiently extract QS molecules from culture supernatants (Pearson *et al.*, 1994; Shaw *et al.*, 1997; Pierson *et al.*, 1998; Holden *et al.*, 1999; Marketon and González, 2002). Supernatant samples were mixed using a Blood Tube Rotator, (SB1, Jencons [Scientific] Ltd., Bedfordshire), with an equal

volume of ethyl acetate (HPLC grade) for 20 min. After a 5 min settling period, the top phase was transferred to a concentrating tube whilst the remaining bottom phase was again mixed for 20 min with an equal volume of ethyl acetate using the Blood Tube Rotator. Following a second 5 min settling period, the top phase was again transferred to the concentrating tube. The combined ethyl acetate fractions were concentrated by evaporating the ethyl acetate under nitrogen (oxygen free), aided by a Techne Dri-block® sample concentrator at 40°C, to produce a dried extract which was then resolubilised (5 x concentrated) with ethyl acetate (HPLC grade, for thin layer chromatography (TLC)) or ddH_2O (for bioassays), filter sterilised (0.2 µm) and stored at 4°C.

2.2.4.2 Dichloromethane extraction

The organic solvent dichloromethane has been used to extract QS molecules from culture supernatants (McClean *et al.* 1997; Yates *et al.*, 2002; Llamas *et al.*, 2004). This method was adapted for this study. Supernatant samples were mixed using a test tube rotator, (SB1, Jencons [Scientific] Ltd., Bedfordshire), with an equal volume of dichloromethane (HPLC grade) for 20 min. After a 5 min settling period the bottom phase was transferred to a concentrating tube whilst the remaining top phase was again mixed with an equal volume of dichloromethane and mixed for a further 20 min. Following a second 5 min settling period, the bottom phase was transferred to the concentrating tube. The combined dichloromethane fractions were then concentrated under nitrogen (oxygen free) in a Techne Dri-block® sample concentrator at 40°C.

The extract from the first tube was then resolubilised (5 x concentrated) with ddH_2O (for the bioassay) or ethyl acetate (HPLC grade, for TLC) and added to the second fraction. This was then filter sterilised and stored at 4°C.

2.2.5 French press

Following centrifugation (MISTRAL 3000i, 6000 x g, 4°C, 15 min), cell pellets were re-suspended (5 x concentrated) in fresh sterile culture medium and then passed through a pre-chilled (12 h at 4°C) French Press (Aminco, Silver Spring, Maryland) at 150 Pascalś. The broken cell samples were then centrifuged (MISTRAL 3000i, at 6000 x g, 4°C, 15 min) to remove the debris and the pellet was discarded. The supernatant (the pressed conditioned medium) was then extracted with ethyl acetate as in 2.2.5.1 and rehydrated with ddH_2O (for the bioassay), filter sterilised and stored at 4°C. This method for the preparation of bacterial cell extracts was adapted from Yates *et al.* (2002).

2.2.6 Bioassays

Three different bioassay reporter strains were employed for this study.

2.2.6.1 *Chromobacterium* plate bioassay

C. violaceum is a Gram-negative, non-sporing, rod or coccobacillus shaped bacterium, which normally produces the purple compound violacein in response to the presence of *N*-hexanoyl homoserine lactone, (HHL) (McClean *et al.*, 1997). *C. violaceum* CV026, is a mini-T5 mutant of *C. violaceum* ATCC 31532 (Latifi *et al.*, 1995; Throup *et al.*, 1995; Winson *et al.*, 1995). This white mutant does not produce HHL so violacein synthesis only occurs in the presence of exogeneous HHL and other short-

chain AHLs (Figure 2.2.1; Throup *et al.*, 1995; McClean *et al.*, 1997). Therefore, this strain can be used as an indicator organism in a plate bioassay to detect a structurally diverse series of natural and synthetic short chain AHLs, (Figure 2.2.1; McClean *et al.*, 1997). This reporter strain was LP41 from the University Culture Collection.

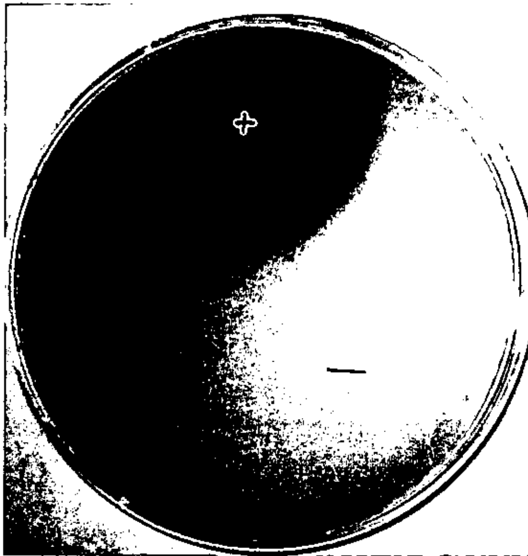


Figure 2.2.1: *Chromobacterium* plate bioassay for the positive and negative controls

The diffuse purple zone surrounding the HHL positive control (marked as +) indicates violacein production, whereas the absence of a visible zone for the negative control (marked as -) suggests that this extract does not contain any compounds capable of inducing violacein production by the *Chromobacterium* plate bioassay reporter strain LP41.

2.2.6.1.1 *Chromobacterium* plate bioassay growth conditions

The *Chromobacterium* plate bioassay used in this study was adapted from McClean *et al.* (1997).The presence of a putative AHL in the extracts produced from the *E. coli* culture supernatants (2.2.4) was assessed via violacein synthesis by the reporter strain LP41. Pre-dried nutrient agar plates were spread with 100 μl of LP41 stationary phase culture. Aliquots of sample culture supernatant extracts (5 μl) were then applied to the plates (maximum of 3 unknown extracts per plate). HHL was used as a positive control with aliquots spotted onto each plate before incubation at 30°C, for 16 h. A diffuse purple zone spreading out from the sample indicated a positive reaction and therefore the possible presence of a putative AI in the sample.

2.2.6.2 *Agrobacterium* plate bioassay

A. tumefaciens is a Gram-negative, capsulated, motile, rod shaped bacterium with an optimum growth temperature of 25-28°C and it induces crown gall tumours in a wide range of dicotyledonous plants (Singleton and Sainsbury, 1999). *A. tumefaciens* NT1(pTiC58Δ*accR*), designated UL33, contains a *tra*^{c} Ti plasmid. This strain synthesises the *Agrobacterium* AI, OOHL, and therefore was the positive control for this bioassay. The negative control strain, UL32, lacks the Ti plasmid and therefore cannot produce AI detectable by this bioassay (Watson *et al.*, 1975). The reporter strain NTL4(pZLR4) is a clone, that contains inserts from pTiC58, that encode for *traR*, and a *traG*::*lacZ* fusion which confers resistance to gentamicin (30 μg/ml) and carbenicillin (100 μg/ml) (Piper *et al.*, 1993; Farrand *et al.*, 1996; Piper *et al.*, 1999; Luo *et al.*, 2001; Luo *et al.*, 2003). As this reporter strain, designated UL34, was incapable of synthesising the *Agrobacterum* AHL AI, OOHL, the *lacZ* fusion was only expressed in the presence of exogenous medium-long chain AI (Farrand, 1998;

Luo *et al.*, 2003). The β-galactosidase activity of the *lacZ* reporter fusion was detected by the incorporation of 5'-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-GAL) in an overlay containing UL34 (Cha *et al.*, 1998; Farrand, 1998; Luo *et al.*, 2003).

2.2.6.2.1 *Agrobacterium* plate bioassay growth conditions

The presence of putative AHL in the extracts produced from the *E. coli* culture supernatants (2.2.4) was determined on plates containing X-GAL via differences in *lacZ* gene expression. ABMM base plates containing X-GAL (40 μg/ml) were prepared and pre-dried. Molten soft agar (5 ml, 0.7 % w/v) containing X-GAL (40 μg/ml) was inoculated with 0.5 ml culture UL34 (6 h) and mixed carefully to avoid air bubble formation. The overlay was then poured over the base plate and allowed to set. Aliquots (5 μl) of the sample culture supernatant extract, *A. tumefaciens* UL32 (negative control) extract and the *A. tumefaciens* UL33 (positive control) extract were applied to the plate, which was then incubated in the dark at 25°C for 16 h. A diffuse blue/green zone spreading out from the sample indicated a positive reaction and therefore the possible presence of putative AHL in the sample (Figure 2.2.2). Duplicate plates, excluding the reporter strain were produced to ensure that any X-GAL cleavage observed on the reporter plates was due to the induction of the *lacZ* fusion rather than any β-galactosidase produced and secreted by the *E. coli* isolates (Cha *et al.*, 1998; Farrand, 1998; Luo *et al.*, 2003).

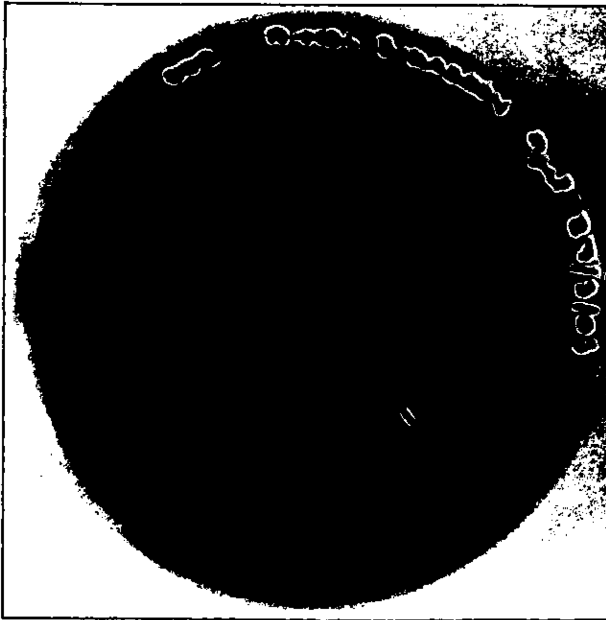


Figure 2.2.2: ***Agrobacterium*** **plate bioassay for the *A. tumefaciens* positive control UL33 and the *A. tumefaciens* negative control UL32 extracts**

The diffuse blue/green zone surrounding the *A. tumefaciens* positive control, UL33 supernatant extract (marked as +) indicates a positive reaction whereas the lack of a visible zone surrounding the *A. tumefaciens* negative control, UL32 supernatant extract (marked as -), suggests that the extract does not contain any compounds capable of inducing β-galactosidase activity in the *Agrobacterium* plate bioassay reporter strain.

2.2.6.3 *Agrobacterium* liquid culture bioassay

Zhu *et al.* (2003) adapted the bacteriophage T7 expression system to express TraR in *A. tumefaciens*. Plasmid pJZ384 confers spectinomycin resistance and contains a T7 promoter *traR* fusion (Hajdukiewicz *et al.*, 1994; Zhu and Winans, 2001; Zhu *et al.*, 2003). Plasmid pJZ410 confers gentamicin resistance and contains both the RNA polymerase gene of bacteriophage T7 and the *cI857* gene of bacteriophage *lambda*

(Tabor and Richardson, 1985; Kovach *et al.*, 1995; Zhu *et al.*, 2003). Plasmid pJZ372 contains a *traI-lacZ* reporter fusion and confers resistance to tetracycline (Chen and Winans, 1991; Fuqua and Winans, 1996; Zhu *et al.*, 2003). These plasmids were introduced into the *A. tumefaciens* strain KYC55, which lacks the Ti plasmid and therefore cannot produce detectable AHL AIs, to produce the final reporter strain *A. tumefaciens* KYC55 (pJZ372); (pJZ384); (pJZ410) (Cho *et al.*, 1997: Zhu *et al.*, 2003). This reporter strain, designated UL225, is reported to be highly sensitive to a wide variety of AHLs, from C4-HSL to C18-HSL, some at extremely low concentrations and it is therefore ideal for detection of novel AHL mediated cell-to-cell signalling systems. For the purposes of this study the *Agrobacterium* liquid culture bioassay used, was adapted from Zhu *et al.* (2003).

2.2.6.3.1 *Agrobacterium* liquid culture bioassay growth conditions

Double strength ATMM (0.25 ml) was inoculated to an optical density of 0.05 ($OD_{600\ nm}$) with a 12 h culture of UL225 in the presence of 0.25 ml sterile extract. Three controls per extract were prepared (Appendix 2, Table A2.1), resulting in a set of 4 samples for each extract. These controls ensured that any differences observed in the $OD_{420\ nm}$ measurements, were due to the supernatant extract induction of β-galactosidase activity. All samples within a set (the sample supernatant extract and the three controls) were processed concurrently and subsequently incubated at 25°C, agitated (180 rpm, Gallenkamp orbital incubator) for 24 h. Following the incubation period the optical density ($OD_{600\ nm}$) was measured and samples (0.5 ml) were centrifuged at 13,000 rpm for 5 min to pellet the cells (MSE Micro Centaur microfuge). Samples were then stored at -20°C for later determination of β-galactosidase activity via the adapted Miller assay (2.2.6.3.2).

2.2.6.3.2 Adapted Miller assay

The presence of a putative AHL in the extracts was determined through differences in β-galactosidase activity encoded by *lacZ*. This activity was determined by a colorimetric enzyme assay, adapted from Miller (1992). Each set of samples was processed concurrently. To maintain the cell pellet, samples were thawed by centrifuging at 13,000 rpm for 15 min in a MSE Micro Centaur microfuge. The supernatatant was discarded, and the pellet was thoroughly resuspended in sterile distilled water (0.5 ml) and Z buffer (0.5 ml) by vortexing (10 seconds). A control was prepared by the addition of 0.5 ml sterile distilled water to 0.5 ml Z-buffer; this control also served as the blank for the optical density reading at 420 nm. Cells were disrupted by the addition of 2 drops of chloroform and 1 drop of 0.1 % (w/v) sodium dodecyl sulphate (SDS), and then vortexed for 10 seconds. Samples sets were then incubated at 28°C (Haake G/D8 waterbath) for 5 min. The reaction was initiated by the consecutive addition of 200 μl of *o*-nitrophenyl-β-D-galactoside solution (ONPG) (4 mg/ml), to each sample at 10 second intervals. The reaction was stopped, after sufficient yellow colour had developed (ideal readings were between 0.3 and 1.2) in any of the samples in the set, by the addition of 0.5 ml of 1 M Na_2CO_3 solution (Eastman Kodak Company). Samples were then centrifuged at 13,000 rpm for 5 min to pellet any precipitate/cell debris. The absorbance of the *o*-nitrophenol produced in the supernatant was measured against the blank control at 420 nm. β-galactosidase activity was calculated using the following equation (expressed in Miller units) for each set of samples with the control samples providing a background reading for comparison to the extract sample (Appendix 2, Table A2.1).

$$\frac{1000 \times OD^{420\,nm}}{t \times V \times OD^{600\,nm}} = \text{Miller units of } \beta\text{-galactosidase}$$

Where t = Reaction time (minutes)
V = Culture volume (ml)

To ensure that any results were due to the bacterial conditioning of the growth media, samples of sterile medium were extracted (see 2.2.5.1) and assayed with *A. tumefaciens* UL225 (2.2.7.3).

2.2.7 TLC

According to Brelles-Mariño and Bedmar (2001), TLC coupled with a specific biosensor assay is an easy and useful technique for monitoring the purity of signalling molecules that can also provide preliminary information about the number and structure of active compounds present in the bacterial culture supernatants. This method of TLC coupled to the *Agrobacterium* plate bioassay reporter strain, UL34, was adapted from Shaw *et al.* (1997). As there is limited control of the development environment, with regards to atmospheric saturation, TLC plates are not directly comparable. For this reason a vertical moat (10 mm) was cut into a Baker Si. C_{18} reverse-phase TLC plate (Mallinckrodt Baker, Inc.), dividing the plate into two equal sections-one for the bioassay and one for sample separation that can be developed concurrently and which are therefore comparable. The extract was applied with a syringe (Hamilton 1710RNR syringe), to each section of the plate in equivalent positions resulting in two replica samples, (25 μl for the bioassay side and 50 μl for the separation side). The plate was developed in a mixture of methanol and ddH_2O (30:20, v/v), air-dried and then surrounded by molten agar (0.7 % w/v) in a Nunc Bio-

assay dish (Intermed, Denmark). Before the agar surround was set, a sterile barrier was positioned along the moat. Molten ABMM (1.6 g/100 ml agar) containing 0.15 ml X-GAL (40 μg/ml) was inoculated with 50 ml of the UL34 *Agrobacterium* plate bioassay reporter strain (6 h culture) and then poured over the reporter section of the TLC plate (Figure 2.2.3). Once the overlay had set, the barrier was carefully removed and the lid of the bioassay dish replaced. The plates were incubated at 25°C in the dark for 18 h.

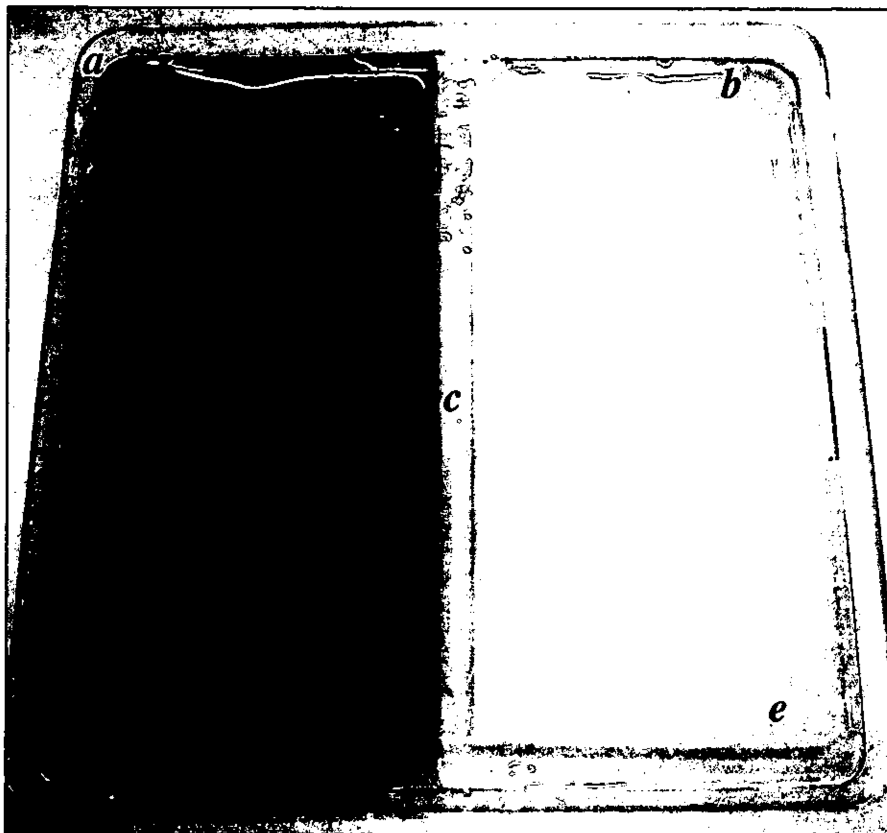


Figure 2.2.3 TLC plate in a Nunc Bio-assay dish with vertical moat, agar surround and ABMM agar overlay The Baker Si. C_{18} reverse-phase TLC plate is held in place in the Nunc bio-assay dish (*a*) by the agar surround (*b*). The vertical moat (*c*) cut into the TLC plate, divides the plate into two equal sections that can be developed concurrently-one for the bioassay (*d*) and one for sample separation (*e*).

A diffuse blue/green zone, as observed for the *Agrobacterium* plate bioassay, spreading out from the sample, indicated a positive reaction and therefore the possible presence of putative AHL AIs in that fraction. The distance was measured on the bioassay side from the centre of the original sample spot, to the top and bottom of the blue/green zone. These measurements were then superimposed onto the other side of the plate and the silica gel from the corresponding area was carefully removed from the TLC plate and transferred to a sterile universal for further analysis using high performance liquid chromatography time of flight-mass spectroscopy (LC/TOF-MS).

2.2.8 LC/TOF-MS

LC/TOF-MS is a form of high resolution HPLC-mass spectroscopy (LC-MS) that is frequently used in the pharmaceutical industry to elucidate structures and to determine the purity of a sample (Michelsen and Karlsson, 1999). Electrospray ionisation (ESI) is an atmospheric pressure ionisation technique that is often used in biochemical analyses to produce ions and is especially useful in producing ions from macromolecules, as it overcomes the propensity of these molecules to fragment when ionised. In ESI the sample (the analyte) is dissolved in a volatile solvent and passed through a charged capillary. This results in the production of an aerosol of highly charged droplets from which the neutral solvent is evaporated, forcing the charged analyte molecules closer together. Thus, the similarly charged analyte molecules become unstable, causing the droplets to explode resulting in the eventual production of the lone analyte ion. As with most ionisation methods, ESI can produce both positively and negatively charged ions. In the positive ionisation mode, samples (M) with molecular masses up to approximately 1200 Da give rise to singly charged (protonated) molecular-related ions of the formula $(M + H)^+$.

TOF analysers separate the ions formed via ESI, according to their mass/charge ratios. TOF mass spectrometers operate on the principle that when a group of ions of differing mass/charge ratios (*m/z*) are subjected to the same applied electric field and allowed to drift in a region of constant electric field, their velocities will depend only on their mass/charge ratio. LC/TOF-MS analysis was performed at Liverpool John Moores University, Liverpool by Nicola Dempster. The active compound was desorbed from the TLC plate silica gel samples with a mixture of acetonitrile and ddH_2O (50:50 v/v, 200 μl). The ionisation method was ESI, positive mode and samples were introduced into the ESI using direct injection at a flow rate of 10 μl min^{-1}. The TOF-MS analysis was performed on a Micromass LCT (Micromass, UK) and Masslynx software was used for both the operation and data collection.

2.3 RESULTS

2.3.1 Screening various *E. coli* strains and isolates for AHL production

The initial screen of the *E. coli* blood isolate culture supernatant extracts and a selection of *E. coli* laboratory isolate culture supernatant extracts (produced with ethyl acetate) was performed with both the *Chromobacterium* and *Agrobacterium* plate bioassays.

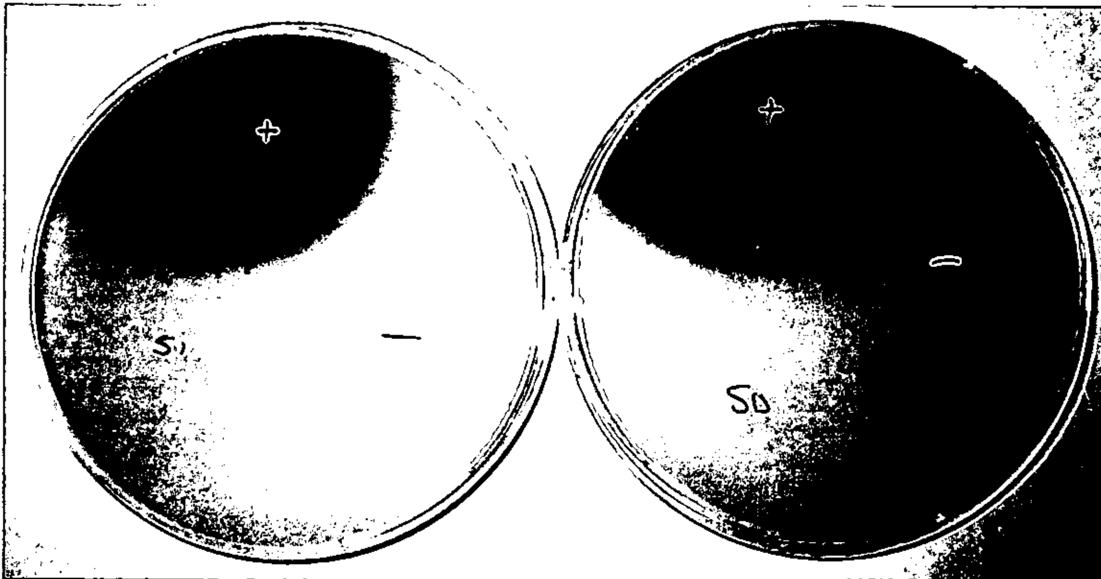


Figure 2.3.1 *Chromobacterium* plate bioassay for the *E. coli* clinical isolate UL51 and the *E. coli* laboratory strain LP50 extracts

Purple zones were not observed for the *E. coli* blood isolate, UL51, (marked as 51) or the *E. coli* laboratory strain, LP50, (marked as 50) extracts. Purple zones, indicating violacein production were observed for the HHL positive control (marked as +) but were not observed for the negative control (marked as -).

2.3.1.1 *Chromobacterium* plate bioassay

Purple zones were not observed for this initial screen (Figure 2.3.1). These results indicated that none of the *E. coli* extracts produced with ethyl acetate (2.2.4.1) from the *E. coli* blood isolates and the *E. coli* laboratory strain cultures (2.2.3) induced violacein production by the *Chromobacterium* plate assay reporter strain.

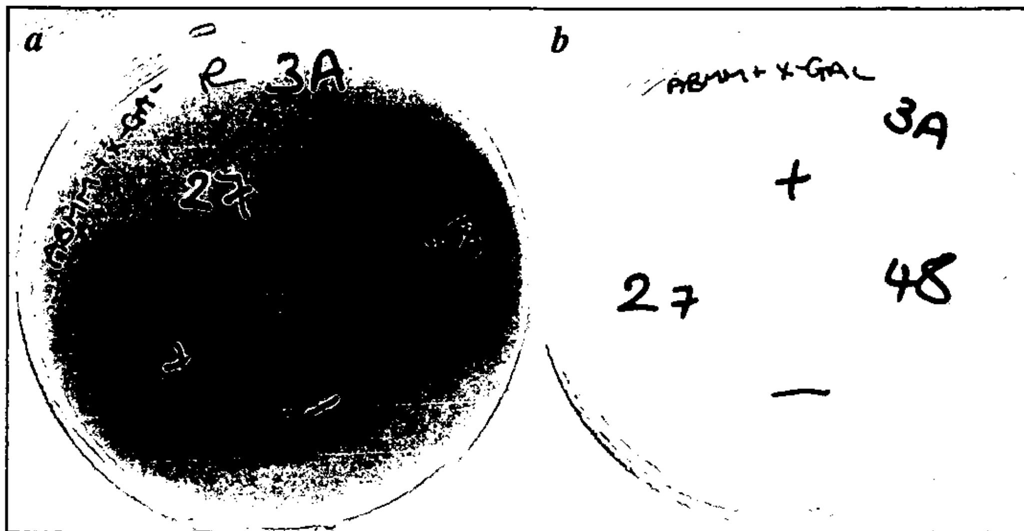


Figure 2.3.2 ***Agrobacterium*** **plate bioassay for the** ***E. coli*** **clinical isolate UL48 and the** ***E. coli*** **laboratory strain LP27 extracts**

(a) Blue/green zones spreading out from the initial extract sample for the *E. coli* blood isolate, UL48, extract (marked as 48) and the *A. tumefaciens* positive control, UL33, extract (marked as +) on the reporter plate indicated a positive reaction. Blue/green zones were not observed for the *E. coli* laboratory strain UL27 extract (marked as 27) or the *A. tumefaciens* negative control, UL32, extract (marked as -). ***(b)*** Blue/green zones were not observed for any of the extracts on the duplicate control plate, indicating that the X-GAL cleavage observed on the reporter plate was due to the induction of the *lacZ* fusion rather than any β-galactosidase produced and secreted by the *E. coli* isolates.

2.3.1.2 *Agrobacterium* plate bioassay

The initial screen of the ethyl acetate produced extracts with the *Agrobacterium* plate bioassay, indicated that all of the *E. coli* blood isolate extracts induced β-galactosidase activity resulting in a blue/green zone surrounding the sample (Figure 2.3.2*a*), whereas the extracts from the *E. coli* laboratory strains did not induce β-galactosidase activity. Importantly, extracts of several batches of sterile unconditioned A-MOPS growth media did not induce β-galactosidase activity indicating that the positive results obtained for the *E. coli* blood isolate extracts are due to bacterial conditioning of the medium. Additionally, no blue/green zones were observed on the duplicate control plates which lacked the reporter strain (Figure 2.3.2*b*). These results indicate that the X-GAL cleavage zones observed on the reporter plates were due to the induction of the *lacZ* fusion of the reporter strain rather than any β-galactosidase produced and secreted by the *E. coli* isolates. The *E. coli* blood isolate UL51 which had already been used in several pilot studies at the University of Central Lancashire, Preston, UK (unpublished data), was selected for further analysis via TLC.

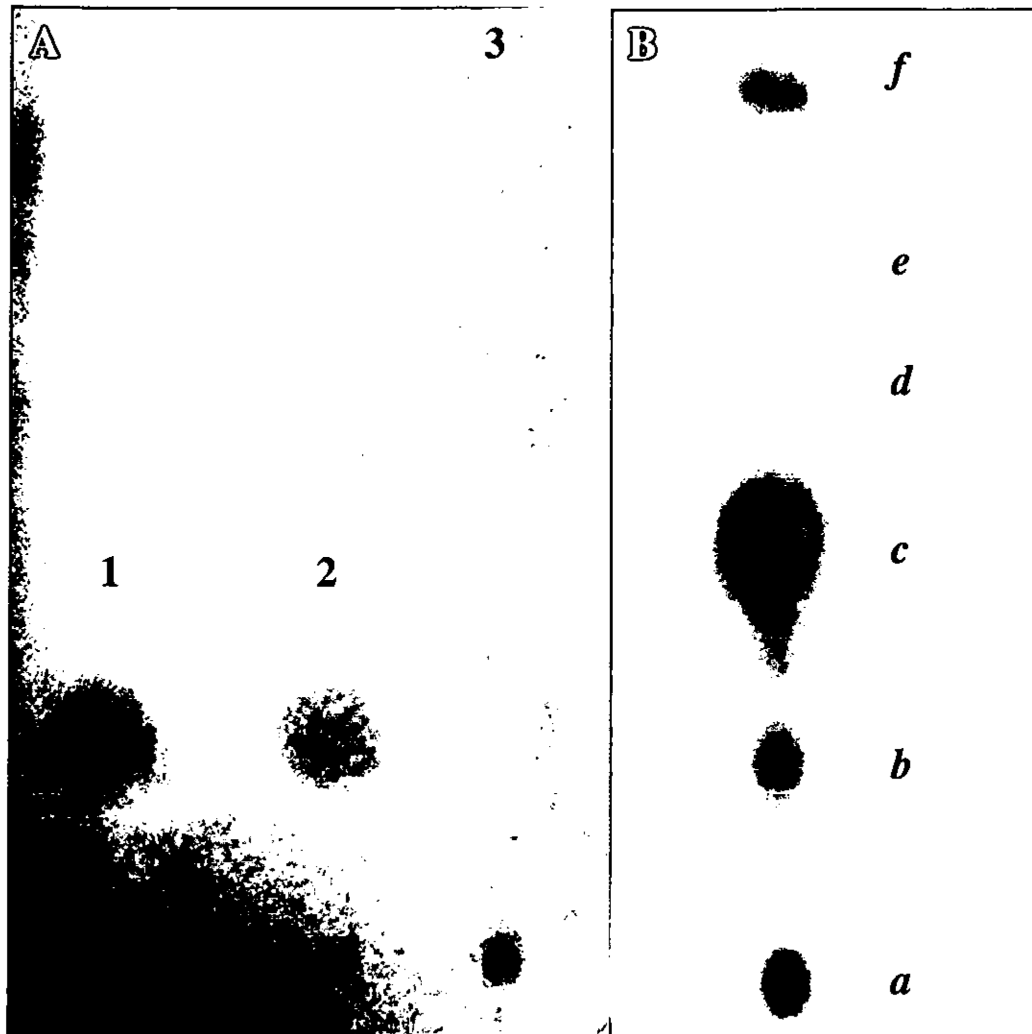


Figure 2.3.3 Analysis of ethyl acetate extracted material derived from various bacterial culture supernatants by TLC

Culture supernatants from the *E. coli* blood isolate, UL51, and the *A. tumefaciens* positive control were extracted with ethyl acetate. The extracts were then analysed by TLC, using a methanol/water (30:20 v/v) development system. Active components were visualised with the *Agrobacterium* plate bioassay reporter strain, UL34. **(A)** Tracks **1** and **2** are duplicate extracts from the *E. coli* blood isolate UL51; Track **3** is an extract from the positive control, UL33. **(B)** R*f* values calculated for the active components derived from the *A. tumefaciens* positive control, UL33, extract: ***a***, 0.04; ***b***, 0.25; ***c***, 0.51; ***d***, 0.64; ***e***, 0.75; ***f***, 0.91.

2.3.2 TLC analysis of active extracts

Once developed on the reverse-phase TLC plate, the ethyl acetate derived culture supernatant extracts from both the *A. tumefaciens* positive control, UL33 and *E. coli* blood isolate, UL51, induced β-galactosidase activity in the *A. tumefaciens* reporter strain, UL34. One diffuse blue/green zone was produced by the extracts derived from the *E. coli* blood isolate, UL51 culture suggesting that under the growth conditions used (2.2.4), the *E. coli* blood isolate, UL51 produced at least one component capable of inducing β-galactosidase activity in the *A. tumefaciens* reporter strain, UL34, (Figure 2.3.3 A). Several distinct zones were produced by the *A. tumefaciens* positive control, UL33, extract (Figure 2.3.3 B).

The Retention factor (R*f*), is defined as the maximum distance travelled by each active component, divided by the distance travelled by the solvent. The R*f* was calculated for all the UL33 and *E. coli* UL51 active fractions. The duplicate UL51 extracts both contained one active component with an R*f* value of 0.25 (Figure 2.3.3 A; spots 1 and 2). The multiple active components produced by the UL33 extract, demonstrated a wide range of R*f* values, one of which (***b***) was comparable with the UL51 R*f* value of 0.25 (Figure 2.3.3 B).

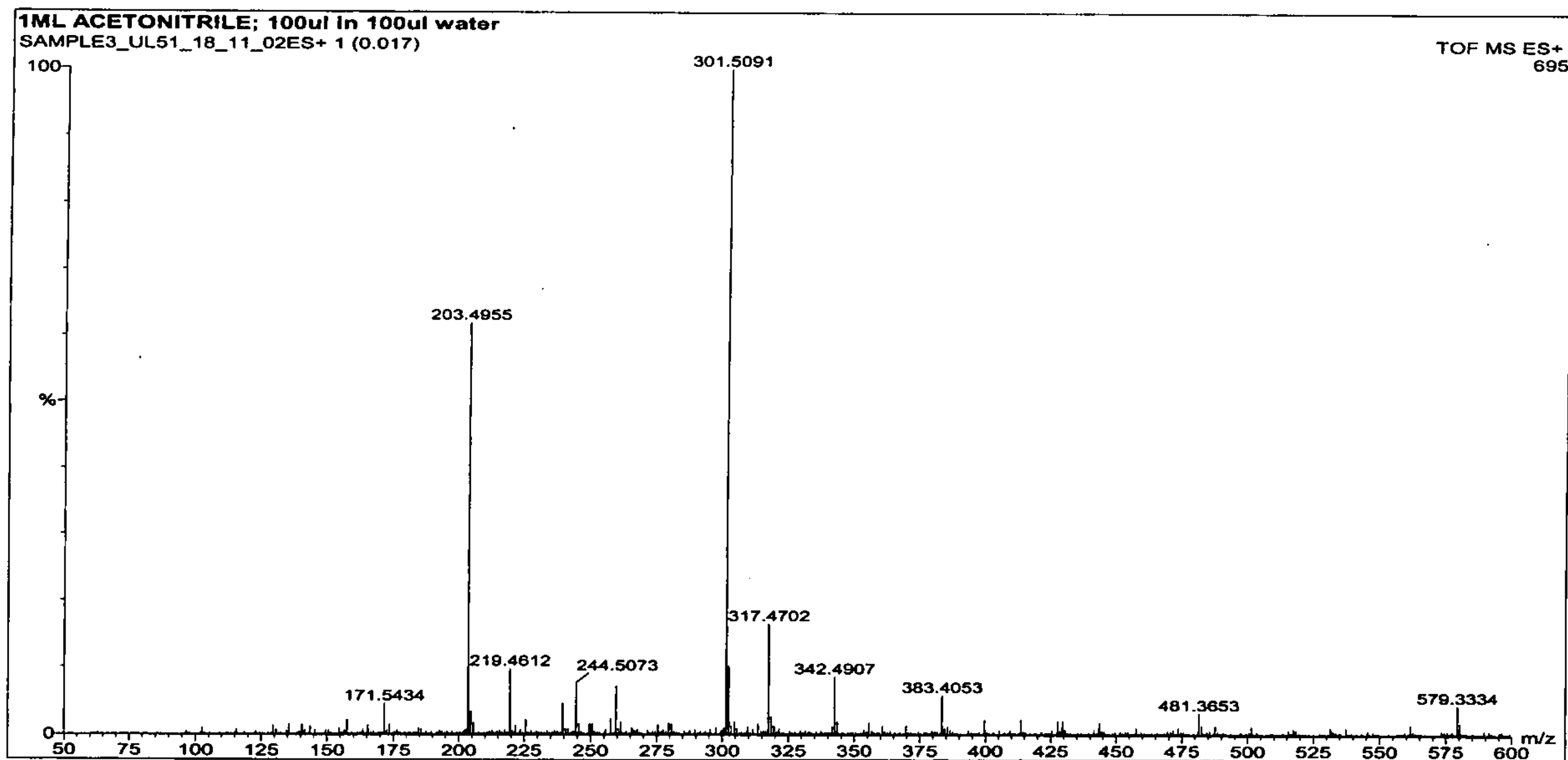


Figure 2.3.4 Analysis of ethyl acetate extracted material derived from culture supernatants of the *E. coli* blood isolate by LC/TOF-MS

The preliminary mass spectrum for the *E. coli* blood isolate UL51 extract indicates 2 substantial peaks, a peak of specific interest at 203.4955 m/z and one at 301.5091 m/z, a silica artefact from the TLC separation technique.

2.3.3 LC/TOF-MS

Mass spectroscopic analysis was performed for the TLC separated active components from the *A. tumefaciens* positive control, UL33, and the *E. coli* blood isolate, UL51, ethyl acetate extracts and the following synthetic standards: *N*-butyryl-L-homoserine lactone; hexanoyl-homoserine lactone; octanoyl-homoserine lactone; 5-methyl-4-hydroxy-3(2H)-furanone. The preliminary spectrum for the *E. coli* blood isolate UL51 extract (Figure 2.3.4) indicates two substantial peaks, a peak of specific interest at 203.4955 m/z and one at 301.5091 m/z, a silica artefact from the TLC separation technique. However, further analysis of this active fraction has not been possible as it was not possible to isolate further samples of the active fraction.

2.3.4 Reproducibility of the ethyl acetate extraction procedure

Despite the initial positive results for several of the extracts derived from the *E. coli* blood isolate culture supernatants, subsequent attempts at further replica extractions using ethyl acetate failed to isolate any components from the culture supernatants capable of inducing β-galactosidase activity in the *Agrobacterium* reporter strain, UL34.

2.3.4.1 Additional extraction method

As previously stated, the subsequent attempts to isolate fractions by ethyl acetate extraction, that induced β-galactosidase activity in the *Agrobacterium* plate bioassay, were unsuccessful. Thus replica supernatants were also extracted with the alternative solvent dichloromethane. These dichloromethane-produced extracts also failed to induce β-galactosidase activity in the *Agrobacterium* plate bioassay reporter strain,

UL34, whereas extracts from the *A. tumefaciens* positive control, UL33, produced with either solvent, induced β-galactosidase activity.

2.3.4.2 Effect of cell lysis on the release of an active fraction capable of inducing β-galactosidase activity

Both the organic solvent extraction methods have failed to consistently produce an active fraction from the *E. coli* blood isolates. One possible reason for this lack of reproducibility is that there may be a variation in the release of the active component from the bacterial cells into the supernatant. Thus cells from various *E. coli* cultures were lysed using a French Press. Neither the extracts of the lysed cells nor the extracts of the supernatants for the various replica *E. coli* cultures induced β-galactosidase activity in the *Agrobacterium* plate bioassay reporter strain, UL34.

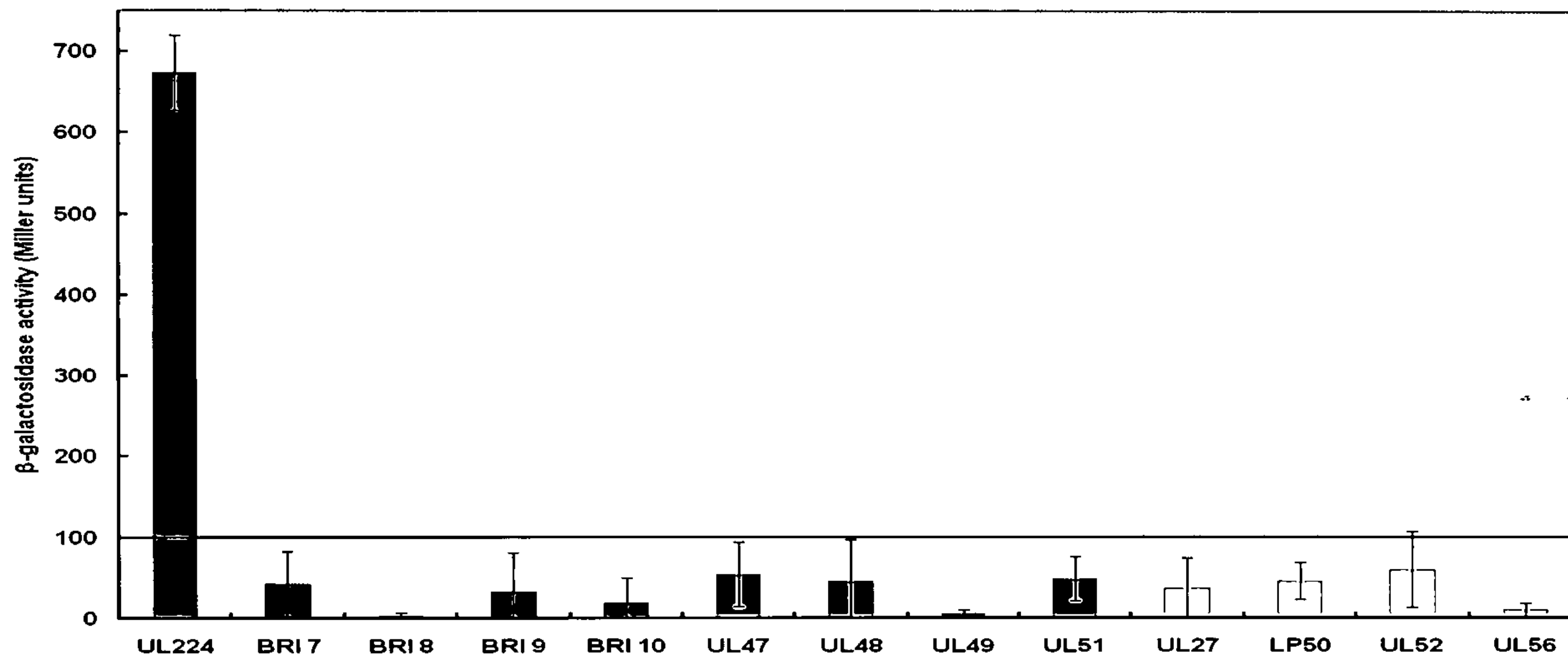


Figure 2.3.5 ***Agrobacterium*** **liquid culture bioassay of** ***E. coli*** **isolate culture supernatant extracts**

β-galactosidase activity was induced by extracts derived from the *A. tumefaciens* positive control (green) and various *E. coli* strains/isolates: urine isolates (blue); blood isolates (orange); laboratory strains (yellow). The base line at 100 Miller units indicates the considered level of importance for this study. Data are mean ± standard deviation, (n = 3).

2.3.5 Application of the ultrasensitive *Agrobacterium* liquid culture bioassay

A. tumefaciens UL225, the reporter strain used for the *Agrobacterium* liquid culture bioassay (2.2.6.3), is highly sensitive to a wide variety of AHLs, some at extremely low concentrations (Zhu *et al.*, 2003). Therefore this highly sensitive bioassay system was used to determine the presence of active components in the ethyl acetate produced extracts from the replica *E. coli* cultures.

The *E. coli* blood isolate UL47, UL48 and UL51 extracts and *E. coli* laboratory isolate LP50 and UL52 extracts induced very low levels of β-galactosidase activity in the ultrasensitive *A. tumefaciens* reporter strain, UL225 (Figure 2.3.5). The *E. coli* urine isolate, *E. coli* blood isolate UL49 and *E. coli* laboratory strain UL27 and UL56 extracts did not induce β-galactosidase activity (Figure 2.3.5). Extracts of several batches of sterile A-MOPS growth medium, produced with ethyl acetate, induced negligible levels of β-galactosidase activity (data not presented). As the levels of β-galactosidase activity induction for all the *E. coli* isolate extracts tested were below the base line of considered importance no further attempts were made to isolate the active component.

2.4 DISCUSSION

2.4.1 Screening various *E. coli* strains and isolates for AHL production

Each bioassay reporter strain varies in detection sensitivity to different AHL acyl chain lengths and carbon 3 substitutions. Therefore the use of multiple bioassay reporter strains with different sensitivities and specificities is highly recommended when screening for novel QS molecules and systems (Shaw *et al.*, 1997). According to Cha *et al.* (1998), the *Agrobacterium* plate bioassay is superior in both sensitivity and specificity when compared with the *Chromobacterium* plate bioassay. However, the *Agrobacterium* plate bioassay is not particularly sensitive to short chain AHLs whereas, the *Chromobacterium* plate bioassay is (Cha *et al.*, 1998). Therefore these two different bioassay strains were used in tandem for the initial screening process.

2.4.1.1 *Chromobacterium* plate bioassay

None of the *E. coli* culture supernatant extracts tested induced violacein synthesis in the *C. violaceum* reporter strain, LP41, suggesting that they do not contain any exogenous putative short chain AHLs compatible with this reporter strain. However, as LP41 cannot detect any of the 3-hydroxy derivatives and lacks sensitivity to most of the 3-oxo derivatives, this result does not exclude the possibility of the presence of other types of AHL molecules (McClean *et al.*, 1997).

2.4.1.2 *Agrobacterium* plate bioassay

All of the culture supernatant extracts from the *E. coli* blood isolates (UL47, UL48, UL49 and UL51) induced production of the *lacZ* fusion product by the *A. tumefaciens* reporter strain, UL34, suggesting the presence of an exogenous putative AHL in these extracts. As the extracts of samples taken from several batches of sterile A-MOPS medium did not induce β-galactosidase activity, it is possible to conclude that the

positive results for the *E. coli* blood isolate extracts are due to the bacterium conditioning the growth medium by producing and secreting an active compound.

Interestingly, none of the culture supernatant extracts from the *E. coli* laboratory isolates (UL27, LP50, UL52 and UL56) induced β-galactosidase activity, indicating that under the culture conditions used in this study, these laboratory *E. coli* strains do not produce any active components that activate this bioassay reporter strain. Many laboratory strains are essentially non-pathogenic and they have been sub-cultured for many decades in standardised and idealised laboratory conditions. Fux *et al.* (2005) proposed that the striking differences of *in vitro* monocultures compared with *in vivo* ecosystems may lead to laboratory adaptation and the possible loss of important pathophysiological characteristics within laboratory reference strains.

It is therefore not surprising that the laboratory strains and the blood isolates differ in their active component producing capabilities as it is possible that the laboratory isolates have lost the ability to produce the active compound over many successive laboratory passages.

2.4.2 TLC analysis of active extracts

According to Shaw *et al.* (1997), the TLC migration of each AHL is dependent upon the acyl chain length and carbon 3 substitution. Each active component will have a particular R*f* value representing a different AHL. Therefore comparing the R*f* values for the extract derived from the *E. coli* blood isolate culture supernatant, UL51, extract against the *A. tumefaciens* positive control, UL33, extract values (Figure 2.3.3 B (*b*)), suggests that under the particular growth conditions used the two bacterial species may be producing the same AHL (2.2.4; Figure 2.3.3). However, this is only

conjecture and it is not possible to conclude that the active compound is an AHL from these results alone, as although these bioassays are specifically designed to detect AHLs, DKPs have also been shown to activate similar TraR-based bioassays in a concentration-dependent manner (Holden *et al.*, 1999). Therefore despite the acyl chain length and carbon 3 substitution affecting the R*f* value, the R*f* value alone is not sufficient for structural identification and the active compound must be separated and chemically characterised (Holden *et al.*, 1999; Holden *et al.*, 2000).

2.4.3 LC/TOF-MS

According to Brelles-Mariño and Bedmar, (2001) mass spectroscopy is a valuable technique for the identification of chemical structures of QS signalling molecules in that it can detect even picomoles of sample. As previously stated TOF analysers use an electric field to accelerate a group of ions of differing mass/charge ratios (*m/z*). When the particles all have the same charge, their kinetic energies are identical and their velocities will depend only on their mass/charge ratio. LC/TOF-MS was employed for the structural identification of the active compound isolated from the *E. coli* blood isolate, UL51, culture supernatant extract. A possible peak of interest was identified with a mass/charge ratio of 203.4955 *m/z*, (Figure 2.3.2). This may pertain to the singly charged (protonated) molecular-related ion $(M + H)^+$ of the active component isolated from the *E. coli* blood isolate UL51 culture supernatant extract. Therefore as AHL are organic compounds, the odd mass/charge ratio of the protonated molecular-related ion suggests that the compound isolated contains an even number of nitrogen atoms and therefore it is unlikely to be an AHL, but could possibly be a DKP. However, the failure to isolate further active samples has prohibited investigation of this possibility.

2.4.4 Reproducibility of the ethyl acetate extraction procedure

Although all the media constituents and the incubation conditions were kept constant throughout this study, two different bottles of ethyl acetate were used for the extraction process (both from Sigma, HPLC grade). It is important to note that the initial *E. coli* blood isolate supernatant extracts that induced β-galactosidase activity in the *Agrobacterium* plate bioassay, were produced with the original bottle of ethyl acetate. It is unlikely that these results were due to contamination of that bottle of ethyl acetate, as that same bottle of ethyl acetate was used to produce extracts from the negative control, *A. tumefaciens* UL33, and the *E. coli* laboratory isolates, which all failed to induce β-galactosidase activity in the *Agrobacterium* plate bioassay. However, to ensure that the change in ethyl acetate was not affecting the extraction procedure and causing the lack of reproducibility in the results, dichloromethane, which has been used by many researchers to isolate QS signalling molecules including AHLs and DKPs, was used as an alternative extraction solvent (McClean *et al.* 1997; Holden *et al.*, 1999; Yates *et al.*, 2002).

None of the dichloromethane or the new ethyl acetate extracted supernatants from any of the replica *E. coli* cultures induced β-galactosidase activity in the *Agrobacterium* plate bioassay reporter strain, UL34. However, analogous extracts derived from the culture supernatants of the *A. tumefaciens* positive control, UL33, isolated with either solvent, induced β-galactosidase activity in the reporter strain, indicating that the inconsistent results and failure to produce additional active AHL-like fractions were unlikely to be due to problems with the extraction procedure.

2.4.5 Effect of cell lysis on the release of an active fraction capable of inducing β-galactosidase activity

As both the organic solvent extraction methods failed to consistently produce an active fraction from the *E. coli* blood isolates, a French press was used to lyse the bacterial cells by applied pressure. This allowed both the pressed cells and the spent media to be extracted and assessed for activity (2.2.4.1; 2.2.6.2). This method was adapted from Yates *et al.* (2002) who used it to disrupt *P. aeruginosa* cells when preparing cell extracts to research lactonolysis of AHLs. Both the extracts for the pressed cells and the spent media from the replica *E. coli* cultures, failed to induce β-galactosidase activity in the *Agrobacterium* plate bioassay reporter strain, UL34, demonstrating that the putative signalling molecule was not being discarded with the bacterial cell pellet prior to solvent extraction. Thus, this eliminated the possibility, that the lack of reproducibility was a result of variation in the release of the active component from the bacterial cells.

2.4.6 *Agrobacterium* liquid culture bioassay

According to Zhu *et al.* (2003), UL225 is highly sensitive to a wide variety of AHLs, some at extremely low concentrations and it is therefore ideal for the detection of novel AHL mediated cell-cell signalling systems. Due to this sensitivity, the very low/negligible levels of β-galactosidase activity induced by some of the *E. coli* supernatant extracts, indicate that very low/negligible levels of the active compound are being produced by these isolates.

Although the initial *Agrobacterium* plate bioassay results for the *E. coli* blood isolate culture supernatant extracts indicated the presence of an active component within the derived extracts, several bioassays, of various sensitivities for a wide range of AHLs,

have not detected this active component in the extracts produced from subsequent cultures. These results suggest that under the stated conditions this active component is no longer being produced by these blood isolates.

As previously mentioned the sub-culture of bacteria in standardised and idealised laboratory conditions may lead to laboratory adaptation and possibly the loss of important pathophysiological characteristics (Fux *et al.*, 2005). It is therefore possible that the loss of active molecule production by the *E. coli* blood isolates may be due to laboratory conditioning (2.4.1.2).

To test this theory new clinical *E. coli* isolates were obtained. However, in the time span of this study the only new clinical *E. coli* isolates available were urine isolates and therefore were not directly comparable with the *E. coli* blood isolates previously examined. None of the new clinical *E. coli* isolate extracts were able to induce violacein production in the *Chromobacterium* plate bioassay or β-galactosidase activity in the two *A. tumefaciens* bioassays. As previously stated, the UPEC CFT073 genome is particularly rich in genes that encode potential fimbrial adhesins, autotransporters and iron-sequestration systems (Welch *et al.*, 2002). It is therefore potentially very different to that of SEPEC. Thus the negative results for the *E. coli* urine isolate extracts do not indicate whether the original *E. coli* blood isolates used in this study have lost the ability to produce the active compound. However, they do demonstrate that these particular UPEC isolates cannot produce any components capable of inducing violacein production in the *Chromobacterium* plate bioassay or β-galactosidase activity as detected by the two *A. tumefaciens* bioassays used.

2.4.7 Discussion summary

- Extracts derived from all of the *E. coli* culture supernatants tested, were unable to induce violacein synthesis in the reporter strain, LP41, suggesting that they do not contain any exogenous putative AHL compatible with this reporter strain.
- Initial results from the *Agrobacterium* plate bioassay indicated that all the extracts derived from the *E. coli* blood isolate (UL47, UL48, UL49 and UL51) culture supernatants induced β-galactosidase activity, suggesting the presence of an active component in these extracts.
- Extracts derived from the *E. coli* laboratory isolate culture supernatants were unable to induce β-galactosidase activity, indicating that under the culture conditions used in this study, these laboratory *E. coli* strains do not produce any molecules that activate the *Agrobacterium* plate bioassay reporter strain.
- TLC analysis indicated that the *E. coli* blood isolate UL51 was producing one type of active compound that could be detected by the *A. tumefaciens* bioassay plate reporter strain, UL34.
- R*f* values from the TLC analyses of the *E. coli* UL51 active extract and the *Agrobacterium* plate bioassay positive control, UL33, active extract (Figure 2.3.3 B (*b*)) were comparable suggesting that under the conditions used in this study, both *A. tumefaciens* UL33 and *E. coli* UL51 may be producing the same AHL (Figure 2.3.3).
- Mass spectroscopic analysis of the *E. coli* UL51 active extract produced a possible peak of interest with a mass/charge ratio of 203.4955 m/z, (Figure 2.3.4) suggesting that the compound isolated contains an even number of nitrogen atoms and therefore is unlikely be an AHL.

- Due to reproducibility issues it was not possible to obtain further analysis of this active component.
- Negative results were obtained for the *E. coli* culture supernatant extracts produced with the new batch of ethyl acetate and the alternative solvent, dichloromethane. It is therefore unlikely that the reproducibility problems were linked to the use of a new batch of ethyl acetate.
- A French press was used to lyse the *E. coli* cultures, releasing the cells' contents. Extracts derived from the lysed cells and the spent culture media, failed to induce β-galactosidase activity in the *Agrobacterium* plate bioassay reporter strain, UL34. It is therefore unlikely that the lack of reproducibility was a result of variation in the release of the active compound from the bacterial cells.
- The negligible levels of β-galactosidase activity induction detected by the highly sensitive *Agrobacterium* liquid culture bioassay for all the *E. coli* isolate derived extracts, indicated that under the stated culture conditions none of the *E. coli* laboratory, blood or urine isolates were producing any active components.
- These results indicate that the *E. coli* blood isolates may have adapted to the laboratory conditions and lost the ability to produce the active compound detected in the previous pilot studies and the initial preliminary screen of this study (unpublished data; 2.4.1.2).

Due to the aforementioned problems encountered with the *E. coli* isolates and to maintain the focus on foodborne pathogens the rest of this study will concentrate on putative signalling molecule production by *C. jejuni*.

CHAPTER 3

Campylobacter jejuni

3.1 INTRODUCTION

3.1.1 Morphology and metabolism

Campylobacter jejuni from the delta-epsilon group of proteobacteria, is a Gram-negative, non-spore forming, chemoorganotroph that does not oxidise or ferment carbohydrates and has a propensity to invade the intestinal mucosa. Cells are typically highly motile, slender rods, with comma, S, or "gull-wing" shapes. Their characteristic corkscrew-like motion is due to the single polar flagellum (occasionally multiple) at one or both cell poles (Franco and Williams, 2001; van Vliet and Ketley, 2001).

Members of the genus *Campylobacter* are strict microaerophiles, exhibiting oxygen dependent growth, with oxygen being the final electron acceptor in the respiration process. However, they do not tolerate a normal atmospheric oxygen concentration (21 % v/v) (Barros-Velázquez *et al.*, 1999). Energy is obtained through the respiratory chain, from the metabolism of amino acids, and from the metabolism of intermediates of the tricarboxylic acid cycle. Furthermore, thermotolerant microorganisms do not produce indole, are oxidase and catalase positive and methyl red and Voges-Proskauer negative (Barros-Velázquez *et al.*, 1999).

Campylobacter spp. are generally considered to be relatively fragile, and sensitive to environmental stresses, such as drying, heating, disinfectants and acidic conditions, with pH 6-8 being the optimum range for survival. Thermal treatment for more than 15 min at 60°C or 30 min at 57.5°C destroys these microorganisms (Barros-Velázquez *et al.*, 1999). Epidemiological studies have indicated that the digestive tract of several breeding animals, poultry in particular, represent the natural habitat of this organism. The release of the organism into the environment presents it with a series of stressful conditions, such as nutrient starvation, osmotic stress and temperature

variation, forcing the bacterium into active survival strategies, including entering the coccoid, viable but non-culturable (VBNC) state (Korhonen and Martikainen, 1991; Mason, *et al.* 1996; Baffone *et al.*, 2006).

The pathogenic potential of the coccoid/VBNC state of *C. jejuni* has yet to be proven. Moran and Upton (1986; 1987) concluded that the coccoid/VBNC form of *C. jejuni* is a degenerative form, and yet, other researchers have claimed recovery of the organism from the coccoid form by passage through various rodent models. However, this recovery has not yet been demonstrated for passage through the digestive tract of humans or chicks (Hazeleger *et al.*, 1994). Therefore, the role of this morphological change, specifically with regard to pathogenicity, and the possible formation of the VBNC state, remains uncertain (Barros-Velázquez *et al.*, 1999).

3.1.2 Isolation and culture

The isolation and identification of *C. jejuni* is primarily reliant upon culture characteristics. This thermotolerant microorganism grows well at 42-43°C however, growth also occurs at 37°C, but not below 30°C or above 47°C. For isolation of this organism from complex samples such as faeces, or raw meat, where a high microbial load of other bacteria is probable, 42°C is favoured for its selectivity (Barros-Velázquez *et al.*, 1999). Incubation of the organisms at 42 °C on selective media, such as Skirrow's medium, which contains the antibiotics vancomycin, polymyxin B, and trimethoprim at 42°C, can be used to differentiate *C. jejuni* from other *Campylobacter* spp. that would be isolated at 37°C. Colonies are usually colourless or grey with a metallic sheen, and may be watery and spreading or round and convex (Brookes *et al.*, 2001).

Microaerophilic bacteria are unusual in that they require oxygen for growth, yet are poisoned by the levels of oxygen present in air. This toxicity is a result of the increased sensitivity of microaerophilic bacteria, when compared with oxygen-dependent bacteria, to toxic forms of oxygen (including superoxide anions and peroxide) that form in the culture medium (Hoffman *et al.*, 1979). Complex culture media contain various amino acids and flavins which are photosensitive and 'could' generate superoxide anions and hydrogen peroxide. The reduction of oxygen during autooxidation in the environment generates a variety of toxic oxygen derivatives including superoxide anions, hydroxyl radicals, singlet oxygen and hydrogen peroxide (Hoffman *et al.*, 1979). Therefore to counteract this toxic effect, media designed for the culture of microaerophilic bacteria must contain compounds, such as sodium metabisulphite and sodium pyruvate, that are capable of quenching these toxic oxygen derivatives.

Optimal culture conditions for the isolation of *C. jejuni* from environmental samples include a reduced oxygen atmosphere (5 % (v/v) O_2), with added carbon dioxide (10 % (v/v) CO_2). This is usually achieved by commercial kits such as the CampyGen atmospheric generation system (Oxoid) which provides a selective microaerophilic atmosphere introduced into an anaerobic jar system (Barros-Velázquez *et al.*, 1999; Nachamkin *et al.*, 2000).

Bolton Broth (BB, Oxoid) is a liquid medium designed to aid recovery of injured cells and to avoid the need for a microaerobic atmosphere via the inclusion of haemin, sodium metabisulphite and sodium pyruvate which are known to act as oxygen quenchers (Bolton *et al.*, 1984*a*; Corry *et al.*, 1995; Humphrey, 1990). Therefore, when incubated in a small vessel with a reduced headspace, *Campylobacter* can be

cultured in this medium without the need for a microaerobic atmosphere (Baylis *et al.*, 2000). BB also contains sodium carbonate which may prevent acidification and/or provide a source of carbon dioxide (Corry *et al.*, 1995).

Blood is frequently used as a supplement for *Campylobacter* media, due to its ability to neutralize hydrogen peroxide and quench/detoxify other toxic oxygen products (Bolton *et al.*, 1984*b*). BB can be used without the inclusion of horse blood, offering greater convenience to the user. However, although the oxygen quenching agents included in the base medium should counteract the toxic effects of oxygen, it is currently unclear whether this is sufficient, or if the omission of blood causes problems with oxygen quenching and therefore affects the performance of the medium (Baylis *et al.*, 2000).

Several enrichment broths specifically designed for the isolation and culture of *Campylobacter* spp. from foods are now available commercially. As with other foodborne pathogens, the isolation of *Campylobacter* spp. from food can be problematic as the pathogen is likely to be present at low levels and accompanied by high levels of competitor organisms. *Campylobacter* enrichment broths and selective media usually suppress competing organisms via antibiotic supplements (Baylis *et al.*, 2000). Unfortunately these antibiotics can also be inhibitory to sub-lethally injured cells, which exhibit greater sensitivity to such conditions (Humphrey, 1986; Humphrey, 1990). Therefore it is necessary to achieve a compromise between selectivity of the medium, via the inhibition of competitor organisms and the recovery and growth of the target organism, for example via a pre-enrichment at 37°C prior to the addition of the antibiotic supplements (Humphrey, 1986; Humphrey, 1990). For example, when using BB to isolate *Campylobacter* spp. from environmental samples

it is advisable to use the antibiotic selective supplement designed to complement this media which contains cefoperazone, vancomycin, trimethoprim and cycloheximide. However, when using pure *C. jejuni* cultures the addition of the antibiotic selective supplement is not required.

3.1.3 History

In 1886 Theodor Escherich published a series of papers in the *Münchener Medizinishe Wochenschrift* describing a spiral bacterium that he had observed in the colons of children that had died from 'cholera infantum' and in stool samples from several infants suffering from enteric disease. Unfortunately these articles, published in German, remained unrecognised for many decades until Kist reported Escherich's findings at the Third international *Campylobacter* Workshop in Ottawa in 1985 (Kist, 1985). According to Kist, Escherich was unable to culture this organism on solid agar and so concluded that the bacterium played no aetiological role and its presence was prognostic rather than causative (Kist, 1985). Despite being well-documented as an animal pathogen and this early work by Escherich and several other German researchers, *Campylobacter* was not recognised as a human pathogen for many decades.

Originally classified as a *Vibrio*, *Campylobacter* was only initially associated with human disease when it was isolated from blood samples of children with diarrhoea, by King (1957), who referred to it as a 'related vibrio' distinguishing it from the classical Vibrios by its higher optimum growth temperature of 42°C. In 1963, Sebald and Véron, proposed the removal of King's 'related vibrios' from the *Vibrio* genus and the

introduction of a new genus, *Campylobacter*, from the Greek *campylo* (curve-shaped) and *bacter* (rod) (Sebald and Véron, 1963; Véron and Chatelain, 1973).

However, it was the isolation of Campylobacters from faeces by Dekeyser and Butzler's research groups (Dekeyser *et al.*, 1972; Butzler *et al.*, 1973) and the subsequent development of a selective medium by Skirrow (1977), that led to routine stool sample testing in laboratories and the first full account of *Campylobacter* enteritis in humans (Butzler and Skirrow, 1979). By the late 1980s, *Campylobacter* was established as a common human pathogen and is now considered a leading cause of bacterial gastroenteritis.

3.1.4 Pathogenesis/virulence factors

The mechanism of pathogenesis employed by *C. jejuni*, including the induction of diarrhoea, is as yet undetermined (Park, 2005). Numerous studies have resulted in conflicting data and theories, indicating that pathogenesis is multifactorial and that many pathogenic mechanisms may yet remain unidentified. Potential virulence properties that have been identified include cell attachment and colonisation, invasiveness, motility and toxin production (Wassenaar, 1997).

Motility is required to enable the bacterium to reach the attachment sites and for subsequent penetration into intestinal cells. The combination of the polar flagellum and 'cork-screw' cell shape (see 3.1.1) are thought to confer an unusually high level of motility in viscous environments such as the mucus that overlays the intestinal epithelium (Ketley, 1997).

Adhesion of the bacterial cell to the epithelial surface may be an important but not crucial determinant for colonization and may enable the accumulation of secreted

bacterial products (Ketley, 1997; Wassenaar, 1997). Following adhesion, a sub-population of *C. jejuni* invades the epithelial cells, leading to mucosal damage and inflammation, characteristic of *Campylobacter* infection (van Vliet and Ketley, 2001). However, the roles of these virulence properties in human disease, and the importance of strain variation and host factors such as immune status, are still enigmatic (Ketley, 1997).

There have been numerous studies of toxin production by *C. jejuni*, which have reported the production of several toxins, including a cytolethal distending toxin (CDT), a cholera toxin-like enterotoxin (CTLT) and a number of cytotoxins. Although their role in human disease remains uncertain, current belief is that CTLT may contribute to watery diarrhoea and cytotoxins to inflammatory diarrhoea (Ketley, 1997; Wassenaar, 1997).

Despite numerous reports concluding that at least some *C. jejuni* strains can produce enterotoxic activity, the variation in methods employed and the strains used in the studies make any generalisations from them unreliable (Wassenaar, 1997). Current *C. jejuni* toxin research is focused on an exotoxin that is a member of the CDT family of toxins which affect the epithelial cell layer (Johnson and Loir, 1988; Parkhill *et al.*, 2000; Pickett and Lee, 2005). CDT is encoded by three adjacent genes, termed *cdtA*, *cdtB* and *cdtC* and appears to be toxic to many cell lines including HeLa, Chinese hamster ovary and Vero cells causing them to slowly distend and die (Pickett *et al.*, 1996; Pickett and Lee, 2005). However, the lack of animal models that reliably mimic *C. jejuni* diarrhoeal disease has impeded research into possible roles for CDT in human disease (Pickett and Lee, 2005). Only the gene homologous to the *cdt* gene was found in the *C. jejuni* NCTC 11168 genome sequence and therefore great debate

remains with regard to the existence of a *C. jejuni* CTLT in other *C. jejuni* strains (Parkhill *et al.*, 2000 and Pickett, 2000).

3.1.5 Infection

Campylobacter is the primary source of sporadic bacterial gastroenteritis in the majority of the developed world, including the UK and the Republic of Ireland, with an estimated 2-3 million *Campylobacter*-related illnesses per year in the United States alone (Miller and Mandrell, 2005). Large outbreaks are generally considered rare, however, as clinical isolates of *Campylobacter* are not usually identified beyond genus level or serotyped it is therefore possible that many outbreaks go undetected (Pearson and Healing, 1992). Of those that are identified, approximately 90 % are associated with *C. jejuni* (Pearson and Healing, 1992; Tam *et al.*, 2003).

The majority of outbreaks that are detected are small and confined to one family. In most cases, the suspect food/vehicle will have been consumed in its entirety, and there is often little opportunity to establish microbiologically the source of infection and the route of transmission (Pearson and Healing, 1992). Untreated surface water and raw (unpasteurised) milk have previously been identified as common causes of outbreaks involving members of more than one family, as with the large outbreak at the Glastonbury Music Festival in 1992, which yielded 72 laboratory confirmed *Campylobacter* cases. The source of which was identified as the consumption of raw milk purchased from a local farmer (Anonymous, 1992).

3.1.5.1 Seasonality

Campylobacter infections in humans in temperate countries follow a striking seasonal pattern (Nylen *et al.*, 2002). Countries with milder winters have been shown to have peaks of infection early in the year, with the time of the peak being weakly associated with high temperatures 3 months previously (Kovats *et al.*, 2005). This suggests that climate may be a contributory factor to *Campylobacter* transmission, linking the seasonal increase with environmental factors as opposed to a food source. However, the main determinant of seasonality in *Campylobacter* infection levels and the main source of human infection remains to be identified (Louis *et al.*, 2005). It is important to note that the aforementioned studies all considered *Campylobacter* infection at the species level. The application of advanced genotyping methods (see 3.1.6) to seasonality studies may enable the identification of separate seasonal profiles with individual infection peaks for specific clonal complexes and therefore indicate potential infection sources.

3.1.5.2 Reservoirs and potential infection sources

Direct person-to-person transmission of *C. jejuni* infection between adults appears to be uncommon, although this is dependent upon the personal hygiene of individual parties (Pearson and Healing, 1992; Ekdahl *et al.*, 2005). Due to its uniquely fastidious growth requirements *Campylobacter* does not easily grow on food, but the critical infective dose of *Campylobacter* appears to be low, with doses as low as 500 bacteria resulting in human infection (Skirrow and Blaser, 2000). Many researchers speculate that this infective dose may be decreased further by the transmission vehicle, for example milk exerting a buffering action and therefore protecting the

bacteria from the gastric acid encountered in the human stomach (Skirrow and Blaser, 2000).

3.1.5.2.1 Environmental reservoirs

Campylobacter is regularly isolated from the gastrointestinal tract of birds (especially poultry) and cattle. Historically this natural locale has been considered an important potential reservoir for human infections and as an entry point into the food chain. For this reason, raw or undercooked meat (especially poultry) has often been epidemiologically associated with human disease. Thermophilic strains of *Campylobacter* are ubiquitous in the environment. Marine and fresh water, including streams, canals, ponds and ornamental lakes are regularly contaminated with animal and/or avian faeces from either direct or indirect sources, including agricultural run-off, sewage effluent and faeces deposition from large wildfowl populations (Jones, 2001). Other potential sources include unpasteurised milk, 'bird-pecked milk' (on doorsteps), untreated water, domestic pets and person-to-person contact if personal hygiene is poor (Franco and Williams, 2001; Jacobs-Reitsma, 2000). Although pet ownership has also been demonstrated to be a significant risk factor for *Campylobacter* infections in humans, and direct transmission of *C. jejuni* between humans and dogs has been demonstrated, its importance as an infection source remains unclear (Wolfs *et al.*, 2001; Damborg *et al.*, 2004).

Ekdahl *et al.* (2005), recently proposed that flies play an important role in the transmission of *Campylobacter*, suggesting that they could act as mechanical vectors carrying the bacteria on their bodies and glandular hairs on their feet. It was also suggested that flies could play a linking role, transmitting *Campylobacter* from

animals to human food. However, all the evidence so far presented in support of this hypothesis, including that of the seasonal summer increase in infection rates, is circumstantial and actual experimental investigation is required (Ekdahl *et al.*, 2005).

3.1.5.2.2 Non-environmental reservoirs

According to the Public Health Laboratory Service (PHLS), raw (unpasteurised) milk, untreated water and undercooked poultry are the most commonly reported sources of *Campylobacter* infection (Pearson and Healing, 1992). Approximately 80 % of raw chickens sold in the UK in 2001 were found to be contaminated with thermophilic *Campylobacter* spp. and studies have also demonstrated contamination of the outer wrapping of chicken purchased from retail outlets (Corry and Atabay, 2001; Humphrey *et al.*, 2001). Therefore the preparation of raw chicken may result in the widespread dissemination of *Campylobacter* spp. in the food-preparation environment and the contamination of kitchen surfaces (Humphrey *et al.*, 2001; Cogan *et al.*, 1999).

Numerous studies have highlighted the potential for cross-contamination in the kitchen, originating from raw poultry and transferring to ready-to-eat products via hands, food preparation utensils and surfaces (including chopping boards), washing-up water, sponges, cleaning cloths and even tea-towels (Hilton and Austin, 2000; Humphrey *et al.*, 2001; Cogan *et al.*, 2002; Kusumaningrum *et al.*, 2002; Kusumaningrum *et al.*, 2003; Mattick *et al.*, 2003*a*, Mattick *et al.*, 2003*b*; Cools *et al.*, 2005).

Despite being sensitive to desiccation and rendered non-culturable upon exposure to air and light, *C. jejuni* has been shown to persist on contaminated surfaces in the

inhospitable kitchen environment (Humphrey *et al.*, 1995; Kusumaningrum *et al.*, 2003; Cools *et al.*, 2005). Both Kusumaningrum *et al.* (2003) and Cools *et al.* (2005) have successfully isolated *C. jejuni* under test conditions from a variety of kitchen surfaces (stainless steel, beech wood and polypropylene) demonstrating prolonged *C. jejuni* survival ranging from 2 to 4 hours after the initial contamination. Humphrey *et al.* (1995) not only highlighted the sensitivity of *C. jejuni* to desiccation but also the prolonged viability induced by moisture, such as droplets of horse blood, provided the droplets remained liquid.

However, the survival time of *C. jejuni* on dry surfaces is dependent upon the initial loading/level of contamination and may also be affected by the surface integrity. Kusumaningrum *et al.* (2002), found cells in crevices on stainless steel surfaces and demonstrated that the porosity of a surface (for example porous wood compared with polypropylene, which is considered to be non-porous) also affected survival (Cools *et al.*, 2005). Food residues, whilst slowly drying, may also form a layer protecting the *C. jejuni* cells on the surface, resulting in prolonged survival, and an increased potential for cross-contamination and re-infection of food, presenting a considerable hazard to the consumer (Cools *et al.*, 2005).

The average UK washing-up water temperature is 48°C, and as this water often contains low levels of detergent and relatively high levels of protective/nutritional organic matter, it is conceivable that *C. jejuni* could survive a typical washing-up process (Mattick *et al.*, 2003*a*). Cogan *et al.* (2002) demonstrated that detergent-based cleaning using a typical bowl-wash routine without rinsing was insufficient to reliably and consistently restore cloths, chopping boards and hands to a hygienic state, highlighting rinsing as a critical step in achieving hygiene within the kitchen

environment. Although the isolation rate appears to be dependent upon high contamination levels, work surfaces and even freshly washed plates, may be an important vehicle in cross-contamination (Kusumaningrum *et al.*, 2003).

Considering the potential for *Campylobacter* spp. survival in the average washing-up water, it is of particular concern that frequently throughout Britain, this luke warm, dirty water from the end of the washing-up process, is used to clean kitchen counters and other surfaces, smearing them with protective/nutritional organic matter (Humphrey, 2001). This standard cleaning process, following the preparation of a high-risk product, such as chicken, has been shown to have no effect on the prevalence of contaminated sites (Humphrey, 2001). However, the use of hypochlorite disinfectant in addition to detergent and hot water cleaning may improve the cleaning procedure, decreasing the number of bacteria isolated, although this, as with all cleaning routines, is dependent upon the rigour of application by the general public (Cogan *et al.*, 1999).

3.1.5.2.3 Potential novel reservoir

In a recent study Champion *et al.* (2005), conducted whole-genome analysis of 111 *C. jejuni* variants isolated from diverse animal and environmental sources, and a range of disease outcomes and clinical presentations in humans, using a gene-specific *C. jejuni* NCTC 11168 microarray. The data produced enabled the researchers to identify two distinct clades: a livestock clade and a 'non-livestock associated clade' (Champion *et al.*, 2005). Interestingly the majority of the *C. jejuni* human isolates were found in the non-livestock clade, indicating that most *C. jejuni* infections maybe from non-

livestock (and possibly non-agricultural) sources and the presence of a previously unidentified *C. jejuni* reservoir (Champion *et al.*, 2005).

Direct microbiological evidence connecting risk factors with disease is scarce and contradictory. Therefore the precise contribution of poultry to human infection remains unclear (Hudson *et al.*, 1999; Corry and Atabay, 2001). Despite substantial research the epidemiology, including the mode of transmission of this significant risk to public health, is still poorly understood, with the majority of infections remaining unexplained by recognised risk factors (Franco and Williams, 2001). However, with the application of more sensitive sampling techniques *Campylobacter* spp. can be recovered at low levels providing a more accurate understanding of the cross-contamination potential and the infection profile associated with this pathogen. Thus research such as that conducted by Bull *et al.* (2006), aimed at identifying the sources of *Campylobacter* spp. colonising commercial poultry flocks and potentially the food chain, is vital.

3.1.5.3 *Campylobacter* enteritis

Gastroenteritis is the most common clinical syndrome associated with *C. jejuni* infection of humans (Blasser, 1997; Young and Mansfield, 2005). The typical incubation period is 2-5 days, although it has been reported to occasionally extend from 1 to 11 days (Young and Mansfield, 2005; www.hpa.org.uk). It is usually an unpleasant but self-limiting disease, characterised by profuse diarrhoea, severe cramps/abdominal pain, headache and malaise, with extreme cases developing into profuse, grossly bloody diarrhoea and fever. Diarrhoea may be acute, generally lasting for 2-3 days with patients typically having 8-10 bowel movements per day at the peak

of illness. However, dehydration is usually only a complication in the young or elderly (Young and Mansfield, 2005). Many patients suffer from nausea but only 15 % actually vomit. Other symptoms such as myalgia and fatigue have also been reported, as have variations in symptoms from person-to-person, presumably due to variations in the immune status of the host, the virulence of the infective organism and the challenge dose, all of which will also affect the duration of the illness (Skirrow and Blaser, 2000). Resolution of symptoms is usually experienced within a week, although recurrence of symptoms, especially of abdominal pain, is possible. For individuals with additional underlying immune deficiencies, extended illness is particularly common (Young and Mansfield, 2005).

Due to the self-limiting aspect of *Campylobacter* enteritis, treatment beyond fluid and electrolyte restoration is not usually required. Antimicrobial agents may eliminate the *Campylobacter* from the patient's faeces but does not reduce the duration or severity of the symptoms and are therefore only required in extreme cases (Barros-Velázquez *et al.*, 1999). Fatalities are rare and usually confined to elderly patients, the immunocompromised or those already suffering from another serious disease (Skirrow and Blaser, 2000).

3.1.5.4 Bacteraemia

Bacteraemia due to *C. jejuni* is generally considered to be rare. Although it is speculated to occur commonly as a transient event in the early stages of infection, blood cultures are rarely performed that close to disease onset and therefore the actual incidence of *C. jejuni* bacteraemia is unknown (Wang *et al.*, 1998; Skirrow and Blaser, 2000). *C. jejuni* is susceptible to the bactericidal activity of normal human

serum, which may account for the low occurrence of *C. jejuni* mediated bacteraemia, although the poor growth of *C. jejuni* in blood culture may also partly explain its infrequent detection in bacteraemia (Wang *et al.*, 1998; Skirrow and Blaser, 2000). *C. jejuni* bacteraemia has been shown to be a more common manifestation in patients over 65 years of age and in individuals that are immunocompromised including concurrent HIV infection (Wang *et al.*, 1998; Young and Mansfield, 2005).

3.1.5.5 Guillain-Barré syndrome (GBS)

Although *Campylobacter* spp. may cause severe gastrointestinal infection that may last more than a week, they do not usually cause major complications. However, reactive arthritis, Reiter's syndrome, abortion in pregnant women, rheumatic manifestations, Miller-Fisher syndrome and GBS are all associated complications (Barros-Velázquez *et al.*, 1999).

GBS is considered the most common cause of acute neuromuscular paralysis with a relatively uniform yearly incidence of 1-2/100 000 throughout the world (Hughes and Cornblath, 2005; Young and Mansfield, 2005). GBS is characterised by temporary paralysis due to an immunological attack upon peripheral nerve myelin. Symptoms vary from individual to individual, but generally begin with motor and sensory disturbance and pain in the lower limbs, progressing to the upper extremities and trunk, resulting in acute flaccid paralysis. Onset is usually gradual and paralysis is at times extreme with 20-30 % of cases requiring assisted mechanical ventilation (Pearson and Healing, 1992).

GBS is self-limiting, with muscle strength usually reaching a nadir within 2-4 weeks and muscle wastage occurring after approximately 2 weeks (Hughes and Cornblath,

2005). Following a variable plateau phase, recovery generally occurs over a period of several weeks to months. Approximately 20 % are still disabled after one year. Many factors affect the speed and extent of recovery. Age is a factor, with children generally experiencing a more rapid and complete recovery than the elderly. Another factor is the speed of onset. A rapid onset phase and/or the need for artificial ventilation, generally precedes a prolonged ineffectual recovery period (Visser *et al.*, 1999; Hughes and Cornblath, 2005).

Although GBS appears to have multiple aetiologies, *C. jejuni* is generally considered to be the primary associated infectious agent, with up to 40 % of cases being linked to preceding campylobacteriosis (Yan *et al.*, 2005). Hadden *et al.* (2001) concluded that GBS patients with preceding *C. jejuni* infections were more likely to suffer more severe disease symptoms and a delayed recovery, in comparison with other patients. Fortunately only a relatively small proportion of *C. jejuni* enteritis cases develop GBS. However, the severity of disabilities experienced, the long-term recovery, the potential for relapse and emotional disturbances, including anxiety, depression and post traumatic stress disorder, reflect the severity of this condition (Pearson and Healing, 1992).

The GBS mortality rate varies throughout the world, dependening upon the health care provision, but it is generally considered to be 5-8 % (Pearson and Healing, 1992; Nachamkin *et al.*, 2000). Mortality is dramatically increased by respiratory failure and is mainly due to associated medical complications, with optimal outcomes depending on prompt implementation of immunomodulatory therapy (Yavagal and Mayer, 2002).

Studies of *C. jejuni* associated GBS in Japan, Germany, Mexico, South Africa and the United States of America have highlighted the over-representation of several heat

stable serotypes, primarily HS:19 and to a lesser extent, HS:41 (Kuroki *et al.*, 1993; Lastovica *et al.*, 1997; Allos *et al.*, 1998; Nachamkin *et al.*, 1998; Fitzgerald *et al.*, 2005). However, HS:19 is also known to cause diarrhoea in patients without subsequent GBS development indicating that disease occurrence is multifactorial, and that host susceptibility, is in addition to strain characteristics, an important factor (Nachamkin *et al.*, 2000; Yan *et al.*, 2005).

3.1.5.6 GBS and pregnancy

During pregnancy *C. jejuni* induced bacteraemia and infection of the newborn during the birth process or shortly after birth, may be hazardous to the newborn, with complications ranging from neonatal bacteraemia to still birth or early neonatal death. GBS during pregnancy generally does not affect foetal or infant development and or increase the risk of spontaneous abortion or foetal death, although it may induce spontaneous delivery during the third trimester in severe cases. It is also important to note that the physical impairments induced by GBS may render a new mother incapable of caring fully for a newborn infant (Smith, 2002).

3.1.6 *C. jejuni* genome

C. jejuni has a relatively small genome (approximately 1600-1700 kb), when compared with that of other enteric pathogens such as *Escherichia coli* (approximately 4500 kb) (Bergthorsson and Ochman, 1998; Parkhill *et al.*, 2000; Dobrindt, 2005; Fouts *et al.*, 2005). It has been suggested that the small size of the *Campylobacter* genome is reflected in their requirement for complex media and their

inability to ferment carbohydrates and degrade complex substances (Griffiths and Park, 1990; Ketley, 1997).

Although it has been proposed that large regions of the *C. jejuni* genome are genetically stable, there are some highly divergent genes and high levels of intraspecies variability exist (Taboada *et al.*, 2004; Champion *et al.*, 2005). Dingle *et al.* (2001) provided evidence for extensive horizontal genetic exchange, including the import of alleles from at least two other *Campylobacter* species, including *C. coli* and suggesting that *C. jejuni* has a weakly clonal population structure. Several *Campylobacter* strains contain extrachromosomal elements in the form of conjugative plasmids and/or bacteriophages (Taylor, 1992; van Vliet and Ketley, 2001).

3.1.6.1 *C. jejuni* NCTC 11168

C. jejuni NCTC 11168 was originally isolated by Skirrow in 1977 from the faeces of a diarrhoeic patient. The genome sequence published in 2000 is 1,641,481 base pairs in length, with 25 polymorphic regions (Parkhill *et al.*, 2000). The DNA is AT rich with a GC ratio of approximately 30 %-46 % (Barros-Velázquez *et al.*, 1999). As 94.3 % of the genome codes for proteins it is one of the densest bacterial genomes sequenced so far (Parkhill *et al.*, 2000).

3.1.6.2 *C. jejuni* RM1221 (ATCC BAA-1062)

Originally isolated from a NaCl wash of a chicken carcass (bought from a shop) by the Food Safety Research Information Office, *C. jejuni* RM1221 (ATCC BAA-1062) has been minimally passaged and is therefore believed to be a more accurate representation of environmental *C. jejuni* isolates compared with the multi-passaged *C. jejuni* NCTC 11168 (Miller *et al.*, 2000; Dorrell *et al.*, 2005). The genome sequence of RM1221 was completed in 2005 and is a single circular chromosome, 1,777,831 bp in length, with an average GC content of 30.31 % (Fouts *et al.*, 2005).

3.1.6.3 Comparative genome analysis

Initial microarray analysis suggested that several *C. jejuni* NCTC 11168 genes are absent or are highly divergent in the RM1221 strain and with the completion of the sequence, three major regions of variation have been observed: in the capsule, lipo-oligosaccharide and flagellar loci (Dorrell *et al.*, 2005). The genomic structure of *C. jejuni* RM1221 is similar to the genome of *C. jejuni* NCTC 11168, the major difference between the two being the presence within the RM1221 genome of four large integrated elements and open reading frames within the capsular (extracellular) polysaccharide loci in NCTC 11168 (Fouts *et al.*, 2005).

This apparent variation within the *C. jejuni* genome has been proposed as an important survival strategy, with the continuous variation of surface structure and the regulation of the expression of surface determinants such as flagella, when required (Wren *et al.*, 2001).

3.1.6.4 Genetic diversity

The *C. jejuni* strain 81-176 has been extensively studied by numerous researchers and has been shown to carry two large plasmids *pVir* and *pTet*. Poly *et al.* (2005) used a shotgun DNA microarray approach to compare this non-sequenced strain with the *C. jejuni* NCTC 11168 genome. Up to 63 kb of unique chromosomal DNA sequences were obtained from the non-sequenced strain, providing further evidence of substantial genetic diversity among isolates of *C. jejuni* (Poly *et al.*, 2005). Pearson *et al.* (2003) investigated the genetic diversity of 18 *C. jejuni* strains identifying 7 hypervariable regions in the *C. jejuni* genome and finding that, when compared with the *C. jejuni* NCTC 11168 genome sequenced strain, 16.3 % of the genes were variable.

This genetic diversity might enable *Campylobacter* to colonise various environmental niches, including those highly restricted in oxygen and possibly contribute to the reported variation of virulence potential and invasive abilities (Pearson *et al.*, 2003). The study of additional strains and the identification of additional *Campylobacter* genes, would allow the construction of a composite *Campylobacter* microarray which could then be used to identify unique and shared genes between *C. jejuni* strains of various origins and for the development of genome-based molecular typing methods providing invaluable epidemiology and evolutionary information (Poly *et al.*, 2005).

3.1.6.5 Strain variation

Frequent sub-culturing of laboratory strains, combined with the extraordinary plasticity of bacterial genomes, has raised concerns with regard to the use of laboratory-adapted bacterial reference strains in place of 'real-world' isolates. Following their initial isolation, some standard laboratory reference strains undergo decades of sub-culturing and innumerable laboratory passages under strikingly different conditions to the *in vivo* ecosystems from which they were originally isolated. Fux *et al.* (2005) compared genomic differences in laboratory reference strains and low-passage clinical isolates and concluded that bacteria rapidly adapt to *in vitro* conditions and therefore no bacterial strain can truly represent its species.

The original *C. jejuni* NCTC 11168 isolate was archived (strain number 5636/77), frozen and then donated to the *Campylobacter* strain collection of the Veterinary Laboratories Collection. In a recent comparative study, Gaynor *et al.* (2004), demonstrated that there are significant differences between this original isolate and the genome-sequenced NCTC 11168 strain. The genome-sequenced variant has previously been shown to be a very poor coloniser of one day old chicks, following oral challenge, whereas the original clinical isolate from the Veterinary Laboratories Collection was found to be an excellent coloniser of chickens (Gaynor *et al.*, 2004). Further detailed comparison of these organisms by the same group demonstrated dramatic differences in numerous virulence-associated phenotypes, including colonisation, invasion, translocation, motility and morphology which appear to be the result of very subtle genetic changes that were only detectable by direct sequencing (Gaynor *et al.*, 2004).

In conclusion, involuntary laboratory conditioning may render the substitution of the study of actual pathogens with laboratory-adapted reference strains inadequate and inappropriate as important pathophysiological characteristics may have been lost (2.4.1.2).

3.1.7 Stress response

Campylobacter spp. are generally considered to be relatively fragile, and unusually sensitive to environmental stress. Despite this and the apparent lack of adaptive mechanisms and stress response factors, (including the global stationary phase stress response factor RpoS and the oxidative stress response factor SoxRS), *Campylobacter* is the most common bacterial cause of acute gastroenteritis world-wide (Park, 2002). Little is known about the response of *Campylobacter* spp. to starvation and other stressful environmental conditions. *C. jejuni* possess a homologue of CsrA (carbon storage regulator), which in many other bacteria controls the expression of certain genes that are repressed in the stationary phase of growth. Park (2005) hypothesised that given the lack of other key starvation and regulatory phase regulators, this CsrA homologue (Cj1103) plays a key role in modulating gene expression in *C. jejuni* in response to starvation and entry into stationary phase. Murphy *et al.* (2003*a*) suggested that *C. jejuni* (more specifically *C. jejuni* CI 120, a natural isolate) has the ability to induce an adaptive tolerance response to sublethal treatments of acid and/or aerobic conditions, which confers resistance to lethal pH. Murphy *et al.* (2003*b*) also isolated an extracellular protein that provides protection to acid and heat stress indicating that *C. jejuni* CI 120 has the ability to use a novel, stress resistance, extracellular signalling mechanism to induce tolerance to stress factors (Murphy *et al.*, 2003*b*).

Assuming this organism does possess a diminished stress response, the prevalence of this pathogen is remarkable. The frequent isolation of *C. jejuni* from the environment and at several points in the food chain suggests that this organism is tolerant to the wide variety of inimical conditions that they will encounter as a consequence of food processing, the application of food preservation practices, the environment and the low pH of the stomach (Murphy *et al.*, 2003*b*). Since adaptability is a crucial characteristic of bacteria that are capable of survival in such a wide variety of environmental conditions, it is conceivable that this organism possesses novel stress responses that are as yet to be elucidated (Park, 2005).

3.1.8 Phenotyping and genotyping

Campylobacter is a highly diverse genus which currently contains 16 species, six sub-species and several biovars (On, 2005). As *C. jejuni* is a genotypically diverse species, reliable epidemiological data will enable the determination of the genetic relationships between isolates and therefore identify potential sources of human infection and the identification of outbreaks with a wide geographical distribution (Manning *et al.*, 2001). However, epidemiological investigation of *Campylobacter* infection has been greatly hampered by the lack of widely used sub-species typing, prohibiting identification of isolates beyond species level and the absence of a national database (Frost, 2001). *C. jejuni* has a weakly clonal population with genetic exchange occurring at both a species and sub-species level (Dingle *et al.*, 2001; Wassenaar, 2002). Therefore several of the typing methods applied to *Salmonella* and similar bacteria are unsuitable and may provide unreliable, unrepeatable and/or misleading results (Wassenaar *et al.*, 2000)

The vast majority of *Campylobacter* infections are reported as sporadic. Tracing the relative contributions of human infections, variations in pathogenicity and factors controlling temporal and geographical variations in the distribution of specific strains, is dependent upon the availability of good typing techniques and specific markers (Frost, 2001). Original identification schemes based on biochemical tests, although complex, were until recently the most widely employed identification method applied to *Campylobacter*. The recent advent of molecular techniques including phenotyping and genotyping, is proving to be a valuable tool for identification at species and sub-species levels.

A number of sub-typing methods, both phenotypic and genotypic, have been applied to *Campylobacter* spp. including serotyping, phage typing, pulse-field gel electrophoresis (PFGE), polymerase chain reaction-restriction fragment length polymorphism, analysis of the flagellin locus (*fla* typing), and more recently amplified fragment length polymorphism (AFLP) analysis (Manning *et al.*, 2001). Different genotyping methods have different degrees of discrimination. Isolates belonging to one genotype as determined with a low-discriminatory method can be further differentiated by a higher-discriminatory method (Wassenaar, 2002).

3.1. 8.1 Serotyping

Historically, the two main types of serotyping methods applied to *Campylobacter* employ either heat-stable (HS) or heat-labile (HL) antigens.

The most widely accepted and well-evaluated method for the typing of *C. jejuni* strains is the Penner serotyping technique, which is based on passive haemagglutination, differentiating the strains on the basis of HS antigens (Penner and

Hennessy, 1980; Nakari *et al.*, 2005). The major disadvantage of this system is the high level of non-typeability (up to 63 %) of human isolates from some countries, although, in general, the rate of non-typeability is less than 20 % (Nakari *et al.*, 2005).

The Lior serotyping system exploits agglutination of HL antigens. Based on a slide agglutination procedure, this method considers whole cells as antigenic material (Lior *et al.*, 1982). However, multiple reactions are not unusual with this method, which is labour intensive, restricting its use to larger laboratories and preventing its routine application for isolate identification (Barros-Velázquez *et al.*, 1999; Fitzgerald *et al.*, 2005).

3.1.8.2 PFGE

PFGE is a modification of restriction fragment length polymorphism. 'Rare cutting' enzymes (usually *SmaI* for genotyping *Campylobacter*) are used to cut the target DNA into relatively few fragments of comparatively large size, which are then separated with high resolution by PFGE, resulting in clear restriction profiles. The discriminatory potential of PFGE is excellent, but the lengthy preparation procedure for the DNA samples and its dependence upon specialized and expensive electrophoresis apparatus is prohibitive with regard to wide-scale screening (Frost, 2001).

3.1.8.3 *fla* typing

C. jejuni carries two adjacent flagellin genes, *flaA* and *flaB*, where *flaA* is the major gene for flagellin production and motility (Guerry *et al.*, 1990; Nuijten *et al.*, 1990; Wassenaar *et al.*, 1991). *fla* typing is a PCR based technique, that produces high typeability for human, veterinary and environmental isolates. This technique is less labour intensive and uses equipment that is more widely available. However, as this technique only samples a very small proportion of the genome and there is potential for recombination between the *flaA* genes of different strains and between the *flaA* and *flaB* genes of individual strains, this technique cannot be considered to be a particularly stable typing method (Frost, 2001).

3.1.8.4 AFLP

AFLP is a high-resolution genotyping method that involves the digestion of chromosomal DNA with two restriction enzymes. Banding patterns from AFLP can be used to determine genetic relationships between strains from diverse populations (Frost, 2001). This method, which has been used for high-resolution typing of several bacteria in both epidemiological and taxonomic studies, has been adapted for epidemiological typing of *C. jejuni* and other *Campylobacter* spp., enabling subtyping of different strains and differentiation between species (Duim *et al.*, 1999; Kokotovic and On, 1999; Savelkoul *et al.*, 1999; On and Harrington, 2000; Duim *et al.*, 2001) AFLP analysis is very sensitive, highly discriminatory and reflects the total genome of the organism (Manning *et al.*, 2001).

3.1.8.5 Multilocus sequence typing (MLST)

MLST uses automated DNA sequencing to provide a discriminatory molecular profile of 7 house keeping genes. The genetic variation present between strains for these genes produces different sequences, which are assigned as distinct alleles and, for each isolate the allelic profile or sequence type, is identified (Dingle *et al.*, 2001). This technique is reproducible, relatively easy and directly comparable among laboratories, via online electronic storage and distribution systems such as http://mlst.zoo.ox.ac.uk (Dingle *et al.*, 2001) or http://pubmlst.org/campylobacter (Barros-Velázquez *et al.*, 1999). Additionally as nucleotide sequence determination can be achieved from killed-cell suspensions, purified DNA, or clinical material, the need to transport live bacteria is diminished (Dingle *et al.*, 2001). This method identifies variation which is accumulating slowly within a population and it is therefore particularly suited to long-term and global epidemiological studies, but it can also be used in the investigation of individual outbreaks (Dingle *et al.*, 2001).

3.1.8.6 Genomotyping

Despite being a relatively novel technique, comparative genomic studies of *C. jejuni* using microarrays have already enabled the identification of several important regions and core genes that mainly encode housekeeping functions and others that are indispensable for human disease, such as the cytolethal distending toxin and flagella structural proteins (Dorrell *et al.*, 2001; Champion *et al.*, 2005). Additionally Pearson *et al.* (2003) used whole genome microarrays to identify 7 hypervariable plasticity regions in the *C. jejuni* genome that contain many important genes, including ones linked to the utilisation of alternative electron receptors for respiration that may

confer a selective advantage to strains in restricted environments. Many other membrane and periplasmic proteins and hypothetical proteins of unknown function that might be linked to phenotypic variation and adaptation, were also included in these hypervariable regions (Pearson *et al.*, 2003). As previously stated, Champion *et al.* (2005) used whole-genome comparison of isolates using DNA microarrays (genomotyping), in conjunction with complex mathematical analysis, to determine the phylogeny of 111 *C. jejuni* isolates from a wide variety of human, livestock and environmental sources and they identified two distinct clades. These studies indicate the potential of genomotyping which will hopefully provide a sensitive and robust method to examine the genetic relatedness of bacterial populations (Champion *et al.*, 2005).

3.1.8.7 Typing problems/issues

Although DNA-based systems are reliable and rapid, both *fla* typing and PFGE are affected by the instability and plasticity of the *Campylobacter* genome, causing a lack of reproducibility in molecular typing patterns and variation of expression with phenotypic methods (Frost, 2001; Manning *et al.*, 2001). Each typing technique has both advantages and disadvantages and is suitable for different situations and applications. Considering the large number of *Campylobacter* infections occurring each year, for wide scale surveillance the cost and ease of use of a method are of prime importance, whereas for many research purposes discriminatory power is of greater importance (Frost, 2001).

Although the majority of these systems can be used independently, it is advisable to use them in conjunction with each other (as in supplementary typing) to obtain

complementary information and avoid confusion with regard to the assignment of different serotypes to the same strain by the different methods. This is of particular relevance when dominant serotypes or strains not amenable to serotyping, are implicated in epidemiological investigations (Barros-Velázquez *et al.*, 1999).

3.1.9 QS in *C. jejuni*

The QS-mediated production of virulence factors and biofilm formation by certain Gram-negative oxidase positive bacteria such as *P. aeruginosa* is well documented and indicates the potential for QS systems with regard to any bacterial pathogen. As previously stated AI activity has been demonstrated for many foodborne pathogens *in vitro* (1.1.11). However, little is known about the roles of QS in the growth and survival of bacteria in food environments *in vivo*, and its significance to the food industry with regard to food spoilage, food toxicity and the virulence of the foodborne pathogen *C. jejuni*.

3.1.9.1 LuxS/AI-2 and *C. jejuni*

Analysis of the genome sequence of *C. jejuni* NCTC 11168 by Elvers and Park (2002), has revealed a hypothetical protein (Cj1198), with extensive amino acid similarity to LuxS from several other bacteria (71 % identity to LuxS from *E. coli*). AI-2 like activity, which is dependent upon the product of this *LuxS* homologue, has been demonstrated for both the laboratory *C. jejuni* isolate NCTC 11168 and the clinical *C. jejuni* isolate 81116 (Elvers and Park, 2002; Jeon *et al.*, 2003). Cloak *et al.* (2002) reported an increase in AI-2 activity in Brucella broth supplemented with glucose or foetal bovine serum and AI-2 activity in both milk and chicken broth at a

range of temperatures, indicating the potential for AI-2-mediated QS in food-borne *C. jejuni*. Elvers and Park (2002), concluded that *C. jejuni* NCTC 11168 uses an AI-2-like-cell-signalling system which they linked to motility regulation. They also suggested that the system may serve as a global regulatory mechanism for basic physiological functions and virulence factors, as in other bacterial pathogens (Elvers and Park, 2002). Subsequent research has indicated that this putative AI-2-mediated QS influences the transcription of *flaA* but not *flaB*, with a *luxS* mutant exhibiting reduced *flaA* transcription and motility whilst having the same level of total flagellin protein as the wild-type (Jeon *et al.*, 2003). As the agglutination capability was also reduced in the mutant strain, Jeon *et al.* (2003) concluded that this putative AI-2-mediated QS system might influence the regulation of both motility and surface structure formation. However, as both of these studies used *luxS* mutants, which as previously stated, will also have altered bacterial metabolism, it is possible that these changes in metabolism are actually responsible for the changes in motility and agglutination (1.1.3.3).

3.1.9.2 AHL/AI-1 and *C. jejuni*

The genome sequence of *C. jejuni* does not contain any gene predicted to encode an acyl homoserine lactone synthase (Elvers and Park, 2002). Many researchers therefore believe that it is unlikely to produce acyl homoserine lactone based signalling molecules (Park, 2002; Park, 2005). However, *C. jejuni* is currently a poorly understood microorganism that provides an alternative model in comparison to the current enterobacterial standard for both virulence and stress response and therefore

its study is an exciting challenge with the potential to change the current understanding of QS (Ketley and Konkel, 2005).

3.1.10 Aim of the research

Autoinducer activity has been demonstrated in many foodborne pathogens and it is frequently postulated that autoinducer production may be exploited to control bacterial growth and survival in foods. However, without a complete understanding of the QS systems of each pathogen, development in this field is stunted. The aim of this research is to assess putative AHL production by both clinical and laboratory isolates of *C. jejuni*, including the genome sequenced strain NCTC 11168. Assuming verification of putative AHL production, the effect of various conditions, including medium supplementation, growth period and oxygen concentration on putative AHL production, will also be determined. *C. jejuni* conditioned, supernatant extracts will be assessed for putative AHL production via an *Agrobacterium* liquid culture bioassay (Zhu *et al.*, 2003) and a modified Miller assay (Miller, 1992). Further analysis will employ HPLC and LC-MS.

3.2 METHODS

3.2.1 Reagents

All chemicals were purchased from Sigma-Aldrich Company Ltd (Poole, Dorset, UK) or VWR International (Lutterworth, Leicestershire, UK) unless otherwise stated.

3.2.2 *A. tumefaciens* strains and culture conditions

The *A. tumefaciens* reporter strain KYC55 (pJZ372); (pJZ384); (pJZ410), which contains 3 plasmids, was assigned the culture collection designation UL225 (Table 2.2.1). This strain cannot synthesise the *Agrobacterium* autoinducer (OOHL), therefore the *lacZ* fusion product, β-galactosidase, is only expressed when exogenous autoinducer is present (Cho *et al.*, 1997: Zhu *et al.*, 2003). The *A. tumefaciens* positive control, UL224 (Table 2.2.1), contains a *tra*^{c} Ti plasmid and synthesises the *Agrobacterium* AHL autoinducer (Zhu *et al.*, 1998). Both strains were kindly donated by Prof. Stephen Winans, Cornell University, Ithaca.

A. tumefaciens strains UL224 and UL225 were grown at 25°C, 180 rpm (Gallenkamp orbital incubator) in 1 ml aliquots of ATMM (Appendix 1, Table A1.3). Strain UL225 required the addition of gentamicin and spectinomycin (both to a final concentration of 100 μg/ml) and tetracycline (to a final concentration of 2 μg/ml), and strain UL224 required tetracycline (1 μg/ml) and spectinomycin (100 μg/ml) to prevent plasmid loss. Both UL224 and UL225 were stored on ATMM slopes containing the appropriate antibiotics for each strain at 4°C and subcultured every 28 days. Resuscitation of both strains consisted of one 16 h passage in the appropriate conditions as stated above.

3.2.3 *C. jejuni* strains and culture conditions

The *C. jejuni* strains used in this study are described in Table 3.2.1.

Table 3.2.1: *Campylobacter jejuni* strains used in this study (table adapted from Wareing *et al.*, 2003).

NCTC	Isolation Year	HS serotype	Isolation Source	Clonal Complex	Allelic profile						
					aspA	*glnA*	*gltA*	*glyA*	*pgm*	*tkt*	*uncA*
11168-GS	1977	2	Human stool	ST-43	0	0	0	0	0	0	0
11160-O	1977	2	Human stool	ST-43	0	0	0	0	0	0	0
13254	1998	50	Beef offal	ST-21	2	1	1	3	2	1	5
13255	1991	19	Human stool	ST-22	1	3	6	4	3	3	3
13256	1991	23	Human stool	ST-42	1	2	3	4	5	9	3
13257	1999	57	Human stool	ST-45	4	7	10	4	1	7	1
13258	1998	50	Lamb offal	ST-48	2	4	1	2	7	1	5
13259	1991	18	Human stool	ST-49	3	1	5	17	11	11	6
13260	1998	5	Lamb offal	ST-52	9	25	2	10	22	3	6
13261	1998	50	Beef offal	ST-61	1	4	2	2	6	3	17
13262	1994	NT	Sand	ST-177	17	2	8	5	8	2	4
13263	1991	NT	Human stool	ST-206	2	21	5	37	2	1	5
13264	1999	11	Human stool	ST-257	9	2	4	62	4	5	6
13265	1991	53	Human stool	ST-354	8	10	2	2	11	12	6
13266	1994	41	Human stool	ST-362	1	2	49	4	11	66	8

C. jejuni isolate serotypes (excluding NCTC 11168-GS and 11168-O) were identified using various methods by Wareing *et al.* (2003).

Sequence type (ST); Not typeable (NT).

All *C. jejuni* strains (excluding NCTC 11168-GS and 11168-O) were provided by Dr. D. Wareing (Dynal).

C. jejuni strain NCTC 11168-O was provided by Dr. Anne Ridley (Veterinary Laboratory Agency, Weybridge).

For *C. jejuni*, the microaerophilic environment was produced by a CampyGen™ atmospheric generation system, (2.5 L [Oxoid] in a 2.5 L anerobic jar [Oxoid]). All *C. jejuni* strains used in this study were stored in 1 ml brain heart infusion broth (Oxoid) containing glycerol to a final concentration of 20 % (v/v) at -80°C. *C. jejuni* strains were resuscitated on pre-dried Blood Agar Base (Oxoid) medium containing lysed horse blood (7.0 % v/v) (Oxoid). The cultures were incubated in a microaerophilic environment at 37°C (Status® static incubator, The Northern Media Supply Ltd., North Humberside) for 72 h, then subcultured to fresh Blood Agar plates for 40 h. The resuscitated *C. jejuni* cultures were then transferred completely from the plate via a sterile loop to 2 ml BB, (Oxoid) and carefully emulsified to avoid an excessive increase in oxygen levels. For standard conditions this suspension was then used to inoculate 100 ml BB in a glass screw top Duran bottle (total capacity 130 ml) to an optical density at 600 nm ($OD_{600\ nm}$) of 0.05, measured using a spectrophotometer (Pharmacia LKB Novaspec II). Cultures were then incubated under aerobic conditions statically at 37°C for 48 h (Tran, 1998).

3.2.4 Growth period length and β-galactosidase activity induction

Like most signalling mechanisms QS mechanisms are cell-density dependent. To assess whether any changes in β-galactosidase activity induction is related to cell density, culture samples from several different time period lengths were taken and compared with growth measured by $OD_{600\ nm}$.

3.2.4.1 Growth period length (24 h, 48 h and 96 h)

Variation in the growth period length was achieved by varying the length of incubation from 24 to 48 and 96 h. Cultures were then sampled (3.2.6) and extracted as detailed in 2.2.4.1.

3.2.4.2 *C. jejuni* growth curves

C. jejuni culture growth, measured as $OD_{600\ nm}$ was assessed at regular time points over a 96 h period. To ensure accuracy, cultures having optical density readings of 0.3 or more, were diluted in sterile BB and the optical density was re-measured at 600 nm.

3.2.5 Dissolved oxygen levels and β-galactosidase activity induction

3.2.5.1. Percentage headspace air volume

Variation in the dissolved oxygen concentration of BB was achieved by changing the final culture volume of 100 ml to 117 ml in a screw top Duran medium bottle (total capacity 130 ml), thereby limiting the headspace air to either 23 % or 10 % of the bottle's total capacity (Tran, 1998).

3.2.5.2 Culture supplementation

Supplemented growth conditions were produced by the addition of lysed horse blood (7.0 % v/v) and $FeSO_4.7H_2O$ (0.2 % w/v) (Appendix 2.1) to 100 ml BB which was then inoculated and incubated as stated in 3.2.3. The pH of all supplemented media and the BB alone was 7.3 ± 0.2.

3.2.6 Culture sampling

Following incubation, *C. jejuni* cultures were thoroughly shaken to ensure that the bacterial cells were suspended in the medium and not attached to the culture vessel. The culture was transferred to sterile centrifuge tubes and centrifuged at 6000 x g, 4°C for 15 min (MSE MISTRAL 3000i). The pellet was then discarded and the supernatant was stored at -80°C for future extraction.

3.2.7 Ethyl acetate extraction

The method was performed as detailed in 2.2.4.1.

3.2.8 *Agrobacterium* liquid culture bioassay

The method was performed as detailed in 2.2.6.3. Microsoft Excel was used to statistically assess the effect of a change in condition (growth period length, percentage headspace air volume or culture supplementation) on β-galactosidase activity induction. A one-tailed, dependent student t-test was applied to the data (paired samples, 3 replicates) to compare the actual difference between the two means (that one is larger or smaller that the other) in relation to the variation in the data.

3.2.9 Viable cell counts

Viable cell counts were performed for every *C. jejuni* culture concurrent to sampling for ethyl acetate extraction. For each culture, serial dilutions (10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7}) were produced in sterile ¼ strength ringers solution (Oxoid) and 100 μl was spread onto pre-dried blood agar plates (7.0 % v/v lysed horse blood) which were then incubated at 37°C for 48 h. Plates containing between 30 and 300 colonies were used to obtain the viable cell counts. Experiments were carried out in triplicate.

3.2.10 Separation and structure characterisation

To ensure that the molecule activating this bioassay is an AHL, the active component(s) must be separated and chemically characterised.

3.2.10.1 Serum enzyme activity assay

Yang *et al.* (2005) demonstrated that horse serum displays strong enzymatic inactivation activity against a range of AHLs with varying efficiency and it is proposed that paraoxonase(s), a lactonase-like enzyme(s), contributes to this inactivation by hydrolysing the lactone ring of AHL to produce acyl homoserine. The serum enzyme activity assay exploits this inactivation activity and indicates whether the active component contained within the supernatant extracts, is likely to be an AHL. This method was adapted from Yang *et al.* (2005).

The reaction mixture containing the horse serum (2 % v/v) and the sterile culture supernatant extract (6 μl) or HHL synthetic control (20 μM) in 0.6 ml phosphate buffered saline (PBS) incubated at 37°C (Status® static incubator, The Northern Media Supply Ltd., North Humberside) for 12 h. An additional control, as above but excluding the serum, was incubated simultaneously under the same conditions. The reaction was subsequently stopped by heating at 95°C (Haake G/D8 waterbath) for 3 min. Controls were prepared to ensure that any differences observed were due to the effect of the serum and not the PBS or the heat treatment. β-galactosidase activity induction was then assessed via the *Agrobacterium* liquid culture bioassay (2.2.6.3).

3.2.10.2 HPLC separation

Reverse phase HPLC separates compounds based on their hydrophobic character, in that hydrophobic analytes are retained on the column, due to interactions between the alkyl chains of the stationary phase and the analyte. The analytes elute more readily as the proportion of the hydrophobic component in the mobile phase is increased. QS AIs are usually small, low molecular weight molecules therefore a stationary phase with an alkyl chain length of C_{18} was chosen for the HPLC separation.

Extract samples were further concentrated by freeze drying for 12 h at -80°C and rehydrated with sterile ddH_2O (20 x original concentration, 50 μl). The concentrated extracts were applied via a Series 200 Pump (Perkin Elmer, USA) onto a S5ODS2 C_{18} reverse-phase semi-preparative column (Hichrom Ltd., Berkshire). The elution procedure was adapted from Bainton *et al.* (1992) and Pearson *et al.* (1994) and consisted of a ddH_2O:methanol gradient, for the first 15 min 25 % (v/v), then increasing to 60 % (v/v) by 35 min and 100 % (v/v) by 50 min. This gradient separated the analyte mixture as a function of how well the changing solvent mobilised the analyte. The more hydrophilic components were eluted near the start of the profile under conditions of relatively low methanol concentrations and hydrophobic components eluted later under the higher methanol concentrations. Fractions were collected at every peak detected by a Perkin Elmer LC 90 BIO Spectrophotometric UV detector (at a wavelength of 210 nm) and the fraction time and peak area was recorded using a Hewlett Packard HP3396A integrator. Fractions were collected for each peak, freeze dried for 12 h at -80°C and rehydrated with 0.7 ml sterile ddH_2O. β-galactosidase activity induction was then assessed via the *Agrobacterium* liquid culture bioassay (2.2.6.3).

3.2.10.3 LC-MS

LC-MS analysis of the active HPLC fractions was performed at Quay Pharmaceuticals Limited, Wirral by Dr. Mark Powell using a Hewlett-Packard 1050 series HPLC system comprising of an in-line vacuum degasser, quarternary pump, autosampler, variable wavelength UV detector and Dionex model STH5585 column oven. The mass spectrometer used was a Finnigan Aqa single quadrupole LC-MS system equipped with both ESI and atmospheric pressure chemical ionisation and positive or negative ion detection (2.2.8). The active HPLC separated fractions from the *C. jejuni* NCTC 13255 culture supernatant extract (10 µl), were applied onto a S5ODS2 C_{18} reverse-phase semi-preparative column (Hichrom Ltd., Berkshire). The ionisation method was ESI, positive mode and the elution procedure consisted of a single ramp methanol:ddH_2O (0.02 % v/v formic acid) gradient at a flow rate of 1 ml min^{-1}. The probe temperature was 380°C and peaks were detected with the variable wavelength UV detector at 210 nm. Peak areas were recorded using the Dionex Chromeleon (version 6.50) data system at a rate of 1 scan/second.

In selective ion monitoring (SIM) the mass spectrometer scans a very small mass range. SIM is more sensitive than a full scan as only the compounds with the selected mass are detected and plotted allowing the mass spectrometer to dwell for a longer time over a smaller mass range. Thus the smaller the SIM range, the more specific the SIM assay. For this study the SIM assay (mass range 339.75 - 340.25 *m/z*) employed the same ionisation method, elution procedure and probe temperature as the full scale scan (Appendix 2.2).

3.3 RESULTS

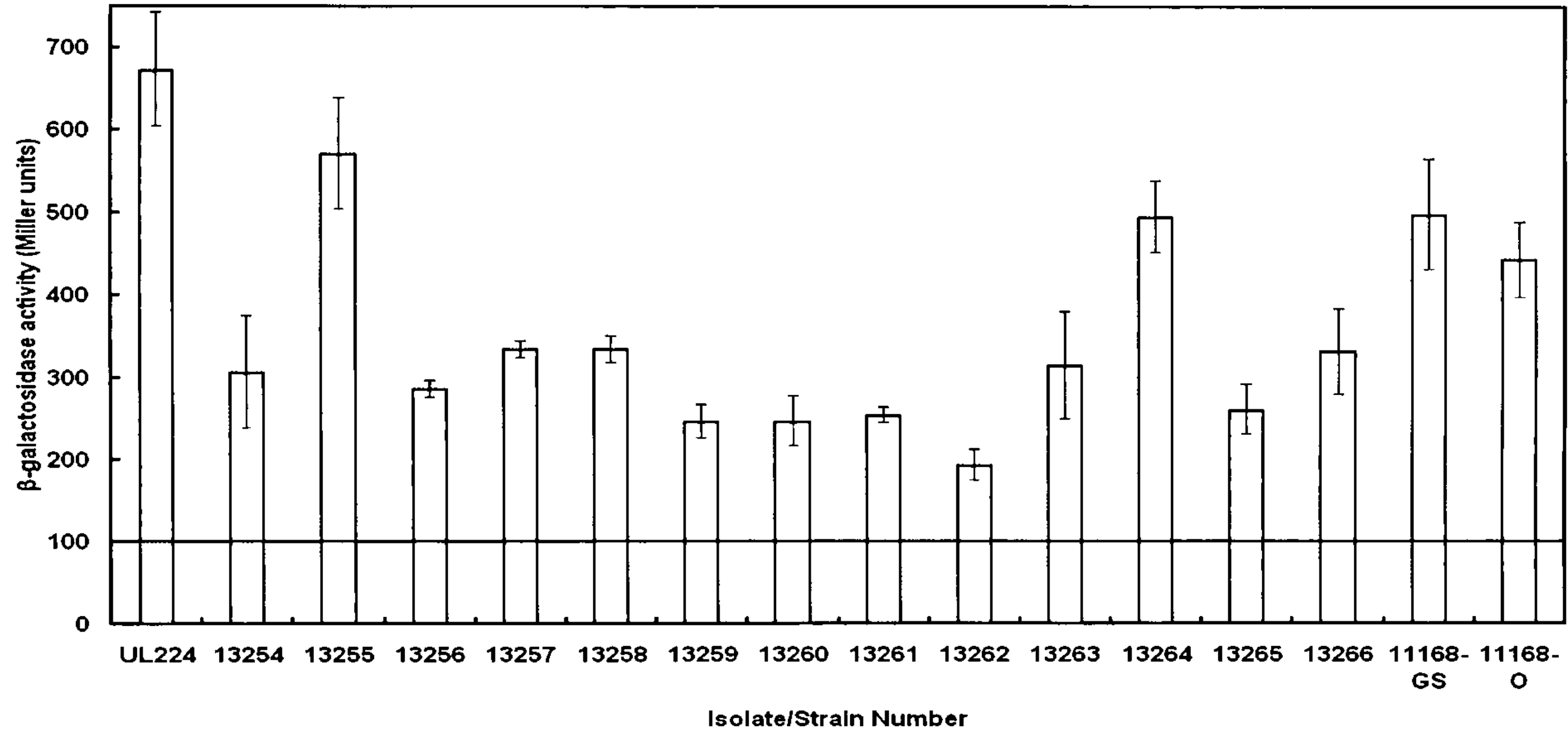


Figure 3.3.1 ***Agrobacterium*** **liquid culture bioassay of** ***C. jejuni*** **isolate culture supernatant extracts**

β-galactosidase activity induction by supernatant extracts from 15 isolates of *C. jejuni* (blue) and the *A. tumefaciens* positive control, UL224 (green). The base line at 100 Miller units indicates the considered level of importance for this study. Data are mean ± standard deviation, (n = 3).

3.3.1 Induction of β-galactosidase activity by *C. jejuni* isolate extracts

All the *C. jejuni* isolate supernatant extracts tested in this preliminary screen induced β-galactosidase activity in the *A. tumefaciens* reporter strain, UL225, above the considered level of importance (Figure 3.3.1), whereas extracts of several batches of sterile BB growth media induced negligible levels of β-galactosidase activity (data not presented). Growth was measured as optical density ($OD_{600\ nm}$) for all the *C. jejuni* isolates (Appendix 3, Table A3.1). All final 48 h *C. jejuni* optical densities were within a 0.03 OD range ($OD_{600\ nm}$) and therefore considered normalised. The extract derived from each isolate culture supernatant demonstrated an individual activation level, with *C. jejuni* NCTC 13255, 13264, 11168-GS and 11168-O extracts exhibiting the most activity under the stated culture conditions and the *C. jejuni* NCTC 13262 extract the least. Interestingly extracts derived from *C. jejuni* isolates NCTC 11168-GS and 11168-O culture supernatants induced comparable levels of β-galactosidase activity. The extract derived from the *C. jejuni* NCTC 13255 culture supernatant induced similar levels of β-galactosidase activity as found in the positive control, *A. tumefaciens* UL224 extract (Figure 3.3.1). However, it should be noted that these results are not directly comparable as different growth conditions were used to culture *A. tumefaciens* and *C. jejuni.*

C. jejuni isolates NCTC 11168-GS and 11168-O were selected for further analysis to enable the comparison of a laboratory 'acclimatised' isolate with the non-acclimatised 'clinical' version. *C. jejuni* NCTC 13255 was also studied as it demonstrated a high level of activity and is serotype HS:19 which has been associated with GBS.

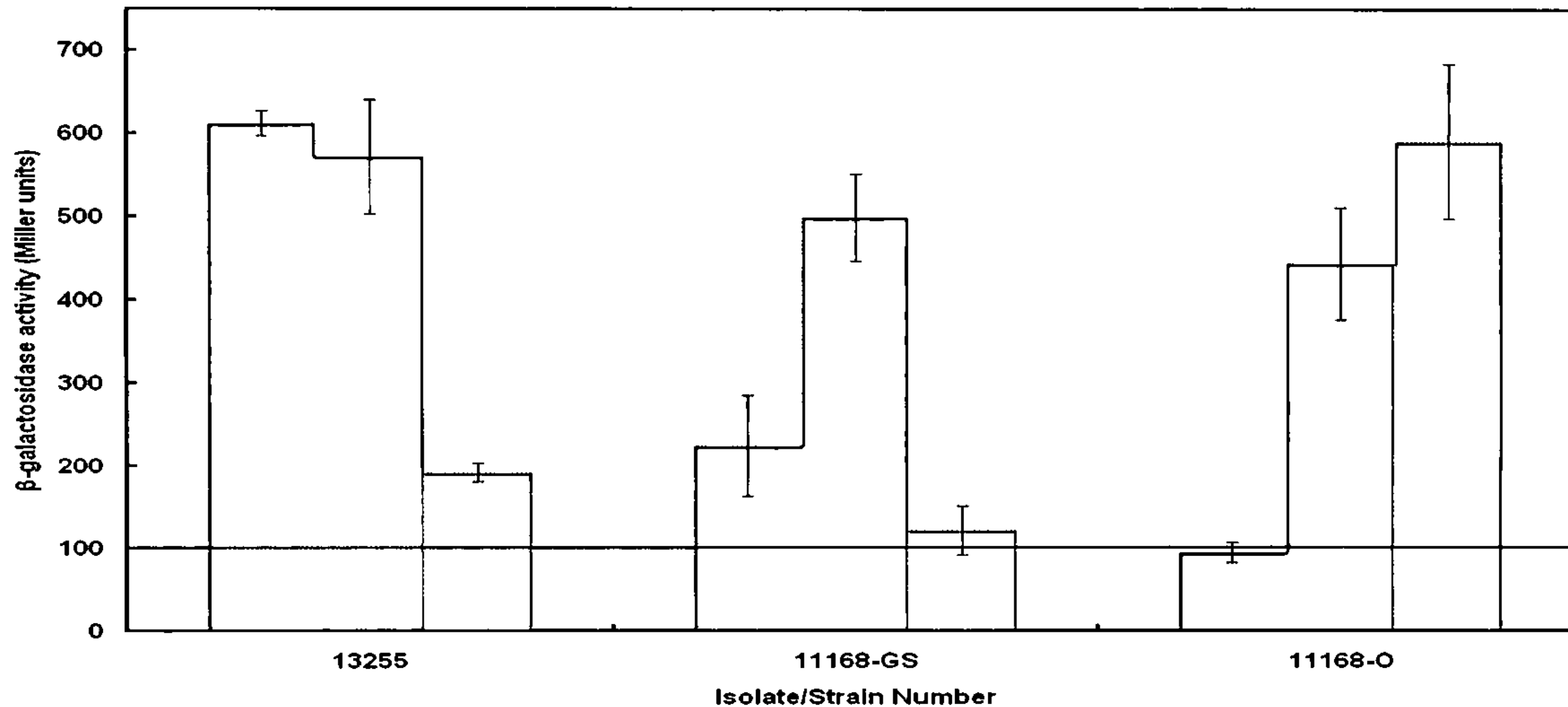


Figure 3.3.2 *Agrobacterium* liquid culture bioassay of *C. jejuni* isolate culture extracts from various growth period lengths

β-galactosidase activity induction by extracts derived from *C. jejuni* NCTC 13255, 11168-GS and 11168-O culture supernatants from three separate growth periods; 24 h (green), 48 h (blue) and 96 h (orange). The base line at 100 Miller units indicates the considered level of importance for this study. Data are mean ± standard deviation, (n = 3).

Replicate Number	β-galactosidase activity induction levels (Miller units)								
	24h			48h			96h		
	13255	**11168-GS**	**11168-O**	**13255**	**11168-GS**	**11168-O**	**13255**	**11168-GS**	**11168-O**
1	627.86	291.95	108.10	504.65	438.01	367.58	204.34	93.23	680.38
2	598.65	172.57	85.22	568.86	523.16	494.81	184.30	152.72	494.87
3	610.96	207.26	89.16	641.27	532.86	469.50	186.74	117.37	597.09
Mean	612.49	223.93	94.16	571.59	498.01	443.96	191.79	121.10	590.78
SD	14.67	61.41	12.23	68.35	52.19	67.35	10.94	29.92	92.91
Statistical Comparison	**13255 vs 11168-GS**	**13255 vs 11168-O**	**11168-GS vs 11168-O**	**13255 vs 11168-GS**	**13255 vs 11168-O**	**11168-GS vs 11168-O**	**13255 vs 11168-GS**	**13255 vs 11168-O**	**11168-GS vs 11168-O**
p value	**0.00**	**0.00**	**0.02**	**0.03**	**0.02**	**0.03**	**0.05**	**0.01**	**0.01**

Replicate Number	β-galactosidase activity induction levels (Miller units)								
	13255			11168-GS			11168-O		
	24h	48h	96h	24h	48h	96h	24h	48h	96h
1	627.86	504.65	204.34	291.95	438.01	93.23	108.10	367.58	680.38
2	598.65	568.86	184.30	172.57	523.16	152.72	85.22	494.81	494.87
3	610.96	641.27	186.74	207.26	532.86	117.37	89.16	469.50	597.09
Mean	612.49	571.59	191.79	223.93	498.01	121.10	94.16	443.96	590.78
SD	14.67	68.35	10.94	61.41	52.19	29.92	12.23	67.35	92.91
Statistical Comparison	24 vs 48h	**24 vs 96h**	**48 vs 96h**	**24 vs 48h**	**24 vs 96h**	**48 vs 96h**	**24 vs 48h**	**24 vs 96h**	48 vs 96h
p value	0.23	**0.00**	**0.01**	**0.03**	**0.09**	**0.00**	**0.01**	**0.00**	0.12

Table 3.3.1 Student t-test analysis (one-tailed) of *Agrobacterium* liquid culture bioassay of *C. jejuni* isolate culture extracts from various growth period lengths

β-galactosidase activity induction levels (Miller units) for replica extracts derived from *C. jejuni* NCTC 13255, 11168-GS and 11168-O culture supernatants from three separate growth periods; 24 h, 48 h and 96 h. All significant comparisons are highlighted in bold ($p < 0.05$).

3.3.2 Growth period length and β-galactosidase activity induction

A major defining characteristic of QS is that signalling molecule production and secretion is cell density dependent. Therefore the effect of growth period length on the amount of active component(s) present in the *C. jejuni* culture supernatant extracts was determined via induction of β-galactosidase activity in the *Agrobacterium* liquid culture bioassay.

3.3.2.1 The effect of growth period length (24 h, 48 h and 96 h) on β-galactosidase activity induction

Figure 3.3.2 indicates that each of the three *C. jejuni* isolate supernatant extracts tested exhibit a unique growth period induction profile. For *C. jejuni* NCTC 13255 the levels of induction appear to remain constant for both the 24 and 48 h supernatant extracts, whereas the 96 h supernatant extract results show a significant decrease ($p < 0.05$) in induction level (Figure 3.3.2, Table 3.3.1). The induction levels for *C. jejuni* NCTC 11168-GS increases significantly ($p < 0.05$) between 24 and 48 h but subsequently significantly decreases ($p < 0.05$) by 96 h to a level significantly lower ($p < 0.05$) than that observed for the 24 h extract (Figure 3.3.2, Table 3.3.1). Induction levels significantly increase ($p < 0.05$) between 24 and 48 h for *C. jejuni* NCTC 11168-GS and *C. jejuni* NCTC 11168-O. However, for *C. jejuni* NCTC 11168-O there is no subsequent significant decrease ($p < 0.05$) in the levels of induction after 96 h of growth as compared to *C. jejuni* NCTC 11168-GS (Figure 3.3.2, Table 3.3.1).

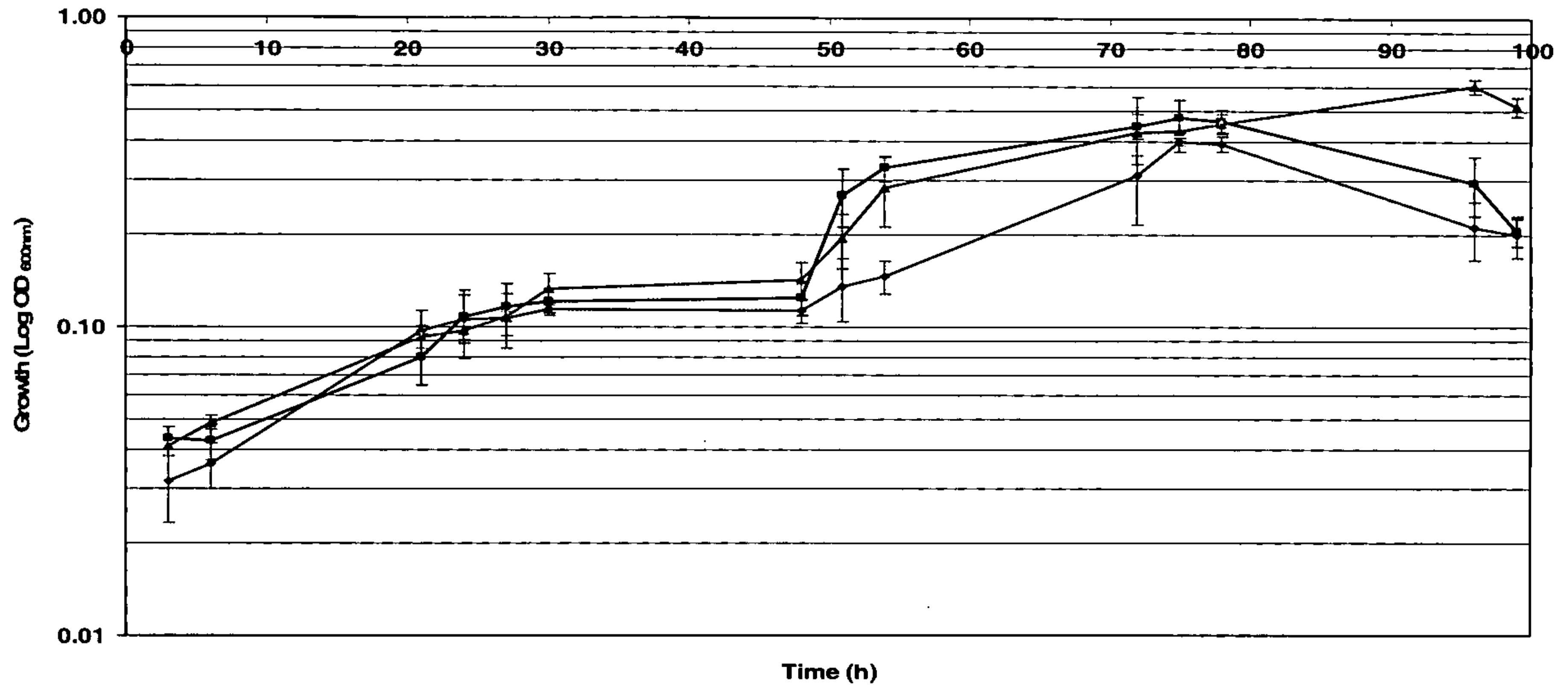


Figure 3.3.3 Growth of *C. jejuni* in stationary culture

Growth was measured as optical density $OD_{600\ nm}$ for *C. jejuni* NCTC 13255 (green), 11168-GS (blue) and 11168-O (orange). Data are mean ± standard deviation, (n = 3).

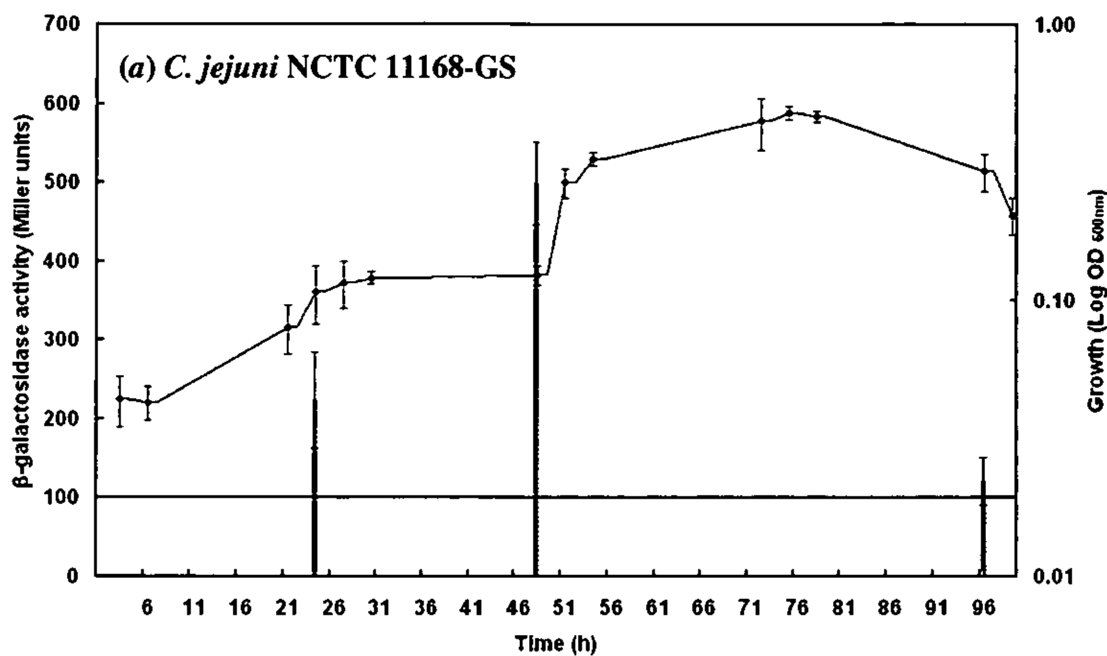
3.3.2.2 Growth curves of *C. jejuni* isolates

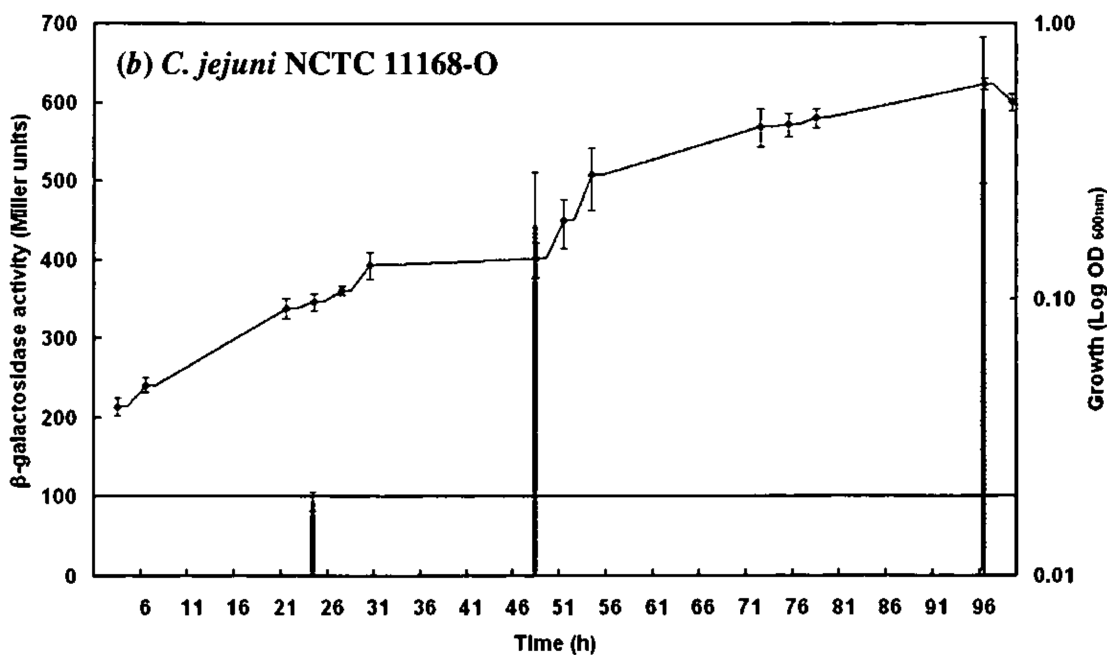
The previous experiment (3.3.2.1) indicated that the level of β-galactosidase induction by *C. jejuni* culture supernatant extracts, varied with the length of the bacterium's growth period. Thus, the growth curves of *C. jejuni* NCTC 13255, 11168-GS and 11168-O were determined, to see if the component(s) inducing β-galactosidase activity in the *Agrobacterium* liquid culture bioassay were synthesised/released into the growth medium at a particular phase in the bacterium's growth cycle.

Firstly, the growth curves of *C. jejuni* NCTC 13255, 11168-GS and 11168-O were determined (Figure 3.3.3, Appendix 3, Table A3.2.1-3).

All three *C. jejuni* cultures exhibit a long lag phase, with the optical density increasing dramatically from 48 h for *C. jejuni* NCTC 11168-GS and 11168-O, whereas for *C. jejuni* NCTC 13255 the optical density appears to steadily increase from 48 h onwards (Figure 3.3.3).

The optical density readings for both *C. jejuni* NCTC 11168-GS and 13255 decrease after 75 h, indicating the end of stationary phase and a possible decline in cell number for these isolates. On the other hand, the optical density of *C. jejuni* NCTC 11168-O cultures continues to increase until 96 h resulting in a far greater final optical density than the other two *C. jejuni* isolates (Figure 3.3.3).


(a) C. jejuni NCTC 11168-GS
β-galactosidase activity (Miller units)
Growth (Log OD 600nm)
Time (h)
700
600
500
400
300
200
100
0
1.00
0.10
0.01
6 11 16 21 26 31 36 41 46 51 56 61 66 71 76 81 86 91 96


(b) C. jejuni NCTC 11168-O
β-galactosidase activity (Miller units)
Growth (Log OD 600nm)
Time (h)
700
600
500
400
300
200
100
0
1.00
0.10
0.01
6 11 16 21 26 31 36 41 46 51 56 61 66 71 76 81 86 91 96

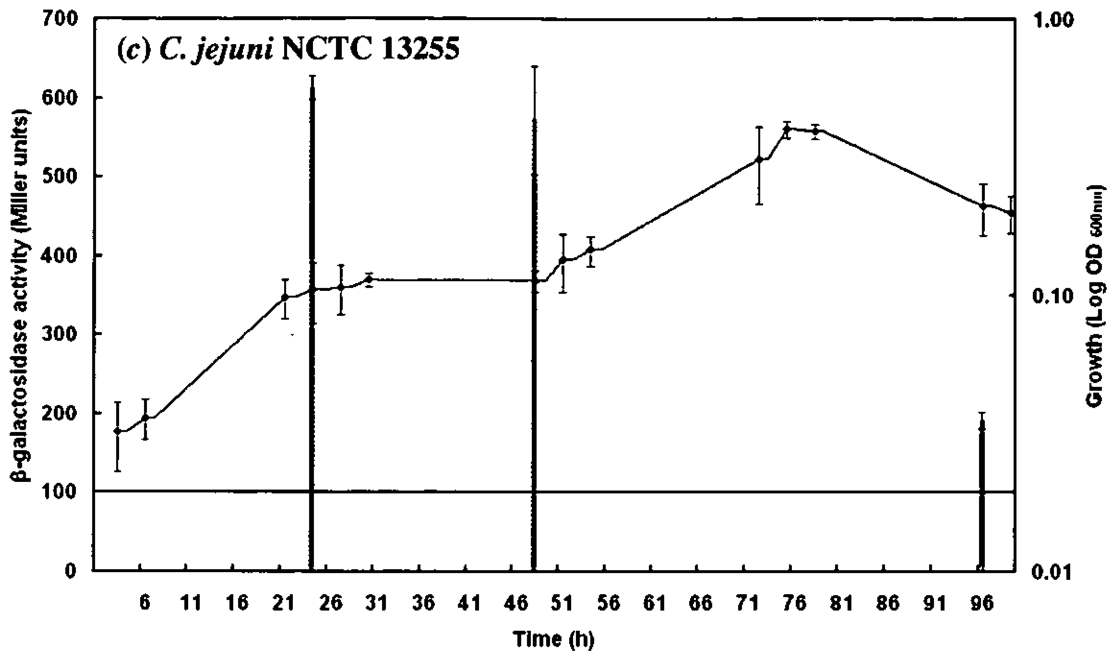


Figure 3.3.4 The induction of β-galactosidase activity during the growth of three *C. jejuni* isolates

β-galactosidase activity induction by extracts derived from *C. jejuni* NCTC 13255, 11168-GS and 11168-O culture supernatants from three separate growth periods (24 h, 48 h and 96 h, orange) were compared with growth which was measured as optical density $OD_{600\,nm}$ for the same *C. jejuni* isolates ($OD_{600\,nm}$, green). The base line at 100 Miller units indicates the considered level of importance for this study. Data are mean ± standard deviation, (n = 3).

(*a*) *C. jejuni* isolate NCTC 11168-GS.

(*b*) *C. jejuni* isolate NCTC11168-O.

(*c*) *C. jejuni* isolate NCTC13255.

3.3.2.3 Growth and β-galactosidase activity induction

The extracts derived from the 24 h *C. jejuni* culture supernatants induced very different levels of β-galactosidase activity, ranging from approximately 100 to 600 Miller units, even though the $OD_{600\ nm}$ readings at that time point are similar (~ 0.1). The extracts derived from the 48 h *C. jejuni* culture supernatants induced relatively similar levels of β-galactosidase activity in the *Agrobacterium* liquid culture bioassay, ranging from approximately 450 to 550 Miller units. Again the $OD_{600\ nm}$ readings at that time point were similar (all ~0.1-0.14). Although, the extracts derived from the 96 h *C. jejuni* culture supernatants induced very different levels of β-galactosidase activity, (*C. jejuni* NCTC 11168-GS ~ 120 Miller units; 11168-O ~ 590 Miller units; and 13255 ~ 190 Miller units) the $OD_{600\ nm}$ measurements for this time point also varied, perhaps reflecting the differences in β-galactosidase induction (*C. jejuni* NCTC 11168-GS ~ 0.3; 11168-O ~ 0.6; and 13255 ~ 0.2).

The long lag phase exhibited by all three of the *C. jejuni* isolates tested indicated by the low optical densities, suggests low cell densities up to 48 h of growth. These low cell density levels complement the relatively low induction levels of β-galactosidase activity for the *C. jejuni* NCTC 11168-GS and 11168-O supernatant extracts for this growth period (Figure 3.3.4 (*a*) and (*b*)) but not the *C. jejuni* NCTC 13255 culture supernatant extract, which induced a high level of β-galactosidase activity for the same growth period (Figure 3.3.4 (*c*)).

The decrease in $OD_{600\ nm}$ for *C. jejuni* NCTC 13255 and 11168-GS from 78 h appears to correspond with the decrease in β-galactosidase activity, induced by the supernatant extracts derived from the 96 h culture supernatants (Figure 3.3.4 (*c*) and (*a*)). *C. jejuni* NCTC 11168-O appears to enter stationary phase later than the other isolates and does not experience the same drop in β-galactosidase activity induction (Figure 3.3.4(*b*)).

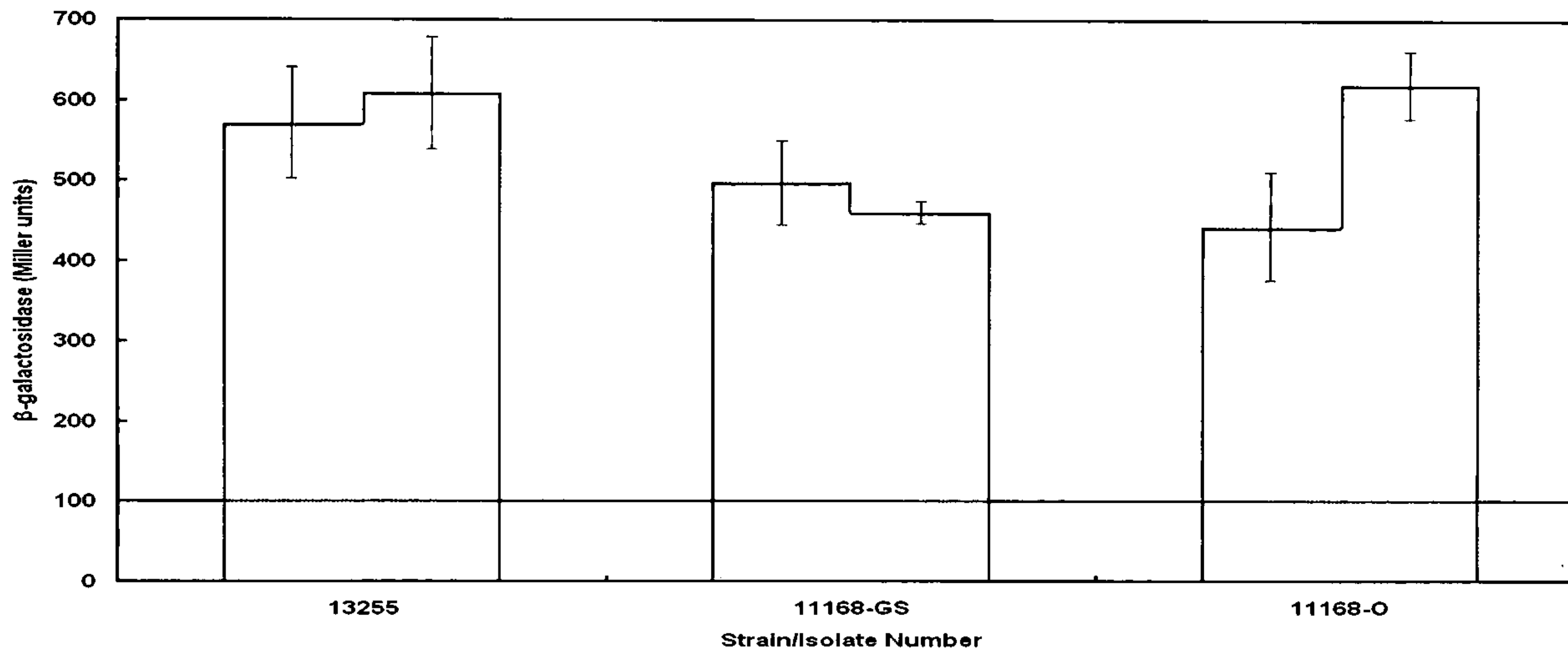


Figure 3.3.5 ***Agrobacterium*** **liquid culture bioassay of** ***C. jejuni*** **isolate culture extracts from various percentage headspace air volumes**

β-galactosidase activity induction by extracts derived from *C. jejuni* NCTC 13255, 11168-GS and 11168-O culture supernatants, were determined at two different percentage headspace air volumes; 23 % (by volume) headspace air (green) and 10 % (by volume) headspace air (blue). The base line at 100 Miller units indicates the considered level of importance for this study. Data are mean ± standard deviation, (n = 3).

	β-galactosidase activity induction levels (Miller units)					
Replicate Number	**23 % (v/v) headspace**			**10 % (v/v) headspace**		
	13255	**11168-GS**	**11168-O**	**13255**	**11168-GS**	**11168-O**
1	504.65	438.01	365.08	559.22	463.90	591.68
2	564.86	523.16	484.81	688.66	474.74	551.48
3	642.27	535.86	479.50	581.02	446.39	508.51
Mean	570.59	499.01	443.13	609.63	461.68	550.56
SD	68.99	53.21	67.65	69.30	14.31	41.59
Statistical Comparison	**13255 vs 11168-GS**	**13255 vs 11168-O**	**11168-GS vs 11168-O**	**13255 vs 11168-GS**	13255 vs 11168-O	**11168-GS vs 11168-O**
p value	**0.03**	**0.02**	**0.02**	**0.03**	0.18	**0.02**

	β-galactosidase activity induction levels (Miller units)					
Replicate Number	**13255**		**11168-GS**		**11168-O**	
	23 % (v/v)	**10 % (v/v)**	**23 % (v/v)**	**10 % (v/v)**	**23 % (v/v)**	**10 % (v/v)**
1	504.65	559.22	438.01	463.90	365.08	591.68
2	564.86	688.66	523.16	474.74	484.81	551.48
3	642.27	581.02	535.86	446.39	479.502	508.51
Mean	570.59	609.63	499.01	461.68	443.13	550.56
SD	68.99	69.30	53.21	14.31	67.65	41.59
Comparison	23 % (v/v) vs 10 % (v/v)		23 % (v/v) vs 10 % (v/v)		23 % (v/v) vs 10 % (v/v)	
p value	0.27		0.19		0.11	

Table 3.3.2 Student t-test analysis (one-tailed) of *Agrobacterium* liquid culture bioassay of *C. jejuni* isolate culture extracts from various percentage headspace air volumes

β-galactosidase activity induction levels (Miller units) for replica extracts derived from *C. jejuni* NCTC 13255, 11168-GS and 11168-O culture supernatants from two separate % headspace volumes; 10 % (by volume) headspace air and 23 % (by volume) headspace air. All significant comparisons are highlighted in bold ($p < 0.05$).

3.3.3 Aerotolerance and β-galactosidase activity induction

As previously stated *C. jejuni* is a strict microaerophile and therefore the effect of changing the level of available oxygen and the levels of toxic oxygen derivatives within the growth medium was determined (3.1.1).

3.3.3.1 The effect of the percentage headspace air volume on β-galactosidase activity induction

The decrease in dissolved oxygen concentration produced by decreasing the volume of headspace air from 23 % to 10 % appears to have no effect on the activity of the supernatant extracts of the *C. jejuni* laboratory isolates (NCTC 13255 and NCTC 11168-GS) (Figure 3.3.5, Table 3.3.2). The extract from the decreased dissolved oxygen concentration culture of *C. jejuni* NCTC 11168-O, (the clinical isolate), appears to have a more positive effect on β-galactosidase activity induction, (Figure 3.3.5). However, the statistical analysis indicates that this increase is not significant at the $p < 0.05$ level (Table 3.3.2). Interestingly the *C. jejuni* NCTC 11168-O 23 % headspace extract induced significantly lower levels ($p < 0.05$) of β-galactosidase activity than the *C. jejuni* NCTC 11168-GS 23 % headspace extract, where as the *C. jejuni* NCTC 11168-O 10 % headspace extract induced significantly higher levels ($p < 0.05$) of β-galactosidase activity than the equivalent NCTC 11168-O extract (Figure 3.3.5, Table 3.3.2).

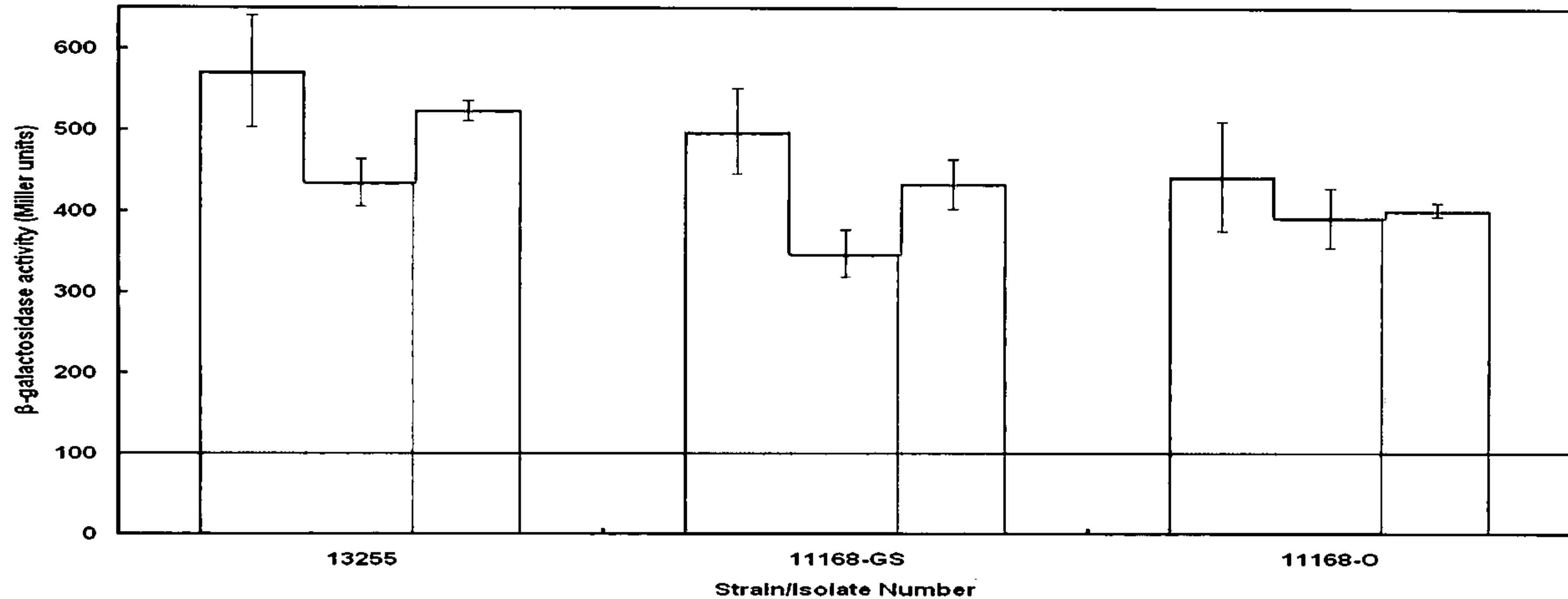


Figure 3.3.6 ***Agrobacterium*** **liquid culture bioassay of** ***C. jejuni*** **isolate culture extracts from supplemented culture conditions**

β-galactosidase activity induction was determined for extracts derived from *C. jejuni* NCTC 13255, 11168-GS and 11168-O culture supernatants produced from three culture conditions; BB alone (control) (green); BB supplemented with 7.0 % (v/v) blood (blue); and BB supplemented with 0.2 % w/v $FeSO_4.7H_2O$ (orange). The base line at 100 Miller units indicates the considered level of importance for this study. Data are mean ± standard deviation, (n = 3).

	β-galactosidase activity induction levels (Miller units)								
Replicate Number	**BB**			**BLD**			**IRON**		
	13255	**11168-GS**	**11168-O**	**13255**	**11168-GS**	**11168-O**	**13255**	**11168-GS**	**11168-O**
1	495.65	479.21	365.08	403.38	342.38	352.26	510.13	462.37	395.06
2	584.86	458.16	492.81	460.06	322.65	424.06	527.89	401.92	400.66
3	633.27	556.86	468.50	442.68	379.41	401.13	532.69	439.18	412.54
Mean	571.26	498.08	442.13	435.37	348.15	392.48	523.57	434.49	402.75
SD	69.81	51.99	67.83	29.04	28.82	36.67	11.89	30.50	8.93
Statistical Comparison	13255 vs 11168-GS	**13255 vs 11168-O**	11168-GS vs 11168-O	**13255 vs 11168-GS**	**13255 vs 11168-O**	11168-GS vs 11168-O	**13255 vs 11168-GS**	**13255 vs 11168-O**	11168-GS vs 11168-O
p value	0.07	**0.013**	0.17	**0.04**	**0.01**	0.13	**0.03**	**0.00**	0.12

	β-galactosidase activity induction levels (Miller units)								
Replicate Number	**13255**			**11168-GS**			**11168-O**		
	BB	**BLD**	**IRON**	**BB**	**BLD**	**IRON**	**BB**	**BLD**	**IRON**
1	495.65	403.38	510.13	479.21	342.38	462.37	365.08	352.26	395.06
2	584.86	460.06	527.89	458.16	322.65	401.92	492.81	424.06	400.66
3	633.27	442.68	532.69	556.86	379.41	439.18	468.50	401.13	412.54
Mean	571.26	435.37	523.57	498.08	348.15	434.49	442.13	392.48	402.75
SD	69.81	29.04	11.89	51.99	28.82	30.50	67.83	36.67	8.93
Statistical Comparison	**BB vs bld**	**Bld vs Iron**	BB vs iron	**BB vs bld**	**Bld vs iron**	BB vs iron	BB vs bld	Bld vs iron	BB vs iron
p value	**0.02**	**0.01**	0.15	**0.00**	**0.02**	0.08	0.06	0.32	0.20

Table 3.3.3 Student t-test analysis (one-tailed) of *Agrobacterium* liquid culture bioassay of *C. jejuni* isolate culture extracts from supplemented culture conditions

β-galactosidase activity induction levels (Miller units) for replica extracts derived from *C. jejuni* NCTC 13255, 11168-GS and 11168-O culture supernatants from three separate supplementary conditions; BB alone, BB supplemented with 7.0 % (v/v) blood (Bld); and BB supplemented with 0.2 % w/v $FeSO_4.7H_2O$ (Iron). All significant comparisons are highlighted in bold ($p < 0.05$).

3.3.3.2 The effect of culture supplementation on β-galactosidase activity induction

The previous experiments (Figure 3.3.5) indicated that decreasing the % headspace air volume had no effect on the level of β-galactosidase induction in the *Agrobacterium* liquid culture bioassay by any of the extracts derived from the three *C. jejuni* clinical isolate culture supernatants tested. Therefore the effect of culture supplementation with blood and ferrous sulphate which quench toxic oxygen derivatives on the induction levels of β-galactosidase activity was also determined.

Supplementation of BB with 7.0 % (v/v) blood appears to significantly decrease ($p < 0.05$) the induction of β-galactosidase activity in comparison with BB medium alone, for the extract from *C. jejuni* NCTC 13255 and 11168-GS culture supernatants, the two laboratory isolates, but it has no significant effect ($p < 0.05$) on β-galactosidase activity induction for the *C. jejuni* NCTC 11168-O (clinical isolate) supernatant extract (Figure 3.3.6, Table 3.3.3). Interestingly ferrous sulphate supplementation (0.2 % w/v) of the culture medium appears to have no significant effect ($p < 0.05$) on β-galactosidase activity induction in comparison with BB alone for all three of the *C. jejuni* isolates tested (Figure 3.3.6, Table 3.3.3).

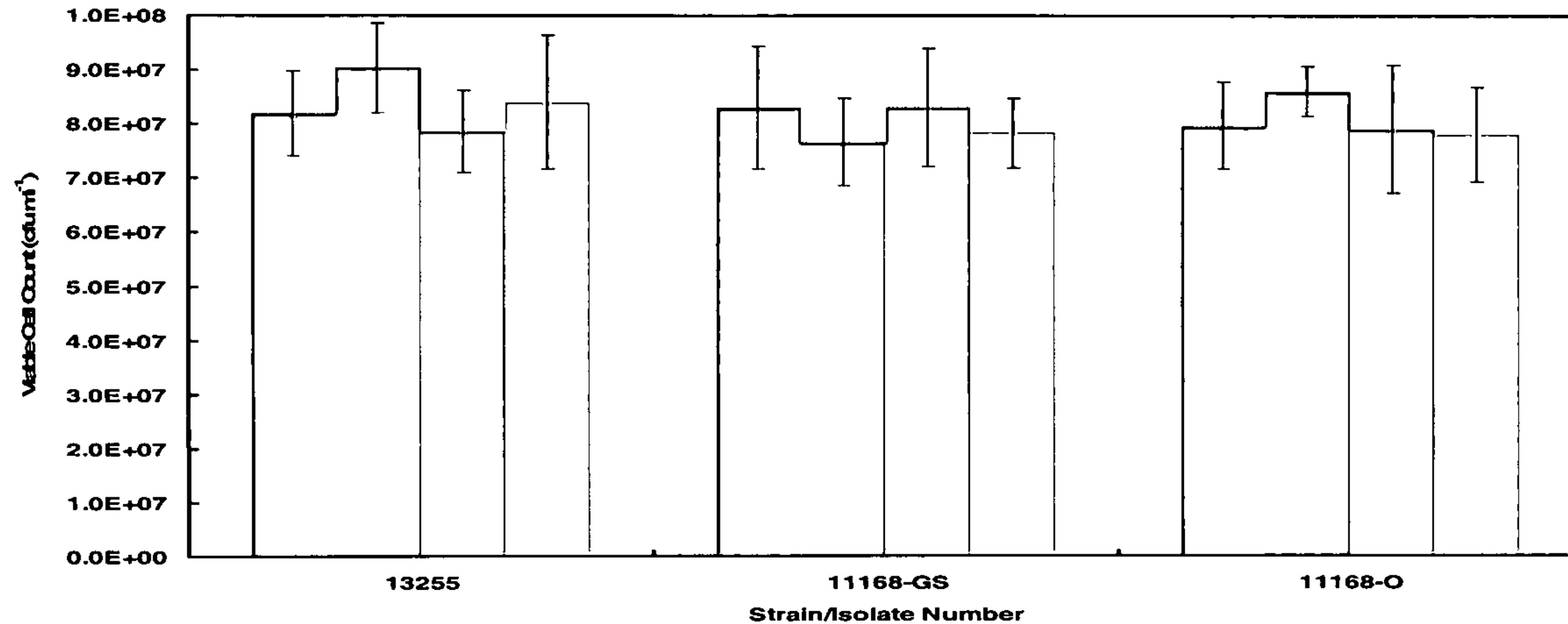


Figure 3.3.7 ***C. jejuni*** **viable cell counts (cfu ml^{-1}) under various growth conditions**

Viable cell counts for four different culture conditions (supplementation and % headspace air volume) were determined after 48 h of growth. Standard conditions (no supplementation, 23 % headspace (v/v) (green); blood supplementation (7.0 % v/v) 23 % headspace air (v/v), (blue); Ferrous sulphate supplementation (0.2 % v/v) 23 % headspace air (v/v) (orange); no supplementation, 10 % headspace air (v/v) (yellow). Data are mean ± standard deviation, (n = 3).

3.3.4 Viable cell counts

One explanation for the varying levels of β-galactosidase induction by the various *C. jejuni* culture supernatant extracts is that they may reflect variations in viability of the *C. jejuni* cell cultures under the conditions used. Thus, viable cell counts were performed on the *C. jejuni* cultures, under the different growth conditions (Figure 3.3.7). There was no difference in the number of viable cells detected for any of the *C. jejuni* isolates or for *C. jejuni* NCTC 13255, 11168-GS or 11168-O when grown in either 23 % (v/v) or 10 % (v/v) culture volumes or when supplemented with lysed horse blood (7.0 % [v/v]) or ferrous sulphate (0.2 % [w/v]) (Figure 3.3.7).

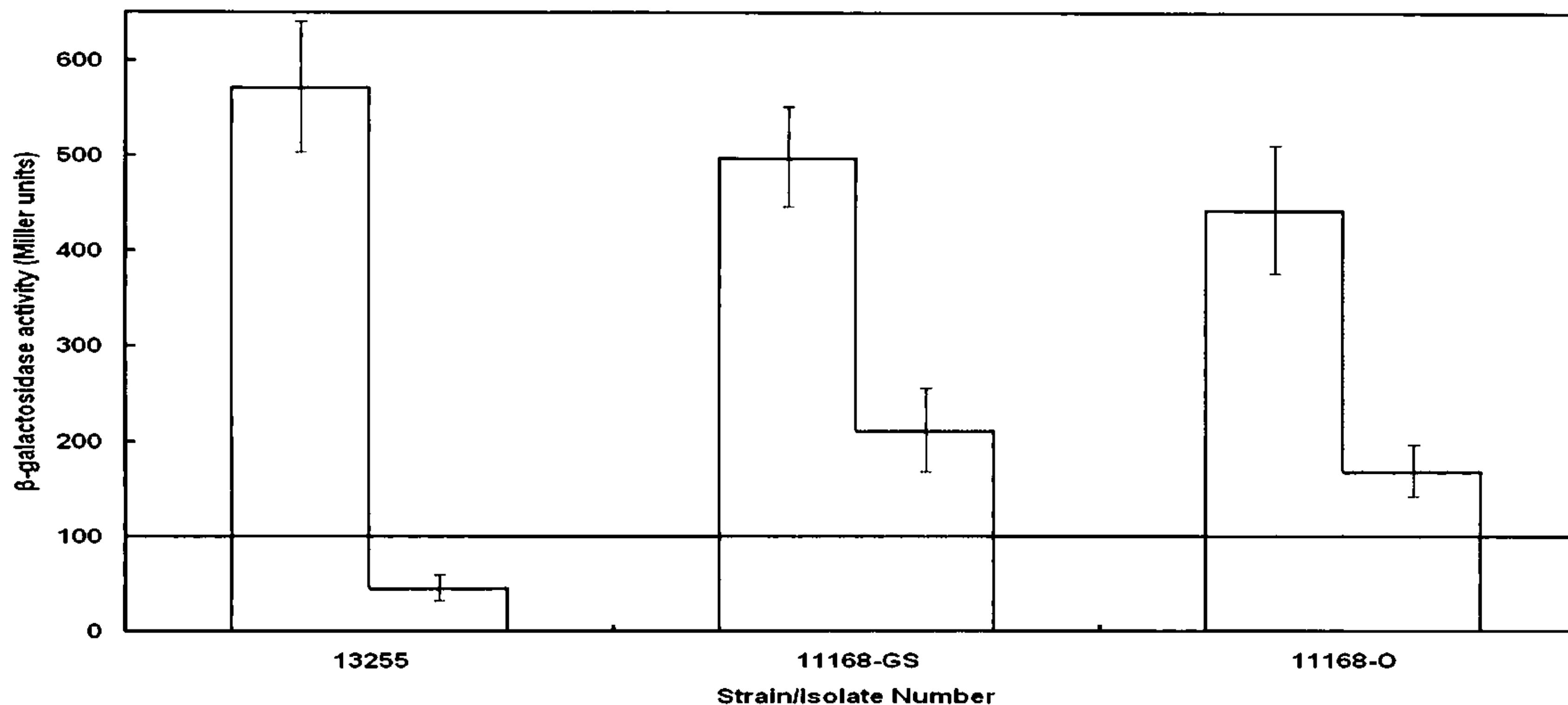


Figure 3.3.8 Effect of incubation with serum on the induction of β-galactosidase activity

β-galactosidase activity induced by non-treated (blue) and serum treated (green) extracts derived from *C. jejuni* NCTC 13255, 11168-GS and 11168-O culture supernatants was determined. The base line at 100 Miller units indicates the considered level of importance for this study. Data are mean ± standard deviation, (n = 3).

Replicate Number	β-galactosidase activity induction levels (Miller units)					
	Normal			Treated		
	13255	11168-GS	11168-O	13255	11168-GS	11168-O
1	499.65	438.51	366.68	33.38	258.81	138.03
2	581.86	518.16	491.81	60.00	172.10	187.91
3	636.27	538.86	471.50	44.69	205.46	182.97
Mean	572.59	498.51	443.33	46.02	212.12	169.64
SD	68.78	52.98	67.15	13.36	43.74	27.49
Statistical Comparison	**13255 vs 11168-GS**	**13255 vs 11168-O**	**11168-GS vs 11168-O**	**13255 vs 11168-GS**	**13255 vs 11168-O**	11168-GS vs 11168-O
p value	**0.01**	**0.01**	**0.03**	**0.02**	**0.00**	0.20

	β-galactosidase activity induction levels (Miller units)					
Replicate Number	**13255**		**11168-GS**		**11168-O**	
	Normal	**Treated**	**Normal**	**Treated**	**Normal**	**Treated**
1	499.65	33.38	438.51	258.81	366.68	138.03
2	581.86	60.00	518.16	172.10	491.81	187.91
3	636.27	44.69	491.81	205.46	471.50	182.97
Mean	572.59	46.02	482.83	212.12	443.33	169.64
SD	68.78	52.98	67.16	13.36	43.74	27.48
Comparison	**Normal vs Treated**		**Normal vs Treated**		**Normal vs Treated**	
p value	**0.00**		**0.02**		**0.00**	

Table 3.3.4 Student t-test analysis (one-tailed) of the effect of incubation with serum on the induction of β-galactosidase activity

β-galactosidase activity induction levels (Miller units) for replica extracts derived from *C. jejuni* NCTC 13255, 11168-GS and 11168-O culture supernatants were determined from two separate conditions; serum treated (treated) and non-treated (normal). All significant comparisons are highlighted in bold ($p < 0.05$).

3.3.5 Active molecule separation and structural assessment

To further characterise this putative AI the active compound(s) extracted from the *C. jejuni* culture supernatants was separated and purified enabling chemical structural analysis.

3.3.5.1 The effect of incubation with serum on the induction of β-galactosidase activity

Previous work by Yang *et al.* (2005) has shown that AHL activity can be inactivated by incubation with sera from a range of mammalian species. Draganov *et al.* (2005) demonstrated that this inactivation may be due to paraoxonase enzymes in the serum. Thus, extracts derived from the *C. jejuni* NCTC 13255, 11168-GS and 11168-O 48 h culture supernatants were incubated with horse serum to see if the induction activity of the extracts was affected.

The serum treated extracts demonstrated significantly lower levels ($p < 0.05$) of β-galactosidase activity induction when compared with the levels of β-galactosidase activity induced by the standard, untreated extracts for all three *C. jejuni* isolates (Figure 3.3.8, Table 3.3.4). The controls indicated that the differences observed were due to the effect of the serum and not the PBS or the heat treatment (data not presented). Interestingly the serum treated extracts from NCTC 11168-GS and NCTC 11168-O induced significantly higher levels ($p < 0.05$) of β-galactosidase activity induction when compared with the β-galactosidase activity induction level for the serum treated *C. jejuni* NCTC 13255 extract (Figure 3.3.8, Table 3.3.4).

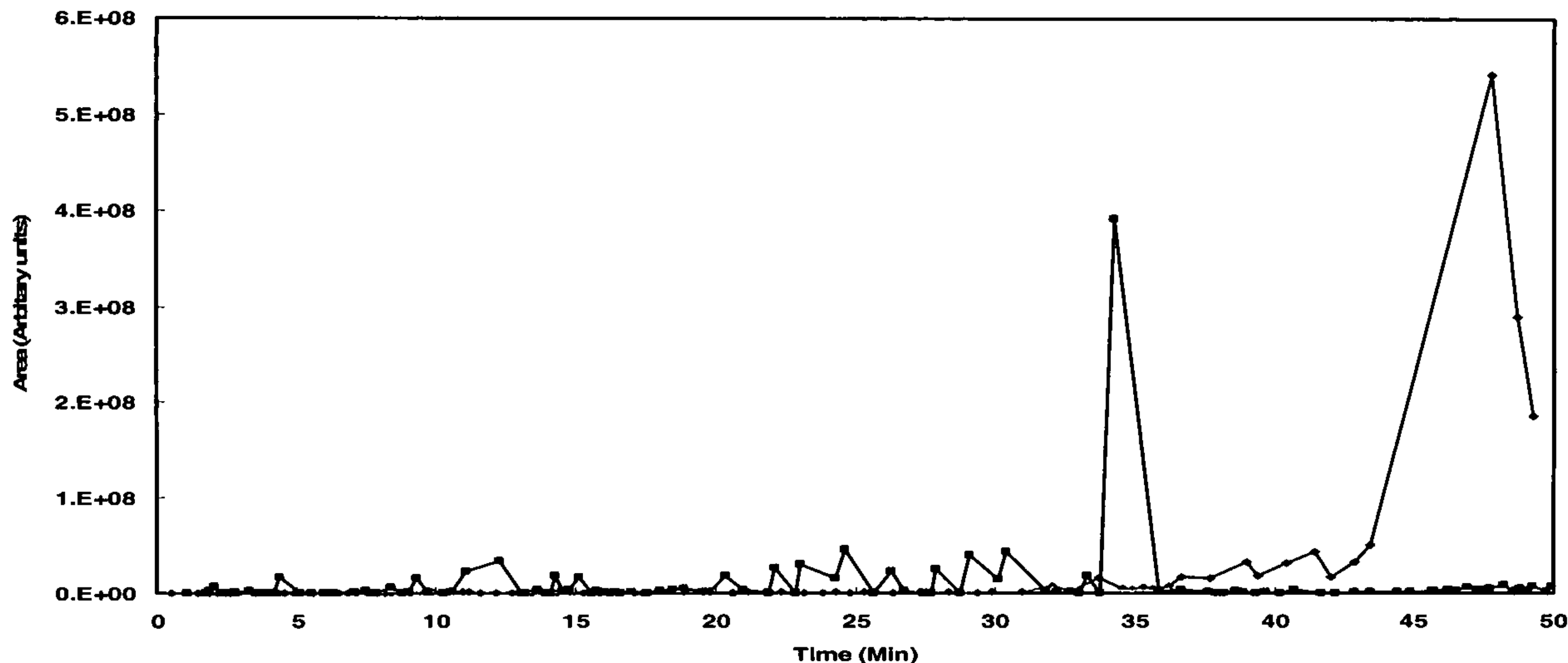


Figure 3.3.9 HPLC analysis of a concentrated extract derived from *C. jejuni* NCTC 13255 (blue) and *A. tumefaciens* UL224 (orange) supernatants The concentrated (x 20) sample was applied to a S5ODS2 C_{18} reverse-phase semi-preparative column. The elution procedure consisted of a ddH_2O:methanol gradient-up to 25 % (v/v) for the first 15 min, then increasing to 60 % (v/v) by 35 min and 100 % (v/v) by 50 min. Fractions were collected at every $OD_{210\,nm}$ peak (Section 3.2.10.2).

3.3.5.2 HPLC separation

As substantial evidence has been obtained in this study that the test isolates of *C. jejuni* can produce a compound capable of inducing β-galactosidase activity in the *Agrobacterium* liquid culture bioassay, supernatants of *C. jejuni* NCTC 13255 cultures were extracted with ethyl acetate, concentrated further (x 20) by freeze drying and then analysed by HPLC and compared with analogous extracts from *A. tumefaciens* UL224, the positive control. A substantial peak was eluted between 43.50 and 49.25 min for the *A. tumefaciens* positive control (UL224) supernatant extract and between 33.74 and 35.75 min for the *C. jejuni* NCTC 13255 supernatant extract (Figure 3.3.9). The activity of these fractions was then determined with the *Agrobacterium* liquid culture bioassay.

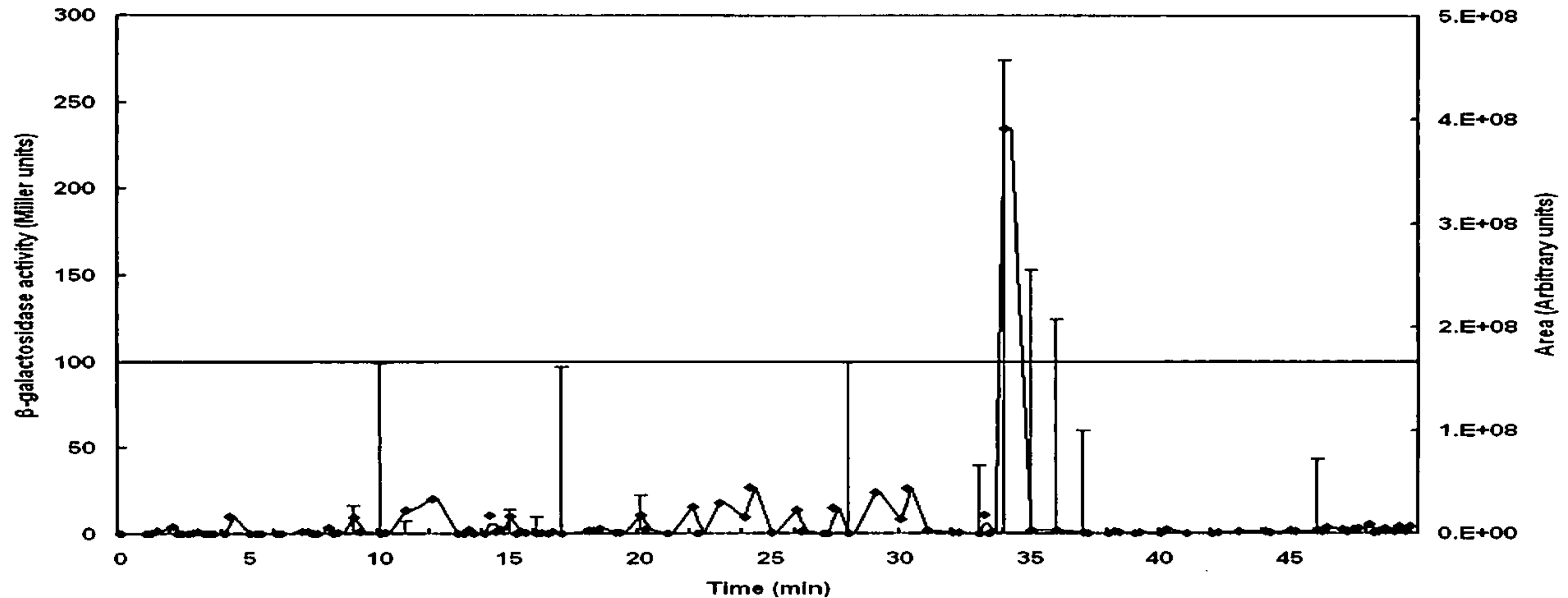


Figure 3.3.10 ***Agrobacterium*** **liquid culture bioassay of various fractions produced by the HPLC analysis of a concentrated extract derived from** ***C. jejuni*** **NCTC 13255**

β-galactosidase activity induction (orange) by fractions eluted from the HPLC separation of *C. jejuni* NCTC 13255 supernatant extracts (blue) were determined as indicated in the HPLC elution profile detailed above. The base line at 100 Miller units indicates the considered level of importance for this study.

3.3.5.3 The induction of β-galactosidase activity by HPLC separated fractions

When the *Agrobacterium* liquid culture bioassay was performed on the fractions produced by the HPLC analysis of the concentrated *C. jejuni* NCTC 13255 culture supernatant extract, the main UV absorbance peak corresponds with the highest induction levels of β-galactosidase activity indicating that this fraction contains the highest concentrations of the active component (Figure 3.3.10). It is clear that the active component(s) derived from the *C. jejuni* NCTC 13255 culture supernatant has been enriched and fractionated by the HPLC. Therefore LC-MS was used to further aid in characterising the active component(s) in the fraction eluted between 33.74 and 35.75 min.

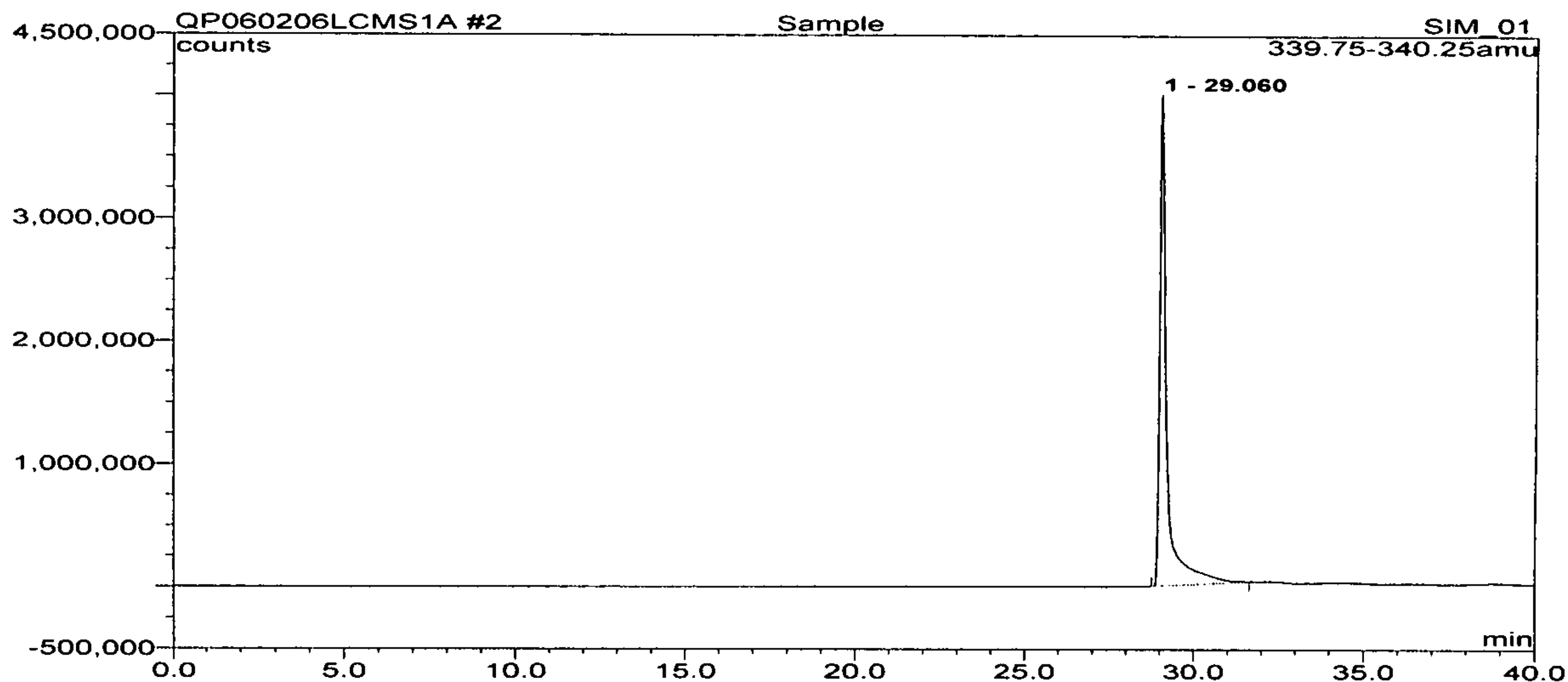


Figure 3.3.11 LC-MS SIM

A typical LC-MS SIM spectrum (ESI, positive mode) for the major active fraction from the concentrated *C. jejuni* NCTC 13255 culture supernatant extract, identified by HPLC and the *Agrobacterium* liquid bioassay in the previous experiments (3.3.5.2). This selected ion monitoring spectrum shows a peak between 339.75 - 340.25 *m/z*, eluting at 29.06 min.

3.3.5.4 LC-MS

The major fraction identified by HPLC in the previous experiments (3.3.5.2; 3.3.5.3) eluting between 33.74 and 35.75 min, was analysed by LC-MS. LC-MS full scan analysis of that fraction from the *C. jejuni* NCTC 13255 culture supernatant extract, indicated a peak at ~ 340 *m/z* which eluted after approximately 30 min (data not shown). SIM, where the mass spectrometer scans a very small mass range only detecting and plotting compounds with a selected mass, allows the mass spectrometer to dwell for a longer time over a smaller mass range. Thus, SIM LC-MS is more sensitive than a full LC-MS scan. SIM (339.75 - 340.25 *m/z*, EPI, positive mode) of several repeat HPLC separated samples eluted over the same time period identified peaks in all the HPLC separated samples tested (Figure 3.3.11).

3.4 DISCUSSION

3.4.1 Induction of β-galactosidase activity of *C. jejuni* isolate extracts

The induction of β-galactosidase activity in the *Agrobacterium* liquid culture bioassay (Zhu *et al.*, 2003) by all of the *C. jejuni* supernatant extracts tested in this preliminary screen, suggests the presence of an active component in the *C. jejuni* culture supernatant extracts and therefore the conditioned medium (Figure 3.3.1). The negligible levels of β-galactosidase activity induced by extracts of several batches of sterile BB medium, indicated that the β-galactosidase activity induction is due to an active compound extracted from the *C. jejuni* supernatants rather than any active compounds already present in the growth medium. These results indicate putative production of an AI-like active compound by all the *C. jejuni* isolates tested, including the genome sequenced strain NCTC 11168-GS, providing preliminary evidence of a previously unidentified signalling system in some *C. jejuni* strains that is not just limited to a few unrepresentative isolates. Interestingly, under the conditions used in this study, there appears to be no difference in the induction levels for *C. jejuni* NCTC 11168-GS and 11168-O, suggesting comparable concentrations of the active compound in the extracts (Figure 3.3.1).

3.4.2 Growth period length and β-galactosidase activity induction

3.4.2.1 The effect of growth period length (24 h, 48 h and 96 h) on β-galactosidase activity induction

The individual induction profiles of the three *C. jejuni* strains tested, indicate that each isolate has different kinetics of active compound(s) production and therefore generalisation between these isolates is inappropriate (Figure 3.3.2). As all three of the isolates tested have a relatively high activity level for the 48 h supernatant

extracts, all subsequent results will be reported for 48 h extracts. Interestingly there is a significant difference ($p < 0.05$) between the levels of β-galactosidase activity induced by the *C. jejuni* NCTC 11168 variants for all three time periods, suggesting that as well as exhibiting different virulence profiles (Gaynor *et al.*, 2004), these isolates are also able to produce different *lacZ* fusion induction profiles in the *Agrobacterium* liquid culture bioassay, over a 96 h period (Figure 3.3.2).

3.4.2.2 Growth curves of *C. jejuni* isolates

All three *C. jejuni* isolates tested exhibited a long lag phase which is likely to be a result of the different chemical compositions of the media used; BA medium was used for the initial "resuscitation culture", in comparison with BB medium, which was used to grow the isolates (Figure 3.3.3). This lag in cell division is often associated with a physiological adaptation to a new environment by the cells, prior to their resumption of cell division. Therefore the different atmosphere generation methods used for the two media and the change in growth state (solid to liquid), may also be associated with this lag in cell division. Previous growth studies of the two *C. jejuni* NCTC 11168 variants have not highlighted a sustained lag phase, but in these studies, the atmosphere generation method and the medium constituents remained constant (Gaynor *et al.*, 2004).

Comparison of the $OD_{600\ nm}$ levels as a measure of growth for the *C. jejuni* isolates NCTC 11168-GS and 11168-O indicated that *C. jejuni* isolate NCTC 11168-GS initially demonstrated similar growth kinetics to the *C. jejuni* isolate NCTC 11168-O, but eventually the latter isolate reached a higher optical density (Figure 3.3.3). Gaynor *et al.* (2004) also compared the growth of *C. jejuni* isolates NCTC 11168-GS and 11168-O but found that the *C. jejuni* isolate NCTC 11168-GS grew faster and reached

a higher optical density. However, as the two studies utilised different media and atmosphere generation methods, it is probably inappropriate to compare the results.

3.4.2.3 Growth and β-galactosidase activity induction

All three of the *C. jejuni* strains tested exhibited a long lag phase indicated by the relatively low optical densities before 48 h, suggesting low cell densities for all three cultures (Figure 3.3.3). Interestingly these low cells densities reflect the low induction levels of β-galactosidase activity induced by the extracts derived from the *C. jejuni* NCTC 11168-GS and 11168-O 24 h culture supernatants, suggesting a link between cell density and active compound production (Figure 3.3.4 (*a*) and (*b*)). However, the *C. jejuni* NCTC 13255 extract for this growth period exhibited a high level of β-galactosidase activity, suggesting a high concentration of active compound in the 24 h supernatant extract from this strain (Figure 3.3.4 (*c*)). As yet it is unclear as to why *C. jejuni* isolate NCTC 13255 appears to produce high levels of the active compound during the prolonged lag phase when the cell density is low. This result may be a peculiarity specific to this strain emphasising the inappropriateness of trying to generalise amongst *C. jejuni* strains.

The reduction in optical density $OD_{600\ nm}$ for both the *C. jejuni* laboratory isolates, NCTC 13255 and 11168-GS at 78 h, indicates the end of stationary phase and a possible decline in cell number for these strains (Figure 3.3.4 (*c*) and (*a*)), whereas the optical density of the *C. jejuni* clinical isolate (NCTC 11168-O) continued to increase beyond 78 h and appeared to peak at 96 h (Figure 3.3.4 (*b*)). This sustained growth again seems to correlate with the maintenance of β-galactosidase activity induction by the *C. jejuni* NCTC 11168-O supernatant extract (Figure 3.3.4 (*b*). Again this result and the decrease in β-galactosidase activity induction by both *C. jejuni* NCTC 11168-

GS and 13255, suggest that the production of the active compound is linked to cell density.

3.4.3 Aerotolerance and β-galactosidase activity induction

C. jejuni is microaerophilic and as such is capable of oxygen-dependent growth, using oxygen as a terminal electron acceptor for energy-yielding respiration. However, oxygen and its derivatives including superoxide anions, hydroxyl radicals, singlet oxygen and hydrogen peroxide are frequently toxic to microaerophiles and although optimum oxygen levels are species and strain specific, atmospheric oxygen concentrations (21 % v/v) are generally considered to be inhibitory for *C, jejuni* growth (Hoffman *et al.*, 1979; Krieg and Hoffman, 1986).

3.4.3.1 The effect of the percentage headspace air volume on β-galactosidase activity induction

As for all liquid medium, the dissolved oxygen levels for BB are naturally relatively low. BB is specifically designed for microaerophilic growth, with the inclusion of haemin, sodium metabisulphite and sodium pyruvate which act as oxygen quenchers (Morris, 1976; Bolton *et al.*, 1984*a*; Bolton *et al.*, 1984*b*; Humphrey, 1990; Corry *et al.*, 1995; Mohammed *et al.*, 2005).

The potential decrease in the dissolved oxygen concentration in the media, induced by the decrease in the % headspace air volume from 23 % to 10 %, appears to have had no significant effect ($p < 0.05$) on the induction of β-galactosidase activity within the bioassay by extracts from the *C. jejuni* laboratory isolate supernatants (NCTC 13255 and NCTC 11168-GS) (Figure 3.3.5).

Although the extract derived from the clinical isolate *C. jejuni* NCTC 11168-O supernatant, under the same conditions, appears to induce an increase in β-galactosidase activity, suggesting an increase in the amount of active compound present in the extract (Figure 3.3.5) this difference is not significant ($p < 0.05$, Table 3.3.2). Significantly lower levels ($p < 0.05$) of β-galactosidase activity were induced by the *C. jejuni* NCTC 11168-O 23 % headspace extract, when compared to the levels induced by the *C. jejuni* NCTC 11168-GS 23 % headspace extract. This is surprising as the *C. jejuni* NCTC 11168-O 10 % headspace extract induced significantly higher levels ($p < 0.05$) of β-galactosidase activity than the equivalent NCTC 11168-O extract (Figure 3.3.5, Table 3.3.2). This indicates that the two variants are able to produce different *lacZ* fusion induction profiles in the *Agrobacterium* liquid culture bioassay.

This lack of a significant difference ($p < 0.05$) in active compound production for both *C. jejuni* NCTC 11168-GS and 11168-O as a consequence of a change in the dissolved oxygen concentration, is of particular interest, considering that Gaynor *et al.* (2004) demonstrated dramatic expression differences for several gene families, under microaerobic and severely oxygen-limited conditions in *C. jejuni* NCTC 11168-GS and 11168-O. Gaynor *et al.* (2004) also suggested that adaptation to different oxygen tensions may influence colonisation potential given that anaerobic priming/aerobic passage can result in an increase/decrease, in colonisation compared with the parent strains. Additionally it is conceivable that different stress responses are linked to the production of this putative AI in these strains.

3.4.3.2 The effect of culture supplementation on β-galactosidase activity induction

BB is frequently supplemented with lysed horse blood, which acts to prevent the build up of toxic oxygen products that may hinder *Campylobacter* growth. Horse blood and other *Campylobacter* growth medium supplements are generally considered to act as quenching or detoxifying agents rather than as enrichment factors (Bolton *et al.*, 1984*a*). Bolton *et al.* (1984*a*) demonstrated that blood is an excellent supplement for *Campylobacter* growth medium in that it was effective in neutralising the effects of toxic oxygen-derived substances such as superoxide anions, hydroxyl radicals, singlet oxygen and hydrogen peroxide (Hoffman *et al.*, 1979).

Supplementation of BB medium with 7.0 % (v/v) lysed horse blood appears to decrease the induction of β-galactosidase activity in comparison with unsupplemented BB, for the extracts derived from the supernatants of the two *C. jejuni* laboratory isolates (NCTC 13255 and 11168-GS) (Figure 3.3.6). This apparent decrease may be due to reduced levels of toxic oxygen derivatives.

Supplementation of BB with 7.0 % (v/v) lysed horse blood appears to have no significant effect ($p < 0.05$) on β-galactosidase activity induced by the extract produced from the clinical isolate, *C. jejuni* NCTC 11168-O supernatant (Figure 3.3.6). This suggests that this variant of *C. jejuni* NCTC 11168 may be more tolerant of the toxic oxygen derivatives, which may be advantageous in a clinical setting. Both QS and AHL production have been linked to biofilm formation in many bacterial species, including *P. aeruginosa* (Favre-Bonté *et al.*, 2003). Recent research has indicated that supplementation of BB with 7.0 % (v/v) lysed horse blood increases biofilm formation by both *C. jejuni* NCTC 11168-GS and 11168-O (Dowling, 2006). Therefore, in the light of this research, the apparent difference in response to

supplementation with 7.0 % (v/v) lysed horse blood in comparison with unsupplemented BB medium by the two *C. jejuni* NCTC 11168 variants, is of particular interest. These results again highlight the differences between the two *C. jejuni* NCTC 11168 variants.

Ferrous sulphate is also known to prevent the build up of toxic oxygen products that may hinder *Campylobacter* growth. Thus, the effect of supplementing BB with ferrous sulphate on the level of active compound in the extract was determined. Ferrous sulphate supplementation (0.2 % w/v) of BB appears to have no significant effect ($p < 0.05$) on β-galactosidase activity induction, in comparison with extracts derived from unsupplemented BB cultures, for all three of the *C. jejuni* isolates tested (Figure 3.3.6). Bolton *et al.* (1984*a*) demonstrated that ferrous sulphate (0.025 % w/v) is less effective than lysed horse blood in preventing the formation and build up of toxic substances as it does not completely neutralise all the toxic products. In light of this, the absence of a significant change ($p < 0.05$) in the levels of β-galactosidase activity induction and therefore the active compound concentrations, when comparing ferrous sulphate supplementation with unsupplemented BB for all three *C. jejuni* isolates, is unsurprising (Figure 3.3.6).

As previously stated, the pH of the supplemented and unsupplemented BB growth medium was 7.3 ± 0.2, therefore it is unlikely that any changes in the level of β-galactosidase induction observed for the supplemented *C. jejuni* culture supernatant extracts were due to chemical lactonolysis or any other pH induced degradation of the active compound (1.1.9.3.4; 3.2.5.2).

3.4.3.3 Percentage headspace air volume, culture supplementation and β-galactosidase activity induction

Despite being generally considered as an obligate microaerophile, *C. jejuni* must be able to survive (although not necessarily grow) within a wide variety of environmental niches, including the microaerobic and often severely oxygen-limited environment of the avian and mammalian GI tract and the aerobic conditions in the general environment (Gaynor *et al.*, 2004).

Decreasing the % headspace air volume from 23 % to 10 % and potentially the available oxygen and the dissolved oxygen levels, appears to have no significant effect ($p < 0.05$) on the composition of extracts from the any of the *C. jejuni* isolate cultures. However, significantly lower levels ($p < 0.05$) of β-galactosidase activity were induced by the *C. jejuni* NCTC 11168-O 23 % headspace extract, when compared to the levels induced by the *C. jejuni* NCTC 11168-GS 23 % headspace extract, whereas the *C. jejuni* NCTC 11168-O 10 % headspace extract induced significantly higher levels ($p < 0.05$) of β-galactosidase activity than the equivalent NCTC 11168-O extract (Figure 3.3.5, Table 3.3.2). This indicates that the two variants are able to produce different *lacZ* fusion induction profiles in the *Agrobacterium* liquid culture bioassay.

For both the *C. jejuni* laboratory strains (NCTC 11168-GS and NCTC 13255) , culture supplementation (7.0 % (v/v) lysed horse blood) appears to significantly decrease ($p < 0.05$) the induction of β-galactosidase activity when compared with the induction levels for unsupplemented BB, supernatant but appears to have no significant effect ($p < 0.05$) on β-galactosidase activity induction for the *C. jejuni* NCTC 11168-O clinical variant supernatant extract (Figure 3.3.6, Table 3.3.2). These results suggest that the clinical variant, *C. jejuni* NCTC 11168-O is more tolerant of

the toxic oxygen derivatives than the laboratory variant *C. jejuni* NCTC 11168-GS. A balance is required for microaerophilic organisms, ensuring that sufficient oxygen is available for energy production and DNA synthesis but that the levels of toxic oxygen products are not inhibitory. However, it is important to note that optimum levels of oxygen and tolerance of toxic oxygen derivatives will vary for both strain and isolate, as does the ability of *Campylobacter* to utilise alternative electron acceptor for respiration under highly restricted oxygen conditions (Pearson *et al.*, 2003).

3.4.4 Viable cell counts

As there was no difference in the number of viable cells detected for any of the *C. jejuni* strains under any of the growth conditions, the differences in β-galactosidase activity can be attributed to variations in the concentration of the active compound(s) in the supernatant extracts (Figure 3.37).

Gaynor *et al.* (2004) found that neither variant of *C. jejuni* NCTC 11168 grew well under severely oxygen-limited conditions. Therefore the lack in variation in the viable cell counts for the change in the % headspace air volume (from 23 % v/v to 10 % v/v) is of specific interest, indicating that the culture environment is not anoxic or even severely lacking in dissolved oxygen. This finding also indicates that active compound production is not solely linked to cell density but it may also be linked to environmental conditions such as dissolved oxygen levels and levels of toxic oxygen derivatives. This is particularly interesting in light of a recent study by Medina-Martínez *et al.* (2006) which showed an effect of environmental conditions, more specifically, temperature and glucose constraints, on BHL production by *Aeromonas hydrophila*.

3.4.5 Active compound separation and structural assessment

Although the *Agrobacterium* liquid culture bioassay and the *A. tumefaciens* reporter strain (UL225) used in this study are specifically designed to detect AHLs, cyclic dipeptides (DKPs) have been shown to activate similar TraR based bioassays in a concentration-dependent manner, although generally much higher concentrations are required than for the natural activator (Holden *et al.*, 1999; Wolf-Rainer, 2005). Therefore the bioassay alone cannot be relied upon for AHL identification.

3.4.5.1 The effect of incubation with serum on the induction of β-galactosidase activity

Horse serum has been shown to display strong enzymatic inactivation activity against a range of AHLs with varying efficiency (Yang *et al.*, 2005). Yang *et al.* (2005) proposed that paraoxonase(s), lactonase-like enzyme(s), contribute to this inactivation by hydrolysing the lactone ring of AHL to produce acyl homoserine. Incubation of extracts derived from the 48 h culture supernatants of the three *C. jejuni* strains with horse serum for 12 h significantly decreased ($p < 0.05$) the induction of β-galactosidase activity suggesting that the active compound has been inactivated by the horse serum and therefore may well be an AHL (Figure 3.3.8). The levels of β-galactosidase activity induced by the serum treated extracts from both variants of *C. jejuni* NCTC 11168 were above the level of considered importance for this study, whereas the level of β-galactosidase activity induced by the equivalent extract from NCTC 13255 was below this level. Additionally, as there is no significant difference in the levels of inactivation of NCTC 11168-GS or NCTC 11168-O, it would seem that the concentration and structure of the putative AHL(s) is similar in both of these *C. jejuni* extracts. However, as current research suggests that AHLs and DKPs

compete for the same binding site and as the paraoxonases are generic hydrolyases, known to hydrolyse various esters and lactones, there is the possibility that DKPs may also be inactivated by serum (Wolf-Rainer, 2005; Dong and Zhang, 2005). Therefore although these results do suggest that the active compound may be AHL, they do not completely exclude the possibility that the active compound is a DKP and further structural identification is required (Wolf-Rainer, 2005).

3.4.5.2 HPLC separation

The substantial peaks eluted between 43.50 and 49.25 min for the *A. tumefaciens* positive control (UL225) supernatant extract and between 33.74 and 35.75 min for the *C. jejuni* NCTC 13255 supernatant extract, correspond with the highest induction levels of β-galactosidase activity, indicating that these fractions contain the highest concentration of active compound (Figure 3.3.9 and Figure 3.3.10). These single peaks indicate the presence of one main active compound for each extract and the different elution times for each of the active fractions suggest that the active compounds of the fractions have different hydrophobic properties and thus different molecular structures.

3.4.5.3 LC-MS

The LC-MS peak identified in all the HPLC-separated active samples suggests a potential mass/charge ratio ~ 340 *m/z* (Figure 3.3.11). This mass/charge ratio corresponds to that of *N*-hexadecanoyl-homoserine lactone (HDHL), an AHL with the molecular formula $C_{20}H_{37}NO_3$ (Figure 3.4.1). HDHL has previously been isolated from *Rhodobacter capsulatus* cultures by Schaefer *et al.* (2002) and may potentially be the active compound present in the *C. jejuni* NCTC 13255 supernatant.

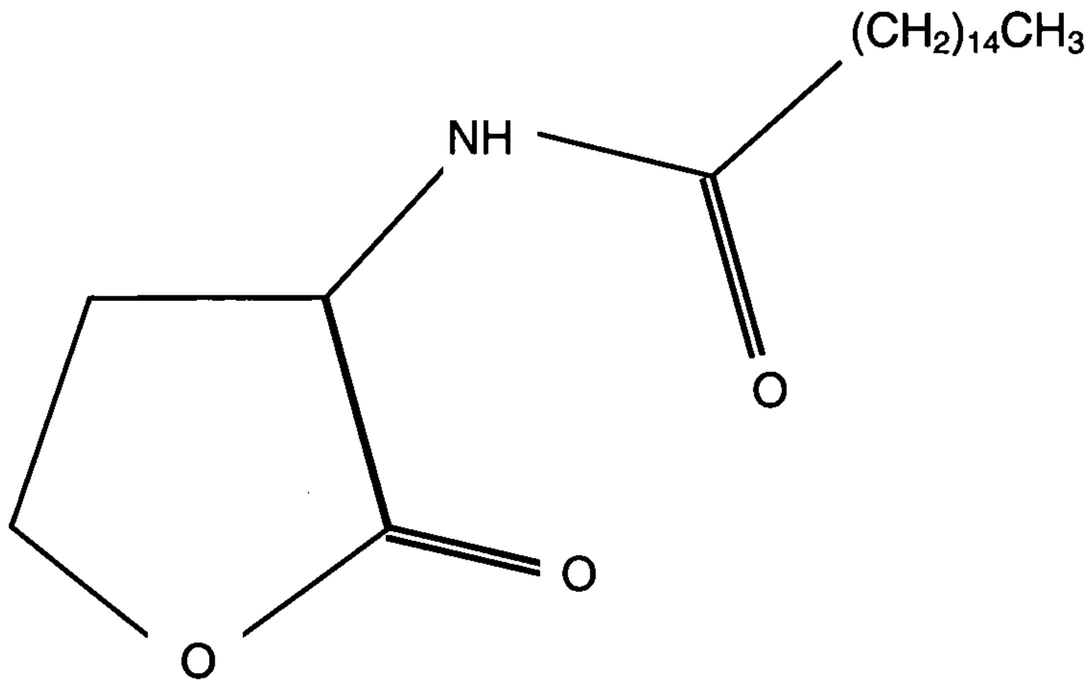


Figure 3.4.1 Proposed structure of the putative AHL

The AHL $C_{20}H_{37}NO_3$, which with a mass/charge ratio of ~ 340 *m/z*, may potentially be the active compound, isolated from the *C. jejuni* NCTC 13255 supernatant extract.

3.4.6 Discussion summary

- All of the *C. jejuni* isolates tested in the preliminary screen induced levels of β-galactosidase activity in the *A. tumefaciens* reporter strain (UL225), greater than the level of importance for this study, indicating the presence of an autoinducer-like active compound(s) in all the culture supernatant extracts (Figure 3.3.1).
- These results highlight the potential for a previously unidentified signalling mechanism in *C. jejuni* (Figure 3.3.1).
- The decline of β-galactosidase activity induction at the end of stationary phase and the onset of the decline phase indicates that the levels of the active compound present in the supernatants and therefore in the supernatant extracts, may be linked to cell density and cell viability (Figure 3.3.4 (a), (b) and (c)).
- Decreasing the % headspace air volume (23 to 10 % (v/v)) had no significant effect ($p < 0.05$) on β-galactosidase activity induction for any of the three *C. jejuni* isolates, (NCTC 13255, NCTC 11168-GS and NCTC 11168-O), (Figure 3.3.5, Table 3.3.2).
- Supplementing BB with lysed horse blood (7.0 % v/v) significantly decreased ($p < 0.05$) β-galactosidase activity induction by extracts from both the *C. jejuni* laboratory isolates (NCTC 13255 and 11168-GS). However, this supplementation did not significantly ($p < 0.05$) affect the β-galactosidase induction level for the extract from the *C. jejuni* clinical variant, NCTC 11168-O supernatant, suggesting that this variant is more tolerant of the toxic oxygen derivatives and therefore is less affected by the changes in their concentrations (Figure 3.3.6, Table 3.3.3).

- Supplementing BB with ferrous sulphate (0.2 % w/v), had no significant effect ($p < 0.05$) on the β-galactosidase activity induction for any of the *C. jejuni* isolate supernatant extracts, concurring with previous research by Bolton *et al.* (1984*a*), which indicated that ferrous sulphate, is less effective than lysed horse blood at neutralising toxic oxygen derivatives (Figure 3.3.6, Table 3.3.3).
- The significant decrease ($p < 0.05$) in β-galactosidase activity induction, indicating the inactivation of the active compound by incubation with horse serum, suggests that the active compound could be an AHL (Figure 3.3.8, Table 3.3.4). However, as the basis of the serum inactivation is believed to be due to the presence of a lactonase(s), which hydrolyses the lactone ring (Dong and Zhang, 2005), there is the possibility that a DKP would also be inactivated by serum. Despite being structurally distinct from AHLs, DKPs compete for the same binding site and can demonstrate activity in several TraR based bioassays. Therefore although these results do suggest that the active compound may be AHL, they do not completely exclude the possibility that the active compound is a DKP and further structural identification is required (Holden *et al.*, 1999; Wolf-Rainer, 2005).
- The inactivation of the active compound by horse serum also indicates the potential for the targeted disruption of this putative signalling mechanism, which may be of particular importance considering that many QS networks have been found to regulate virulence genes, including biofilm formation (Figure 3.3.8).
- The HPLC separation results indicate the presence of one main active compound for both the extract from the *A. tumefaciens* positive control (UL224) and *C. jejuni* NCTC 13255 (Figure 3.3.9 and Figure 3.3.10). The different elution times for

each of the active fractions indicate that the active compounds contained within the fractions have different molecular properties and structures (Figure 3.3.9).

- LC-MS analysis indicates that the active compound in the *C. jejuni* NCTC 13255 supernatant extract has a mass/charge ratio of ~ 340 *m/z* (Figure 3.3.11) and these results combined with those of the *Agrobacterium* liquid bioassay and the serum inactivation assay, suggest that the active compound in this extract may well be an AHL, such as HDHL.
- The combined results of this study provide preliminary evidence of a previously unidentified AHL-mediated QS mechanism within the Epsilon *Proteobacteria* class.

CHAPTER 4

General Discussion

4.1 *E. coli*

The initial results from this research concur with those of several pilot studies carried out at the University of Central Lancashire, Preston, (unpublished data) in that they indicate the presence of an active AI compound in all the *E. coli* blood isolate (UL47, UL48, UL49 and UL51) culture supernatant extracts, as they induced β-galactosidase activity in the *Agrobacterium* plate bioassay reporter strain UL34. TLC coupled to the *Agrobacterium* plate bioassay was used to separate the active component from the *E. coli blood* isolate, UL51, supernatant extract. The R*f* value of this fraction was equal to that of an *A. tumefaciens* positive control active fraction, suggesting that it may contain similar, if not the same active component(s). However, the lack of replication of this result with subsequent UL51 culture supernatant extracts has prohibited comparison of the UL51 active fraction with synthetic standards, without which it is impossible to confirm this result. The preliminary mass spectroscopic data for UL51 (Figure 2.2.2) indicated one peak of interest (203.4955 *m/z*) which suggested that the active component isolated from the *E. coli* UL51 culture supernatant extract contained an even number of nitrogen atoms. Therefore it was unlikely to be an AHL and may have been an alternative active compound such as an autoinducing peptide (AIP) or a DKP (McClean *et al.*, 1997; Holden *et al.*, 1999).

The common pathogen *Staphylococcus aureus* secretes AIPs, regulating virulence factors such as capsule and toxin production, via AIP-mediated QS (McDowell *et al.*, 2001; Dunman *et al.*, 2001; Novick, 2003; Cheung *et al.*, 2004). However, many AIPs, including *N*-acylhomocysteine thiolactone induce violacein production by the *Chromobacterium* plate bioassay reporter strain (LP41) and none of the *E. coli* supernatant extracts induced violacein production (McClean *et al.*, 1997). It is

therefore unlikely that the active component isolated from the *E. coli* blood isolate, UL51, culture supernatant extract is an AIP.

As previously stated, the mass spectroscopic analysis of the active component isolated from the *E. coli* blood isolate, UL51, culture supernatant extracts suggested that the active compound was unlikely to be an AHL and may well be a DKP (2.4.3). Skwierczynski and Connors (1993) demonstrated that DKPs can be generated via the non-enzymatic cyclisation of linear dipeptides at extremes of temperature and pH. Therefore DKPs can be formed in microbiological media as a result of the high temperatures attained during the sterilisation process. However, extracts of samples taken from several batches of sterile A-MOPS growth medium did not induce β-galactosidase activity in the *Agrobacterium* plate bioassay. It is thus possible to conclude that the positive results for the *E. coli* blood isolate supernatant extracts were due to the bacterium conditioning the growth medium, by producing and secreting an active component, rather than the non-enzymatic production of an active component.

These preliminary results indicating putative-DKP production and secretion by the *E. coli* blood isolates are intriguing in that Holden *et al.* (1999; 2000), reported that many Gram-negative bacteria including *P. aeruginosa, Proteus mirabilis, Citrobacter frundii, Enterobacter agglomerans* and *E. coli* produce DKPs or related molecules. In light of this, the problems experienced in repeating initial results are both disappointing and frustrating, especially considering that it is still unclear whether the production and release of DKPs by bacteria actually constitutes a QS-signalling mechanism (Holden *et al.*, 2000).

4.1.1 Reproducibility

As previously stated the reproducibility issues encountered in this section of the study hindered progress with the identification of the active component isolated from the *E. coli* UL51 culture supernatant extract. Two different bottles of ethyl acetate were used for the extraction process (both from Sigma, HPLC grade). The initial *E. coli* blood isolate supernatant extracts that induced β-galactosidase activity in the *Agrobacterium* plate bioassay, were produced with the "original bottle" of ethyl acetate whereas the subsequent replica *E. coli* extracts were produced with a different bottle of ethyl acetate. However, contamination of the original bottle is unlikely, as the extracts from the negative control, *A. tumefaciens* UL33, and the *E. coli* laboratory isolates, and they all failed to induce β-galactosidase activity in the *Agrobacterium* plate bioassay, were produced with the same bottle of ethyl acetate.

The organic solvent dichloromethane can also be used to isolate QS signalling molecules including AHLs and DKPs, (McClean *et al.*, 1997; Holden *et al.*, 1999; Yates *et al.*, 2002). However, none of the dichloromethane or the subsequent ethyl acetate produced extracts from any of the replica *E. coli* culture supernatants induced β-galactosidase activity in the *Agrobacterium* plate bioassay, indicating that the inconsistent results and failure to produce additional active fractions were unlikely to be due to problems with the extraction procedure.

The experiment involving cell breakage using a French press indicated that the reproducibility issues were not a result of the active component remaining inside the bacterial cells and therefore being discarded with the pellet.

As the reporter strain was still responding appropriately to the *A. tumefaciens* positive and negative control supernatant extracts, it is reasonable to conclude that the reproducibility issues were unlikely to be due to mutation of the reporter strain. It is

possible that the *E. coli* blood isolates, which originally had a low-passage level, had adapted to the idealised conditions of the laboratory and in doing so had lost the ability to produce the active component initially isolated at the start of the study and in several pilot studies at the University of Central Lancashire, Preston. Although stocks of UL51 and the other blood isolates were available from the University of Central Lancashire culture collection, new freeze dried vials had recently been prepared from fresh cultures and they may have carried the mutation. Thus, it was therefore impossible to resolve the basis of the irreproducibility.

The replica *E. coli* blood isolate supernatant extracts were also tested for active component production with the highly sensitive *Agrobacterium* liquid culture bioassay using the *A. tumefaciens* reporter strain, UL225. Additional clinical *E. coli* (urine) isolates were obtained, and screened along with *E. coli* blood isolate and laboratory strain supernatant extracts. None of the *E. coli* urine isolate supernatant extracts or the extracts from the *E. coli* laboratory isolate UL27 and UL56 culture supernatants, induced β-galactosidase activity in the *Agrobacterium* liquid culture bioassay. The very low levels of β-galactosidase activity induced by the *E. coli* blood isolates UL47 and UL51 supernatant extracts and the *E. coli* laboratory isolates LP50 and UL52 supernatant extracts, indicate that very low/negligible levels of active component were being produced and secreted by these isolates. Without additional samples of the active component it was not possible to proceed any further with this section of the study.

4.2 *E. coli* and *C. jejuni*

Both *E. coli* and *C. jejuni* are Gram-negative, enteric, foodborne pathogens capable of causing serious GI disease and even death. Unsurprisingly, as natural inhabitants of the GI tract of many animals, *E. coli* and *C. jejuni* are widely distributed throughout the environment and therefore both food and water supplies are susceptible to contamination. The genome of sequences of several strains of *C. jejuni* and *E. coli* have been published (Blattner *et al.*, 1997; Parkhill *et al.*, 2000; Perna *et al.*, 2001; Welch *et al.*, 2002; Fouts *et al.*, 2005). *C. jejuni* has a relatively small genome (approximately 1600 - 1700 kb), when compared with that of *Escherichia coli* (approximately 4500 kb) (Bergthorsson and Ochman, 1998; Parkhill *et al.*, 2000; Dobrindt, 2005; Fouts *et al.*, 2005). *E. coli* possess a LuxR homologue termed SdiA which binds specific AHL AI molecules when a threshold concentration is attained. However, no *E. coli* strains have, as yet, been found to produce these AHLs and analysis of the genome sequence of *C. jejuni* has indicated that it does not contain any gene predicted to encode an AHL synthase (Elvers and Park, 2002; Williams, 2006).

4.3 *C. jejuni*

All of the *C. jejuni* isolate supernatant extracts tested in this study induced β-galactosidase activity in the *A. tumefaciens* reporter strain, UL225 (Figure 3.3.1). These results highlight the potential for the production and secretion of an AI-like active compound by *C. jejuni* and provide preliminary evidence of a previously unidentified signalling system in this organism.

Comparison of culture growth (cell density) with induction levels of β-galactosidase activity for *C. jejuni* NCTC 11168-GS and 11168-O supernatant extracts suggest a link between cell density and active compound production (Figure 3.3.4 (*a*) and (*b*)).

The sudden decline in the levels of putative AHL seen in *C. jejuni* NCTC 11168-GS and 13255 is characteristic of many QS systems where it has been linked to both enzymatic and pH influenced chemical degradation of AHL (Figure 3.3.4 (*a*) and (*c*)) (Yates *et al.*, 2002).

4.3.1 Oxygen Tolerance

The liquid growth medium BB is specifically designed to aid recovery of injured *C. jejuni* cells. This growth medium contains the oxygen quenchers, haemin, sodium metabisulphite and sodium pyruvate. Therefore the use of this growth medium in a small vessel with a reduced headspace avoids the need for a microaerobic atmosphere when culturing *Campylobacter* spp. (Bolton *et al.*, 1984*a*; Bolton *et al.*, 1984*b*; Humphrey 1990; Corry *et al.*; 1995). When the volume of headspace air was decreased from 23 % to 10 % there was no significant change ($p < 0.05$) in the induction of β-galactosidase activity in the *Agrobacterium* liquid culture bioassay by the extracts derived from any of the three *C. jejuni* isolate (NCTC 13255, NCTC 11168-GS and NCTC 11168-O) culture supernatants (Figure 3.3.5, Table 3.3.2).

BB is frequently supplemented with lysed horse blood, which acts to prevent the build up of toxic oxygen products that may hinder *Campylobacter* growth. The effect of supplementing *C. jejuni* cultures with lysed horse blood (7.0 % v/v) on the induction of β-galactosidase activity by *C. jejuni* culture supernatant extracts was assessed with the *Agrobacterium* liquid culture bioassay. A significant decrease ($p < 0.05$) in β-galactosidase activity induction was observed for the *C. jejuni* laboratory isolate, NCTC 13255 and 11168-GS, supplemented culture (7.0 % (v/v) horse blood) supernatant extracts. No significant effect ($p < 0.05$) on β-galactosidase activity

induction was observed for *C. jejuni* NCTC 11168-O, the clinical isolate supplemented culture supernatant extract (Figure 3.3.6). These results suggest that *C. jejuni* NCTC 11168-O is more tolerant to toxic oxygen derivatives.

The results for *C. jejuni* laboratory isolates (NCTC 11168-GS and 13255) suggest that the addition of blood and therefore the neutralisation of the toxic oxygen derivatives significantly reduces ($p < 0.05$) active molecule production, whereas a decrease in % headspace air (v/v) does not sufficiently affect the levels of dissolved oxygen, so a significant change ($p < 0.05$) in the levels of active component production was not observed. These results indicate that *C. jejuni* NCTC 11168-O (the clinical variant) is more tolerant to toxic oxygen derivatives than the *C. jejuni* laboratory strains, NCTC 11168-GS and NCTC 13255. *C. jejuni* is a microaerophile rather than a strict anaerobe and as such requires oxygen for DNA synthesis and as a terminal electron acceptor for respiration but cannot neutralise the toxic oxygen derivatives produced (Sellars *et al.*, 2002). Previous research has indicated that *C. jejuni* is incapable of growth during anoxia and that neither *C. jejuni* NCTC 11168 variant grows well under severely oxygen-limited conditions (Sellars *et al.*, 2002; Gaynor *et al.*, 2004). Therefore a balance is required to ensure on one hand sufficient oxygen for energy production and to satisfy normal cellular processes, whilst on the other hand limiting the levels of toxic oxygen derivatives which are obviously detrimental to the cell. As the ability of *Campylobacter* spp. to utilise alternative electron receptors for respiration under highly restricted oxygen conditions, varies with both strain and isolate, it is highly likely that this balance between minimising the production of toxic oxygen derivatives and optimising the levels of oxygen for energy production, will also vary (Pearson *et al.*, 2003).

Despite the naturally relatively low levels of dissolved oxygen in liquid medium and the decrease in the % headspace air volume, the dissolved oxygen levels seem to be sufficient to support growth in all the experiments, indicating that neither of the % headspace air volumes induced severely oxygen limited conditions (Morris, 1976; Mohammed *et al.*, 2005). However, the change in the % headspace air volume will have affected the dissolved oxygen levels in relation to the optimum level for each *C. jejuni* strain, which in turn affects the dissolved oxygen/toxic oxygen derivative balance and therefore the levels of active molecule production.

Additional consideration must be given to the interpretation of these results in that the decrease in the % headspace air volume will not only decrease the level of dissolved oxygen available, but may also decrease the level of toxic oxygen derivatives in the medium. Interestingly different manufacturers of medium constituents, such as tryptone, are now known to affect oxygen tolerance in *C. jejuni* and the presence of nutrients (dissolved mineral salts, amino acids and peptides), in biological media affects the subsequent levels of dissolved oxygen (Hodge and Krieg, 1994). Therefore, supplementing a medium with lysed horse blood (7.0 % v/v) or ferrous sulphate (0.2 % w/v) will also affect the level of dissolved oxygen and, although these supplements are generally considered to act as a detoxifying agents rather than enrichment factors, they may also potentially provide additional nutrients for the bacteria so affecting the level of active compound produced (Bolton *et al.*, 1984*a*; Ivanova *et al.*, 1985; Mohammed *et al.*, 2005).

Furthermore, according to Krieg and Hoffman (1986), the protective effect of a deep layer of broth may also be an important factor for growth during aerobic incubation. All of the *C. jejuni* cultures (both laboratory and clinical isolates) formed a substantial biofilm towards the bottom of the culture vessel (Appendix 3, Figure A3.3). This may have been due to the effects of gravity, but *C. jejuni* is highly motile and aerotaxis (chemotaxis with oxygen/air as a stimulant) has previously been observed in *C. jejuni* (Hazeleger *et al.*, 1998). The biofilm formation may therefore indicate that *C. jejuni* was positioned for the most appropriate oxygen levels within the culture vessel.

Bolton *et al.* (1984*a*) demonstrated that although supplementation with ferrous sulphate (0.025 % w/v) helps to prevent the formation and build up of toxic substances, it is less effective than lysed horse blood as it does not completely neutralise all the toxic products. Therefore the effect of ferrous sulphate supplementation (0.2 % w/v) of the growth medium on β-galatsosidase activity induction was also assessed. No significant change ($p < 0.05$) in β-galactosidase activity induction, in comparison with extracts derived from unsupplemented BB cultures, was observed for any of the three of the *C. jejuni* isolates tested (Figure 3.3.6). Intriguingly, Ulitzur and Dunlap (1995), demonstrated that both iron and oxygen limitation lead to the early induction of *V. fischeri* luminescence and a link between QS and iron/oxygen regulation has also been established for *P. aeruginosa* (Kim *et al.*, 2005). In light of these studies, the negligible effect of ferrous sulphate supplementation and the various effects of culture supplementation (7.0 % horse blood) on active compound concentration in the supernatant extracts is particularly interesting.

The results of this study confirm that BB in a closed vessel with a restricted headspace (either 23 or 10 % v/v) provides a suitable environment for the growth of

the *C. jejuni* strains used in this study. Previous aerobic liquid culture studies by Tran (1998) and Mohammed *et al.* (2005) have also indicated that a restricted headspace air volume of 50 % v/v for brain heart infusion broth and 16 - 17 % v/v for blood free enrichment broth can also sustain *C. jejuni* growth. Mohammed *et al.* (2005) also demonstrated that such culture conditions did not affect the growth rates, protein profiles or substrate utilisation, when compared with cells grown under anaerobic conditions. The results of altering the % headspace air volume experiments from this study indicate that for all three *C. jejuni* isolates, (NCTC 11168-O, 11168-GS and 13255) changing the % headspace volume does not significantly affect ($p < 0.05$) active component production (Table 3.3.2). Although these studies utilise different media and toxic oxygen derivative quenching agents, the results still indicate that it is of vital importance that the % headspace air volume (v/v) and concentration/type of supplementation of any *C. jejuni* culture, is conserved throughout and between any experiments.

4.3.2 The plasmid theory

Analysis of the *C. jejuni* NCTC 11168 genome sequence has indicated that it does not contain any genes predicted to encode any known AHL synthase and therefore is assumed unlikely to produce AHL-based signalling molecules (Elvers and Park, 2002; Park, 2005). Many studies have indicated that a significant proportion of *C. jejuni* strains harbour plasmids, some of which are cryptic, raising the possibility that the synthase gene that encodes the production of this putative AHL, may be plasmid based (Austen and Trust, 1980; Taylor *et al.*, 1981; Bradbury *et al.*, 1983; Bopp *et al.*, 1985; Bradbury and Munroe, 1985; Sagara *et al.*, 1987). However, as both the *C. jejuni* NCTC 11168 strains seem to produce the active compound, yet do not contain a

plasmid, the synthase for the active molecule, for these two variants at least, cannot be plasmid encoded (Gaynor *et al.*, 2004). Therefore if the active compound is an AHL there must be a novel, unidentified, AHL synthase present in the *C. jejuni* NCTC 11168 genome.

4.3.3 Implications in context

The protective effect of blood supplementation upon *C. jejuni* may have implications for survival of *C. jejuni* in aerobic environments such as the kitchen, the slaughter house or in meat products potentially leading to prolonged survival of *C. jejuni*. This in turn would increase the potential for cross-contamination of previously uncontaminated chicken products (in the slaughter house) or other foods (in the domestic kitchen) and its subsequent delivery to the GI tract. The effect of blood supplementation of the growth medium and changes in the % headspace air volume on active component production, may well have implications with regard to these aerobic environments, providing some strains with a selective advantage in specific ecological niches. It is therefore of vital importance that research elucidates as much detail as possible about the QS mechanisms of this pathogen and the roles that they may play in virulence factor regulation. This knowledge is particularly important considering the prevalence of antibiotic resistant strains infecting commercial poultry flocks and therefore the potential for the entry of these strains into the food chain (Humphrey *et al.*, 2005).

4.4 Generalisation between clinical and laboratory acclimatised bacterial variants

Both *E. coli* and *C. jejuni* are enteric pathogens that colonise the GI tract and can exist as commensals in a symbiotic relationship with the host or as pathogens resulting in disease. As previously stated AHL-mediated QS systems have been identified in many Gram-negative bacteria and as such AHL-mediated QS should not be assumed absent from *Campylobacter* spp.. Pathogenic and commensal variants of these two bacterial strains vary immensely as demonstrated in this study and by Gaynor *et al.* (2004). Therefore the failure to identify a QS system in a laboratory isolate should not automatically exclude the possibility of the presence of a QS system in a commensal or pathogenic isolate.

The initial screening procedure using the *Agrobacterium* plate bioassay indicated that under the stated conditions, the *E. coli* blood isolates were capable of producing and releasing compound(s) that induced β-galactosidase activity in the reporter strain, UL34, whereas the laboratory *E. coli* isolates did not. Although the 48 h supernatant extracts from the clinical and laboratory variants of *C. jejuni* NCTC 11168 cultures induced comparable levels of β-galactosidase activity in the *Agrobacterium* liquid culture bioassay, the overall profile of active molecule production by these two isolates differed with time, and media supplementation (7.0 % lysed horse blood (v/v)). Therefore the generalisation of results between clinical isolates and laboratory isolates would at least be misleading and potentially incorrect.

4.5 Closing statement

Whilst there is no denying the enormous amount of information that a completed genome sequence can provide, it is equally important that researchers do not lose sight of the fact that a large percentage of proposed genes in any given sequence have either been assigned putative (purely speculative) functions or quite simply have been catergorised as "function unknown". The study of QS has expanded at a phenomenal rate going through the three phases every novel scientific idea is said to pass:- disbelief, belief but under-appreciation, and finally belief, combined with an understanding of importance but a lack of novelty (Harold, 1986; Greenberg, 2003). However, it is vital that researchers do not forget the novelty of the original discovery of QS or the mistakes of previous research and fail to learn from them. The assumption that all bacteria that possess *luxS* are capable of QS is a misnomer. Of the many bacteria that do possess this gene, only the *Vibrio* spp. have a defined mechanism. This does not mean that the mechanisms do not exist in other bacterial species, just that they have not yet been fully characterised. Equally the assumption that the failure to identify a gene known to encode an AHL synthase in many bacteria, including *E. coli* and *C. jejuni*, proves that these bacteria are incapable of producing AHL, is foolhardy and as such, may hinder the progress of QS research and the full understanding of the virulence potential of these pathogens. As previously stated a completed genome sequence does provide a wealth of information about any given organism, but it is vital that additional, more basic, traditional research methods are also applied to enhance the understanding of the genomic sequence and to prevent the assumption of errors. This study has provided some novel information regarding AHL production and preliminary evidence of a previously unknown QS system within *C.*

jejuni. It may therefore indicate the potential for other unrecognised QS systems in *E. coli* and many other bacteria throughout the microbial world.

CHAPTER 5

Future Work

5.1 Structural identification

This study has highlighted the potential for QS molecule production (be it a DKP or a previously unidentified compound) by *E. coli* and the potential for differences in its production between laboratory organisms and those isolated from infections of humans (urine and blood isolates). There remains a pressing need for a large scale screening programme of clinical *E. coli* isolates, more specifically blood isolates, employing a wide range of bioassays (preferably including the three bioassays used in this study) in an attempt to isolate any active molecules produced. This work would very much mirror the work of Cha *et al.* (1998) who used TLC coupled to an *A. tumefaciens* reporter bioassay system, to test 106 isolates from seven genera of plant-associated Gram-negative bacteria for AHL production.

Although this study has isolated a putative AHL produced by all the *C. jejuni* strains tested, confirmation of its molecular structure is still required. Chemical structures of any active compounds isolated can be assigned via spectroscopic properties. As previously stated mass spectrometry detects even picomoles of samples and can be coupled to HPLC or GC. Nuclear magnetic resonance spectroscopy (NMR) is also a valuable tool for identifying structures of active compounds as the hydrogen and the carbon atoms in the structure will resonate at different chemical shifts, depending upon their environment. They will also appear as singlet, doublet, triplet etc. by coupling to the neighbouring nuclei. Holden *et al.* (1999) used high resolution electron impact mass spectrometry (EI-MS) to identify the structure of several DKPs produced by *P. aeruginosa* and Degrassi *et al.* (2002) used NMR to elucidate the structure of four DKPs produced by *Pseudomonas putida* WCS358. Bainton *et al.* (1992) also used EI-MS and NMR to identify the structure of the antibiotic regulating, *E. carotovora* AHL (*N*-(3-oxo-hexanoyl)-L-homoserine lactone).

Additional techniques that can be employed in structural analysis include infrared spectroscopy (IR), which Zhang *et al.* (1993) used to identify the functional groups of OOHL from *A. tumefaciens*, and matrix-assisted laser desorption ionisation-time of flight mass spectroscopy which Huang *et al.* (2006) used to identify a novel AHL acylase from *P. aeruginosa* strain PAO1. Final confirmation of the structure can be obtained via the comparison of the spectrometric properties of the natural active compound and a chemically synthesised version.

As analysis of the genome sequence of *C. jejuni* NCTC 11168 has indicated that it does not contain any genes predicted to encode any known AHL synthase, identification of the synthase and receptor protein for the AHL-like molecule isolated from the *C. jejuni* culture supernatant extracts is also vital, as is the experimental determination of the genes regulated by this potential QS system. The combination of this data with the identification of the synthase and the receptor protein would then enable a screening programme for genes regulated by this QS mechanism (Whiteley *et al.*, 1999).

5.2 QS in pathogens

The further study and characterisation of QS mechanisms and the genes that they regulate in pathogens such as *C. jejuni* and *E. coli*, would enhance the understanding of how these microorganisms survive a variety of extreme environments and induce disease in host systems. Research involving mixed cultures would also provide insight as to how QS mechanisms and the genes they regulate, influence competition and survival in habitats such as the GI tract where numerous bacterial species are present. A better understanding of cross-species and cross-kingdom signalling within host systems such as the GI tracts or the lungs would also be of great interest. This work

would have implications for medical research with specific regard to the prospect of quorum quenching as a mechanism for the prevention of infection through virulence factor production inhibition, and the inhibition of biofilm formation which could potentially reduce antibiotic resistance and long-term colonisation.

APPENDICES

Appendix 1: Growth media, antibiotic and indicator stock solutions and Z buffer

A1.1 Reagents

All chemicals were purchased from Sigma-Aldrich Company Ltd (Poole, Dorset, UK) or VWR International (Lutterworth, Leicestershire, UK) unless otherwise stated.

Table A1.1: Supplied growth media

Medium	Method
Nutrient Broth No. 2 [a]	Culture conditions (2.2.4)
Nutrient Agar [a]	*Chromobacterium* plate bioassay (2.2.7.1)
Agar Bacteriological (Agar No. 1) [a]	*Agrobacterium* plate bioassay (2.2.7.2)
Brain Heart Infusion Broth [a]	*C. jejuni* strains and culture conditions (3.2.4)
Blood Agar Base Medium [a b]	*C. jejuni* strains and culture conditions (3.2.4)
Bolton Broth [a b]	*C. jejuni* strains and culture conditions (3.2.4)

Supplied growth medium was prepared as instructed by the supplier and autoclaved for 20 minutes at 15 psi (121°C).

[a] Supplied by Oxoid Ltd., Basingstoke, Hampshire, UK.

[b] Once autoclaved the medium was stored at 4°C in the dark.

Table A1.2: Concentrations of ABMM components (adapted from Chilton *et al.*, 1974)

Component	Formula	g/l
D (+) -glucose	$C_6H_{12}O_6$	2.00
Potassium hydrogen phosphate [a]	K_2HPO_4	3.00
Sodium dihydrogen orthophosphate [a]	NaH_2PO_4	1.00
Ammonium chloride	NH_4Cl	1.00
Magnesium sulphate	$MgSO_4.7H_2O$	0.30
Potassium chloride	KCl	0.15
Calcium chloride	$CaCl_2.2H_2O$	0.01
Iron (II) sulphate [a]	$FeSO_4.2H_2O$	2.5×10^{-3}
Yeast extract [b]	N/A	1.00

Components were made up with ddH_2O, adjusted to pH 7.2 with NaOH and autoclaved for 20 minutes at 15 psi (121°C).

[a] Supplied by BDH Laboratory Supplies, Poole, UK.

[b] Supplied by Becton, Dickinson and Company, Sparks, USA.

Table A1.3: Concentrations of ATMM components (adapted from Fuqua and Winans, 1994)

Component	**Formula**	**g/l**
Potassium dihydrogen orthophosphate [a]	KH_2PO_4	10.70
Magnesium sulphate	$MgSO_4.7H_2O$	0.16
Calcium chloride	$CaCl_2.2H_2O$	0.01
Iron (II) sulphate [a]	$FeSO_4.7H_2O$	5×10^{-3}
Manganese sulphate	$MnSO_4.H_2O$	1.13×10^{-3}
Ammonium sulphate	$(NH_4)_2SO_4$	2.00
D (+) -glucose	$C_6H_{12}O_6$	5.00

Components were made up with ddH_2O, adjusted to pH 7.2 with NaOH and autoclaved for 20 minutes at 15 psi (121°C).

[a] Supplied by BDH Laboratory Supplies, Poole, UK.

Table A1.4: Concentrations of A-MOPS growth medium components (adapted from Neidhardt *et al.*, 1974)

Component	Formula	g/l
MOPS	$C_7H_{15}NO_4S$	8.36
Tricine	$C_6H_{13}NO_5$	7.16×10^{-1}
Sodium chloride	$NaCl$	2.92
D (+) -glucose	$C_6H_{12}O_6$	3.50
Potassium sulphate	K_2SO_4	0.05
Magnesium chloride	$MgCl_2.6H_2O$	0.11
Ammonium chloride	NH_4Cl	0.51
Potassium hydrogen phosphate [a]	K_2HPO_4	0.23
Trace element solution [b]	N/A	0.50 ml

Components were made up with ddH_2O and adjusted to pH 7.4 with NaOH. The components were all autoclaved together for 20 minutes at 15 psi (121°C), unlike the instructions stated by Neidhardt *et al.* (1974).

[a] Supplied by BDH Laboratory Supplies, Poole, UK.

[b] As detailed in Appendix 1, Table A1.5.

Table A1.5: Concentration of trace element solution components for A-MOPS growth medium

Component	Formula	g/l
Ammonium paramolybdate	$(NH_4)_6Mo_7O_{24}$	8.00×10^{-3}
Boric acid	H_3Bo_3	0.05
Cobalt chloride [a]	$CoCl_2.6H_2O$	1.43×10^{-2}
Copper sulphate	$CuSO_4.5H_2O$	5.00×10^{-3}
Manganese chloride	$MnCl_2.4H_2O$	3.20×10^{-2}
Zinc sulphate [a]	$ZnSO_4.7H_2O$	6.00×10^{-3}
Calcium chloride	$CaCl_2.4H_2O$	1.47×10^{-1}
Iron (II) sulphate [a]	$FeSO_4.7H_2O$	5.56

Components were made up with ddH_2O, and stored at 20°C.

[a] Supplied by BDH Laboratory Supplies, Poole, UK.

Table A1.6: Antibiotic stock concentrations

Antibiotic	Solute	mg/ml
Gentamicin	ddH_2O	10 & 100
Kanamycin	ddH_2O	50
Spectinomycin	ddH_2O	10 & 100
Tetracycline	Ethanol:ddH_2O, 50:50	1.50

All antibiotic stock solutions were filter sterilised through 0.2 μm filters and stored at -20°C.

Table A1.7: Indicator stock solutions

Indicator	Solvent	mg/ml
5'-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-GAL) [a b]	Dimethyl formamide	40
o-nitrophenyl-β-D-galactoside (ONPG) [a b]	Z buffer (Table A1.8)	4

[a] Light sensitive.

[b] Freshly prepared when needed, although it could be stored at -20°C for a maximum of 7 days.

Table A1.8: Concentrations of Z buffer components (Miller, 1992)

Component	Formula	g/l
Sodium hydrogen phosphate	$Na_2HPO_4.7H_2O$	16.10
Sodium dihydrogen orthophosphate [a]	NaH_2PO_4	4.80
Potassium chloride	KCl	0.75
Magnesium sulphate	$MgSO_4.7H_2O$	2.46 5 x 10^{-1}
β-mercaptoethanol	$HSCH_2CH_2OH$	2.70 ml

Components were made up with ddH_2O, adjusted to pH 7.0 with NaOH, filter sterilised (0.2 μm) and stored at 4°C for a maximum of 28 days.

[a] Supplied by BDH Laboratory Supplies, Poole, UK.

Appendix 2: Additional method details

A2.1 $FeSO_4.7H_20$ calculation

- Haemoglobin (Hb) is a tetramer (made up of four identical units).
- Each unit has 1 atom of iron.
- The atomic weight of a haemoglobin molecule is ~64000.
- The atomic weight of iron is 56.85.

Mass of Fe / Mass of Hb x 100 = % of iron in Hb

$$\approx \mathbf{0.35\ \%}$$

- ⅓ of red blood cells (RBC) is Hb.
- 44 % of blood is RBC.
- So ⅓ of the 44 % is Hb.

Therefore 44 % / 3 of the blood is Hb ≈ 15 % of blood is Hb.

So 15 % (Hb) of the 7 % (horse blood supplement) is Hb.

$$\approx \mathbf{1.05\ \%}$$

So 0.35 % x 1.05 % = Amount of iron added...

0.0035 x 0.0105 = **3.675×10^{-5}** for HUMAN BLOOD

- HORSE BLOOD contains ~ 10 x Hb

Therefore

$3.675 \times 10^{-5} \times 10 = 3.675 \times 10^{-4}$

= 3.675×10^{-2} % of horse blood supplement is iron.

Table A2.1 Controls prepared for each crude extract during the *Agrobacterium* liquid culture bioassay

Control	Media Composition	UL225
Extract control	Extract + double strength ATMM	No inoculum
Water control	Water + double strength ATMM	No inoculum
Inoculum control	Water + double strength ATMM	With inoculum

A2.2 LC-MS SIM

Program File: HSL SIM_340
Operator: MarkPowell

Commands, Page 1 of 1
Printed: 08-Feb-06 8:38:16 AM

Title: HSL SIM 340m/z
Datasource: LAB_local
Location: Quay Sequences\MS\QP060206LCMS1A.SEQ
Timebase: Aqa

Created: 02-Feb-06 4:57:04 PM by MarkPowell
Changed: 03-Feb-06 8:41:08 AM by MarkPowell

```
        Temperature.Nominal =        30
        Temperature.LowerLimit =     15
        Temperature.UpperLimit =     50
        Pressure.LowerLimit =        0
        Pressure.UpperLimit =        300
        %A.Equate =                  "%A"
        %B.Equate =                  "Water + 0.02% formic acid"
        %C.Equate =                  "Methanol + 0.02% formic acid"
        %D.Equate =                  "%D"
        Range =                      10
        Smoothing =                  None

-6.000  Flow =                       0.800
        %B =                         100.0
        %C =                         0.0
        %D =                         0.0

 0.000  Wait                         MS.Ready
        Inject
        RELAY1050S_1.On              Duration=0.10
        RELAY1050S_2.On              Duration=0.10
        %B =                         100.0
        %C =                         0.0

40.000  ; Acquisition Off
        %B =                         30.0
        %C =                         70.0
        Flow =                       0.800

        End
```

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Program File: HSL SIM_340
Operator: MarkPowell

Finnigan AQA, Page 1 of 1
Printed: 08-Feb-06 8:38:16 AM

Title: HSL SIM 340m/z
Datasource: LAB_local
Location: Quay Sequences\MS\QP060206LCMS1A.SEQ
Timebase: Aqa

Created: 02-Feb-06 4:57:04 PM by MarkPowell
Changed: 03-Feb-06 8:41:08 AM by MarkPowell

General Details
Method Type: AQA
Method Version: 3

Ionization Mode Settings
Ionization Mode: Electrospray

Analysis Settings
Probe Temperature (Celsius): 380.0

Tune File: D:\Xcalibur\methods\HSL.tun

Acquisition Settings
Acquisition Type: SIM

Retention Window 1
Start Time (min): 0.00
End Time (min): 60.00
Polarity: +ve
Points per Second: 1.00

#	Mass	AQAmax (V)	Dwell Time	Mass Span
1	340.00	30	1.000	0.50

Event Control
Stop acquiring after (min): 40.0

Chromeleon © Dionex Corporation, Version 6.50 SP4 Build 1000

Appendix 3 Additional results

Table A3.1 ***C. jejuni*** **isolate optical density ($OD_{600\ nm}$)**

Strain/isolate number (NCTC)	$OD_{600\ nm}$	SD
11168-GS	0.124	0.005
11160-O	0.141	0.004
13254	0.138	0.005
13255	0.113	0.008
13256	0.131	0.003
13257	0.126	0.004
13258	0.116	0.007
13259	0.138	0.008
13260	0.141	0.008
13261	0.132	0.006
13262	0.117	0.006
13263	0.129	0.008
13264	0.135	0.004
13265	0.116	0.006
13266	0.122	0.003

Data are mean ± standard deviation, (n = 3).

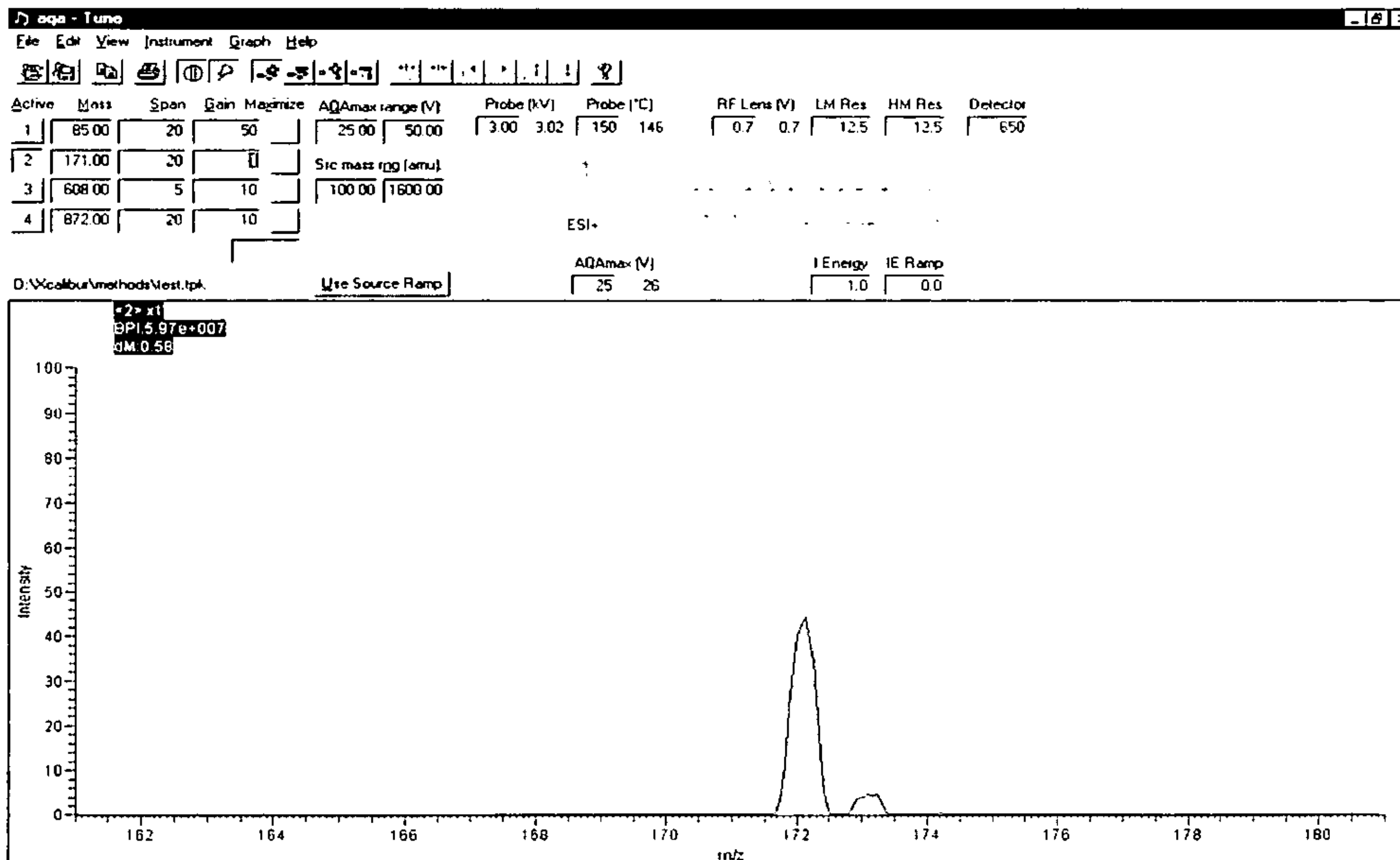

Figure A3.1: *N*-butyryl-L-homoserine lactone LC-MS control 50:50 methanol:H_2O + 0.02 % (v/v) formic acid. ESI^+

Table A3.2.1 Growth Curve Data *C. jejuni* NCTC 13255

Time (h)	Replicate 1	Replicate 2	Replicate 3	Mean	SD
3	0.023	0.040	0.032	0.032	0.009
6	0.029	0.039	0.040	0.036	0.006
21	0.089	0.116	0.088	0.098	0.016
24	0.086	0.135	0.095	0.105	0.026
27	0.090	0.131	0.099	0.107	0.022
30	0.110	0.121	0.112	0.114	0.006
48	0.121	0.102	0.116	0.113	0.010
51	0.104	0.134	0.167	0.135	0.032
54	0.160	0.151	0.126	0.146	0.018
72	0.335	0.205	0.393	0.311	0.096
75	0.402	0.371	0.427	0.400	0.028
78	0.413	0.368	0.398	0.393	0.023
96	0.261	0.196	0.174	0.210	0.045
99	0.164	0.225	0.207	0.199	0.031

Table A3.2.2 Growth Curve Data *C. jejuni* NCTC 11168-GS

Time (h)	Replicate 1	Replicate 2	Replicate 3	Mean	SD
3	0.041	0.042	0.048	0.044	0.004
6	0.049	0.040	0.039	0.043	0.006
21	0.064	0.082	0.093	0.080	0.015
24	0.105	0.090	0.128	0.108	0.019
27	0.091	0.124	0.133	0.116	0.022
30	0.109	0.129	0.126	0.121	0.011
48	0.109	0.140	0.124	0.124	0.016
51	0.295	0.306	0.202	0.268	0.057
54	0.309	0.364	0.308	0.327	0.032
72	0.454	0.333	0.553	0.447	0.110
75	0.521	0.401	0.516	0.479	0.068
78	0.498	0.421	0.483	0.467	0.041
96	0.362	0.286	0.233	0.294	0.065
99	0.224	0.182	0.207	0.204	0.021

Table A3.2.3 Growth Curve Data *C. jejuni* NCTC 11168-O

Time (h)	Replicate 1	Replicate 2	Replicate 3	Mean	SD
3	0.040	0.039	0.044	0.041	0.003
6	0.048	0.047	0.052	0.049	0.003
21	0.091	0.102	0.086	0.093	0.008
24	0.090	0.103	0.100	0.098	0.007
27	0.109	0.112	0.104	0.108	0.004
30	0.135	0.147	0.117	0.133	0.015
48	0.128	0.132	0.164	0.141	0.020
51	0.150	0.225	0.205	0.193	0.039
54	0.252	0.231	0.366	0.283	0.073
72	0.419	0.495	0.364	0.426	0.066
75	0.451	0.462	0.382	0.432	0.043
78	0.483	0.417	0.473	0.458	0.036
96	0.610	0.573	0.630	0.604	0.029
99	0.480	0.537	0.544	0.520	0.035

Figure A3.2: Appearance of a *C. jejuni* NCTC 13255 culture after 96 h of growth

This photograph of *C. jejuni* NCTC 13255 culture shows the clumping and attachment of the culture to the bottom of the culture vessel.

Reference List

Allos, B. M., Lippy, F. T., Carlsen, A., Washburn, R. G. and Blaser, M. J. (1998) *Campylobacter jejuni* strains from patients with Guillain-Barré syndrome. *Emerging Infectious Diseases* **4**: 263-268

Anand, S. K. and Griffiths, M. W. (2003) Quorum sensing and expression of virulence in *Escherichia coli* 0157:H7. *International Journal of Food Microbiology* **85**: 1-9

Andersson, R. A., Eriksson, A. R. B. Heikinheimo, R., Mäe, A., Pirhonen, M. Kõiv, V., Hyytiäinen, H., Tuikkala, A. and Palva, E. T. (2000) Quorum sensing in the plant pathogen *Erwinia carotovora* subsp. *carotovora*: the role of $expR_{Ecc}$. *Molecular Plant-Microbe Interactions* **13**: 384-393

Andreoli, S. P., Trachtman, H., Acheson, D. W. K., Siegler, R. L. and Obrig, T. G. (2002) Hemolytic uremic syndrome: epidemiology, pathophysiology, and therapy. *Pediatric Nephrology* **17**: 293-298

Angus, D., Linde-Zwirble, W., Lidlicker, J., Clermont, G., Carcillo, M. and Pinsky, M. (2001) Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. *Critical Care Medicine* **29**: 1303-1310

Anonymous (1992) *Campylobacter* surveillance. *Communicable Disease Report* **2**: 163-166

Austen, R. A. and Trust, T. J. (1980) Detection of plasmids in the related group of the genus *Campylobacter. Federation of European Microbiological Societies Microbiology Letters* **8**: 201-204

Baca-DeLancey, R. R., South, M. M. T., Ding, X. and Rather, P. N. (1999) *Escherichia coli* genes regulated by cell-to-cell signaling. *Proceedings of the National Academy of Sciences USA* **96**: 4610-4614

Bachmann, B. J. (1996) Derivations and genotypes of some mutant derivatives of *Escherichia coli* K-12. In: *Escherichia coli* and *Salmonella typhimurium*: Cellular and Molecular Biology Vol 2 (Eds) Neidhardt, F. C., Ingraham, J. L., Low K. B., Magasanik B., Reznikoff W., Riley M., Schaechter M., and Umbarger H. E.. American Society for Microbiology, Washington.

Baffone, W., Casaroli, A., Citterio, B., Pierfelici, L., Campana, R., Vittoria, E., Guaglianone, E. and Gianfranco, D. (2006) *Campylobacter jejuni* loss of culturability in aqueous microcosms and ability to resuscitate in a mouse model. *International Journal of Food Microbiology* **107**: 83-91

Bainton, N. J., Stead, P., Chhabra, S. R., Bycroft, B. W., Salmond, G. P., Stewart, G. S. A. B. and Williams, P. (1992) *N*-(3-oxohexanoyl)-L-homoserine lactone regulates carbapenem antibiotic production in *Erwinia carotoovora. Biochemistry Journal* **288**: 997-1004

Barras, F. F., van Gijsegem, F. and Chatterjee, A. K. (1994) Extracellular enzymes and pathogenesis. *Annual Review of Phytopathology* **32**: 201-234

Barros-Velázquez, J., Jiménez and Villa, T. G. (1999) Isolation and typing methods for the epidemiologic investigation of thermotolerant campylobacters. *International Microbiology* **2**: 217-226

Bassler, B. L., Wright, M. and Silverman, M. R. (1994) Multiple signalling systems controlling expression of luminescence in *Vibrio harveyi*: sequence and function of genes encoding a second sensory pathway. *Molecular Microbiology* **13**: 273-286

Baylis, C. L., MacPhee, S., Martin, K. W., Humphrey, T. J. and Betts, R. P. (2000) Comparison of three enrichment media for the isolation of *Campylobacter* spp. from foods. *Journal of Applied Microbiology* **89**: 884-891

Bell, B. P., Goldoft, M., Griffin, P. M., Davis, M. A., Gordon, D. C., Tarr, P. I., Bartleson, C. A., Lewis, J. H., Barrett, T. J. and Wells, J. G. (1994) A multistate outbreak of *Escherichia coli* O157:H7-associated bloody diarrhea and hemolytic uremic syndrome from hamburgers. The Washington experience. *Journal of the American Medical Association* **272**: 1349-1353

Bergsten, G., Wullt, B. and Svanborg, C. (2005) *Escherichia coli*, fimbriae, bacterial persistence and host response induction in the human urinary tract. *International Journal of Medical Microbiology* **295**: 487-502

Bergthorsson, U. and Ochman, H. (1998) Distribution of chromosome length variation in natural isolates of *Escherichia coli*. *Molecular and Biology Evolution* **15**: 6-16

Bielaszewska, M., Janda, J., Blahova, K., Minarikova, H., Jikova, E., Karmali, M. A, Laubova, J., Sikulova, J., Preston, M. A., Khakhria, R., Karch, H., Klazarova, H. and Nyc, O. (1997) Human *Escherichia coli* O157:H7 infection associated with the consumption of unpasteurized goat's milk. *Epidemiology and Infection* **119**: 299-305

Black, R. E., Merson, M. H., Rowe, B., Taylor, P. R., Alim, A. R. M. A., Gross, R. J. and Sack, D. A. (1981) Enterotoxigenic *Escherichia coli* diarrhoea: acquired immunity and transmission in an endemic area. *Bulletin World Health Organisation* **59**: 263-268

Blanc-Potard, A. B., Tinsley, C., Scaletsky, Le Bouguenec, C., Guignot, J., Servin, A. L., Nassif, X. and Bernet-Camard, M.-F. (2002) Representational difference analysis between Afa/Dr diffusely adhering *Escherichia coli* and non-pathogenic *E. coli* K-12. *Infection and Immunity* **70**: 5503-5511

Blank, T. E., Nougayrede, J-P. and Donnenberg, M. S. (2002) *Enteropathogenic Escherichia coli.* In *Escherichia coli*: Virulence Mechanisms of a Versatile Pathogen (Ed) Donnenberg M. S. Academic Press

Blasser, M. J. (1997) Epidemiologic and clinical features of *Campylobacter jejuni* infections. *The Journal of Infectious Diseases* **176**: (Suppl. 2) S103-S105

Blattner, F. R., Plunkett, G., III, Bloch, C. A., Perna, N. T., Burland, V., Riley, M., Collado-Vides, J., Glasner, J. D., Rode, C. K., Mayhew, G. F., Gregor, J., Davis, N. W., Kirkpatrick, H. A., Goeden, M. A., Rose, D. J., Mau, B. and Shao, Y. (1997) The Complete Genome Sequence of *Escherichia coli* K-12. *Science* **277**: 1453-1475

Blomberg, B., Jureen, R., Manji, K. P., Tamim, B. S., Mwakagile, D. S., Urassa, W. K., Fataki, M., Msangi, V., Tellevik, M. G., Maselle, S. Y. and Langeland, N. (2005) High rate of fatal cases of pediatric septicemia caused by Gram-negative bacteria with extended-spectrum beta-lactamases in Dar es Salaam, Tanzania. *Journal of Clinical Microbiology* **43**: 745-749

Bolton, F. J., Coates, D. and Hutchinson, D. N. (1984*a*) The ability of campylobacter media supplements to neutralize photochemically induced toxicity and hydrogen peroxide. *Journal of Applied Bacteriology* **56**: 151-157

Bolton, F. J., Hutchinson, D. N. and Coates, D. (1984*b*) Blood-free selective medium for isolation of *Campylobacter jejuni* from feces. *Journal of Clinical Microbiology* **19**: 169-171

Bopp, C. A, Birkness, K. A., Wachsmuth, I. K. and Barrett, T. J. (1985) In vitro antimicrobial susceptibility, plasmid analysis, and serotyping of epidemic-associated *Campylobacter jejuni*. *Journal of Clinical Microbiology* **21**: 4-7

Bradbury, W. C., Marko, M. A., Hennessy, J. N. and Penner, J. L. (1983) Occurrence of plasmid DNA in serologically defined strains of *Campylobacter jejuni* and *Campylobacter coli*. *Infection and Immunity* **40**: 460-463

Bradbury, W. C. and Munroe, D. L. G. (1985) Occurrence of plasmids and antimicrobial resistance among *Campylobacter jejuni* and *Campylobacter coli* isolated from healthy and diarrheic animals. *Journal of Clinical Microbiology* **22**: 339-346

Brandt, J. R., Joseph, M. W., Fouser, L. S., Tarr, P. I., Zelikovic, I., McDonald, R. A., Avner, E. D., McAfee, N. G. and Watkins, S. L. (1998) Cholelithiasis following *Escherichia coli* O157:H7-associated hemolytic uremic syndrome. *Pediatric Nephrology* **12**: 222-225

Branger, C., Zamfir, O., Geoffroy, S., Laurans, G., Arlet, G., Thien, H. V., Gourior, S., Picard, B. and Denamur, E. (2005) Genetic background of *Escherichia coli* and extended-spectrum beta-lactamase type. *Emerging Infectious Diseases* **11**: 54-61

Bray, J. (1945) Isolation of antigenically homogeneous strains of *Bact. coli neapolitanum* from summer diarrhea of infants. *Journal of Pathology and Bacteriology* **57**: 239-247

Bray, J. and Beaven, T. E. D. (1948) Slide agglutination of *Bacterium coli* var. *neapolitanum* in summer diarrhea. *Journal of Pathology and Bacteriology* **60**: 395-401

Brelles-Mariño, G. and Bedmar, E. J. (2001) Detection, purification and characterisation of quorum-sensing signal molecules in plant-associated bacteria. *Journal of Biotechnology* **91**: 197-209

Brookes, G. F., Butel, J. S. and Morse, S. A. (2001) Medical Microbiology: 22nd Edition Appleton and Lange

Bruhn, J. B., Christensen, A. B., Flodgaard, L. R., Nielsen, K. F., Larsen, T. O., Givskov, M. and Gram, L. (2004) Presence of acylated homoserine lactones (AHLs) and AHL-producing bacteria in meat and potential role of AHL in spoilage of meat. *Applied and Environmental Microbiology* **70**: 4293-4302

Buch, C., Sigh, J., Nielsen, J., Larsen, J. L. and Gram, L. (2003) Production of acylated homoserine lactones by different serotypes of *Vibrio anguillarum* both in culture and during infection of rainbow trout. *Systematic and Applied Microbiology* **26**: 338-349

Bull, S. A., Allen, V. M., Domingue, G., Jørgensen, F., Frost, J. A., Ure, R., Whyte, R., Tinker, D., Corry, J. E. L., Gillard-King, J. and Humphrey, T. J. (2006) Sources of *Campylobacter* spp. colonising housed broiler flocks during rearing. *Applied and Environmental Microbiology* **72**: 645-652

Butzler, J-P, Dekeyser, P., Detrain, M. and Dehaen, F. (1973) Related vibrio in stools. *Journal of Pediatrics* **82**: 493-495

Butzler, J. P. and Skirrow, M. B. (1979) Campylobacter enteritis. *Clinics in Gastroenterology* **8**: 737-765

Byers, J. T., Lucas, C., Salmond, G. P. and Welch, M. (2002) Nonenzymatic turnover of an *Erwinia carotovora* quorum-sensing signaling molecule. *Journal of Bacteriology* **184**: 1163-1171

Càmara, M., Williams, P. and Hardman, A. (2002) Controlling infection by tuning in and turning down the volume of bacterial small-talk. *The Lancet Infectious Diseases* **2**: 667-676

Cao, J.-G. and Meighen, E. A. (1989) Purification and structural identification of an autoinducer for the luminescence system of *Vibrio harveyi*. *Journal of Biological Chemistry* **264**: 21670-21676

Cha, C., Gao, P., Chen, Y. C., Shaw, P. D. and Farrand, S. K. (1998) Production of acyl-homoserine lactone quorum-sensing signals by Gram-negative plant-associated bacteria. *Molecular Plant-Microbe Interactions* **11**: 1119-1129

Chambers, C. E., Visser, M. B., Schwab, U. and Sokol, P. A. (2005) Identification of *N*-acylhomoserine lactones in mucopurulent respiratory secretions from cystic fibrosis patients. *Federation of European Microbiological Societies Microbiology Letters* **244**: 297-304

Champion, O. L., Gaunt, M. W., Gundogdu, O., Elmi, A., Witney, A. A., Hinds, J., Dorrell, N. and Wren, B. W. (2005) Comparative phylogenomics of the food-borne pathogen *Campylobacter jejuni* reveals genetic markers predictive of infection source. *Proceedings of the National Academy of Sciences USA* **102**: 16043-16048

Charlton, T. S., de Nys, R., Netting, A., Kumar, N., Hentzer, M., Givskov, M. and Kjelleberg, S. (2000) A novel and sensitive method for the quantification of *N*-3-oxoacyl homoserine lactones using gas chromatography-mass spectrometry: application to a model bacterial biofilm. *Environmental Microbiology* **2**: 530-541

Chen, C. Y. and Winans, S. C. (1991) Controlled expression of the transcriptional activator gene *virG* in *Agrobacterium tumefaciens* by using the *Escherichia coli lac* promoter. *Journal of Bacteriology* **173**: 1139-1129

Chen, X., Schauder, S., Potier, N., Dorsselaer, V., Pelczer, A., Bassler, B. L. and Hughson, F. M. (2002) Structural identification of a bacterial quorum-sensing signal containing boron. *Nature* **415**: 545-549

Cherla, R. P., Lee, S.-Y. and Tesh, V. L. (2003) Shiga toxins and apoptosis. *Federation of European Microbiological Societies Microbiology Letters* **228**: 159-166

Cheung, A., Bayer, A., Zhang, G., Gresham, H. and Xiong, Y.-Q. (2004) Regulation of virulence determinants in vitro and in vivo in *Staphylococcus aureus*. *Federation of European Microbiological Societies Immunology and Medical Microbiology* **40**: 1-9

Chilton, M.-D., Currier, T. C., Farrand, S. K., Bendich, A. J., Gordon, M. P. and Nester, E. W. (1974) *Agrobacterium tumefaciens* DNA and PS8 bacteriophage DNA not detected in crown gall tumors. *Proceedings of the National Academy of Sciences USA* **71**: 3672-3676

Cho, K., Fuqua, C. and Winans, S. C. (1997) Transcription regulation and locations of *Agrobacterium tumefaciens* genes required for complete catabolism of octopine. *Journal of Bacteriology* **179**: 1-8

Chuanchuen, R., Beinlich, K., Hoang, T. T., Becher, A., Karkhoff-Schweizer, R. R. and Schweizer, H. P. (2001) Cross-resistance between Triclosan and antibiotics in *Pseudomonas aeruginosa* is mediated by multidrug efflux pumps: exposure of a susceptible mutant strain to triclosan selects *nfxB* mutants overexpressing MexCD-OprJ. *Antimicrobial Agents and Chemotherapy* **45**: 428-432

Chun, C. K., Ozer, E. A., Welsh, M. J., Zabner, J. and Greenberg, E. P. (2004) Inactivation of a *Pseudomonas aeruginosa* quorum-sensing signal by human airway epithelia. *Proceedings of the National Academy of Sciences USA* **101**: 3587-3590

Clarke, S. C., Haigh, R. D., Freestone, P. P. E. and Williams, P. H. (2002) Enteropathogenic *Escherichia coli* infection: history and clinical aspects. *British Journal of Biomedical Sciences* **59**: 123-127

Cloak, O. M., Solow, B. T., Briggs, C. E., Chen, C-Y. and Fratamico, P. M. (2002) Quorum sensing and production of autoinducer-2 in *Campylobacter* spp., *Escherichia coli* O157:H7, and *Salmonella enterica* serovar Typhimurium in foods. *Applied and Environmental Microbiology* **68**: 4666-4671

Cogan, T. A., Bloomfield, S. F. and Humphrey, T. J. (1999) The effectiveness of hygiene procedures for the prevention of cross-contamination from chicken carcases in the domestic kitchen. *Letters in Applied Microbiology* **29**: 354-358

Cogan, T. A., Slader, J., Bloomfield, S. F. and Humphrey, T. J. (2002) Achieving hygiene in the domestic kitchen: the effectiveness of commonly used cleaning procedures. *Journal of Applied Microbiology* **92**: 885-892

Cohen, M. B. and Gianella, R. A. (1995) Enterotoxigenic *Escherichia coli* In: Infections of the Gastrointestinal Tract. (Eds) Blaser, M. J., Smith, P. D., Ravdin, J. I., Greenberg, H. B. and Guerrant, R. L. Raven Press, New York

Connell, H., Agace, W., Klemm, P. (1996) Type 1 fimbrial expression enhances *Escherichia coli* virulence for the urinary tract. *Proceedings of the National Academy of Sciences USA* **93**: 9827-9832

Cools, I., Uyttendaele, M., Cerpentier, J., D'Haese, E. D., Nelis, H. J. and Debevere, J. (2005) Persistence of *Campylobacter jejuni* on surfaces in a processing environment and on cutting boards. *Letters in Applied Microbiology* **40**: 418-423

Corry, J. E. L. and Atabay, H. I. (2001) Poultry as a source of Campylobacter and related organisms. *Journal of Applied Microbiology* **90**: 69S-114S

Corry, J. E. L., Post, D. E. Collin. P. and Laisney, M. J. (1995) Culture media for the isolation of campylobacters. *International Journal of Food Microbiology* **26**: 43-76

Coulthurst, S. J., Whitehead, N. A., Welch, M. and Salmond, G. P. C. (2002) Can boron get bacteria talking? *Trends in Biochemical Sciences* **27**: 217-219

Cravioto, A., Tello, A., Navarro, A., Ruiz, J., Villafán, H., Uribe, F. and Eslava, C. (1991) Association of *Escherichia coli* HEp-2 adherence patterns with type and duration of diarrhoea. *The Lancet* **337**: 262-264

Damborg, P., Olsen, K. E. P., Nielson, E. M. and Guardabassi, L. (2004) Occurrence of *Campylobacter jejuni* in pets living with human patients infected with *C. jejuni*. *Journal of Clinical Microbiology* **42**: 1363-1364

Daniels, R., Vanderleyden, J. and Michiels, J. (2004) Quorum sensing and swarming in bacteria. *Federation of European Microbiological Societies Microbiology Reviews* **28**: 261-289

Davies, D. G., Parsek, M. R., Pearson, J. P., Iglewski, B. H., Costerton, J. W. and Greenberg, E. P. (1998) The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science* **280**: 295-298

Dawson, K. G., Emerson, J. C. and Burns, J. L. (1999) Fifteen years of experience with bacterial meningitis. *Pediatric Infectious Disease Journal* **18**: 816-822

Degrassi, G., Aguilar, C., Bosco, M., Zahariev, S., Pongor, S. and Venturi, V. (2002) Plant growth-promoting *Pseudomonas putida* WCS358 produces and secretes four cyclic dipeptides: cross-talk with quorum-sensing bacterial sensors. *Current Microbiology* **45**: 250-254

Dekeyser, P., Gossuin-Detrain, M., Butzler, J. P. and Sternon, J. (1972) Acute enteritis due to related Vibrio: first positive stool cultures. *The Journal of Infectious Disease* **125**: 390-392

DeLoney, C.R., Bartley, T. M. and Visick, K. L. (2002) Role for photoglucomutase in *Vibrio fischeri-Eurymna scolopes* symbiosis. *Journal of Bacteriology* **184**: 5121-5129

de Louvois, J. (1994) Acute bacterial meningitis in the newborn. *Journal of Antimicrobial Chemotherapy* **34**: (suppl. A) 61-73

de Louvois, J., Blackburn, J., Hurley, R. and Harvey, D. (1991) Infantile meningitis in England and Wales: a two year study. *Archives of Childhood Diseases* **66**: 603-607

DiMango, E., Zar, H. J., Bryan, R. and Prince, A. (1995) Diverse *Pseudomonas aeruginosa* gene products stimulate respiratory epithelial cells to produce interleukin-8. *Journal of Clinical Investigation* **96**: 2204-2210

Dingle, K. E., Colles, F. M., Wareing, D. R. A., Ure, R., Fox, A.J., Bolton, F. E., Bootsma, H. J., Willems, R. J. L., Urwin, R. and Maiden, C. J. (2001) Multilocus sequence typing system for *Campylobacter jejuni*. *Journal of Clinical Microbiology* **39**: 14-23

Dobrindt, U. (2005) (Patho-)genomics of *Escherichia coli*. *International Journal of Medical Microbiolgy* **295**: 357-371

Dong, Y-H., Gusti, A. R., Zhang, Q., Xu, J-L. and Zhang, L-H. (2002) Identification of quorum-quenching *N*-acyl homoserine lactonases from *Bacillus* species. *Applied and Environmental Microbiology* **68**: 1754-1759

Dong, Y-H, Xu, J-L., Li, X-Z. and Zhang, L-H. (2000) AiiA, an enzyme that inactivates the acylhomoserine lactone quorum-sensing signal and attenuates the virulence of *Erwinia carotovora*. *Proceedings of the National Academy of Sciences USA* **97**: 3526-3531

Dong, Y-H. and Zhang, L-H. (2005) Quorum sensing and quorum-quenching enzymes. *The Journal of Microbiology* **43**: special issue (No. S) 101-109

Donnenberg, M. S. (2002) Introduction. In: *Escherichia coli*: Virulence Mechanisms of a Versatile Pathogen (Ed.) Donnenberg M. S. Academic Press

Donnenberg, M. S. and Whittam, T. S. (2001) Pathogenesis and evolution of virulence in enteropathogenic and enterohemorrhagic *Escherichia coli*. *The Journal of Clinical Investigation* **107**: 539-548

Dorrell, N., Champion, O. L. and Wren, B. W. (2005) Advances in *Campylobacter jejuni* comparative genomics through whole genome DNA microarrays. In *Campylobacter*: Molecular and Cellular Biology (Ed.) Ketley, J. M. and Konkel, M. E. Horizon Bioscience

Dorrell, N., Mangan, J. A., Laing, K. G., Hinds, J., Linton, D., Al-Ghusein, H., Barrell, B. G., Parkhill, J., Stoker, N. G., Karlyshev, A. V., Butcher P. D. and Wren, B. W. (2001) Whole genome comparison of *Campylobacter jejuni* human isolates using a low-cost microarray reveals extensive genetic diversity. *Genome Research* **11**: 1706–1715

Dowling, R. (2006) University of Central Lancashire, Preston, UK. *Personal Communication*

Dragonov, D. I., Teiber, J. F., Speelman, A., Osawa, Y., Sunahara, R. and La Du, B. N. (2005) Human paraoxonases (PON1, PON2, and PON3) are lactonases with overlapping and distinct substrate specificities. *Journal of Lipid Research* **46**: 1239-1247

Duim, B., Vandamme, P. A. R., Rigter, A., Laevens, S., Dijkstra, J. R. and Wagnaar, J. A. (2001) Differentiation of *Campylobacter* species by AFLP fingerprinting. *Microbiology* **147**: 2729-2737

Duim, B., Wassenaar, T. M., Rigter, A. M. and Wagenaar, J. A. (1999) High-resolution genotyping of *Campylobacter* strains isolated from poultry and humans with AFLP fingerprinting. *Applied and Environmental Microbiology.* **65**: 2369-2375

Dundas, S., Todd, W. T. A., Stewart, A. I., Murdoch, P. S., Chaudhuri, A. K. R. and Hutchinson, S. J. (2001) The Central Scotland *Escherichia coli* O157:H7 outbreak: risk factors for the hemolytic uremic syndrome and death among hospitalized patients. *Clinical Infectious Diseases* **33**: 923-931

Dunman, P. M., Murphy, E., Haney, S., Palacios, D., Tucker-Kellogg, G., Wu, S., Brown, E. L., Zagursky, R. J., Shlaes, D. and Projan, S. J. (2001) Transcription profiling-based identification of *Staphylococcus aureus* genes regulated by the *agr* and/or *sarA* loci. *Journal of Bacteriology* **183**: 7341-7353

Eberl, L. and Tümmler, B. (2004) *Pseudomonas aeruginosa* and *Berkholderia cepacia* in cystic fibrosis: genome evolution, interactions and adaptation. *International Journal of Medical Microbiology* **294**: 123-131

Ekdahl, K., Normann, B. and Andersson, Y. (2005) Could flies explain the elusive epidemiology of campylobacteriosis? *BMC Infectious Diseases* **5**: 11

Elvers, K.T. and Park, S. F. (2002) Quorum sensing in *Campylobacter jejuni*: detection of a *luxS* encoded signalling molecule. *Microbiology* **148**: 1475-1481

Erickson, D. L., Endersby, R., Kirkham, A., Stuber, K., Vollman, D. D., Rabin, H. R., Mitchell, I. and Storey, D. G. (2002) *Pseudomonas aeruginosa* quorum-sensing systems may control virulence factor expression in the lungs of patients with Cystic Fibrosis. *Infection and Immunity* **70**: 1783-1790

Fagundes-Neto, U. and Scaletsky, A. I. C. (2000) The gut at war: The consequences of enteropathogenic *Escherichia coli* infection as a factor of diarrhea and malnutrition. *São Paulo Medical Journal* **118**: 21-29

Farthing, M. J. G. (2004) Bugs and the gut: an unstable marriage. *Best Practice and Research: Clinical Gastroenterology* **18**: 233-239

Farrand, S. K. (1998) Petri dish assay for acyl-homoserine lactone production. Departments of Crop Sciences and Microbiolgy, University of Illinois at Urbana-Champaign. *Personal Communication*

Farrand, S. K., Hwang, I. and Cook, D. M. (1996) The *tra* region of the nopaline-type Ti plasmid is a chimera with elements related to the transfer systems of RSF1010, RP4, and F. *Journal of Bacteriology* **178**: 4233-4247

Favre-Bonté, S., Köhler, T. and van Delden, C. (2003) Biofilm formation by *Pseudomonas aeruginosa*: role of the C4-HSL cell-to-cell signal and inhibition by azithromycin. *Journal of Antimicrobial Chemotherapy* **52**: 598-604

Federle, M. J. and Bassler, B. L. (2003) Interspecies communication in bacteria. *The Journal of Clinical Investigation* **112**: 1291-1299

Feng, P. (2001) *Escherichia coli* In Guide to Foodborne Pathogens (Eds.) Labbe, G. G. and Garcia, S. John Wiley and Sons, Inc.

Fitzgerald, C., Sails, A. D. and Fields, P. I. (2005) *Campylobacter jejuni* strain variation. In *Campylobacter*: Molecular and Cellular Biology (Ed.) Ketley, J. M. and Konkel, M. E. Horizon Bioscience

Flodgaard, L. R., Christensen, A. B., Molin, S., Givskov, M. and Gram, L. (2003) Influence of food preservation parameters and associated microbiota on production rate, profile and stability of acylated homoserine lactones from food-derived Enterobacteriaceae. *International Journal of Food Microbiology* **84**: 145-156

Fouts, D. E., Mongodin, E. F., Mandrell, R. E., Miller, W. G., Rasko, D. A., Ravel, J., Brinkac, L. M., DeBoy, R. T., Parker, C. T., Daugherty, S. C., Dodson, R. J., Durkin, A. S., Madupu, R., Sullivan, S. A., Shetty, J. U., Ayodeji, M. A., Shvartsbeyn, A., Schatz, M. C., Badger, J. H., Fraser, C. M. and Nelson, K. E. (2005) Major structural differences and novel potential virulence mechanisms from the genomes of multiple *Campylobacter* species. *Public Library of Science Biology* **3**: 72-85

Franco, D. A. and Williams, C. E. (2001) *Campylobacter jejuni.* In Foodborne Disease Handbook, 2nd Edition Volume 1: Bacterial Pathogens (Ed.) Hui, Y. H., Pierson, M. D. and Gorham, J. R. Marcel Dekker, Inc., New York

Franco, S. M., Cornelius, V. E. and Andrews, B. F. (1992) Long term outcome of neonatal meningitis. *American Journal of Diseases of Children* **146**: 567-571

Frankel, G., Phillips, A. D., Rosenshine, I., Dougan, G., Kaper, J. B. and Knutton, S. (1998) Enteropathogenic and enterohaemorrhagic *Escherichia coli*: More subversive elements. *Molecular Microbiology* **30**: 911-921

Frost, J. A. (2001) Current epidemiological issues in human campylobacteriosis *Journal of Applied Microbiology* **90**: 85S-95S

Fuqua, C. and Eberhard, A. (1999) Signal generation in autoinduction systems: synthesis of acylhomoserine lactones by LuxI-type proteins. In: Cell-Cell Signalling in Bacteria (Ed.) Dunny, G. M. and Winans, S. C. ASM Press, Washington

Fuqua, C. and Greenberg, E. P. (1998) Self perception in bacteria: quorum sensing with acylated homoserine lactones. *Current Opinions in Microbiology* **1**: 183-189

Fuqua, C. and Greenberg, P. (2002) Listening in on bacteria: acyl-homoserine lactone signalling. *Nature Reviews: Molecular Cell Biology* **3**: 685-693

Fuqua, W. C. and Winans, S. C. (1994) A LuxR-LuxI type regulatory system activates *Agrobacterium* Ti plasmid conjugal transfer in the presence of a plant tumor metabolite. *Journal of Bacteriology* **176**: 2796-2806

Fuqua, W. C. and Winans, S. C. (1996) Conserved *cis*-acting promoter elements are required for density-dependent transcription of *Agrobacterium tumefaciens* conjugal transfer genes. *Journal of Bacteriology* **178**: 435-440

Fuqua, W. C., Winans, S. C. and Greenberg, E. P. (1994) Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. *Journal of Bacteriology* **176**: 269-275

Fux, C. A., Shirtliff, M., Stoodley, P. and Costerton, J. W. (2005) Can laboratory reference strains mirror 'real-world' pathogenesis? *Trends in Microbiology* **13**: 58-63

Gallio, M., Sturgill, G., Rather, P. and Kylsten, P. (2002) A conserved mechanism for extracellular signaling in eukaryotes and prokaryotes. *Proceedings of the National Academy of Sciences USA* **99**: 12208-12213

Garg, A. X., Suri, R. S., Barrowman, N., Rehman, F., Matsell, D., Rosas-Arellano, M. P., Salvadori, M., Haynes, R. B. and Clark, W. F. (2003) Long-term renal prognosis of diarrhea-associated hemolytic uremic syndrome: a systematic review, meta-analysis, and meta-regression. *Journal of the American Medical Association* **290**: 1360-1370

Gaynor, E. C., Cawthraw, S., Manning, G., MacKichan, J. K., Falkow, S. and Newell, D. G. (2004) The genome-sequenced variant of *Campylobacter jejuni* NCTC 11168 and the original clonal clinical isolate differ markedly in colonization, gene expression, and virulence-associated phenotypes. *Journal of Bacteriology* **186**: 503-517

Girardeau, J. P., Lalioui, L., Said, A. M., De Champs, C. and Le Bouguenec, C. (2003) Extended virulence genotype of pathogenic *Escherichia coli* isolates carrying the afa-8 operon: evidence of similarities between isolates from humans and animals with extraintestinal infections. *Journal of Clinical Microbiology* **41**: 218-226

Gordillo, M. E., Reeve, G. R., Pappas, J., Mathewson, J. J., DuPoint, H. L. and Murray, B. E. (1992) Molecular characterization of strains of enteroinvasive *Escherichia coli* O143, including isolates from a large outbreak in Houston, Texas. *Journal of Clinical Microbiology* **30**: 889-893

Govan, J. R. W. and Deretic, V. (1996) Microbial pathogenesis in Cystic Fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. *Microbiological Reviews* **60**: 539-574

Gram, L., Christensen, A. B., Ravn, L., Molin, S. and Givskov, M. (1999) Production of acylated homoserine lactones by psychrotrophic members of the *Enterobacteriaceae* isolated from foods. *Applied and Environmental Microbiology* **65**: 3458-3463

Gram, L., Ravn, L., Rasch, M., Bruhn, J. B., Christensen, A. B. Givskov, M. (2002) Food spoilage-interactions between food spoilage bacteria. *International Journal of Food Microbiology* **78**: 79-97

Greenberg, E. P. (2003) Quorum-sensing in Gram-negative bacteria. *American Society for Microbiology News* **63**: 371-377

Greenberg, E. P. (1999) Quorum sensing in Gram-negative bacteria: acylhomoserine lactone signalling and cell-cell communication. pp. 71-84. In: Microbial signalling and communication. R. England, G. Hobs, N. Bainton, D. Roberts (eds.) Cambridge University Press

Griffiths, P. L. and Park, R. W. A. (1990) Campylobacters associated with human diarrhoeal disease. *Journal of Applied Bacteriology* **69**: 281-301

Gruenheid, S. and Finlay, B. B. (2000) Crowd control: quorum sensing in pathogenic *E. coli*. *Trends in Microbiology* **8**: 442-443

Guerry, P., Logan, S. M., Thornton, S. and Trust, T. J. (1990) Genomic organization and expression of *Campylobacter* flagellin genes. *Journal of Bacteriology* **172**: 1853-1860

Hadden, R. D. M., Karch, H., Hartung, H.-P., Zielasek, J., Weissbrich, B., Schubert, J., Weishaupt, A., Cornblath, D. R., Swan, A. V., Hughes, R. A. C. and Toyka, K. V. (2001) Preceding infections, immune factors, and outcome in Guillain-Barré syndrome. *Neurology* **56**: 758-765

Hajdukiewicz, P., Svab, Z. and Maliga, P. (1994) The small, versatile pPZP family of *Agrobacterium* binary vectors for plant transformation. *Plant Molecular Biology* **25**: 989-994

Hale, T. L., Echeverria, P. and Nataro, J. P. (1997) Enteroinvasive *Escherichia coli*. In: *Escherichia coli*: Mechanisms of Virulence (Ed) Sussman, M. Cambridge University Press, UK

Hardie, K. R., Cooksley, C., Green, A. D. and Winzer, K. (2003) Autoinducer 2 activity in *Escherichia coli* culture despite maintenance of an active synthase, LuxS. *Microbiology* **149**: 715-728

Harold, F. M. (1986) The vital force: a study of bioenergetics. W. H. Freeman and Co., New York

Harris, J. R., Mariano, J., Wells, J. G., Payne, B. J., Donnell, H. D. and Cohen, M. L. (1985) Person-to-person transmission in an outbreak of enteroinvasive *Escherichia coli*. *American Journal of Epidemiology* **122**: 245-252

Hayashi, T., Makino, K., Ohnishi, M., Kurokawa, K., Ishii, K., Yokoyama, K., Han, C.-G., Ohtsubo, E., Nakayama, K., Murata, T., Tanaka, M., Tobe, T., Iida, T., Takami, H., Honda, T., Sasakawa, C., Ogasawara, N., Yasunaga, T., Kuhara, S., Shiba, T., Hattori, M. and Shinagawa, H. (2001) Complete genome sequence of enterohemorrhagic *Escherichia coli* O157:H7 and genomic comparison with a laboratory strain K-12. *DNA Research* **8**: 11-22

Hazeleger, W., Arkestijn, C., Toorop-Bouma, A. and Beumer, R. (1994) Detection of the coccoid form of *Campylobacter jejuni* in chicken products with the use of the polymerase chain reaction. *International Journal of Food Microbiology* **24**: 273-281

Hazeleger, W. C., Wouters, J. A., Rombouts, F. M. and Abee, T. (1998) Physiology activity of *Campylobacter jejuni* far below the minimal growth temperature. *Applied and Environmental Microbiology* **64**: 3917-3922

Hentzer, M. and Givskov, M. (2003) Pharmacological inhibition of quorum sensing for the treatment of chronic bacterial infections. *Journal of Clinical Investigation* **112**: 1300-1307

Heuvelink, A. E., Zwartkruis-Nahuis, J. T. M., van der Biggelaar, F. L. A. M., van Leeuwen, W. J. and de Boer, E. (1999) Isolation and characterization of verocytotoxin-producing *Escherichia coli* O157 from slaughter pigs and poultry. *International Journal of Food Microbiology* **52**: 67-75

Hicks, S., Candy, D. C. A. and Phillips, A. D. (1996) Adhesion of enteroaggregative *Escherichia coli* to pediatric intestinal mucosa in vitro. *Infection and Immunity* **64**: 4751-4760

Hilton, A. C. and Austin, E. (2000) The kitchen dishcloth as a source of and vehicle for foodborne pathogens in a domestic setting. *International Journal of Environmental Health Research* **10**: 257-261

Hoang, T. T. and Schweizer, H. P. (1999) Characterisation of the *Pseudomonas aeruginosa* enoyl-acyl carrier protein reductase (FabI): a target for the antimicrobial triclosan and its role in acylated homoserine lactone synthesis. *Journal of Bacteriology* **181**: 5489-5497

Hodge, J. P. and Krieg, N. R. (1994) Oxygen tolerance estimates in *Campylobacter* species depend on the testing medium. *Journal of Applied Microbiology* **77**: 666-673

Hoffman, P. S., George, H. A., Krieg, N. R. and Smibert, R. M. (1979) Studies of the microaerophilic nature of *Campylobacter fetus* subsp. *jejuni*. II. Role of exogenous superoxide anions and hydrogen peroxide. *Canadian Journal of Microbiology* **25**: 8-16

Holden, M. T. G., Chhabra, S. R., de Nys, R., Bainton, N. J., Hill, P. J., Manefeild, M., Kumar, N., Labatte, M., England, D., Rice, S., Givskov, M., Salmond, P. C., Stewart, G. S. A. B., Bycroft, B. W., Kjelleberg, S. and Williams, P. (1999) Quorum-sensing cross-talk: isolation and chemical characterization of cyclic dipeptides from *Pseudomonas aeruginosa* and other Gram-negative bacteria. *Molecular Microbiology* **33**: 1254-1266

Holden, M., Swift, S. and Williams, P. (2000) New signal molecules on the quorum-sensing block. *Trends in Microbiology* **8**: 101-103

Huang, J. J., Petersen, A., Whiteley, M. and Leadbetter, J. R. (2006) Identification of QuiP, the product of gene PA1032, as the second acyl-homoserine lactone acylase of *Pseudomonas aeruginosa* PAO1. *Applied and Environmental Microbiology* **72**: 1190-1197

Hudson, J. A., Nicol, C., Wright, J., Whyte, R. and Hasell, S. K. (1999) Seasonal variation of *Campylobacter* types from human cases, veterinary cases, raw chicken, milk and water. *Journal of Applied Microbiology* **87**: 115-124

Hughes, R. A. C. and Cornblath, D. R. (2005) Guillain-Barré syndrome. *The Lancet* **366**: 1653-1666

Humphrey, T. J. (1986) Injury and recovery in freeze- or heat-damaged *Campylobacter jejuni*. *Letters in Applied Microbiology* **3**: 81-84

Humphrey, T. J. (1990) The synergistic inhibition of *Campylobacter jejuni* by rifampicin and hydrogen peroxide. *Letters in Applied Microbiology* **10**: 97-100

Humphrey, T. (2001) The spread and persistence of *Campylobacter* and *Salmonella* in the domestic kitchen. *Journal of Infection* **43**: 50-53

Humphrey, T. J., Jørgensen, F., Frost, J. A., Wadda, H., Domingue, G., Elviss, N. C., Griggs, D. J. and Piddock, L. J. V. (2005) Prevalence and subtypes of ciprofloxacin-resistant *Campylobacter* spp. in commercial poultry flocks before, during, and after treatment with fluoroquinolones. *Antimicrobial Agents and Chemotherapy* **49** 690-698

Humphrey, T. J., Martin, K. W., Slader, J. and Durham, K. (2001) *Campylobacter* spp. in the kitchen: spread and persistence. *Journal of Applied Microbiology* **90**: 115S-120S

Humphrey, T., Mason, M. and Martin, K. (1995) The isolation of *Campylobacter jejuni* from contaminated surfaces and its survival in diluents. *International Journal of Food Microbiology* **26**: 295-303

Huppertz, H.-I., Ruthkowski, S., Busch, D. H., Eisebit, R., Lissner, R. and Karch, H. (1999) Bovine colostrum ameliorates diarrhea in infection with diarrheagenic *Escherichia coli*, Shiga toxin-producing *E. coli*, and *E. coli* expressing intimin and hemolysin. *Journal of Pediatric Gastroenterology and Nutrition* **29**: 452-256

Hwang, I., Zhang, L. H., Piper, K. R., Cook, D. M., Tate, M. E. and Farrand, S. K. (1994) TraI, a LuxI homologue, is responsible for production of conjugal factor, the Ti plasmid *N*-acylhomoserine lactone autoinducer. *Proceedings of the National Academy of Sciences USA* **91**: 4639-4643

Ivanova, L. G., Vargina, A. K., Gorbachev, I. D., Ershova, Z. I., Yulin, I. I. and Trifonov, V. I. (1985) Content of dissolved oxygen in microbiological culture media. *Zhurnal Mikrobiologii, Epidemiologii, i Immunobiologii* **8**: 26-29

Jackson, L. A., Benson, P., Neuzil, K. M., Grandjean, M. and Marino, J. L. (2005) Burden of community-onset *Escherichia coli* bacteremia in seniors. *The Journal of Infectious Diseases* **191**: 1523-1529

Jacobs-Reitsma, W. (2000) *Campylobacter* in the food supply. In: Campylobacter (2nd Edition) (Ed) Nachamkin, I. and Blaser, M. J. ASM Press, Washington, D.C.

Jeon, B., Itoh, K., Misawa, N. and Ryu, S. (2003) Effects of quorum sensing on *flaA* transcription and autoagglutination in *Campylobacter jejuni*. *Microbiology and Immunology* **47**: 833-839

Johnson, J. R. (2003) Microbial virulence determinants and the pathogenesis of urinary tract infections. *Infectious Disease Clinics Of North America* **17**: 261-278

Johnson, J. R., Kuskowski, M. A., Gajewski, A., Sahm, D. F. and Karlowsky, J. A. (2004) Virulence characteristics and phylogenetic background of multidrug-resistance and antimicrobial-susceptible clinical isolates of *Escherichia coli* from across the United States. *Journal of Infection* **190**: 1739-1744

Johnson, J. R., Kuskowski, M. A., O'Bryan, T. T. and Maslow, J. N. (2002) Epidemiological correlates of virulence genotype and phylogenetic background among *Escherichia coli* blood isolates from adults with diverse-source bacteremia. *The Journal of Infectious Diseases* **185**: 1439-1447

Johnson, J. R., Kuskowski, M. A., Smith, K., O'Bryan, T. T and Tatini, S. (2005) Antimicrobial-resistance and extraintestinal pathogenic *Escherichia coli* in retail foods. *The Journal of Infectious Diseases* **191**: 1040-1049

Johnson, J. R., Orskov, F., Goullet, P., Moseley, S. L., Roberts, P. and Stamm, W. E. (1994) O, K and H antigens predict virulence factors, carboxylesterase B pattern, antimicrobial resistance, and host compromise among *Escherichia coli* strains causing urosepsis. *The Journal of Infectious Diseases* **169**: 119-126

Johnson, J. R. and Stell, A. L. (2000) Extended virulence genotypes of *Escherichia coli* strains from patients with urosepsis in relation to phylogeny and host compromise. *The Journal of Infectious Diseases* **181**: 261-272

Johnson, W. M. and Loir, H. (1988) A new heat-labile cytolethal distending toxin (CLDT) produced by *Campylobacter* spp. *Microbial Pathogenesis* **4**: 115-126

Jones, K. (2001) *Campylobacters* in water, sewage and the environment. *Journal of Applied Microbiology* **90**: 68S-79S

Jones, M. B. and Blaser, M. J. (2003) Detection of a *luxS*-signaling molecule in *Bacillus anthracis*. *Infection and Immunity* **71**: 3914-3919

Jones, M. E., Draghi, D. C., Thornsberry, C., Karlowsky, J. A., Sahm, D. F. and Wenzel, R. P. (2004) Emerging resistance among bacterial pathogens in the intensive care unit-a European and North American surveillance study (2000-2002). *Annals of Clinical Microbiology and Antimicrobials* **3**: 14-25

Kaper, J. B. (2005) Pathogenic *Escherichia coli*. *International Journal of Medical Microbiology* **295**: 355-356

Kaper, J. B., Nataro, J. P. and Mobley, L. T. (2004) Pathogenic *Escherichia coli*. *Nature Reviews Microbiology* **2**: 123-140

Kaper, J. B. and Sperandio, V. (2005) Bacterial cell-to-cell signalling in the gastrointestinal tract. *Infection and Immunity* **73**: 3197-3209

Karch, H., Tarr, P. I. and Bielaszewska, M. (2005) Enterohaemorrhagic *Escherichia coli* in human medicine. *International Journal of Medical Microbiology* **295**: 405-418

Karram, M. M. and Mallipeddi, P. K. (1999) Lower urinary tract infection. In: Urogynecology and Reconstructive Pelvic Surgery 2nd Ed. (Eds) Walters, M. D. and Karram, M. M. Mosby, St. Louis

Keene, W. E., Sazie, E., Kok, J., Rice, D. H., Hancock, D. D., Balan, V. K., Zhao, T. and Doyle, M. P. (1997) An outbreak of *Escherichia coli* O157:H7 infections traced to jerky made from deer meat. *Journal of the American Medical Association* **277**: 1229-1231

Ketley, J. M. (1997) Pathogenesis of enteric infection by *Campylobacter*. *Microbiology* **143**: 5-21

Ketley, J. M. and Konkel, M. E. (2005) Preface. In: *Campylobacter*: Molecular and Cellular Biology (Ed.) Ketley, J. M. and Konkel, M. E. Horizon Bioscience

Kievit, T. R. and Inglewski, B. H. (2000) Bacterial quorum sensing in pathogenic relationships. *Infection and Immunity* **68**: 4839-4849

Kim, E.-J., Wang, W., Deckwer, W.-D. and Zeng, A.-P. (2005) Expression of the quorum-sensing regulatory protein LasR is strongly affected by iron and oxygen concentrations in cultures of *Pseudomonas aeruginosa* irrespective of cell density. *Microbiology* **151**: 1127-1138

Kim, K. S. (2002) Strategy of *Escherichia coli* for crossing the blood-brain barrier. *The Journal of Infectious Diseases* **186**: (Suppl. 2) S220-S224

King, E. O. (1957) Human infections with *Vibrio fetus* and a closely related *Vibrio*. *The Journal of Infectious Diseases* **101**: 119-128

Kist, M. (1985) The historical background of *Campylobacter* infection: new aspects. In: Proceedings of the 3rd International Workshop on *Campylobacter* Infections; Otawa; 1985 Jul 7-10 (Ed) Pearson, A. D. Public Health Service, London

Kokotovic, B. and On, S. L. W. (1999) High-resolution genomic fingerprinting of *Campylobacter jejuni* and *Campylobacter coli* by analysis of amplified fragment length polymorphism. *Federation of European Microbiological Societies Microbiology Letters* **173**: 77-84

Konkel, M. E., Monteville, M. R., Rivera-Amill, V. and Joens, L. A. (2001) The pathogenesis of *Campylobacter jejuni*-mediated enteritis. *Current Issues in Intestinal Microbiology* **2**: 55-71

Koonin, E. V., Makarova, K. S., Rogozin, I. B., Davidovic, L., Letellier, M.-C. and Pellegrini, L. (2003) The rhomboids: a nearly ubiquitous family of intramembrane serine proteases that probably evolved by multiple ancient horizontal gene transfers. *Genome Biology* **4**: R19

Korhonen, L. K. and Martikainen, P. J. (1991) Survival of *Escherichia coli* and *Campylobacter jejuni* in untreated and filtered lake water. *Journal of Applied Bacteriology* **71**: 379-382

Kovach, M. E., Elzer, P. H., Hill, D. S., Robertson, G. T., Farris, M. A., Roop II, R. M. and Peterson, K. M. (1995) Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* **166**: 175-176

Kovats, R. S., Edwards, S. J., Charron, D., Cowden, J., Souza, R. M. D., Ebi, K. L., Gauci, C., Gerne-Smidt, P., Hajat, S., Hales, S., Pezzi, G. H., Kriz, B., Kutsar, K., McKeown, P., Mellou, K., Menne, B., O'Brien, S., van Pelt, W. and Schmid, H. (2005) Climate variability and *Campylobacter* infection: an international study. *International Journal of Biometeorology* **49**: 207-214

Krieg, N. R. and Hoffman, P. S. (1986) Microaerophily and oxygen toxicity. *Annual Reviews of Microbiology* **40**: 107-130

Kudva, I. T., Hatfield, P. G. and Hovde, C. J. (1996) *Escherichia coli* O157:H7 in microbial flora of sheep. *Journal of Clinical Microbiology* **34**: 431-433

Kuroki, S., Saida, T., Nukina, M., Haruta, T., Yoshioka, M., Kobayashi, Y. and Nakanishi, H. (1993) *Campylobacter jejuni* strains from patients with Guillain-Barrié syndrome belong mostly to Penner serogroup 19 and contain β-N-acetylglucosamine residues. *Annals of Neurology* **33**: 243-247

Kusumaningrum, H. D., van Putten, M. M., Rombouts, F. M. and Beumer, R. R. (2002) Effects of antibacterial dishwashing liquid on foodborne pathogens and competitive microorganisms in kitchen sponges. *Journal of Food Protection* **65**: 61-65

Kusumaningrum, H. D., Riboldi, G., Hazeleger, W. C. and Beume, R. R. (2003) Survival of foodborne pathogens on stainless steel surfaces and cross-contamination. *International Journal of Food Microbiology* **85**: 227-236

Lan, R., Lumb, B., Ryan, D. and Reeves, P. R. (2001) Molecular evolution of large virulence plasmid in *Shigella* clones and enteroinvasive *Escherichia coli*. *Infection and Immunity* **69**: 6303-6309

Lastovica, A. J., Goddard, E. A. and Argent, A. C. (1997) Guillain-Barré syndrome in South Africa associated with *Campylobacter jejuni* O:41 strains. *The Journal of Infectious Diseases* **176**: (Suppl. 2) 139-143

Latifi, A., Foglino, M., Tanaka, K., Williams, P. and Lanzdunski, A. (1996) A hierarchial quorum-sensing cascade in *Pseudomonas aeruginosa* links the transcriptional activators LasR and RhiR (VsmR) to expression of the stationary-phase sigma factor RpoS. *Molecular Microbiology* **21**: 1137-1146

Latifi, A., Winson, M. K., Foglino, M., Bycroft, B. W., Stewart, G. S. A. B., Lazdunski, A., and Williams, P. (1995) Multiple homologues of LuxR and LuxI control expression of virulence determinants and secondary metabolites through quorum sensing in *Pseudomonas aeruginosa* PAO1. *Molecular Microbiology* **17**: 333-343

Law, D. and Chart, H. (1998) Enteroaggregative *Escherichia coli. Journal of Applied Microbiology* **84**: 685-697

Leadbetter, J. R. and Greenberg, E. P. (2000) Metabolism of acyl-homoserine lactone quorum-sensing signals by *Variovorax paradoxus. Journal of Bacteriology.* **182**: 6921-6926

Le Bouguénec, C. (1999) Diarrhea-associated diffusely adherent *Escherichia coli. Clinical Microbiology Reviews* **12**: 180-181

Le Bouguénec, C. and Servin, A. L. (2006) Diffusely adherent *Escherichia coli* strains expressing Afa/Dr adhesions (Afa/Dr DAEC): hitherto unrecognized pathogens. *Federation of European Microbiological Societies Microbiological Letters* **256**: 185-194

Lederberg, J. (1951) Genetic studies with bacteria. In: Genetics in the 21st Century (Ed.) Dunn, L. C. Macmillan, New York

Lederberg, J. (2004) *E. coli* K-12. *Microbiology Today* **31**: 116

Lee, S. J., Park, S-Y., Lee, J-J., Yum, D-Y., Koo, B-T. and Lee, J-K. (2002) Genes encoding the *N*-acyl homoserine lactone-degrading enzyme are widespread in many subspecies of *Bacillus thuringiensis*. *Applied and Environmental Microbiology* **68**: 3919-3924

Lin, Y-H., Xu, J-L, Hu, J., Wang, L-H., Ong, S. L., Leadbetter, J. R. and Zhang, L-H. (2003) Acyl-homoserine lactone acylase from *Ralstonia* strain XJ12B represents a novel and potent class of quorum-quenching enzymes. *Molecular Microbiology* **47**: 849-860

Lindberg, U., Claesson, I., Hanson, L. Å. and Jodal, U. (1978) Asymptomatic bacteriuria in schoolgirls. VIII. Clinical course during a 3-year follow-up. *Journal of Pediatrics* **92**: 194-199

Lior, H. Woodward, D. L., Edgar, J. A., Laroche, L. J. and Gill, P. (1982) Serotyping of *Campylobacter jejuni* by slide agglutination based on heat-labile antigenic factors. *Journal of Clinical Microbiology* **15**: 761-768

Llamas, I., Keshavan, N. and González J. E. (2004) Use of Sinohizobium meliloti as an indicator for specific detection of long-chain *N*-acyl homoserine lactones. *Applied and Environmental Microbiology* **70**: 3715-3723

Louis, V. R., Gillespie, I. A., O'Brien, S. J., Russek-Cohen, E., Pearson, A. D. and Colwell, R. R. (2005) Temperature-driven *Campylobacter* seasonality in England and Wales. *Applied and Environmental Microbiology* **71**: 85-92

Luo, Z.-Q., Clemente, T. E. and Farrand, S. K. (2001) Construction of a derivative of *Agrobacterium tumefaciens* C58 that does not mutate to tetracycline resistance. *Molecular Plant-Microbe Interactions* **14**: 98-103

Luo, Z.-Q., Su, S. and Farrand, S. K. (2003) In situ activation of the quorum-sensing transcription factor TraR by the cognate and noncognate acyl-homoserine lactone ligands: kinetics and consequences. *Journal of Bacteriology* **185**: 5665-5672

Manefield, M., de Nys, R., Kumar, N., Reed, R., Givskov, M., Steinberg, P. and Kjelleberg, S. (1999) Evidence that halogenated furanones from *Delisea pulchra* inhibit acylated homoserine lactone (AHL)-mediated gene expression by displacing the AHL signal from its receptor protein. *Microbiology* **145**: 283-291

Manefield, M., Rasmussen, T. B., Henzter, M., Anderson, J. B., Steinberg, P., Kjelleberg, S. and Givskov, M. (2002) Halogenated furanones inhibit quorum sensing through accelerated LuxR turnover. *Microbiology* **148**: 1119-1127

Manefield, M. and Turner, S. (2002) Quorum sensing in context: out of molecular biology and into microbial ecology. *Micriobiology* **148**: 3762-3764

Manefield, M., Turner, S. L., Lilley, A. K. and Bailey, M. J. (2004) Are you out there? Intercellular signalling in the microbial world. In: Microbial Evolution: Gene establishment, Survival and Exchange. Ed. Miller, E. V. and Day, M. J. ASM Press, Washington

Manning, G., Duim, B., Wassenaar, J. A., Ridley, A. and Newell, D. G. (2001) Evidence for a genetically stable strain of *Campylobacter jejuni. Applied and Environmental Microbiology* **67**: 1185-1189

Marketon, M. M. and González, J. E. (2002) Identification of two quorum-sensing systems in Sinorhizobium meliloti. *Journal of Bacteriology* **184**: 3466-3475

Martin, M., Showalter, R. and Silverman, M. (1989) Identification of a locus controlling expression of luminescence genes in *Vibrio harveyi. Journal of Bacteriology* **171**: 2406-2414

Marrs, C. F., Zhang, L. and Foxman, B. (2005) *Escherichia coli* mediated urinary tract infections: Are there distinct uropathogenic *E. coli* (UPEC) pathotypes? *Federation of European Microbiological Societies Microbiolgy Reviews* **252**: 183-190

Maslow, J. N., Lautenbach, E., Glaze, T., Bilker, W. and Johnson, J. R. (2004) Colonization with extraintestinal pathogenic *Escherichia coli* among nursing home residents and its relationship to fluoroquinolone resistance. *Antimicrobial Agents and Chemotherapy* **48**: 3618-3620

Maslow, J. N., Whittam, T. S., Gilks, C. F., Wilson, E. A., Mulligan, M. E., Adams, K. S. and Arbeit, R. D. (1995) Clonal relationship among bloodstream isolates of *Escherichia coli. Infection and Immunity* **63**: 2409-2417

Mason, M. J., Humphrey, T. J. and Martin, K. W. (1996) Isolation of sublethally injured *Campylobacters* from Water. In: *Campylobacters*, *Helicobacters* and Other Related Organisms. (Ed) Newell, D. G., Ketley, J. M. and Feldman, R. A. Plenum Press, New York

Massion, P. P., Inoue, H., Richman-Eisenstat, J., Grunberger, D., Jorens, P. G., Housset, B., Pittet, J.-F., Wiener-Kronish, J. P. and Nadel, J. A. (1994) Novel *Pseudomonas* product stimulates interleukin-8 production in airway epithelial cells *in vitro*. *The Journal of Clinical Investigation* **93**: 26-32

Mattick, K., Durham, K., Domingue, G., Jørgensen, F., Sen, M., Schaffner, D. W. and Humphrey, T. (2003*a*) The survival of foodborne pathogens during domestic washing-up and subsequent transfer onto washing-up sponges, kitchen surfaces and food. *International Journal of Food Microbiology* **85**: 213-226

Mattick, K., Durham, K., Hendrix, M., Slader, J., Griffith, C., Sen, M. and Humphrey, T. (2003*b*) The microbiological quality of washing-up water and the environment in domestic and commercial kitchens. *Journal of Applied Microbiology* **94**: 842-848

Matussek, A., Lauber, J., Bergau, A., Hansen, W., Rohde, M., Dittmar, K. E. J., Gunzer, M., Mengel, M., Gatzlaff, P., Hartmann, M., Buer, J. and Gunzer, F. (2003) Molecular and functional analysis of shiga toxin-induced response patterns in human vascular endothelial cells. *Blood* **102**: 1323-1332

McBean, M. and Rajamani, S. (2001) Increasing rates of hospitalization due to septicemia in the US elderly population, 1986-1997. *The Journal of Infectious Diseases* **183**: 596-603

McClean, K. H., Winson, M. K., Fish, L., Taylor, A., Chhabra, S. R., Camara, M., Daykin, M., Lamb, J. H., Swift, S., Bycroft, B. W., Stewart, G. S. A. B. and Williams, P. (1997) Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of *N*-acylhomoserine lactones *Microbiology* **143**: 3703-3711

McDowell, P., Affas, Z., Reynolds, C., Holden, M. T. G., Wood, S. J., Saint, S., Cockayne, A., Hill, P. J., Dodd, C. E. R., Bycroft, B. W., Chan, W. C. and Williams, P. (2001) Structure, activity and evolution of the group 1 thiolactone peptide quorum-sensing system of *Staphylococcus aureus*. *Molecular Microbiology* **41**: 503-512

Mead, P. S., Slutsker, L., Dietz, V., McCaig, L. F., Bresee, J. S., Shapiro, C., Griffin, P. M. and Tauxe, R. V. (1999) Food-related illness and death in the United States. *Emerging Infectious Diseases* **5**: 607-625

Michelsen, P. and Karlsson, A. Å. (1999) Accurate mass determination of a metabolite of a potential diagnostic imaging drug candidate by high performance liquid chromatography with time-of-flight mass spectrometry. *Rapid Communications in Mass Spectrometry* **13**: 2146-2150

Medina-Martínez, M. S., Uyttendaele, M., Demolder, V. and Debevere, J. (2006) Effect of temperature and glucose concentration on the *N*-butanoyl-L-homoseine lactone production by *Aeromonas hydrophila. Food Microbiology* **23**: 534-540

Middleton, B., Rodgers, H. C., Camara, M., Knox, A. J., Williams, P. and Hardman, A. (2002) Direct detection of *N*-acylhomoserine lactones in cystic fibrosis sputum. *Federation of European Microbiological Societies Microbiology Letters* **207**:1-7

Miller, J. H. (1992) A short course in bacterial genetics: a laboratory manual and handbook for *Escherichia coli* and related bacteria. Cold Spring Harbor Laboratory Press, New York

Miller, M.B. and Bassler, B. L. (2001) Quorum sensing in bacteria. *Annual Review of Microbiology* **55**: 165-199

Miller, W. G., Bates, A. H., Horn, S. T., Brandl, M. T., Wachtel, M. R. and Mandrell, R. E. (2000) Detection on surfaces and in Caco-2 cells of *Campylobacter jejuni* cells with gfp, yfp, and cfp marker plasmids. *Applied and Environmental Microbiology* **66**: 5426-5436

Miller, W. G. and Mandrell, R. E. (2005) Prevalence of Campylobacter in the food and water supply: incidence, outbreaks, isolation and detection. In: *Campylobacter*: Molecular and Cellular Biology. (Ed.) Ketley, J. M. and Konkel, M. E. Horizon Bioscience

Mobley, H. L. T., Green, D. M., Trifillis, A. L., Johnson, D. E., Chippendale, G. R., Lockatell, C. V., Jones, B. D. and Warren, J. W. (1990) Pyelonephriogenic *Escherichia coli* and killing of cultured human renal proximal tubular epithelial cells: role of hemolysin in some strains. *Infection and Immunity* **58**: 1281-1289

Mohammed, K. A. S., Miles, R. J. and Halablab, M. A. (2005) Simple method to grow enteric campylobacters in unsupplemented liquid medium without the need for microaerophilic kits. *Journal of Microbiological Methods* **61**: 273-276

Mol, O. and Oudega, B. (1996) Molecular and structural aspects of fimbriae biosynthesis and assembly in *Escherichia coli. Federation of European Microbiological Societies Microbiology Reviews* **19**: 25-52

Mølbak, K., Mead, P. S. and Griffin, P. M. (2002) Antimicrobial therapy in patients with *Escherichia coli* O157:H7 infection. *Journal of American Medical Association* **288**: 1014-1016

Moran, A. P. and Upton, M. E. (1986) A comparative study of the rod and coccoid forms of *Campylobacter jejuni* ATCC 29428. *Journal of Applied Bacteriology* **60**: 103-110

Moran, A. P. and Upton, M. E. (1987) Effect of medium supplements, illumination and superoxide dismutase on the production of coccoid forms of *Campylobacter jejuni* ATCC 29428. *Journal of Applied Bacteriology* **62**: 43-51

Moré, M. I., Finger, L. D., Stryker, J. L., Fuqua, C., Eberhard, A. and Winans, S. C. (1996) Enzymatic synthesis of a quorum-sensing autoinducer through use of a defined substrates. *Science* **272**: 1655-1658

Morris, J. G. (1976) Fifth Stenhouse-Williams Memorial Lecture: Oxygen and the obligate anaerobes. *Journal of Applied Bacteriology* **40**: 229-244

Mulder, C. J., Carroll, C. and Jordan, K. N. (1984) Neonatal meningitis caused by *Escherichia coli* in The Netherlands. *The Journal of Infectious Diseases* **150**: 935-940

Murphy, C., Carroll, C. and Jordan, K. N. (2003*a*) Induction of an adaptive tolerance response in the foodborne pathogen, *Campylobacter jejuni*. *Federation of European Microbiological Societies Microbiology Letters* **223**: 89-93

Murphy, C., Carroll, C. and Jordan, K. N. (2003*b*) Identification of a novel stress resistance mechanism in *Campylobacter jejuni*. *Journal of Applied Microbiology* **95**: 704-708

Mylotte, J. M., Tayara, A. and Goodnough, S. (2002) Epidemiology of bloodstream infection in nursing home residents: evaluation in a large cohort from multiple homes. *Clinical Infectious Diseases* **35**: 1484-1490

Nachamkin, I., Allos, B. M. and Ho, T. W. (1998) *Campylobacter* species and Guillain-Barré syndrome. *Clinical Microbiology Reviews* **11**: 555-567

Nachamkin, I., Allos, B. M. and Ho, T. W. (2000) *Campylobacter jejuni* infection and the association with Guillain-Barré syndrome. In: Campylobacter (2nd Edition) (Ed) Nachamkin, I. and Blaser, M. J. ASM Press, Washington, D.C.

Nakari, U-M., Laaksonen, K., Korkeila, M. and Siitonen, A. (2005) Comparative typing of *Campylobacter jejuni* by heat-stable serotyping and PCR-based restriction fragment length polymorphism analysis. *Journal of Clinical Microbiology* **43**: 1166-1170

Nataro, J. P., Deng, Y., Cookson, S., Cravioto, A., Savarino, J., Guers, L. D., Levine, M. M. and Tacket, C. O. (1995) Heterogeneity of enteroaggregative *Escherichia coli* virulence demonstrated in volunteers. *The Journal of Infectious Diseases* **171**: 465-468

Nataro, J. P. and Kaper, J. B. (1998) Diarrheagenic *Escherichia coli. Clinical Microbiology Reviews* **11**: 142-201

Nataro, J. P., Steiner, T. and Guerrant, R. L. (1998) Enteroaggregative *Escherichia coli. Emerging Infectious Diseases* **4**: 251-261

Nealson, K. H., Platt, T. and Hastings, J. W. (1970) Cellular control of the synthesis and activity of the bacterial luminescence system. *Journal of Bacteriology* **104**: 313-322

Neddermann, P., Gargioli, C., Muraglia, E., Sambucini, S., Bonelli, F., De Francesco, R. and Cortese, R. (2003) A novel, inducible, eukaryotic gene expression system based on the quorum-sensing transcription factor TraR. *The European Molecular Biology Organization Report* **4**: 159-165

Neidhardt, F. C., Bloch, P. L. and Smith, D. F. (1974) Culture medium for Enterobacteria. *Journal of Bacteriology* **119**: 736-747

Neill, M. A., Tarr, P. I., Taylor, D. N. and Wolf, M. *Escherichia coli* (2001) In: Foodborne Disease Handbook, Second Edition, Volume 1: Bacterial Pathogens. (Ed) Hui, Y. H., Pierson, M. D. and Gorham, J. R. Marcel Dekker, Inc.

Nickel, J. C., Ruseska, I, Wright J. B. and Costerton, J. W. (1985) Tobramycin resistance of *Pseudomonas aeruginosa* cells growing as a biofilm on urinary catheter material. *Antimicrobial Agents and Chemotherapy* **27**: 619-624

Novick, R. P. (2003) Autoinduction and signal transduction in the regulation of staphylococcal virulence. *Molecular Microbiology* **48**: 1429-1449

Nuijten, P. J., van Asten, F. J., Gaastra, W. and van der Zeijst, B. A. (1990) Structural and functional analysis of two *Campylobacter jejuni* flagellin genes. *Journal of Biological Chemistry* **265**: 17798-17804

Nylen, G., Dunstan, F., Palmer, S. R., Andersson, Y., Bager, F., Cowden, J., Feilerl, G., Galloway, Y., Kapperud, G., Megraud, F., Molbak, K., Petersen, L. R. and Ruutu, P. (2002) The seasonal distribution of campylobacter infection in nine European countries and New Zealand. *Epidemiology and Infection* **128**: 383-390

Onkeke, I. N. and Nataro, J. P. (2001) Enteroaggregative *Escherichia coli. Lancet Infectious Diseases* 1: 304-313

On, S. L. W. (2005) Taxonomy, Phylogeny, and Methods for the Identification of *Campylobacter* Species. In: *Campylobacter*: Molecular and Cellular Biology. (Ed.) Ketley, J. M. and Konkel, M. E. Horizon Bioscience

On, S. L. W. and Harrington, C. S. (2000) Identification of taxonomic and epidemiological relationships among *Campylobacter* species by numerical analysis of AFLP profiles. *Federation of European Microbiological Societies Microbiology Letters* **193**: 161-169

Park, S. F. (2005) *Campylobacter jejuni* stress responses during survival in the food chain and colonization. In: *Campylobacter*: Molecular and Cellular Biology. (Ed.) Ketley, J. and Konkel, M. Horizon Bioscience

Park, S. F. (2002) The physiology of *Campylobacter* species and its relevance to their role as foodborne pathogens. *International Journal of Food Microbiology* **74**: 177-188

Park, S., Wolanin, P. M., Yuzbashyan, E. A., Silberzan, P., Stock, J. B. and Austin, R. H. (2003) Motion to form a quorum. *Science* **301**: 188

Parkhill, J., Wren, B. W., Mungall, K., Kentley, J. M., Churcher, C., Basham, D., Chillingworth, T., Davies, R. M., Feltwell, T., Holroyd, S., Jagels, K., Karlyshev, A. V., Moule, S., Pallen, M. J., Penn, C. W., Quail, M. A., Rajandream, M-A., Rutherford, K. M., van Vliet, A. H. M., Whitehead, A. and Barrell, B. G. (2000) The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. *Nature* **403**: 665-668

Parsek, M. R. and Greenberg, E. P. (2000) Acyl-homoserine lactone quorum sensing in Gram-negative bacteria: A signaling mechanism involved in associations with higher organisms. *Proceedings of the National Academy of Sciences USA* **97**: 8789-8793

Parsek, M. R., Val, D. L., Hanzelka, B. L., Cronan, J. E. and Greenberg, E. P. (1999) Acyl homoserine-lactone quorum-sensing signal generation. *Proceedings of the National Academy of Sciences USA*. **96**: 4360-4365

Paton, J. C., Rogers, T. J., Morona, R. and Paton, A. W. (2001) Oral administration of formaldehyde-killed recombinant bacteria expressing a mimic of the Shiga toxin receptor protects mice from fatal challenge with Shiga-toxigenic *Escherichia coli*. *Infection and Immunity* **69**: 1389-1393

Pearson, A. D. and Healing, T. D. (1992) The surveillance and control of campylobacter infection. *Communicable Disease Review* **2**: R133-R138

Pearson, B. M., Pin, C., Wright, J., I'Anson, K., Humphrey, T. and Wells, J. M. (2003) Comparative genome analysis of *Campylobacter jejuni* using whole genome DNA microarrays. *FEBS Letters* **554**: 224-230

Pearson, J. P., Gray, K. M., Passador, L., Tucker, K. D., Eberhard, A., Iglewski, B. H. and Greenberg, E. P. (1994) The structure of the autoinducer molecule required for expression of *Pseudomonas aeruginosa* virulence genes. *Proceedings of the National Academy of Sciences USA* **91**: 197-201

Pearson, J. P., Passador, L., Iglewski, B. H. and Greenberg, E. P. (1995) A second acylhomoserine lactone signal produced by *Pseudomonas aeruginosa*. *Proceedings of the National Academy of Sciences USA* **92**: 1490-1494

Penner, J. L. and Hennessy, J. N. (1980) Passive hemagglutination technique for serotyping *Campylobacter fetus* subsp. *jejuni* on the basis of soluble heat-stable antigens. *Journal of Clinical Microbiology* **12**: 732-737

Peiffer, I., Blanc-Potard, A. B., Bernet-Camard, M. F., Guignot, J., Barbat, A. and Servin, A. L. (2000) Afa/Dr diffusely adhering *Escherichia coli* C1845 infection promotes selective injuries in the junctional domain of polarized human intestinal Caco-2/TC7 cells. *Infection and Immunity* **68**: 3431-3442

Peiffer, I., Bernet-Camard, M. F., Rousset, M. and Servin, A. L. (2001) Impairments in enzyme activity and biosynthesis of brush border- associated hydrolases in human intestinal Caco-2/TC7 cells infected by members of the Afa/Dr family of diffusely adhering *Escherichia coli*. *Cell Microbiology* **3**: 341-357

Perna, N. T., Plunkett, G., III, Burland, V., Mau, B., Glasner, J. D., Rose, D. J., Mayhew, G. F., Evans, P. S., Gregor, J., Kirkpatrick, H. A., Pósfai, G., Hackett, J., Klink, S., Boutin, A., Shao, Y., Miller, L., Grotbeck, E. J., Davis, N. W., Lim, A., Dimalanata, E. T., Potamousis, K. D., Apodaca, J., Anantharaman, T. S., Lin, J., Yen, G., Schwartz, D. C., Welch, R. A. and Blattner, F. R. (2001) Genome sequence of enterohaemorrhagenic *Escherichia coli* O157:H7. *Nature* **409**: 529-533

Pesci, E. C. and Iglewski, B. H. (1997) The chain of command in *Pseudomonas* quorum sensing. *Trends in Microbiology* **5**: 132-134

Picard, B., Sevali, J., Gouriou, S., Duriez, P., Brahimi, N., Bingen, E., Elion, J. and Denamur, E. (1999) The link between phylogeny and virulence in *Escherichia coli* extraintestinal infection. *Infection and Immunity* **67**: 546-553

Pickett, C. L. (2000) *Campylobacter* toxins and their role in pathogenesis. In: Campylobacter (2nd Edition) (Ed) Nachamkin, I. and Blaser, M. J. ASM Press, Washington, D.C.

Pickett, C. L. and Lee, R. B. (2005) Cytolethal distending toxin. In: *Campylobacter*: Molecular and Cellular Biology (Ed.) Ketley, J. M. and Konkel, M. E. Horizon Bioscience

Pickett, C. L., Pesci, E. C., Cottle, D. L., Russell, G., Erdem, A. N. and Zeytin, H. (1996) Prevalence of cytolethal distending toxin production in *Campylobacter jejuni* and relatedness of *Campylobacter* sp. *cdtB* genes. *Infection and Immunity* **64**: 2070-2078

Pierson, E. A., Wood., D. W., Cannon, J. A., Blachere, F. M. and Pierson, L. S. (1998) Interpopulation signalling via *N*-acyl homoserine lactones among bacteria in the wheat rhizosphere. *Molecular Plant-Microbe Interactions* **11**: 1078-1084

Piper, K. R., Beck von Bodman, S. and Farrand, S. K. (1993) Conjugation factor of *Agrobacterium tumefaciens* regulates Ti plasmid transfer by autoinduction. *Nature* **362**: 448-450

Piper, K. R., Beck von Bodman, S., Hwang, S. B. and Farrand, S. K. (1999) Hierarchical gene regulatory systems arising from fortuitous gene associations: controlling quorum sensing by the opine regulon in *Agrobacterium. Molecular Microbiology* **32**: 1077-1089

Poly, F., Threadgill, D. and Stintzi, A. (2005) Genomic Diversity in *Campylobacter jejuni*: Identification of *C. jejuni* 81-176-Specific Genes. *Journal of Clinical Microbiology* **43**: 2330-2338

Rasch, M., Andersen, J. B., Nielsen, K. F., Flodgaard, L. R., Christensen, H., Givskov, M. and Gram, L. (2005) Involvement of bacterial quorum-sensing signals in spoilage of bean sprouts. *Applied and Environmental Microbiology* **71**: 3321-3330

Rasmussen, T. B. and Givskov, M. (2006*a*) Quorum sensing inhibitors: a bargain of effects. *Microbiology* **152**: 895-904

Rasmussen, T. B. and Givskov, M. (2006*b*) Quorum-sensing inhibitors as anti-pathogenic drugs. *International Journal of Medical Microbiology* **296**: 149-161

Rather, P. N., Ding, X., Baca-DeLancey, R. R. and Siddiqui, S. (1999) *Providencia stuartii* genes activated by cell-to-cell signaling and identification of a gene required for production or activity of an extracellular factor. *Journal of Bacteriology* **181**: 7185-7191

Ravn, L., Christensen, A. B., Molin, S., Givskov, M. and Gram, L. (2001) Methods for detecting acylated homoserine lactones produced by Gram-negative bacteria and their application in studies of AHL-production kinetics. *Journal of Microbiological Methods* **44**: 239-251

Reimmann, C. Ginet, N., Michel, L., Keel, C., Michaux, P., Krishnapillai, V., Zala, M., Heurlier, K., Trindafillu, K., Harms, H., Defago, G. and Haas, D. (2002) Genetically programmed autoinducer destruction reduces virulence gene expression and swarming motility in *Pseudomonas aeruginosa* PAO1. *Microbiology* **148**: 923-932

Riley, L. W., Remis, R. S., Helgerson, S. D., McGee, H. B., Wells, J. G., Davis, B. R., Hebert, R. J., Olcott, E. S., Johnson, L. M., Hargett, N. T., Blake, P. A. and Cohen, M. L. (1983) Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. *New England Journal of Medicine* **308**: 681-685

Ron, E. Z. (2006) Host specificity of septicemic *Escherichia coli*: human and avian pathogens. *Current Opinion in Microbiology* **9**: 28-32

Russo, T. A. and Johnson, J. R. (2000) Proposal for a new inclusive designation for extraintestinal pathogenic isolates of Escherichia coli: ExPEC. *The Journal of Infectious Diseases* **181**: 1753-1754

Russo, T. A. and Johnson, J. R. (2003) Medical and economic impact of extraintestinal infections due to *Escherichia coli*: focus on an increasingly important endemic problem. *Microbes and Infection* **5**: 449-456

Sagara, H., Mochizuki, A., Okamura, N. and Nakaya, R. (1987) Antimicrobial resistance of *Campylobacter jejuni* and *Campylobacter coli* with special reference to plasmid profiles of Japanese clinical isolates. *Antimicrobial Agents and Chemotherapy* **31**: 713-719

Savelkoul, P. H., Aarts, H. J., de Hass, J., Dijkshoorn, L., Duim, B., Otsen, M., Rademaker, J. L., Schouls, L. and Lenstra, J. A. (1999) Amplified-fragment length polymorphism analysis: the state of an art. *Journal of Clinical Microbiology* **37**: 3083-3091

Schaechter, M., Beckwith, J., Curtiss, R., Hatfield, G. W., Ingraham, J., Low, K. B., Magasanik, B., Neidhardt, F., Reznikoff, W., Riley, M. and Schaechter, M. (2004) Module 1.4 *Escherichia coli* and *Salmonella* 2000: the view from here. In: EcoSal (Web Edition) www.ecosal.org/ecosal/index.jsp

Schaefer, A. L., Taylor, T. A., Beatty, J. T. and Greenberg, E. P. (2002) Long-chain acyl-homoserine lactone quorum-sensing regulation of *Rhodobacter capsulatus* gene transfer agent production. *Journal of Bacteriology* **184**: 6515-6521

Schaefer, A. L., Val, D. L., Hanzelka, B. L., Eberhard, A. and Greenberg, E. P. (1996*a*) Quorum sensing in *Vibrio fischeri*: probing autoinducer-LuxR interactions with autoinducer analogs. *Journal of Bacteriology* **178**: 2897-2901

Schaefer, A. L., Val, D. L., Hanzelka, B. L., Cronan, J. E., Jr. and Greenberg, E. P. (1996*a*) Generation of cell-to-cell signals in quorum sensing: acyl homoserine lactone synthase activity of a purified *Vibrio fischeri* LuxI protein. *Proceedings of the Nation Academy of Sciences USA* **93**: 9505-9509

Schauder, S. and Bassler, B. L. (2001) The languages of bacteria. *Genes and Development* **15**: 1468-1480

Schauder, S., Shokat, K., Surette, M. G. and Bassler, B. L. (2001) The LuxS family of bacterial autoinducers: biosynthesis of a novel quorum-sensing signal molecule. *Molecular Microbiology* **41**: 463-476

Schuster, M., Lostroh, C. P., Ogi, T. and Greenberg, E. P. (2003) Identification, timing, and signal specificity of *Pseudomonas aeruginosa* quorum-controlled genes: a transcriptome analysis. *Journal of Bacteriology* **185**: 2066-2079

Schweizer, H. P. (2001) Triclosan: a widely used biocide and its links to antibiotics. *Federation of European Microbiological Societies Microbiology Letters* **202**: 1-7

Schweitzer, R. and Shilo, B. Z. (1997) A thousand and one roles for the *Drosophila* EGF receptor. *Trends in Genetics* **13**: 191-196

Sebald, P. M. M. and Véron, M. (1963) Teneur en bases de l'and et classification des vibrions. *Annales de l'Institut Pasteur* **105**: 898-910

Sellars, M. J., Hall, S. J. and Kelly, D. J. (2002) Growth of *Campylobacter jejuni* supported by respiration of fumarate, nitrate, nitrite, trimethylamine-*N*-oxide, or dimethyl sulfoxide requires oxygen. *Journal of Bacteriology* **184**: 4187-4196

Servin, A. L. (2005) Pathogenicity of Afa/Dr diffusely adhering *Escherichia coli*. *Clinical Microbiology Reviews* **18**: 264-292

Shaw, P. D., Ping, G., Daly, S. L., Cha, C., Cronan, J. E., Rinehart, K. L. and Farrand, S. K. (1997) Detecting and characterizing *N*-acyl-homoserine lactone signal molecules by thin layer chromatography. *Proceedings of the National Academy of Sciences USA* **94**: 6036-6041

Shiner, E. K., Reddy, S., Timmons, C., Li, G., Williams, S. C. and Rumbaugh, K. P. (2004) Construction of a bacterial autoinducer detection system in mammalian cells. *Biological Procedures Online* **6**: 268-276

Showalter, R. E., Martin, M. O. and Silverman, M. R. (1990) Cloning and nucleotide sequence of *luxR*, a regulatory gene controlling bioluminescence in *Vibrio harveyi*. *Journal of Bacteriology* **172**: 2946-2954

Siegler, R. L. (1995) The hemolytic uremic syndrome. *Pediatric Clinics of North America* **42**: 1505-1529

Siegman-Igra, Y., Fourer, B., Orni-Wasserlauf, R., Golan, Y., Noy, A., Schwartz, D. and Giladi, M. (2002) Reappraisal of community-acquired bacteremia: a proposal of a new classification for the spectrum of acquisition of bacteremia. *Clinical Infections Diseases* **34**: 1431-1439

Singh, P. K., Schaefer, A. L., Parsek, M. R., Moninger, T. O., Welsh, M. J. and Greenberg, E. P. (2000) Quorum-sensing signals indicate that Cystic Fibrosis lungs are infected with bacterial biofilms. *Nature* **407**: 762-764

Singleton, P. and Sainsbury, D. (1999) Dictionary of Microbiology and Molecular Biology 2nd Edition. John Wiley and Sons

Sitnikov, D. M., Schineller, J. B. and Baldwin, T. O. (1996) Control of cell division in *E. coli*: regulation of transcription of *ftsQA* involves both *rpoS* and SdiA-mediated autoinduction. *Proceedings of the National Academy of Sciences USA* **93**: 336-341

Skirrow, M. B. (1977) Campylobacter enteritis: a "new" disease. *British Medical Journal* **2**: 9-11

Skirrow, M. B. and Blaser, M. J. (2000) Clinical aspects of *Campylobacter* infection. In: Campylobacter (2nd Edition) (Ed) Nachamkin, I. and Blaser, M. J. ASM Press, Washington, D.C.

Skwierczynski, R. D. and Connors, K. A. (1993) Demethylation kinetics of aspartame and L-phenylalanine methyl-ester in aqueous-solution. *Pharmaceutical Research* **10**: 1174-1180

Smith, J. L. (2002) *Campylobacter jejuni* infection during pregnancy: long-term consequences of associated bacteremia, Guillain-Barré syndrome, and reactive arthritis. *Journal of Food Protection* **65**: 696-708

Smith, J. L. and Fratamico, P. M. (2005) Diarrhea-inducing *Escherichia coli*. In: Foodborne Pathogens Microbiology and Molecular Biology (Eds) Fratamico, P. M., Bhunia, A. K. and Smith, J. L.Caister Academic Press, Norfolk

Smith, R. S. and Iglewski, B. H. (2003) *P. aeruginosa* quorum-sensing systems and virulence. *Current Opinions in Microbiology* **6**: 56-60

Sperandio, V., Mellies, J. L., Nguyen, W., Shin, S. and Kaper, J. B. (1999) Quorum sensing controls expression of the type III secretion gene transcription and protein secretion in enterohemorrhagic and enteropathogenic *Escherichia coli*. *Proceedings of the National Academy of Sciences USA* **96**: 15196-15201

Sperandio, V., Torres, A. G., Giron, J. A. and Kaper, J. B. (2001) Quorum sensing is a global regulatory mechanism in enterohemorrhagic *Escherichia coli* O157:H7 *Journal of Bacteriology* **183**: 5187-5197

Sperandio, V., Torres, A. G. and Kaper, J. B. (2002) Quorum sensing *Escherichia coli* regulators B and C (QseBC): a novel two-component regulatory system involved in the regulation of flagella and motility by quorum sensing in *E. coli*. *Molecular Microbiology* **43**: 809-821

Sperandio, V., Torres, A. G., Jarvis, B., Nataro, J. P. and Kaper, J. B. (2003) Bacterial communication: The language of hormones. *Proceedings of the National Academy of Sciences USA* **100**: 8951-8956

Stamm, W. E. (1982) Recent developments in the diagnosis and treatment of urinary tract infections. *Westen Journal of Medicine* **137**: 213-220

Surrette, M. G. and Bassler, B. L. (1998) Quorum sensing in *Escherichia coli* and *Salmonella typhimurium*. *Proceedings of the National Academy of Sciences USA* **95**: 7046-7050

Surrette, M. G., Miller, M. B. and Bassler, B. L. (1999) Quorum sensing in *Escherichia coli*, *Salmonella typhimurium*, and *Vibrio harveyi*: a new family of genes responsible for autoinducer production. *Proceedings of the National Academy of Sciences USA* **96**: 1639-1644

Swartzman, E., Silverman, M. and Meighen, E. A. (1992) The *luxR* gene product of *Vibrio harveyi* is a transcriptional activator of the *lux* promoter. *Journal of Bacteriology* **174**: 7490-7493

Taboada, E. N., Acedillo, R. R., Carrillo, C. D., Findlay, W. A., Medeiros, D. T., Mykytczuk, O. L., Roberts, M. J., Valencia, C. A., Farber, J. M. and Nash, J. H. E. (2004) Large-scale comparative genomics meta-analysis of *Campylobacter jejuni* isolates reveals low level of genome plasticity. *Journal of Clinical Microbiology* **42**: 4566-4576

Tabor, S. and Richardson, C. C. (1985) A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. *Proceedings of the National Academy of Sciences USA* **82**: 1074-1078

Taga, M. E. and Bassler, B. L. (2003) Chemical communication among bacteria *Proceedings of the National Academy of Sciences USA* **100**: 14549-14554

Taga, M. E., Semmelhack, J. L. and Bassler, B. L. (2001) The LuxS-dependent autoinducer AI-2 controls the expression of the ABC transporter that functions in AI-2 uptake in *Salmonella typhimurium*. *Molecular Microbiology* **42**: 777-793

Tam, C. C., O'Brien, S. J., Adak, G. K., Meakins, S. M. and Frost, J. A. (2003) *Campylobacter coli*-an important foodborne pathogen. *Journal of Infection* **47**: 28-32

Tang., H. B., DiMango, E., Bryan, R., Gambello, M., Iglewski, B. H., Goldberg, J. B. and Prince, A. (1996) Contribution of specific *Pseudomonas aeruginosa* virulence factors to pathogenesis of Pneumonia in a neonatal mouse model of infection. *Infection and Immunity* **64**: 37-43

Tarr, P. I. and Neill, M. A. (2001) *Escherichia coli* O157:H7. *Gastroenterology Clinics of North America* **30**: 735-751

Taylor, D. E. (1992) Genetics of *Campylobacter* and *Helicobacter*. *Annual Review of Microbiology* **46**: 35-64

Taylor, D. E., De Grandis, S. A., Karmali, M. A. and Fleming, P. C. (1981) Transmissible plasmids from *Campylobacter jejuni* **19**: 831-835

Throup, J., Winson, M. K., Bainton, N. J., Bycroft, B. W., Williams, P. and Stewart, G. S. A. B. (1995) Signalling in bacteria beyond bioluminescence. In: Bioluminescence and Chemiluminescence: Fundamentals and Applied Aspects. (Ed) Campbell, L. K. and Stanley, P. Chichester: Wiley

Tilden, J., Young, W., McNamara, A-M., Custer, C., Boesel, B., Lambert-Fair, M. A., Majkowski, J., Vugia, D., Werner, S. B., Hollingsworth, J. and Morris, J. G. (1996) A New Route of Transmission for *Escherichia coli*: Infection from Dry Fermented Salami. *American Journal of Public Health* **86**: 1142-1145

Tran, T. T. (1998) A blood-free enrichment medium for growing *Campylobacter* spp. under aerobic conditions. *Letters in Applied Microbiology* **26**: 145-148

Ulitzur, S. and Dunlap, P. V. (1995) Regulatory circuitry controlling luminescence autoinduction in *Vibrio fischeri*. *Photochemistry and Photobiology* **62**: 625-632

Unhanand, M. M., McCracken, G. H. Jr. and Nelsom. J. D. (1993) Gram-negative enteric bacillary meningitis: a twenty-one-year experience. *Journal of Pediatrics* **122**: 15-21

Vallance, B. A., Chan, C., Robertson, M. L. and Finlay, B. B. (2002) Enteropathogenic and enterohemorrhagic *Escherichia coli* infections: Emerging themes in pathogenesis and prevention. *Canadian Journal of Gastroenterology* **16**: 771-778

Vallance, B. A. and Finlay, B. B. (2000) Exploitation of host cells by enteropathogenic *Escherichia coli*. *Proceedings of the National Academy of Sciences USA* **97**: 8799-8806

Vannini, A., Volpari,C., Gargioli,C., Muraglia,E., Cortese,R., Francesco,R.De., Neddermann, P. and Marco, S. Di. (2002) The crystal structure of the quorum sensing protein TraR bound to its autoinducer and target DNA. *The European Molecular Biology Organization Journal* **21**: 4393-4401

van Vliet, A. H. M. and Ketley, J. M. (2001) Pathogenesis of enteric *Campylobacter* infection. *Journal of Applied Microbiology* **90**: (Suppl. 6) 45S-56S

Vendeville, A., Winzer, K., Heurlier, K., Tang, C. M. and Hardie, K. R. (2005) Making 'sense' of metabolism: autoinducer-2, LuxS and pathogenic bacteria. *Nature Reviews Microbiology* **3**: 383-396

Véron, M. and Chatelain, R. (1973) Taxonomic study of the genus *Campylobacter* Sebald and Véron and designation of the neotype strain for the type species, *Campylobacter fetus* (Smith and Taylor) Sebald and Véron. *International Journal of Systematic Bacteriology* **23**: 122-134

Visick, K. L. and Fuqua, C. (2005) Decoding microbial chatter: Cell-cell communication in bacteria. *Journal of Applied Bacteriology* **187**: 5507-5519

Visser, L. H., Schmitz, P. I. M., Meulstee, J., van Doorn, P. A., van der Meché, F. G. A. and the Dutch Guillain-Barré Study Group (1999) Prognostic factors of Guillain-Barré syndrome after intravenous immunoglobulin or plasma exchange. *Neurology* **53**: 598-604

van Delden, C. and Iglewski, B. H. (1998) Cell-to-cell signalling and *Pseudomonas* infection. *Emerging Infectious Diseases* **4**: 551-560

von Baum, H. and Marre, R. (2005) Antimicrobial resistance of *Escherichia coli* and therapeutic implications. *International Journal of Medical Microbiology* **295**: 503-511

Wagner, V. E., Bushnell, D., Passador, L., Brooks, A. I. and Iglewski, B. H. (2003) Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: effects of growth phase and environment. *Journal of Bacteriology* **185**: 2080-2095

Walters, M. and Sperandio, V. (2006) Quorum sensing in *Escherichia coli* and *Salmonella*. *International Journal of Medical Microbiology* **296**: 125-131

Wang, A., Kostandy, G., Weiner, M., Sosler, B. and Pachter, B. R. (1998) Bacteremia caused by *Campylobacter jejuni*. *Infections in Medicine* **15**: 770-772

Wareing, D. R. A., Ure, R., Colles, F. M., Bolton, F. J., Fox, A. J., Maiden, M. C. J. and Dingle, K. E. (2003) Reference isolates for the clonal complexes of *Campylobacter jejuni*. *Letters in Applied Microbiology* **36**: 106-110

Wassenaar, T. M. (1997) Toxin production by *Campylobacter* spp. *Clinical Microbiology Reviews* **10**: 466-476

Wassenaar, T. M. (2002) Genetic differentiation of *Campylobacter jejuni*. *International Journal of Infectious Diseases* **6**: 3S22-3S25

Wassenaar, T. M., Bleumink-Pluym, N. M. and van der Zeijst, B. A. M. (1991) Inactivation of *Campylobacter jejuni* flagellin genes by recombination demonstrates that *flaA* but not *flaB* is required for invasion. *The European Molecular Biology Organization Journal* **10**: 2055-2061

Wassenaar, T. M., On, S. L. W. and Meinersmann, R. J. (2000) Genotyping and the consequences of genetic instability. In: Campylobacter (2nd Edition) (Ed) Nachamkin, I. and Blaser, M. J. ASM Press, Washington, D.C.

Waters, C. M. and Bassler, B. L. (2005) Quorum sensing: cell-to-cell communication in bacteria *Annual Review of Cell Developmental Biology* **21**: 319-346

Watson, B., Currier, T. C., Gordon, M. P., Chilton, M.-D. and Nester, E. W. (1975) Plasmid required for virulence of *Agrobacterium tumefaciens. Journal of Bacteriology* **123**: 255-264

Watson, W. T., Minogue, T. D., Val, D. L., Bodman, B. V. and Churchill, M. E. A. (2002) Structural Basis and Specificity of Acyl-Homoserine Lactone Signal Production in Bacterial Quorum Sensing *Molecular Cell* **9**: 685-694

Welch, R. A., Burland, V., Plunkett, G., III, Redford, P., Roesch, P., Rasko, D., Buckles, E. L., Liour, S.-R., Boutin, A., Hackett, J., Stroud, D., Mayhew, G. F., Rose, D. J., Zhou, S., Schwartz, D. C., Perna, N. T., Mobley, H. L. T., Donnenberg, M. S. and Blattner, F. R. (2002) Extensive mosaic structure revealed by the complete genome sequence of uropathogenic *Escherichia coli. Proceedings of the National Academy of Sciences USA* **99**: 17020-17024

Welsh, M. J., Ramsey, B. W., Accurso, F. and Cutting, G. R. (2001) Cystic Fibrosis. In: Metabolic and Molecular Basis of Inherited Disease, Eds. Scriver, C. R., Beaudet, A. L., Sly, W. S., Valle, D., Childs, B. and Vogelstein, B. (McGraw-Hill, New York), 8th Ed., Vol. 3, Part 21

Whitehead, N. A., Barnard, A. M. L., Slater, H., Simpson, N. J. L. and Salmond, G. P. C. (2001) Quorum-sensing in Gram-negative bacteria *Federation of European Microbiological Societies Microbiology Reviews* **25**: 365-404

Whiteley, M., Lee, K. M. and Greenberg, E. P. (1999) Identification of genes controlled by quorum sensing in *Pseudomonas aeruginosa. Proceedings of the National Academy of Sciences USA* **96**: 13904-13909

Wilkerson, C. and van Kirk, N. (2004) Antibiotic resistance and distribution of tetracycline resistance genes in *Escherichia coli* O157:H7 isolates from humans and bovines. *Antimicrobial Agents and Chemotherapy* **48**: 1066-1067

Williams, P. (2006) Quorum sensing. *International Journal of Medical Microbiology* **296**: 57-59

Williams, P. (2002) Quorum sensing: an emerging target for antimicrobial chemotherapy? *Expert Opinion on Therapeutic Targets* **6**: 257-274

Williams, P., Bainton, N. J., Swift, S., Chhabra, S. R., Winson, M. K., Stewart, G. S. A. B., Salmond, G. P. C. and Bycroft, B. W. (1992) Small molecule-mediated density-dependent control of gene expression in prokaryotes: bioluminescence and the biosynthesis of carbapenem antibiotics. *Federation of European Microbiological Societies Microbiology Letters* **100**: 161-167

Williams, S. C., Patterson, E. K., Carty, N. L., Griswold, J. A., Hamwood, A. N. and Rumbaugh, K. P. (2004) *Pseudomonas aeruginosa* autoinducer enters and functions in mammalian cells. *Journal of Bacteriology* **186**: 2281-2287

Willshaw, G. A., Cheasty, T. and Smith, H. R. (2000) *Escherichia coli.* In: The Microbiological Safety and Quality of Food, Vol II (Eds) Lund, B. M., Baird-Parker, T. C. and Gould, G. W. Aspen Publishers Inc., Maryland

Winson, M. K., Camara, M., Latifi, A., Foglino, M., Chhabra, S. R., Daykin, M., Bally, M., Chapon, V., Salmond, G. P. C., Bycroft, B. W., Lazdunski, A., Stewart, G. S. A. B. and Williams, P. (1995) Multiple *N*-acyl-L-homoserine lactone signal molecules regulate production of virulence determinants and secondary metabolites in *Pseudomonas aeruginosa. Proceedings of the National Academy of Sciences USA* **92**: 9427-9431

Winzer, K., Hardie, K. R., Burgess, N., Doherty, N., Kirke, D., Holden, M. T. G., Linforth, R., Cornell, K. A., Taylor, A. J., Hill, P. J. and Williams, P. (2002*b*) LuxS: its role in central metabolism and the *in vitro* synthesis of 4-hydroxy-5-methyl-3(*2H*)-furanone *Microbiology* **148**: 909-922

Winzer, K., Hardie, K. R. and Williams, P. (2002*a*) Bacterial cell-to-cell communication: sorry, can't talk now-gone to lunch! *Current Opinion in Microbiology* **5**: 216-222

Winzer, K., Hardie, K. R. and Williams, P. (2003) Sensing and metabolism in bacteria. *Advances in Applied Microbiology* **53**: 291-396

Withers, H., Swift, S. and Williams, P. (2001) Quorum sensing as an integral component of gene regulatory networks in Gram-negative bacteria. *Current Opinions in Microbiology* **4**: 186-193

Wolf-Rainer, A. (2005) Controlling pathogenic Gram-negative bacteria by interfering with their biofilm formation. *Drug Design Reviews-Online* **2**: 13-33

Wolfs, T. F. W., Duim, B., Geelen, S. P. M., Rigter, A., Thomson-Carter, F., Fleer, A. and Wagenaar, J. A. (2001) Neonatal sepsis by *Campylobacter jejuni*: genetically proven transmission from a household puppy. *Clinical infectious diseases* **32**: e97-e99

Wong, C. S., Jelacic, S., Habeeb, R. L., Watkins, S. L. and Tarr, P. I. (2000) The risk of haemolytic-uremic syndrome after antibiotic treatment of *Escherichia coli* O157:H7 infections. *New England Journal of Medicine* **342**: 1930-1936

Wren, B. W., Linton, D., Dorrell, N. and Karlyshev, A. V. (2001) Post genome analysis of *Campylobacter jejuni*. *Journal of Applied Microbiology* **90**: 36S-44S

Xie, Y., Kim, K. J. and Kim, K. S. (2004) Current concepts on *Escherichia coli* K1 translocation of the blood-brain barrier. *Federation of European Microbiological Societies Immunology and Medical Microbiology* **42**: 271-279

Xu, J., Bjursell, M. K., Himrod, J., Deng, S., Carmichael, L. K., Chiang, H. C., Hooper, L. V., and Gordon, J. I. (2003) A genomic view of the human-*Bacteroides thetaiotaomicron* symbiosis. *Science* **299**: 2074-2076

Yamagami, S., Motoki, M., Kimura, T., Isumi, H., Takeda, T., Katsuura, Y. and Matsumoto, Y. (2001) Efficacy of postinfection treatment with anti-Shiga toxin (Stx) 2 humanized monoclonal antibody TMA-15 in mice lethally challenged with Stx-producing *Escherichia coli*. *The Journal of Infectious Diseases* **184**: 738-743

Yan, S. S., Pendrak, M. L., Foley, S. L. and Powers, J. H. (2005) Campylobacter infection and Guillain-Barré syndrome: public health concerns from a microbial food safety perspective. *Clinical and Applied Immunology Reviews* **5**: 285-305

Yang, F., Wang, L-H., Wang, J., Dong, Y-H,. Hu, J. Y. and Zhang, L-H. (2005) Quorum quenching enzyme activity is widely conserved in the sera of mammalian species. *Federation of European Biochemical Societies Letters* **579**: 3713-3717

Yates, E. A., Philipp, B., Buckley, C., Atkinson, S., Chhabra, S. R., Sockett, R. E., Goldner, M. Dessaux, Y., Cámara, M., Smith, H. and Williams, P. (2002) *N*-acylhomoserine lactones undergo lactonolysis in a pH-, temperature-, and acyl chain length-dependent manner during growth of *Yersinia pseudotuberculosis* and *Pseudomonas aeruginosa. Infection and Immunity* **70**: 5635-5646

Yavagal, D. R. and Mayer, S. A. (2002) Respiratory complications of rapidly progressive neuromuscular syndromes: Guillain-Barré syndrome and Myasthenia Gravis. *Seminars in Respiratory and Critical Care Medicine* **23**: 221-229

Young, V. B. and Mansfield, L. S. (2005) *Campylobacter* infection- clinical context. In: *Campylobacter*: Molecular and Cellular Biology (Ed.) Ketley, J. M. and Konkel, M. E. Horizon Bioscience

Zhang, H-B., Wang, L-H. and Zhang, L-H. (2002*b*) Genetic control of quorum-sensing signal turnover in *Agrobacterium tumefaciens. Proceedings of the National Academy of Sciences USA* **99**: 4638-4643

Zhang, L-H. and Dong, Y-H. (2004) Quorum sensing and signal interference: diverse implications. *Molecular Microbiology* **53**: 1563-1571

Zhang, L-H. and Kerr, A. (1991) A diffusible compound can enhance conjugal transfer of the Ti plasmid in *Agrobacterium tumefaciens Journal of Bacteriology* **173**: 1867-1872

Zhang, L-H., Murphy, P. J., Kerr, A. and Tate, M. E. (1993) *Agrobacterium* conjugation and gene regulation by *N*-acyl-L-homoserine lactones. *Nature* **362**: 446-448

Zhang, R-G., Pappas, T., Brace, J. L., Miller, P. C., Oulmassov, T., Molyneaux, J. M., Anderson, J. C., Bashkin, J. K., Winans, S. C. and Joachimiak, A. (2002*a*) Structure of a bacterial quorum-sensing transcription factor complexed with pheromone and DNA. *Nature* **417**: 971-974

Zhu, J., Beaber, J., More, M. I., Fuqua, C., Eberhard and Winans, S. C. (1998) Analogs of the autoinducer 3-oxooctanoyl-homoserine lactone strongly inhibit activity of the TraR protein of *Agrobacterium tumefaciens*. *Journal of Bacteriology* **180**: 5398-5405

Zhu, J., Chai, Y., Zhong, Z., Li, S. and Winans, S. C. (2003) Agrobacterium bioassay strain for ultrasensitive detection of *N*-acylhomoserine lactone-type quorum-sensing molecules: Detection of autoinducers in *Mesorhizobium huakuii*. *Applied and Environmental Microbiology* **69**: 6949-6953

Zhu, J. and Winans, S. C. (2001) Autoinducer binding by the quorum-sensing regulator TraR increases affinity for target promoters *in vitro* and decreases TraR turnover rates in whole cells. *Proceedings of the National Academy of Sciences USA* **96**: 4832-4837

Publications and Presentations

Carmichael, S. J., Greenway, D. and England, R. R. (2006) Putative acyl-homoserine lactone production by *Campylobacter jejuni* reference isolates. *Faculty of Science 3rd Annual Research Day*; University of Central Lancashire; Abstract for poster presentation.

Carmichael, S. J., Greenway, D. and England, R. (2005) Putative acyl-homoserine lactone production by *Campylobacter jejuni* reference isolates. *Institute of Biomedical Science Congress*, Birmingham International Convention Centre; Abstract for Poster presentation.

Carmichael, S. J., Greenway, D. and England, R. R. (2005) Putative acyl-homoserine lactone production by *Campylobacter jejuni* reference isolates. *Society for General Microbiology* 157th Meeting, Keele University; Abstract for Poster presentation.

Carmichael, S. J., Greenway, D. and England, R. (2005) Putative acyl-homoserine lactone production by *campylobacter jejuni* reference isolates. *Faculty of Science 2nd Annual Research Day*; University of Central Lancashire; Abstract for oral presentation.

Carmichael, S. J., England, R. R. & Greenway, D. (2005) Putative signalling Molecule production by *Campylobacter jejuni*. *Postgraduate Research in Science Medicine Conference*, University of Central Lancashire; Abstract for oral presentation.

Putative acyl-homoserine lactone production by *Campylobacter jejuni* reference isolates

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Abstract

Campylobacter jejuni is a ubiquitous commensal organism of livestock, domestic/wild animals, birds and poultry. It is the predominant cause of gastrointestinal infection in the UK and is the principle infectious agent associated with the development of the acute demyelinating polyneuropathy, Guillain-Barré Syndrome.

Quorum sensing (QS) mechanisms enable bacteria to regulate density-dependent gene expression by the production, secretion and binding of small chemicals such as acyl-homoserine lactone (AHL), known as autoinducers. AHL mediated QS was originally promoted as a widespread phenomenon in Gram-negative bacteria. However, no known AHL producing bacteria are present in the Epsilon *Proteobacteria* class which contains *C. jejuni*.

Synthesis of a second group of autoinducer is dependent upon the LuxS enzyme in many bacteria. The genome sequence of *C. jejuni* NCTC 11168 contains a gene encoding an orthologue of LuxS but does not contain genes predicted to encode any known AHL synthetase. This is surprising as the majority of AHL QS systems characterised so far occur in Gram-negative bacteria that establish relationships, either pathogenic or symbiotic, with plant or animal hosts.

Putative AHL production by several *C. jejuni* isolates, including NCTC 11168, was assessed through the ability of concentrated conditioned medium to induce β-galactosidase activity in *Agrobacterium tumefaciens* KYC55 (pJZ372)(pJZ384)(pJZ410). These results suggest that AHL mediated QS is present in the Epsilon *Proteobacteria* class.

Initial HPLC separation of *C. jejuni* NCTC 13255 crude extract has provided one main active fraction, however the structure and genes regulated by this putative signalling molecule have yet to be elucidated.

1. Introduction

QS is a cell-to-cell signalling mechanism that regulates bacterial gene expression in a density dependent manner via the production, secretion and binding of autoinducers such as AHL. AHL-mediated QS is known to play a key role in synchronising virulence gene expression in several pathogens.

It has previously been shown that the genome of *C. jejuni* NCTC 11168 contains a gene encoding an orthologue of LuxS and that it is positive for autoinducer-2 production. In this study we tested several *C. jejuni* isolates, including NCTC 11168, for putative AHL production using an *A. tumefaciens* reporter strain and a colorimetric bioassay.

Two different variants of *C. jejuni* NCTC 11168 were screened; the original strain (NCTC 11168-O) and the genome sequenced variant (NCTC 11168-GS) that have previously been shown to express different virulence properties [1].

Sera of several mammalian species, including horse, have been shown to inactivate AHL. This is probably due to the lactonase activity of paraoxonase(s) which hydrolyse the lactone ring, [2].

2. Method

Culture Conditions

C. jejuni strains were grown in 100 ml Bolton Broth at 37°C for 24, 48 or 96 h. Supernatants were extracted with equal quantities of ethyl acetate, concentrated under nitrogen at 40°C and re-hydrated with 20 ml distilled water and filter sterilised.

Serum Assay

The serum assay was adapted from [2] using horse serum.

HPLC

Concentrated crude extract was applied onto a S5ODS2 C18 reverse-phase semi-preparative column (Hichrom Ltd., Berkshire). Fractions were freeze dried overnight at −80°C and re-hydrated with 0.7 ml sterile distilled water.

β-Galactosidase Activity

Putative AHL production was determined by induction of the *lacZ* fusion in the reporter strain *A. tumefaciens* strain KYC55 (pJZ372)(pJZ384)(pJZ410) [3]. β-galactosidase activity was determined as described in [4] and reported in Miller units.

3. Results

All of the *C. jejuni* isolates tested in this study induced β-galactosidase in the reporter strain, suggesting the presence of a putative AHL within the crude extracts and therefore the conditioned medium (Figure 1).

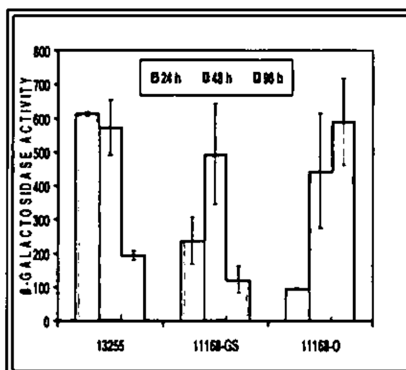

Figure 1: β-galactosidase activity (in Miller units) induced by crude extract from 3 isolates of *C. jejuni* after 24, 48 and 96 h at 37°C in Bolton broth. Data are the mean ± standard deviation, n = 3.

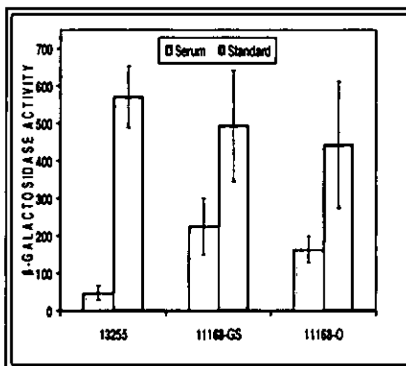

Figure 2: β-galactosidase activity (in Miller units) induced by serum-treated crude extract from 3 isolates of *C. jejuni* after 48 h at 37°C in Bolton broth. Data are the mean ± standard deviation, n = 3.

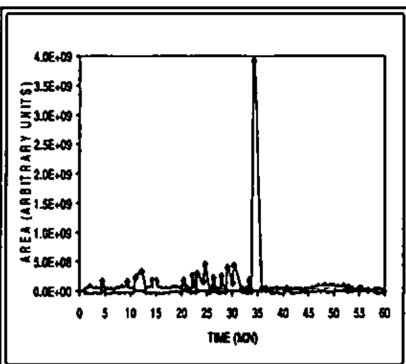

Figure 3: HPLC trace of concentrated crude extract from *C. jejuni* NCTC 13255 (48 h at 37°C in Bolton broth), showing a substantial peak eluted between 33.75 and 35.75 min that corresponds to reporter strain stimulatory activity.

4. Discussion

The ability of the conditioned medium crude extracts to induce the *lacZ* fusion in the reporter strain (Figure 1) suggests putative AHL production in the *C. jejuni* strains tested.

Each *C. jejuni* strain tested exhibits a unique induction profile;

For *C. jejuni* NCTC 13255 levels of induction appear to remain constant for both the 24 and 48 h crude extracts, whereas the 96 h crude extract results suggest a large decrease in induction levels.

Induction levels for *C. jejuni* NCTC 11168-GS appear to increase between 24 and 48 h but subsequently decrease to a similar level as the 24 h crude extract by 96 h.

Like NCTC 11168-GS, *C. jejuni* NCTC 11168-O induction levels appear to increase between 24 and 48 h. However, there is no subsequent decrease in induction at 96 h.

As well as *C. jejuni* NCTC 11168-O and 11168-GS exhibiting different virulence profiles [1], Figure 1 suggests that they also display different *lacZ* fusion induction profiles.

The 48 h crude extracts incubated with horse serum for 12 h appear to show lower levels of induction of the *lacZ* fusion, suggesting that the active molecule, the putative AHL, has been inactivated (Figure 2).

There seems to be little difference in the levels of inactivation between the 3 *C. jejuni* isolates tested (Figure 2), suggesting that the concentration and structure of the active molecule is similar.

Figure 3 shows a substantial peak eluted between 33.75 and 35.75 min which corresponds to reporter strain stimulatory activity.

The results of this study indicate putative AHL production by several isolates of *C. jejuni*, including the genome sequenced strain NCTC 11168, providing preliminary evidence that AHL-mediated QS is present in the Epsilon *Proteobacteria* class.

Further work will include MS-HPLC analysis of the HPLC isolated active fractions to determine the structure of the active molecule.

References

[1] Gaynor, E. C., *et al.* (2004) *Journal of Bacteriology* 186: 503-517

[2]Yang, F., *et al.* (2005) *FEBS Letters* 579: 3713-3717

[3] Zhu, J., *et al.* (2003) *Applied and Environmental Microbiology* 69: 6949-6953

[4] Miller, J. C. (1972) In: Experiments in Molecular Genetics. Cold Springs Harbour Laboratory 325-359

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