Acanthamoeba-Campylobacter interactions

By

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ABSTRACT

Campylobacter jejuni is an avian commensal bacterium and causes gastrointestinal diarrhea in humans called campylobacteriosis. Campylobacteriosis is acquired by consumption of undercooked poultry contamined with *C. jejuni*. Poultry can become colonized from contaminated drinking water. The chicken flock and drinking water of 4 poultry farms in Ontario were sampled and the prevalence of *C. jejuni* in these flocks was determined to be 16.7% over a 1 year sampling period. We determined that contamined-water was a significant risk factor for *Campylobacter*-positive flocks from *flaA* typing, PFGE analysis, and genomotyping several isolated strains. Free living amoebae, such as *Acanthamoeba* species, live in the drinking water of poultry farms. It is hypothesized that *Acanthamoeba* in the drinking water of poultry farms can take up and act as environmental reservoirs of *C. jejuni*. *Acanthamoeba* species were isolated from the drinking water. *Acanthamoeba* strains were found to act as a vehicle for protection, persistence and growth of *C. jejuni* isolated from the farm water. The transcriptome of both *C. jejuni* and *A. castellanii* during the initial stages of *C. jejuni* internalization were described by RNA-seq. *C. jejuni* oxidative defence genes (such as *katA*, *sodB*, *fdxA*) and some other unknown genes (Cj0170, Cj1325, Cj1725) were found to be essential in the interaction with *A. castellanii*. Our findings suggest that *Acanthamoebae* act as a *C. jejuni* reservoir and could be a contributing source of *C. jejuni* in the environment. Through transcriptomics studies, we have begun to uncover some genetic clues involved in this interaction.

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LIST OF ABBREVIATIONS

aadUTP	5-(3-aminoallyl)-2′-deoxyuridine 5′-triphosphate
BCYE	Buffered Charcoal Yeast Extract
CFU	Colony forming unit
CI	Competitive index
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
flaA	flagellin A
MH	Müeller-Hinton
mRNA	messenger RNA
NJ	Neighbour joining
NNA	Non-nutrient agar
PAS	Page's Amoeba Saline
PCR	Polymerase chain reaction
PFGE	Pulse field gel electrophoresis
PYG	Proteose Peptone Yeast Extract Glucose
RNA	Ribonucleic acid
RPKM	Reads per kb per million reads
ROS	Reactive oxygen species
SVR	Super variable region
VBNC	Viable but non culturable

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CHAPTER 1. AMOEBA-BACTERIA INTERACTIONS

1.1 Introduction

Microorganisms, such as bacteria, viruses and eukaryotes, have been described as benefiting from interactions with free-living amoebae (reviewed by (Thomas et al., 2009)). Of particular interests are human pathogens which are able to survive within amoebae. Survival within amoebae gives these bacterial pathogens benefits. These benefits include resistance to predation and to chemical treatments, intracellular survival and/or intracellular proliferation in a protected environment within the amoebal hosts (Thomas et al., 2009).

Free-living amoebae are widespread in the environment and are normally found in freshwater microbial ecosystems (Khan, 2006; Rodriguez-Zaragoza, 1994). They have a major impact on the dynamics of microbial biofilms by feeding on various microorganisms thus contributing to nutrient recycling (Pedersen, 1982). Free living amoebae have been isolated from a number of diverse environments. Some of these environments contain harsh physical and/or chemical conditions such as elevated temperature or a high concentration of biocides (e.g. chlorine). For example, amoebae have been recovered from 20-30% of domestic tap water (Jeong and Yu, 2005; Shoff et al., 2008), hospital water networks (Rohr et al., 1998), swimming pools (Vesaluoma et al., 1995), hydrotherapy baths (Scaglia et al., 1983), dental unit waterlines (Singh and Coogan, 2005), eyewash stations (Paszko-Kolva et al., 1991) and cooling towers (Berk et al., 2006).

Amoebae in domestic water systems mostly belong to the genera *Acanthamoeba*, *Hartmannella* and *Naegleria* (Thomas et al., 2009). Species belonging to the *Echinamoeba*, *Vannella*, *Vahlkampfia* and *Saccamoeba* genera have also been isolated (Thomas et al.,

2009). Most species of these genera can encyst. Encysted amoebae can resist harsh environmental conditions (Thomas et al., 2009). Amoebae themselves can be associated with diseases in humans but mostly affecting immunocompromised individuals (Thomas et al., 2009). For example, *Acanthamoeba* spp. and *Balamuthia mandrillaris* have been associated with encephalitis and *Naegleria fowleri* with meningoencephalitis (Visvesvara et al., 2007).

Amoeba are known to interact with various bacterial species. Association of bacterial species are thought to be common and microscopic observation of cytoplasmic endosymbionts in *Acanthamoeba castellanii* were reported as far back as 1967 (Jeon and Lorch, 1967). When *Legionella pneumophila*, a human bacterial pathogen causing Legionaire's disease, was found to be able to proliferate in various amoebal hosts (Rowbotham, 1980), people became more interested in studying the interactions between free living amoebae and pathogenic bacteria. An extensive review of the literature (Thomas et al., 2009) lists 102 bacterial species capable of interacting with free living amoebae. Of these 102 species, 40 (39.2%) were isolated by amoebal coculture without complete demonstration of intracellular survival, 30 (29.4%) have been demonstrated to survive in one or several amoebal species and 32 (31.4%) have been shown to grow in one or several amoebal species. However, most of these studies have used *Acanthamoeba polyphyga* Linc-Ap1 as a host and some amoeba-bacteria interactions are strain specific (Thomas et al., 2009). It would take many more years of research to uncover more combinations of amoeba bacteria interactions empirically. Some of these amoeba-bacteria interactions are listed in Table 1.1.

Table 1.1. Amoeba-bacteria interactions.

A non-exhaustive list of various amoeba-bacteria interactions previously studied with emphasis on pathogenic bacteria. IS = intracellular survival, IM = intracellular multiplication, ICS = intracyst survival.

Bacterial species	Protozoa species	Type of interaction	Reference
Aeromonas hydrophila	*Acanthamoeba castellanii* ATCC 30234	IS	(Rahman et al., 2008)
Agrobacterium tumefaciens	*Acanthamoeba polyphyga* Linc-Ap1	IM	(Evstigneeva et al., 2009)
Bacillus cereus	*Acanthamoeba polyphyga* Linc-Ap1	IM	(Evstigneeva et al., 2009; Pagnier et al., 2008a)
Burkholderia cepacia	*Acanthamoeba polyphyga* Linc-Ap1, *Acanthamoeba polyphyga* ATCC 50372	IS, IM	(Lamothe et al., 2004; Landers et al., 2000)
Campylobacter coli, C. hypointestinalis, C. jejuni, C. lari	*A. polyphyga* Linc-Ap1, *A. rhysodes*, *A. castellanii*, *Hartmanella vermiformis* CCAP 1534/7A, *Naegleria americana* CCAP 1518/1G, *Euglena gracilis* CCAP 1224/5Z, *Tetrahymena pyriformis*	IM, IS	(Axelsson-Olsson et al., 2007; Axelsson-Olsson et al., 2010)
Coxiella burnetii	*Acanthamoeba castellanii* ATCC 30234	IS	(La Scola and Raoult, 2001)
Enterobacter cloacae	*Acanthamoeba polyphyga* Linc-Ap1, *Acanthamoeba castellanii* ATCC 30234	IS, IM	(Evstigneeva et al., 2009; King et al., 1988)
Escherichia coli (including O157H7)	*A. polyphyga* and *A. castellanii*, *Tetrahymena pyriformis*	IS, IM	(Alsam et al., 2006; Barker et al., 1999; Steinberg and Levin, 2007)
Francisella tularensis	*Acanthamoeba castellanii* ATCC 30234	IM, ICS	(Abd et al., 2003)
Helicobacter pylori	*Acanthamoeba castellanii*	IS	(Winiecka-Krusnell et al., 2002)
Legionella pneumophila	More than 20 amoeba species	IM, ICS	For a review, see (Kuiper, 2006)
Listeria monocytogenes	*Tetrahymena pyriformis*, *Acanthamoeba castellanii* ATCC 30234	IS, IM	(Ly and Muller, 1989; Ly and Muller, 1990)
Mycobacterium avium	*A. castellanii* ATCC 30234 and CCAP 1501/1B, *A. polyphyga* Linc-Ap1 and CCAP 1501/3B, *Dictyostelium discoideum* AX2, *Tetrahymena pyriformis* ATCC 30202	IM	(Adekambi et al., 2004; Cirillo et al., 1997; Mura et al., 2006; Skriwan et al., 2002; Steinert et al., 1998; Strahl et al., 2001; Whan et al., 2006)
Mycobacterium leprae	*Acanthamoeba culbertsoni* ATCC 30171	IS	(Lahiri and Krahenbuhl, 2008)
Pseudomonas aeruginosa	*Acanthamoeba polyphyga* ATCC 30461, *Echinamoeba sp.*	IM	(Michel et al., 1995)
Salmonella typhimurium	*Acanthamoeba polyphyga* Linc-Ap1	IM	(Gaze et al., 2003)
Serratia marcescens	*Acanthamoeba polyphyga* Linc-Ap1	IM	(Pagnier et al., 2008b)
Shigella dysenteriae	*Acanthamoeba castellanii* ATCC 30234	IM	(Saeed et al., 2009)
Shigella sonnei	*Acanthamoeba castellanii* ATCC 30234 and 30010	IM	(Jeong et al., 2007; Saeed et al., 2009)
Staphylococcus aureus (MRSA)	*Acanthamoeba polyphyga*	IM	(Huws et al., 2006)
Streptococcus pneumoniae	*Acanthamoeba polyphyga* Linc-Ap1	IM	(Evstigneeva et al., 2009)
Vibrio cholerae	*Acanthamoeba castellanii* ATCC 30234, *Naegleria gruberi* 1518/1e	IM, ICS	(Abd et al., 2005; Thom et al., 1992)
Yersinia pestis	*Hartmannella rhysodes*	IS	(Nikul'shin et al., 1992)

Some bacterial species have been shown to survive in amoebal cysts (Thomas et al., 2009). Amoebal cysts have a high degree of resistance to environmental and chemical stresses (Thomas et al., 2009). Thus these interactions are important with regards to human health because the use of chemical disinfection would not inactivate these pathogens. For example, mycobacteria (Adekambi et al., 2004; Thomas and McDonnell, 2007), *Francisella tularensis* (Abd et al., 2003; El-Etr et al., 2009), *Legionella pneumophila* (Kilvington and Price, 1990a), and *Vibrio cholerae* (Thom et al., 1992) can survive in amoebic cysts.

1.2 *Legionella-Acanthamoeba* interactions

Although many amoeba-bacteria interactions exist (see Table 1.1), *Legionella-Acanthamoeba* is one of the most well studied interactions. *Acanthamoeba* is a natural host for *Legionella* (Bruggemann et al., 2006). *Legionella pneumophila* is a Gram negative bacterium which causes Legionnaire's disease, a form of respiratory disease (Bruggemann et al., 2006). It is believed that amoebae have exerted selective pressure for the evolution of *L. pneumophila* virulence traits contributing to the infection of human cells (Bruggemann et al., 2006). For example, *L. pneumophila* inhibits the amoebal phagolysosomal degradation pathway, a virulence trait also observed in human phagocytic cells (Gao et al., 1997). This interaction was studied in details to understand the mechanism of persistence of *L. pneumophila* in amoebae in the environment, and to infer mechanisms of pathogenicity in human macrophages in order to understand how *Legionella* causes Legionnaire's disease.

1.2.1 *Acanthamoeba* and other amoebae as a reservoir for *Legionella* species

Legionella was discovered after the initial description of an outbreak of severe respiratory disease occurring at the American Legion conference in Pennsylvania in 1976

(Fraser et al., 1977). This disease was named Legionnaire's disease. Afterwards, reports of *Legionella* infections were also found to be associated with contaminated water in the community (Mahoney et al., 1992) and nosocomial (Blatt et al., 1993) settings. It was observed that *L. pneumophila* is able to multiply intracellularly within protozoa suggesting that free-living amoebae could be an important reservoir for *Legionella* (Rowbotham, 1980).

Amoebae could be an important environmental source of *Legionella* because encysted amoebae provide additional protection against extreme temperature, pH, and osmolarity (Rodriguez-Zaragoza, 1994). Studies have shown that encysted amoebae can protect *L. pneumophila* from the effect of chlorine (Kilvington and Price, 1990b) and other biocides (Berk et al., 1998). These studies suggest that ameobae play an important key role as a host in the survival of *Legionella*.

In general, humans are infected by *L. pneumophila* by inhaling aerosols with *L. pneumopila* residing in amoebae (Rowbotham, 1980). The infective dose might contain as few as 100 bacteria (Rowbotham, 1986). Humans can also get infected with other species of *Legionella*. People attending conferences at a hotel in California in the late 1980's were diagnosed with Pontiac fever, a respiratory tract disease which is characterized by acute fever (Fenstersheib et al., 1990). These people were found to be infected by *L. anisa* (Fenstersheib et al., 1990). *Legionella anisa* and *Hartmanella vermiformis* were isolated from the fountain in the hotel lobby (Fields et al., 1990). In contrast to the wide amoebal host range of *L. pneumophila*, this strain of *L. anisa* grew only within *H. vermiformis* (Fields et al., 1990).

While some species of *Legionella* can be cultured on BCYE (Buffered Charcoal Yeast Extract) agar (standard media used for culturing *Legionella*), several species of *Legionnella* causing pneumonia can only be isolated or grow in co-culture with amoebae.

These species include *L. drozanskii*, *L. lytica, L. rowbothamii,* and *L. fallonii* (Adeleke et al., 2001). This observation highlights the importance of amoebae in the lifecycle of *Legionella* pathogens.

1.2.3 The transcriptome of *Legionella* interacting with *Acanthamoeba*

Microarray-based transcriptomic approaches were recently used to study the interaction of *L. pneumophila* with its natural host *Acanthamoeba castellani* (Bruggemann et al., 2006). The rationale of this study was to understand the regulatory circuits in *L. pneumophila* which allows it to survive and multiply intracellularly. The study also aims to uncover clues pertaining to the metabolic needs of intracellular *L. pneumophila.*

Legionella pneumophila was mixed with *A. castellanii* at a multiplicity of infection (MOI) of 1 to 100. *Legionella pneumophila* life cycle could be described in two phases: replicative and transmissive. Bacteria in replicative phase are avirulent, sodium resistant and not flagellated. In contrast, transmissive phase bacteria are virulent, flagellated and highly motile (Molofsky and Swanson, 2004b). *L. pneumophila*, when co-cultured with *Acanthamoeba*, are in a replicative phase at early time points and transmissive phase in late time points (Molofsky and Swanson, 2004b).

Transcriptional analyses of *L. pneumophila* during infection of *A. castellanii* revealed that 405 genes were upregulated during the replicative phase, and 393 genes were upregulated during the transmissive phase (Jules and Buchrieser, 2007). The replicative phase genes comprised DNA and RNA polymerase complexes, transcription and translation elongation factors, ribosomal proteins, protein secretion and translocation systems (*secAB, secEF, secGY*, etc.), genes coding proteins involved in purine and pyrimidine metabolism and other genes encoding metabolic processes (Bruggemann et al., 2006). Among the

identified transmissive phase genes, several transcriptional regulators (such as *fliA, cpxR, rpoE*), virulence factors (such as *ralF, dotA, letE, enhA, sdeA, sdcA*) and flagella biosynthesis genes (such as *fliS, fliD, fliN*) were overexpressed (Bruggemann et al., 2006). The replicative phase favours expression of genes involved in cell division. The transmissive phase favours expression of genes required to break out of the host cell and to invade a new one.

Genes encoding proteins involved in catabolism and uptake systems of amino acid (serine, threonine, alanine, glycine, tyrosine and histidine) were upregulated in the replicative phase (Bruggemann et al., 2006). Moreover, several aminopeptidase and protease encoding genes were upregulated during the replicative phase of *L. pneumophila* suggesting the acquisition of host amino acids (Jules and Buchrieser, 2007). *Legionella pneumophila* may also use carbohydrate-derivatives as shown by the upregulation of genes coding for proteins involved in the Entner–Doudoroff pathway (lpp0483, lpp0487), a putative glucokinase (lpp0486), a sugar transporter (lpp0488) and an eukaryotic-like glucoamylase (lpp0489) (Bruggemann et al., 2006). Glucoamylase is an enzyme which cleaves glycosidic linkages in complex sugars, like glycogen or starch, yielding simple sugars like glucose. Its up-regulation in co-culture with *A. castellanii* but not in planktonic cultures, suggests the presence of a *Acanthamoeba*-dependent signal that may trigger the use of host glycogen (Jules and Buchrieser, 2007).

Altogether, the transcriptome study shows that *L. pneumophila* expresses different genes in the replicative phase (i.e. cell division and protein synthesis related genes) and the transmissive phase (i.e. flagella synthesis genes) and may steal host amino acids and may use carbohydrates. Transcriptomics have provided clues helping us explain the mechanism of *Legionella* infection and multiplication in eukaryotic cells.

1.3 Introduction to *Acanthamoeba-Campylobacter* interactions

The focus of my study revolves around *Acanthamoeba-Campylobacter* interactions. *Campylobacter jejuni* is an avian commensal bacterium and is considered a food borne pathogen causing gastroenteritis in humans (Galanis, 2007). Consumption of undercooked poultry is a risk factor in acquiring *C. jejuni* infection (Friedman et al., 2000). Poultry may acquire *C. jejuni* by various exposure routes (discussed in Chapter 2). One of the possible routes of infection is by contaminated drinking water.

As discussed earlier in this chapter, many amoebae, such as *Acanthamoeba*, are found in living in water. Since it is well known that amoebae interact with bacteria, providing them protection from harsh environmental conditions, others have studied whether *Acanthamoeba* may interact with *C. jejuni*. These early studies have found that *C. jejuni* have a prolonged survival in co-culture with *Acanthamoeba* compared to planktonic *C. jejuni* and that *C. jejuni* is able to grow under aerobic conditions while in co-culture with *Acanthamoeba* (classically, *C. jejuni* can only be grown under a microaerophillic environment) (Axelsson-Olsson et al., 2005; Axelsson-Olsson et al., 2010a; Snelling et al., 2005). Interestingly, a recent study has found that internalized *C. jejuni* in *Acanthamoeba* can colonize chicks (Snelling et al., 2008).

Taken together, we hypothesized that *Acanthamoeba* may act as a possible waterborne source of *C. jejuni* infection for poultry. To investigate this hypothesis, we started our study by investigating the prevalence of *C. jejuni* in Ontario chicken flocks and by testing for the presence of both *Acanthamoeba* and *Campylobacter*. In one instance, we have isolated *Acanthamoeba* and *Campylobacter* from the same water source. We then confirmed by various approaches that the *C. jejuni* positive flock that drank the water was

colonized by the same *C. jejuni* strain from this water source, suggesting that the flock might have been colonized by *Campylobacter* in contaminated water.

Acanthamoeba and *Campylobacter* strains have never been isolated from the same water source at the farm level. This then led us to hypothesize that the *Acanthamoeba* isolated from this water source might have carried *C. jejuni* intracellularly, which resulted in the colonization of the flock. Early studies have used laboratory strains to test *Acanthamoeba-Campylobacter* interaction. Our objective was to use various assays to test whether *Acanthamoeba* in poultry farm water can interact with *C. jejuni* from the farm in terms of invasion, persistence and growth in similar patterns to laboratory strains. Our results show that indeed the interactions between environmental strains were similar to the interactions between to the laboratory strains. These results further show support for the hypothesis that *Acanthamoeba* may act as an environmental reservoir for *C. jejuni*.

Furthermore, early studies have not determined which genes are necessary for *C. jejuni* to interact with *Acanthamoeba*. No whole transcriptomic studies of *C. jejuni* and *Acanthamoeba* in their interaction with one another had been carried out previously. Our next objective was to study the interaction at a molecular level in order to understand the mechanisms by which these two organisms may interact. Our preliminary results show that *C. jejuni* oxidative defence and *Acanthamoeba* vesicle trafficking, and possibly other cellular mechanisms, are important in this interaction at an early time point.

CHAPTER 2. PREVALENCE OF *CAMPYLOBACTER* IN ONTARIO POULTRY FARMS

2.1 Introduction and research objectives

Campylobacter jejuni is an avian commensal bacterium that can cause food borne illness in humans (Galanis, 2007). This illness is called campylobacteriosis and is characterized by bloody diarrhea, abdominal pain, fever and, rarely, vomiting which can last up to several weeks (Galanis, 2007). In Canada, 9345 cases (30.2 per 100,000) of campylobacteriosis were reported in 2004 compared to 4953 cases of salmonellosis (16.0 per 100,000) (Galanis, 2007). About 95% of these cases are caused by *Campylobacter jejuni*, 4% by *Campylobacter coli* and 1% by other *Campylobacter* species (Galanis, 2007).

Very rarely, *C. jejuni* is linked to the development of the Guillain-Barré syndrome (GBS) which is a an inflammatory disorder affecting the peripheral nerves (Winer, 2008). *C. jejuni* lipooligosaccharide shares epitopes with human gangliosides on peripheral nerve cells (Winer, 2008). Thus, when the immune system produces antibodies against *C. jejuni*, they might cross-react with the GM1, GM1b, GD1a, or GalNAc-GD1a gangliosides leading to paralysis (Yuki, 2001). The incidence of Guillain-Barré syndrome varied from 1.2 per 100,000 to 1.6 per 100,000 (Bogliun et al., 2002; Markoula et al., 2007). Finally, *Campylobacter* is suspected to be one of the possible etiological factors of Crohn's disease and/or ulcerative colitis (Boyanova et al., 2004; Nachamkin, 2002).

The majority of *Campylobacter* cases are sporadic and only few outbreaks have been documented. One example of such outbreak is the incident in 2000 in Walkteron, Ontario, where residents contracted campylobacteriosis by drinking contaminated water (Schuster et al., 2005). Consumption of undercooked poultry is a significant risk factor to acquire *Campylobacter* infection (Friedman et al., 2000). Since *C. jejuni* is a commensal member of

chickens' gut microbiota, chickens remain asymptomatic carriers until slaughter at which point the splitting of the intestines might contaminate the carcasses (Keener et al., 2004). Consequently, understanding the processes by which poultry flocks become colonized by *C. jejuni*, in the first place, is key to design novel prevention measures aimed at controlling this important food borne pathogen.

The European prevalence of *Campylobacter* in poultry products varies from 18% to >90% (Newell and Fearnley, 2003). In Quebec, a recent study has shown that up to 36% of the broiler chicken carcasses are contaminated with *Campylobacter* (Arsenault et al., 2007) . Another Quebec study found that approximately 40% of broiler chickens in 67% of 57 farms were colonized by *Campylobacter* (Nadeau et al., 2002). In Alberta, *Campylobacter* was found in 62% of raw chicken legs from a retail marketplace (Bohaychuk et al., 2006). In Ontario, from 2003 to 2005, 40-47% of retail chicken has been found to be contaminated with *Campylobacter* (Galanis, 2007). Another Ontario study reported a prevalence of 9.7% in raw chicken samples of food service operations (Medeiros et al., 2008). One Ontario study found *Campylobacter* prevalence to be 59.6% in retail chicken with *C. jejuni* making up to 90% of the *Campylobacter* positive samples (Deckert et al., 2010) suggesting a high prevalence of *Campylobacter* positive flocks. Nevertheless, the prevalence of *Campylobacter* in Ontario broiler chicken flocks at the farm level is unknown and needs to be investigated.

The epidemiology of *Campylobacter* with respect to poultry is well studied (see Section 3.1). *Campylobacter* has been routinely identified in environmental surface water, drinking water and private wells (St-Pierre et al., 2009; Van Dyke et al., 2010). The objective of our study was to determine the incidence of *Campylobacter*-positive flocks at

the farm level in Eastern Ontario and to assess if contamined-water could be a significant risk factor for *Campylobacter*-positive flocks.

2.2 Methods

2.2.1 Sample collection, farms, and study design

Four Eastern Ontario poultry farms from different geographic locations were enrolled in the study (Figure 2.1). The samples were collected by Dr. Alain Stintzi. For fecal samples, subsamples of soft, fresh and wet cecal droppings were collected from a minimum of 20 separate areas of the barn floor in a fecal collection tube. Dead birds were collected in clean biohazard bags and brought back to the lab for dissection and collection of their ceca. Water samples were taken from the drinking water systems, at a location near the water source and at the middle connection of the water lines (Figure 2.2). All farms used well water. The water was not treated on Farm 1. The water was acidified to pH 4 and iodine was added on Farm 2. The water was acidified to pH 4 on Farm 3 and Farm 4. Removable filters from the drinking water distribution system were removed and placed in a clean biohazard bag for sample processing at the laboratory.

2.2.2 *Campylobacter* isolation

2.2.2.1 Sample processing

Samples were processed immediately upon arrival to the laboratory. To isolate *Campylobacter* from chicken feces, the feces were homogenized manually in a Ziploc bag by hand massage for 1 min. Approximately 1.0 g of fecal matter was placed in a 15 mL Falcon tube pre-filled with 9 mL of sterilized normal saline solution (0.9% NaCl w/v). Approximately 200 mg of fecal matter was placed in a sterile 2.0 mL microfuge tube and stored at -20ºC for subsequent analysis by molecular biology. The Falcon tube was vortexed for 15 sec to homogenize the sample. The fecal suspensions were serially diluted (1:10,

Figure 2.1. Locations of the four participating farms.

The farms are located in Eastern Ontario. This map was generated using Google Earth.

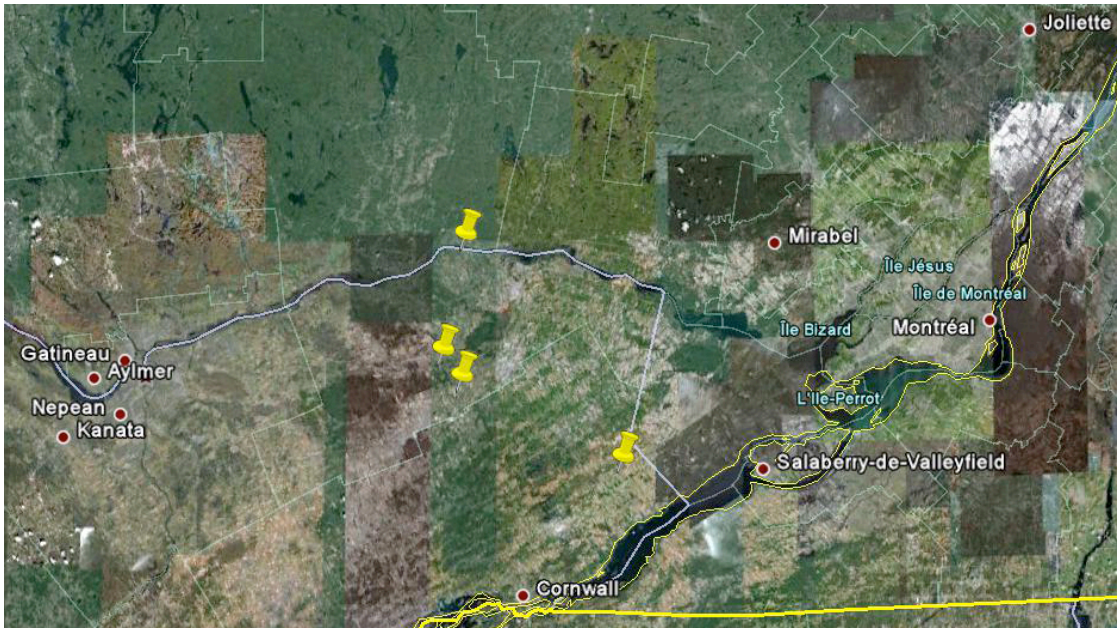
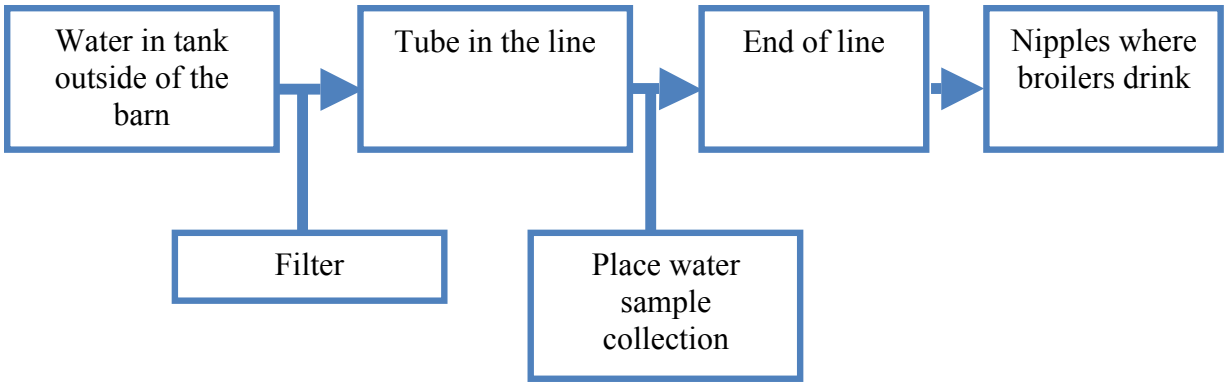

Joliette
Mirabel
Île Jésus
Île de Montréal
Île Bizard
Montréal
L'Île-Perrot
Gatineau
Aylmer
Nepean
Kanata
Salaberry-de-Valleyfield
Cornwall

Figure 2.2. Flow diagram of drinking water systems in the broiler houses of Farm 4.


Water in tank outside of the barn
Tube in the line
End of line
Nipples where broilers drink
Filter
Place water sample collection

1:100, 1:1000 and 1:10000) in sterile normal saline solution and plated on *Campylobacter* selective Karmali agar (Karmali agar (Cat. No. CM0935) prepared with *Campylobacter* selective supplement (Cat. No. SR0167) from Oxoid). Each dilution was plated in two to three replicates. The plates were inverted and incubated at 42ºC under microaerophilic conditions (83% nitrogen, 8% oxygen, 4% hydrogen, 5% carbon dioxide) for 48 h to 72 h. The original fecal sample was frozen and stored at -20ºC.

To isolate *Campylobacter* from chicken ceca, one of the two ceca was excised from the chicken corpse. The cecum was placed in an empty sterile Petri dish and its weight was recorded. Then, 3 mL of MH (Müeller-Hinton) broth was added to the Petri dish. With a flame sterilized scalpel, the cecum was opened by cutting longitudinally followed by scraping of the inside of the cecum to release the bacteria from the mucus layer and from the feces into the broth. The suspension was transferred into a sterile 50 mL Falcon tube. About 200 µL of the suspension was aliquotted to a sterile 2.0 mL microfuge tube and set to be frozen at -20ºC for molecular analysis. The remainder of the sample was plated on Karmali agar following serial dilutions as described above.

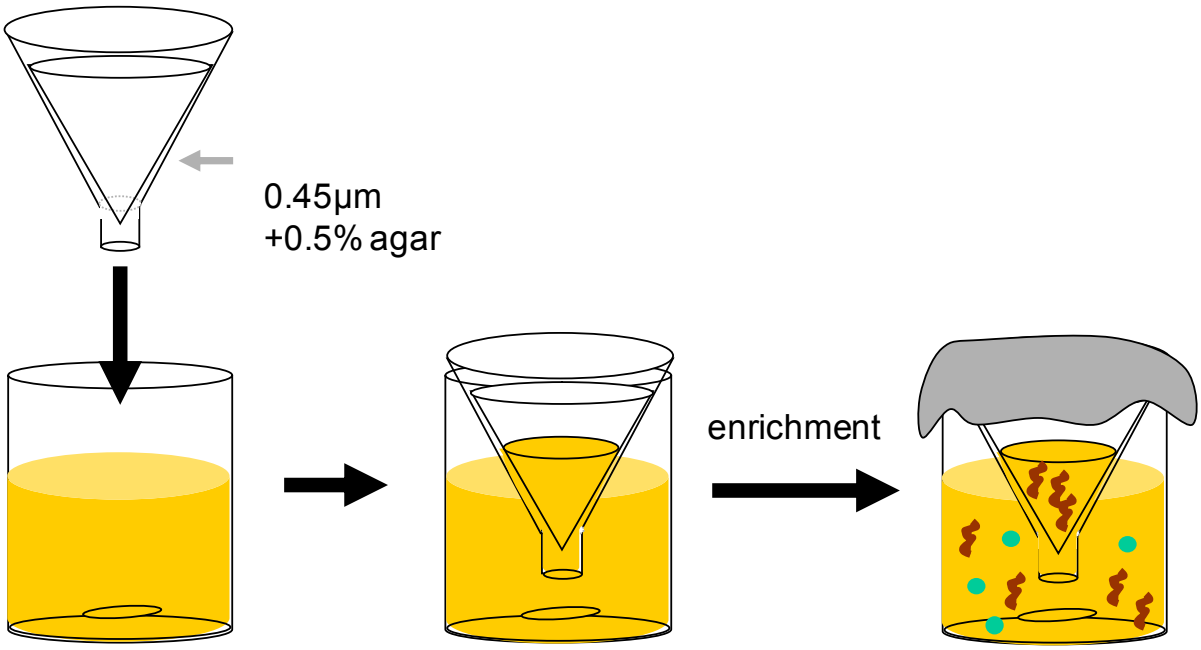
To isolate *Campylobacter* from farm water, a filtering apparatus consisting of a sterile funnel placed on top of a sterile 1 L Erlenmeyer side arm-flask was connected to vacuum by rubber tubing. A sterile 47 mm in diameter nitrocellulose filter with 0.22 µm pore size (Millipore) was placed on the sterile funnel. The water bottle containing the water sample was shaken manually for 15 sec to ensure a homogenous sample. The pH of the water sample was measured using a pH meter (Accumet AR60 model, Fisher Scientific). Afterwards, 500 mL of the water sample was poured into the funnel and the vaccuum was turned on to pass the water through the 0.22 µm filter. With sterile forceps, the filter was removed and dropped in a Falcon tube containing 5 mL of Bolton broth. The tube was

vortexed for 10 sec to help dislodge bacteria and other organisms from the filter into solution. A volume of 200 µL was removed and directly plated on *Campylobacter* selective Karmali agar as described above. The filter and the remaining 4.8 mL of Bolton broth were added to 75 mL of Bolton broth in a sterile beaker. A funnel with a nitrocellulose membrane of 0.45 µm coated with 0.5% agar was placed on top of this beaker and filled with 5 mL of Bolton broth. This enrichment set up (beaker and funnel) is based on a method described and depicted in (Wisessombat et al., 2009) (see Figure 2.3). The remainder of the water sample (500 mL) in the original bottle was used for amoeba isolation (see Chapter 3). The broth was incubated at 42ºC under microaerophilic conditions for 48±4 h. After this enrichment period, the sample was plated on *Campylobacter* selective Karmali agar as described above.

To isolate *Campylobacter* from farms' filter samples, the dirty filters were taken out of the sample bag and placed on clean brown paper. The filter was cut longitudinally using flame sterilized scalpel and scissors. The filter, roughly 10% of the top portion and 10% of the bottom portion, was manually torn into 2 cm^2 pieces. The pieces were placed into a UV sterilized Ziplock bag. About 250 mL of sterile water was added to the bag. The bag was sealed and manually stomached for about 2 min to ensure sample homogenization. The suspension in the bag was poured into a sterile bottle, and 200 µL was removed and directly plated on Karmali agar as described above. Then, 10 mL of the remaining suspension was added to 75 mL of Bolton broth in a beaker with a funnel as above. A 50 mL water sample aliquot from the original bottle was frozen at -20ºC for molecular analysis. The remainder of the sample in the original bottle was used for amoeba isolation (see Chapter 3). The broth was incubated at 42ºC under microaerophilic conditions for 48±4 h. After this enrichment period, the sample was plated on *Campylobacter* selective Karmali agar as described above.

Figure 2.3. Beaker-funnel enrichment setup for *Campylobacter* isolation from water and filter samples.

A beaker filled with Bolton broth was inoculated with the sample. A funnel with a nitrocellulose membrane of 0.45 µm coated with 0.5% agar was placed on top of the beaker. Then 5 mL of Bolton broth was added to the funnel. The beaker and funnel were incubated under microaerophillic conditions for 48 hours to allow for enrichment of *Campylobacter*. Following enrichment, *Campylobacter* will selectively migrate into the funnel allowing pure recovery of *Campylobacter* in the funnel portion. This enrichment set up (beaker and funnel) is based on a method described and depicted in (Wisessombat et al., 2009).


0.45μm
+0.5% agar
enrichment

2.2.2.2 *Campylobacter* isolation, identification and preservation

C. jejuni form smooth and gray colonies of 2-4 mm in diameter on Karmali agar (http://www.hpa-standardmethods.org.uk/documents/bsopid/pdf/bsopid23.pdf; http://www.hc-sc.gc.ca/fn-an/res-rech/analy-meth/microbio/volume3/mflp46-eng.php). Probable *C. jejuni* colonies were counted and reported as CFU/g of fecal or cecal matter. Three to five colonies per sample were re-streaked onto new Karmali agar plates as an additional isolation step and the plates were incubated at 37ºC under microaerophilic conditions for 48±4 h.

Campylobacter isolates were characterized by the following features: curve Gram negative rods, oxidase positive (determined using oxidase disks from Sigma), and absence of growth under aerobic conditions. Finally, the latex agglutination kit (Oxoid) was used as an additional test to identify the colonies as *Campylobacter*.

To preserve the bacterial isolates, identified *C. jejuni* colonies were inoculated into a Nunc flask containing a thin layer of MH agar and 7 mL of MH broth (bi-phasic flask). The bi-phasic flasks were incubated at 37ºC under microaerophilic conditions until turbid growth was observed (16 h to 20 h incubation). Then, 800 µL of the bacterial culture was mixed with 800 µL of 50% sterile glycerol solution in a 2 mL cryovial, resulting in a 25% glycerol solution. The cryovials were preserved at -80ºC for long term storage.

2.2.2.3 *Campylobacter flaA* genotyping

FlaA genotyping was performed according to Callicott et al. (2008). Briefly, the *flaA* short variable region (SVR; a hypervariable part of the flagellin gene) was PCR amplified using primers Fla4F and FlaA625RU (Table 2.1). The PCR master mix consisted of 0.2 mM dNTP's, 0.4 µM forward primer, 0.4 µM reverse primer, 1X PCR buffer, 1.5 mM, $MgCl_2$,

Table 2.1. List of all primers used in this chapter.

Name	Sequence (5' to 3')	Direction	Usage	Reference
Fla4F	GGATTTCGTATTAACACAAATGGTGC	Forward	FlaA PCR/Sequencing	(Callicott et al., 2008)
Fla625RU	CAAGWCCTGTTCCWACTGAAG	Reverse	FlaA PCR	(Callicott et al., 2008)
CC18F	GGTATGATTTCTACAAAGCGAG	Forward	Multiplex PCR	(Linton et al., 1997)
CC519R	ATAAAAGACTATCGTCGCGTG	Reverse	Multiplex PCR	(Linton et al., 1997)
hipO-F	GACTTCGTGCAGATATGGATGCTT	Forward	Multiplex PCR	(Persson and Olsen, 2005)
hipO-R	GCTATAACTATCCGAAGAAGCCATCA	Reverse	Multiplex PCR	(Persson and Olsen, 2005)
16S-F	GGAGGCAGCAGTAGGGAATA	Forward	Multiplex PCR	(Persson and Olsen, 2005)
16S-R	TGACGGGCGGTGAGTACAAG	Reverse	Multiplex PCR	(Persson and Olsen, 2005)

and 2.5 U Taq DNA polymerase enzyme in a final volume of 12.50 µL per reaction. One *Campylobacter* colony from MH agar was touched with a sterile wooden toothpick and transferred into a PCR tube containing the PCR master mix. The PCR reaction was run in a PTC-100 thermal cycler (MJ Research Inc.) with the following cycling conditions: 95°C for 2 min (initial denaturation), then 35 cycles at 94°C for 45 s (denaturation), 55°C for 45 s (annealing), and 72°C for 1 min (extension); and 72°C for 5 min (final extension). Amplicons were analysed by 0.8 % agarose gel electrophoresis. The PCR products were purified with the QIAGEN PCR purification kit and the amplicons were sequenced at the StemCore lab at OHRI (Ottawa Hospital Research Institute) using primer Fla4F. BLAST search was performed against GenBank (megablast) and the *Campylobacter* FlaA/MLST database (http://pubmlst.org/campylobacter/).

2.2.2.4 *Campylobacter* detection and identification by multiplex PCR

Campylobacter species (*C. jejuni* or *C. coli*) were identified using a multiplex PCR assay (Persson and Olsen, 2005). Total genomic DNA was extracted from fecal and cecal samples using the QIAamp DNA stool mini kit (QIAGEN) according to manufacturer's instructions. PCRs were performed in a total reaction volume of 25 µl containing 0.2 mM dNTP's, 1X PCR buffer, 1.5 mM $MgCl_2$, 2.5 U Taq DNA polymerase enzyme, and 0.4 µM asp-primers CC18F and CC519R (Linton et al., 1997), 0.2 µM hipO primers hipO-F and hipO-R, and 0.05 µM 16S rDNA primers 16S-F and 16S-R (Table 2.1), and 40-50 ng of DNA template. Thermocycler conditions for the multiplex PCR were 94°C for 6 min, followed by 35 cycles of 94°C for 50 s (denaturation), 57 °C for 40 s (annealing) and 72°C for 50 s (extension), and finally 72°C for 3 min. *C. jejuni* NCTC11168 and *C. coli* AS43

were used as positive controls. Amplicons were analysed by 0.8 % agarose gel electrophoresis.

2.2.3 *Campylobacter* genomotyping

2.2.3.1 DNA extraction by phenol/chloroform method

Total genomic DNAs were isolated from *C. jejuni* cultures grown in MH in a bi-phasic system as previously described (see Section 2.2.2.2). Briefly, 1.8 mL of *C. jejuni* culture was added to a 2.0 mL sterile microfuge tube in duplicates. The tubes were centrifuged for 10 min at 3,600 x g. The supernatant was discarded and the pellet was resuspended in 100 µL of TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA). Afterwards, 1 mL of extraction buffer (50 mM Tris, pH 8.0, 0.1 mM EDTA, 0.5% SDS) was added followed by the addition of 1 µL of RNAse A (20 mg/mL, Invitrogen). The mixture was incubated at 37°C for 1 hour. Then 15 µL of proteinase K (>600 mAU/ml, QIAGEN) was added to breakdown proteins and the tubes were incubated at 42°C for 3 hours. A volume of 700 µL of phenol (pH 6.6) was added to each tube. The tubes were mixed by inversion followed by an incubation of 15 min at 37°C. The tubes were centrifuged for 5 min at 3,600 x g and the upper layers from each tube were recovered and transferred to new sterile 2.0 mL microfuge tubes. A volume of 1 mL of chlorophorm was added to each tube and the tubes were mixed by inversion. Next, the tubes were centrifuged for 1 min at 4,000 x g. The upper layer was recovered for a second time and transferred to a 10 mL sterile Falcon tube. Finally the DNA was precipitated by adding 0.2 volumes of ammonium acetate (10M) and 2 volumes of 95% ethanol and rolled onto glass Pasteur pipettes. The obtained DNA was washed with 70% ethanol, air-dried, and finally resuspended in 100 µL of distilled water. The amount of DNA

was measured using the Nanodrop 8000 (Thermo Scientific). The genomic DNA was stored at -20°C.

2.2.3.2 Genomic DNA shearing and fragmentation by heat

Genomic DNA was fragmented using a slightly modified method as described by Kreatech Diagnosis (http://www.kreatech.com/Default.aspx?tabid=119). Briefly, 2 µg of genomic DNA was dissolved in 30 µL of sterile distilled water in a 200 µL PCR tube. A total of four tubes were prepared. The samples were boiled at 95°C for up to 40 min. One tube was removed from 95°C every 10 min and cooled at -20°C for at least 10 min. The samples were concentrated to about 10 µL using a vacufuge and a 2 µL aliquot was analyzed by 0.8 % agarose gel electrophoresis to determine the extent of DNA fragmentation. The non heated genomic DNA was also loaded on the agarose gel for comparison. Samples with DNA fragment sizes between 500 to 1,500 bp were selected for downstream steps of DNA labelling with aadUPT's. The DNA concentration was measured with the Nanodrop 8000 spectrophotometer and the fragmented genomic DNA was stored at -20°C until further processing.

2.2.3.3 DNA labelling with aadUTP's

Genomic DNA was labelled with aadUTP's using the BioPrime DNA Labelling kit (Invitrogen) as follows. Briefly, 200 ng of fragmented DNA was dissolved in 5 µL sterile distilled water. Twenty µL of 2.5X Random Primers was added followed by heat denaturation for 5 min at 100°C. Next, the samples were immediately cooled on ice for 5 min. Then, the resulting mixture was brought to a final volume of 50 µL by adding 5 µL of aadUTP containing dNTPs (2 mM dGTP, 2 mM dATP, 2 mM dCTP, 1 mM dTTP, 1 mM

aadUTP), 1 µL of Klenow Fragment and sterile distilled water. Following 2 hours incubation at 37°C, the reaction was stopped by adding 5 µL of Stop buffer (provided in the kit). The samples were purified with a PCR purification kit (Invitrogen Purelink purification kit) and the concentration of the amplified DNA was measured with the Qbit dsDNA BR assay (Invitrogen) according to the manufacturer's instructions. The protocol amplifies the input DNA approximately 10 fold yielding up to 2 µg of amplified DNA. The amplified and labelled DNA was stored at -20°C.

2.2.3.4 Reaction with cyanine dyes and microarray hybridization

Approximately 2 µg of amino-allyl labelled DNA was concentrated to 9 µL under vaccum in a vacufuge. Then, 1 µL of sodium carbonate (1M, pH 9.0) was added to each sample making a total volume of 10 µL. The aminoallyl-labelled DNA was coupled to indocarbocyanine (Cy-3) or indodicarbocyanine (Cy-5) dyes by adding 10 µL of each dye (dissolved in anhydrous DMSO). The samples were incubated at room temperature for 1 hour in the dark. Next, the labelled DNA probes were purified using the Invitrogen PureLink PCR purification kit as follows. Briefly, 55 µL of sterile distilled water, 35 µL of sodium acetate (100 mM at pH 5.2), and 400 µL of binding buffer were added to each tube. The mixture was briefly mixed and transferred to the spin column. The columns were centrifuged at 4,000 x g for 1 min. The flow throughs were discarded and the columns were washed four times by addition of 650 µL of wash buffer. The columns were dried by a final centrifugation at 4,000 x g for 1 min. The purified labelled DNA probes were eluted from the column by addition of 30 µL of sterile distilled water twice.

To prepare the probes for microarray hybridization, the eluted probes were concentrated to about 7 µL under vaccum using a vacufuge, and kept at room temperature in the dark until hybridization onto the microarray slides.

To prepare the microarray slides for hybridization with the probes, the microarray glass slides were incubated in 100 mL pre-warmed hybridization buffer (25% formamide, 5X SSC buffer (20X SSC is 3M NaCl and 0.3M sodium citrate, pH 7), 0.1% SDS, 1% bovine serum albumin) at 42°C for 45 min. Coverslips for the microarray slide were incubated for at least 5 min in 100 mL of 5X SSC and 0.1% SDS pre-warmed at 42°C.

Fluorescently labelled probes were combined in a 36 µL hybridization solution containing 2.5 µL of salmon sperm DNA (10 mg/mL, Ambion), 9 µL of 20X SSC, 0.36 µL of 10% SDS, and 9 µL of formamide (Sigma). The hybridization solution was subsequently denatured for 2 min at 99°C then held at 42°C.

The coverslips were rinsed with 1L of distilled water and dried using pressurized air. The microarray glass slides were washed with distilled water and dried by centrifugation. The microarray glass slide and its coverslip were placed on the PCR machine's heat block set at 42°C. The hybridization solution pre-warmed at 42°C was applied longitudinally on the coverslip using a P10 pipette (Eppendorf). The microarray glass slide was then inverted onto the coverslip and special care was taken to avoid formation of air bubbles (the end of a pipette tip can be used to force out air bubbles that may be produced under the coverslip). Finally, the microarray slide was placed in a humidified chamber pre-heated at 42°C. The chamber was tightly sealed and placed in a 42°C incubator in an upright position. Hybridization was carried out overnight (approximately 16 hours) and in darkness. The microarray slides were then washed in 100 mL of buffer containing 2X SSC and 0.1% SDS for 5 min at 42°C. Next, the microarray slides were consecutively washed in 0.1% SSC and

0.1% SDS for 10 min at room temperature, and 4 times (1 min for each wash) in 400 mL of 0.1% SSC. Finally, the microarray slides were rinsed with 2 L of distilled water and dried by centrifugation.

The microarray slides were scanned at 10 μm resolution using a confocal scanner, ScanArray Gx (Perkin Elmer), equipped with 2 lasers (543 nm for Cy 3 and 633 nm for Cy 5).

2.2.3.5 Data normalization and analysis

Image analysis was performed using the ScanArray software from Perkin-Elmer. The appropriate gene text file was selected to assign *C. jejuni* gene to each spot. The microarray used consisted of 3 meta arrays, each containing 12 subarrays, with each subarray containing 9 rows and 18 columns, altogether representing the entire genome of *C. jejuni* in triplicates. Spots were identified using the ScanArray software and visually inspected for quality assignment. Spots within regions of hybridization abnormalities were removed from further analysis. Next the means of the fluorescence intensities were normalized by applying a locally weighted linear regression (LOWESS) and the data was exported as a CSV file to view in Microsoft Excel. Microsoft Excel was used for subsequent calculations. The $\log_2$ of the normalized ratio of the mean intensities of the two channels was analyzed using the empirical Bayes method (Long et al., 2001). The $\log_{10}$ of the *P* values obtained from the Bayesian analysis and the $\log_2$ of the fold change of each gene were plotted. Genes with *P* value below 10^{-6} and $\log_2$ of the signal intensities ratio (NCTC 11168/C9) below 2 fold were considered absent or divergent.

The GACK software was used to assign genes as present, absent or divergent (Kim et al., 2002). The normalized mean intensity ratio of each gene was log transformed in base 2.

Then the log transformed values were averaged across the 3 subarray sets per microarray slide, creating an average log value for each gene in each microarray slide. These average values were then manually converted into PCL format in Microsoft Excel and exported as a text file. Subsequently, the PCL file was analyzed with the GACK software (Kim et al., 2002). Parameters used in GACK were: no data smoothing, normal curve peak modelling, data histogram with bin size of 0.10, and graded output. After GACK analysis, a graded output showing probability of a gene to be likely present or likely divergent in environmental *C. jejuni* strains was generated. The probabilities were then averaged across all four microarray slide replicates. If a gene was successfully hybridized in less than three microarray slide replicates, it was discarded from further analysis.

2.2.4 *Campylobacter* pulse field gel electrophoresis

C. jejuni C4, C9 and C20 were grown from frozen stocks on MH-agar plates under microaerophillic conditions at 37°C for 3 days. The pulse field gel electrophoresis (PFGE) was carried out as previously described (Chang and Chui, 1998; Ribot et al., 2001). The procedure involes cell lysis, restriction digestion of the DNA, and electrophoresis of the DNA fragments. This procedure was carried out in collaboration with Dr. C. Carrillo (Health Canada, Ottawa).

2.3 Results

2.3.1 *C. jejuni* strains isolated and identified

The objective of our study was to assess if contamined-water could be a significant risk factor for *Campylobacter*-positive flocks and to determine the incidence of *Campylobacter*-positive flocks at the farm level in Eastern Ontario. In collaboration with the chicken farmers of Ontario (CFO) we recruited 4 farms, representing different water supplies (filter/treatment versus no treatment of the drinking water). The 4 farms were located in Eastern Ontario and will be denoted farms 1 to 4 (Figure 2.1). Farm 1 consisted of one single poultry house, farms 2 and 3 consisted of a two-storey poultry house, and farm 4 consisted of an individual poultry house and a two-storey building. Samples were taken from broiler flocks twice per cycle (when the broiler were approximately 20 to 25 days old and just before slaughter at 39 days old). Three to 8 flocks per poultry houses were sampled. Four types of samples have been collected: (1) fecal samples; (2) cecal samples; (3) water samples before entry in the poultry houses; and (4) water samples at the end of the water line (inside the poultry houses (Figure 2.2)). Whenever possible the water filter was removed from the water distribution system of the poultry houses and processed for the presence of *Campylobacter*. These filters allowed us to investigate the contribution of *Campylobacter* biofilm formation in chicken colonization.

A total of 114 samples were collected over the period of October 2008 to November 2009. The breakdown of the type of samples is the following: 39 cecal samples, 37 fecal samples, 36 water samples, and 2 filter samples. Each sample was processed for the presence of *Campylobacter jejuni* using a culture-based approach as described in the Materials and Methods section. A total of 39 *C. jejuni* strains from various sources were

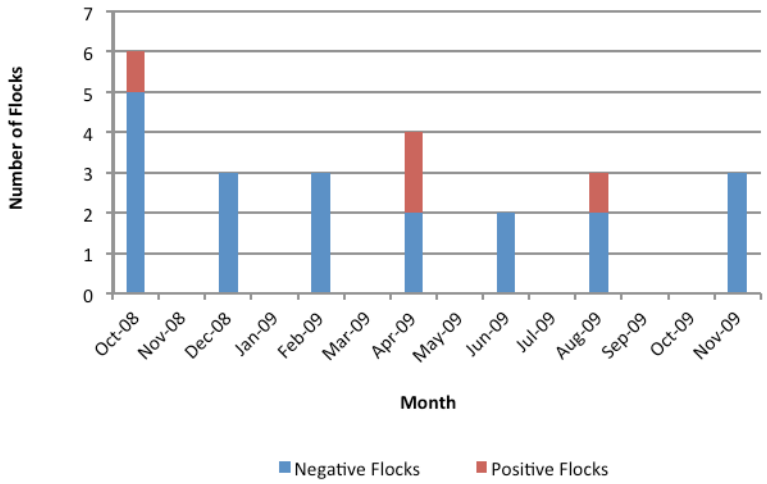
isolated. All strains display *C. jejuni* characteristics: Gram negative, rod like morphology, oxidase positive, microaerophilic growth, no aerobic growth, *flaA* amplification, direct multiplex PCR amplification with the amplicons charactistic of *C. jejuni*, and agglutination positive with Oxoid Latex Agglutination kit. Three distinct *flaA* types were obtained, namely allele 756, allele 1285 and allele 239 (the allele numbering is based on the numbering used in the *Campylobacter flaA* database (Jolley et al., 2004)). A total of 16.7% (4 flocks out of 24 flocks sampled) of the flocks tested were *Campylobacter*-positive, with the infected flocks being raised during the months of October, April, or August (Figure 2.4). Table 2.2 lists the samples that were found to be *Campylobacter*-positive. Farm 4 was characterized by a highest incidence of *Campylobacter*-positive flocks as compared to the 3 other farms, which remained *Campylobacter*-free over the course of this study. The colonization levels ranged from 10^4 to 10^9 CFU per gram of cecal content. *Campylobacter* was detected from the filtered drinking water of one *Campylobacter*-positive flock (October 17, 2008) at a concentration of 10^5 CFU/L of water, while it was not recovered from the drinking water of the 3 other *Campylobacter*-positive flocks. *Campylobacter* was never detected from the drinking water of *Campylobacter*-negative flocks or from the two filter samples tested.

2.3.2 Detection of *Campylobacter* in environmental samples by multiplex PCR

Campylobacter is known to be a fragile bacterium and its viability might reasonably be expected to decline outside from its chicken host, such as during transport from the farms to the laboratory. Therefore, to validate the incidence of *Campylobacter* at the farm level, we also assessed its presence in our samples using a culture-independent molecular-based approach.

Figure 2.4. *Campylobacter jejuni* positive samples and positive flocks.

Twelve out of 114 samples (10.5%) and 4 out of 24 flocks (16.7%) were positive for *C. jejuni*. The samples were collected from October 2008 to November 2009. All positive samples came from Farm 4.


Number of Flocks
7
6
5
4
3
2
1
0
Oct-08
Nov-08
Dec-08
Jan-09
Feb-09
Mar-09
Apr-09
May-09
Jun-09
Jul-09
Aug-09
Sep-09
Oct-09
Nov-09
Month
Negative Flocks
Positive Flocks

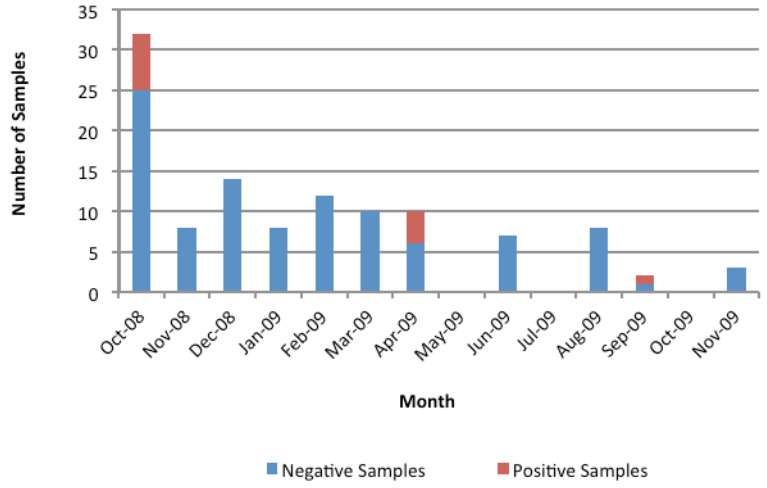

Number of Samples
35
30
25
20
15
10
5
0
Oct-08
Nov-08
Dec-08
Jan-09
Feb-09
Mar-09
Apr-09
May-09
Jun-09
Jul-09
Aug-09
Sep-09
Oct-09
Nov-09
Month
Negative Samples
Positive Samples

Table 2.2. Information on *C. jejuni* positive samples.

C. jejuni was isolated from various sources such as feces, ceca and water. *C. jejuni* was isolated from water on October 17, 2010 without the use of enrichment broth. Farm 4 consisted of an individual poultry house (barn 1) and a two-storey building (barn 2, upper and lower level).

Colonization level	Farm	Barn	Level	Date of Sample Collection	Sample Type	Additional information	Age of flock (days)
10^7 CFU/g	4	1		17-Oct-08	Fecal		17
10^7 CFU/g	4	1		17-Oct-08	Cecal	chicken 1	17
10^7 CFU/g	4	1		17-Oct-08	Cecal	chicken 2	17
10^4 CFU/100mL	4	1		17-Oct-08	Water	pH = 5.9	17
10^8 CFU/g	4	1		31-Oct-08	Fecal		31
10^8 CFU/g	4	1		31-Oct-08	Cecal	chicken 1	31
10^8 CFU/g	4	1		31-Oct-08	Cecal	chicken 2	31
10^6 CFU/g	4	2	upper	25-Apr-09	Fecal		32
10^7 CFU/g	4	2	lower	25-Apr-09	Fecal		26
10^6 CFU/g	4	2	upper	25-Apr-09	Cecal	chicken 2	32
10^7 CFU/g	4	2	lower	25-Apr-09	Cecal	chicken 3	26
10^4 CFU/g	4	2	lower	02-Sep-09	Fecal		35

As a first set of experiments, we determined the sensitivity of a previously described multiplex PCR assay (Persson and Olsen, 2005) to detect and quantify *Campylobacter* in cecal and fecal samples. *Campylobacter*-free samples (as determined by the culture method) were seeded with *C. jejuni* NCTC11168 at densities ranging from 10 to 10^9 cfu / g of cecal or fecal samples. The samples were processed by DNA extraction and the presence of *C. jejuni* was assessed by multiplex PCR as described in the Materials and Methods section. The multiplex assay is a PCR diagnostic assay where different primer sets are used in a single master mix. The detection of *C. jejuni* and/or *C. coli* is determined by the successful amplification of unique loci found in *C. jejuni* or *C. coli*. Primers are directed towards the following loci: the hippuricase gene (*hipO*) characteristic of *C. jejuni* (344 bp amplicon), a sequence partly covering an aspartokinase gene characteristic of *C. coli* (500 bp amplicon), and a universal 16S rDNA gene sequence serving as an internal positive control for the PCR (1062 bp amplicon). Thus, positive samples for *C. jejuni* are expected to produce the 1062 bp and 344 bp amplicons, *C. coli* containing samples are expected to produce the 1062 bp and 500 bp amplicons and samples which are positive for both *C. jejuni* and *C. coli* are expected to produce all 3 amplicons (1062 bp, 500 bp and 344 bp). A single 1062 bp amplicon would be indicative of the presence of other bacteria. As shown in Figure 2.5, the multiplex PCR assay exhibited a detection limit of 10^7 cfu per gram of cecal or fecal sample.

As a second set of experiments, we assessed 53 of our samples for the presence of *Campylobacter* using this multiplex PCR approach. As shown in Figure 2.6, this molecular-based method detected *Campylobacter* in only 1 of the 6 samples known to be *Campylobacter* positive by the culture-based approach. In addition, 5 out of the 53 samples tested did not yield a successful PCR reaction (marked by the absence of the 1062 bp band), indicating a 9.4% failure rate. To note, the sample found to be positive by this multiplex PCR

Figure 2.5. Detection limit of *C. jejuni* by the multiplex PCR assay of cecal and fecal samples.

Cecal (panel A) and fecal (panel B) samples were spiked with varying CFU/g of *C. jejuni* NCTC11168 to determine the limit of detection (shown by the numbers above the lanes of the agarose gel). Controls for the PCR are shown in panel C: *C. jejuni* NCTC11168 (lane 1), *C. coli* AS43 (lane 2), and blank negative control (lane 3).

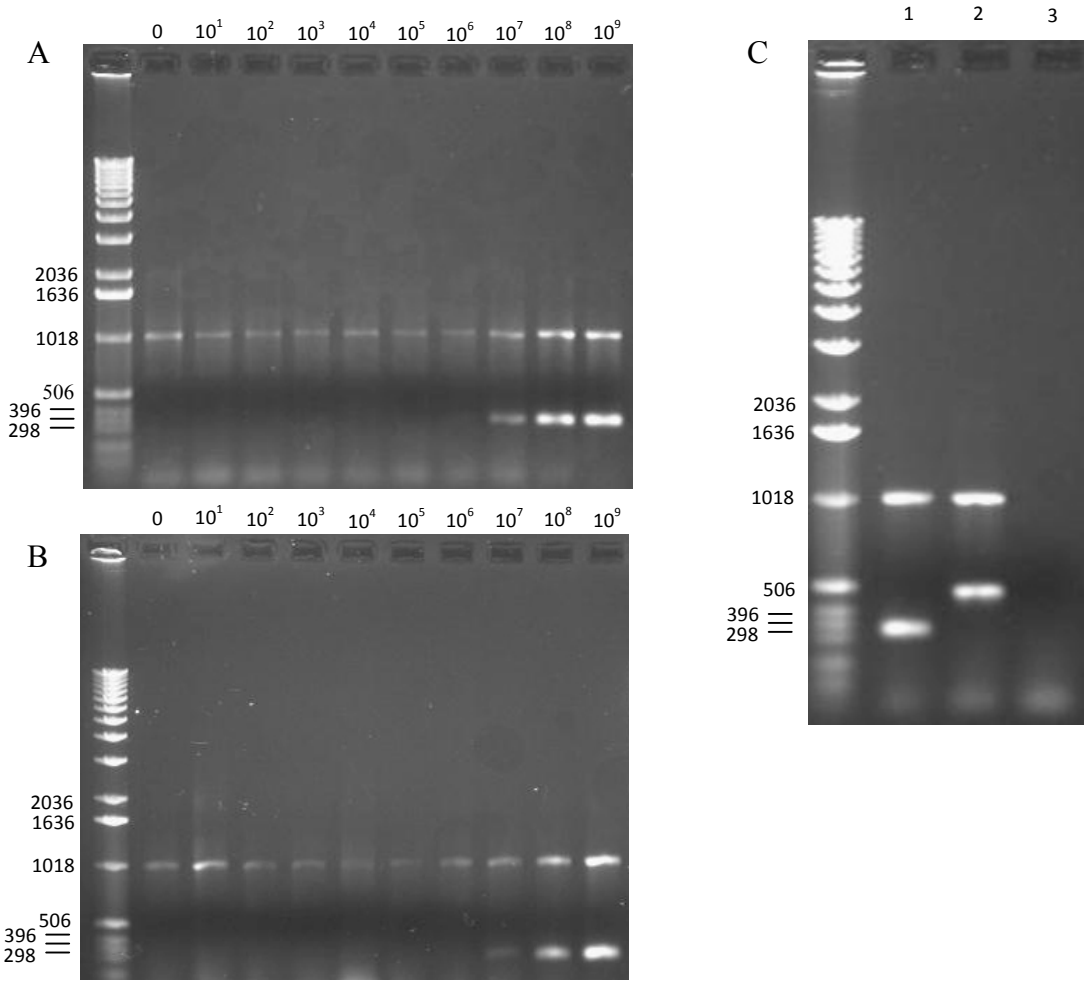
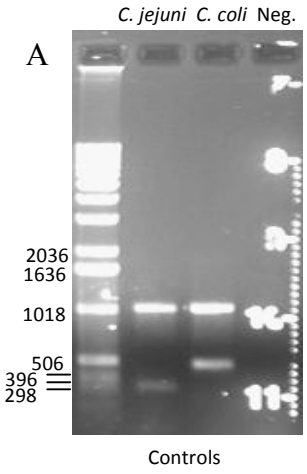
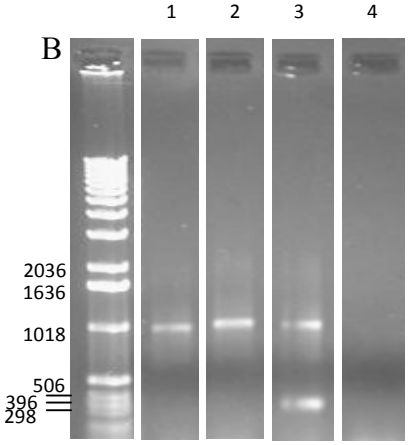

A
0
10^1
10^2
10^3
10^4
10^5
10^6
10^7
10^8
10^9
2036
1636
1018
506
396
298
B
0
10^1
10^2
10^3
10^4
10^5
10^6
10^7
10^8
10^9
2036
1636
1018
506
396
298
C
1
2
3
2036
1636
1018
506
396
298

Figure 2.6. Detection of *Campylobacter* in fecal and cecal samples from farms 1, 2, 3 and 4 by the multiplex PCR assay.

A multiplex PCR was used to detect *Campylobacter* from DNA of the fecal and cecal samples. Panel A shows *C. jejuni* NCTC11168 and *C. coli* AS43 as positive controls and sterile water as a negative control. There were 53 DNA samples tested but panel B shows representative results obtained: true negative (lane 1), false negative (lane 2), true positive (lane 3), failed reaction (lane 4). A true negative is obtained when the sample was negative for *C. jejuni* by culturing and multiplex PCR whereas a false negative is obtained when C. jejuni was recoverd by culture methods but not detected by the multiplex PCR assay. A true positive occurs when both the culture methods and the multiplex PCR assay detects *C. jejuni* in the sample. Our multiplex PCR assay detected *C. jejuni* in only 1 of the 6 samples known to be *Campylobacter* positive by the culture-based approach. In addition, 5 out of the 53 samples tested did not yield a successful PCR reaction.


C. jejuni
C. coli
Neg.
A
2036
1636
1018
506
396
298
Controls


1
2
3
4
B
2036
1636
1018
506
396
298

assay corresponded to the sample with the highest concentration of *C. jejuni* (10^8 cfu/g, Table 2.2). This data suggests that the multiplex PCR assay is less sensitive than the culture-based method and generates a high rate of false negative results (83.3%).

2.3.3 *flaA* typing and genomotyping

The *Campylobacter* isolates from the ceca, fecal and water samples of the positive flocks were characterized by *flaA* typing. Interestingly, *flaA* typing indicated that the same strain of *Campylobacter jejuni* was present in the water distribution system and in the fecal and cecal samples of one of the flocks from farm 4 (samples collected on October 17, 2009; Table 2.2) suggesting that water-borne *Campylobacter* might have been a source of *Campylobacter* for broiler colonization. The presence of the same strain in the drinking water as in the flock was further assessed by whole genome microbial comparison using a previously constructed *C. jejuni* NCTC 11168 microarray. The strains isolated from the water, the cecal and fecal samples were denoted *C. jejuni* C9, C20 and C4 respectively. The genotypic identity of these 3 isolates was further assessed by competitive hybridization of genomic DNA isolated from *C. jejuni* C9 to the array with genomic DNA isolated from *C. jejuni* C20 or C4. Each competitive hybridization experiment was performed in duplicate. Following normalization, the $\log_2$ ratio of the microarray data was statistically analyzed using the empirical Bayes method (Long et al., 2001). The volcano plots for each co-hybridization experiment are shown in Figure 2.7. With a *P* value below 10^{-6} and a fold change threshold of 2, no significant difference could be identified between the 3 strains, indicating that these strains are highly similar (see panels A and B in Figure 2.7).

To further characterize the genotype of the *C. jejuni* isolate obtained from the drinking water, we co-bydridized the genomic DNA from strain *C. jejuni* C9 and *C. jejuni*

NCTC 11168 on the array. The microarray was analyzed as described above and by using the GACK software (Kim et al., 2002). Figure 2.7 (panel C) represents the volcano plot of the microarray data. The asymmetry of the data distribution indicates the genes significantly divergent between the 2 strains. These genes are highlighted in grey and lie above the horizontal *P* value threshold of 10^{-6} and the vertical fold change threshold of 2 (equal to a $\log_2$ of -1). Figure 2.8 represents the complete data set analyzed by GACK on the presence and absence of each gene from C9 with respect to their chromosomal location on the genome of NCTC 11168. The GACK algorithm estimates the probability that any given gene is conserved or present using the shape of the signal distribution. This method has been proposed to be more reliable than approach relying on threshold values to identify which genes are present or absent (Kim et al., 2002). Nevertheless, processing of our microarray data with GACK revealed a near-identical list of absent or divergent genes as compared to the empirical threshold method described above, cross-validating our data analysis. Overall, GACK classified 82 genes as absent or divergent with high certainty in C9 as compared to the reference strain NCTC 11168, representing approximately 4.9% of all genes. As shown Figure 2.8, many of the divergent genes in C9 were found to co-localize in one specific chromosomal region. This region was previously characterized as a placiticity zone in *C. jejuni* genomes and contains the capsule biosynthesis cluster (Cj1421c to Cj1449c). Table 2.3 lists the genes absent in C9 as compared to NCTC 11168.

Genes related to molybdate metabolism, such as *bioF* and *modABC*, were found to be highly likely divergent in *C. jejuni* C9. Notably, the putative type I restriction enzyme locus (Cj1549c to Cj1553c) was also highly likely divergent in *C. jejuni* C9. Other divergent clusters include Cj1721c to Cj1729c, Cj0260c to Cj265c, Cj0809c to Cj0816, and Cj1287c to Cj1298.

Figure 2.7. Bayesian *P* values and fold change of genes in *C. jejuni* from the co-hybridization microarray experiments.

The following co-hybridization experiments were performed: *C. jejuni* C4 and *C. jejuni* C9 (panel A); *C. jejuni* C20 and *C. jejuni* C9 (panel B); *C. jejuni* C9 and *C. jejuni* NCTC11168 (panel C). The red dots represent genes that are below a *P* value of 10^{-6} and a fold change greater than 2. These genes are likely divergent in *C. jejuni* C9 when compared to *C. jejuni* NCTC11168 (panel C). No significant difference could be identified between C4, C9 and C20 (panels A and B).

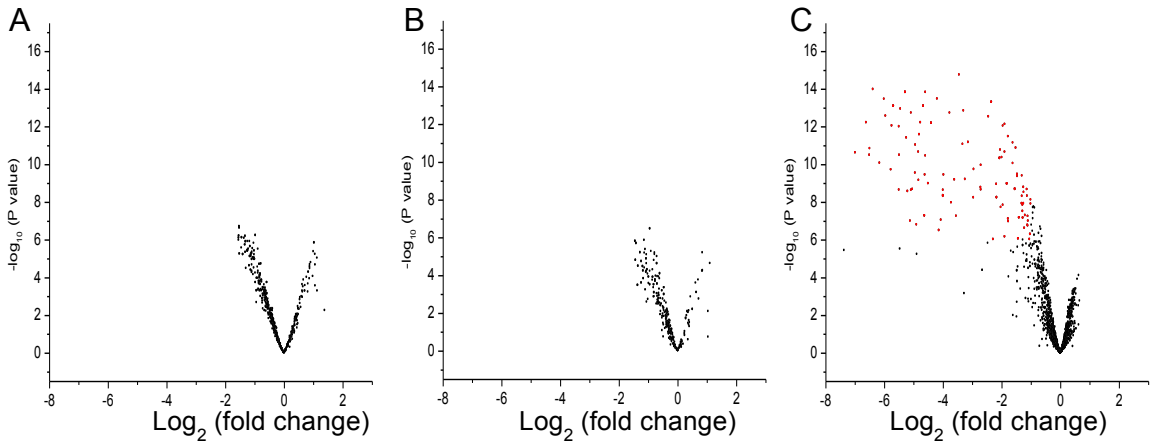

A
B
C
-log10 (P value)
Log2 (fold change)
16
14
12
10
8
6
4
2
0
-8
-6
-4
-2
0
2

Figure 2.8. Presence/absence of each gene from *C. jejuni* C9 with respect to their chromosomal location on the genome of *C. jejuni* NCTC 11168 as a graded scale output.

The graded scale depicts the likelyhood that a gene is divergent or present in *C. jejuni* C9 compared to *C. jejuni* NCTC11168 from the GACK analysis. The yellow colour represents genes that are divergent and the blue colour represents genes that are present. The intensity of the colour is proportional to the likelihood of presence (blue) or divergence/absence (yellow).

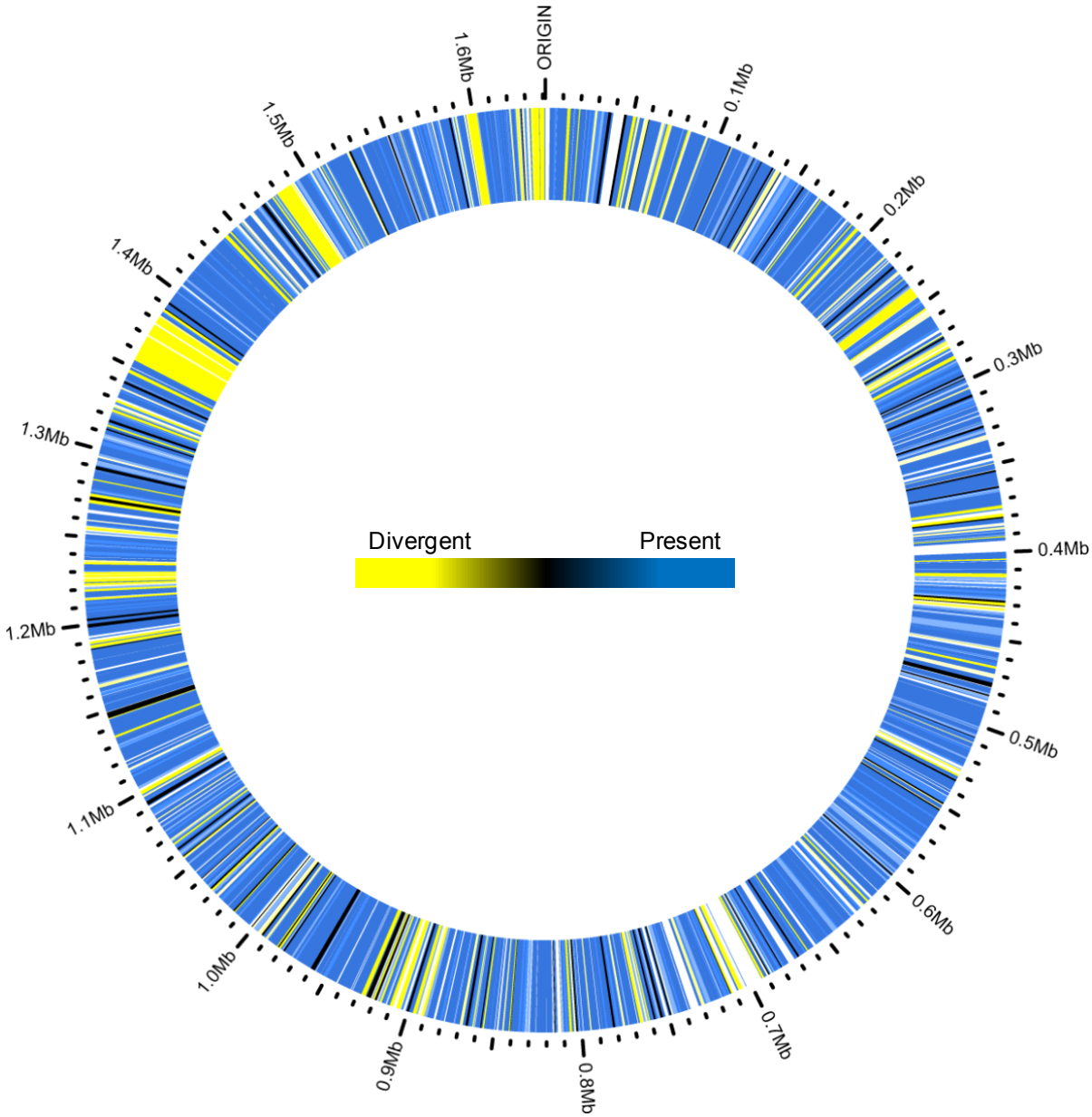

ORIGIN
0.1Mb
0.2Mb
0.3Mb
0.4Mb
0.5Mb
0.6Mb
0.7Mb
0.8Mb
0.9Mb
1.0Mb
1.1Mb
1.2Mb
1.3Mb
1.4Mb
1.5Mb
1.6Mb
Divergent
Present

Table 2.3. Genes that are highly likely absent or divergent in *C. jejuni* C9 grouped according to functional annotation.

Genes absent in *C. jejuni* C9 were grouped by functional annotation.

.

Functional annotation	List of absent genes in *C. jejuni* C9									
Amino acid biosynthesis	*dapB*	*metA*	*metB*							
Antibiotic resistance	Cj1297									
Biosynthesis of cofactors, prosthetic groups and carriers	*folE*	*bioF*	*ribA*							
Broad regulatory functions	Cj1556									
Cell envelope	Cj0967	*omp50*	*porA*	*pseB*	Cj1376	Cj1421c	Cj1422c	*hddC*	*gmhA2*	*hddA*
	Cj1427c	*fcl*	*rfbC*	*hddC*	Cj1432c	Cj1434c	Cj1438c	Cj1440c	Cj1442c	*kpsT*
	kpsM	Cj1544c	Cj1668c	Cj1677	Cj1721c	Cj1723c	Cj1725	*flgE2*		
Central intermediary metabolism	*ald'*	*kfiD*								
Chemotaxis and mobility	Cj0262c									
Conserved hypothetical proteins	Cj0008	Cj1547	*rloH*							
Detoxification	*katA*									
Energy metabolism	Cj0265c	*frdA*								
Fatty acid biosynthesis	*accC*									
Miscellaneous	Cj0264c	Cj0737	Cj1426c	Cj1435c	Cj1437c					
Protein and peptide secretion	Cj0975									
Purines, pyrimidines, nucleosides and nucleotides	*purL*									
Signal transduction	*radA*	*hsdR*	*hsdS*	*hsdM*						
Transport/binding proteins	*zupT*	*modB*	*modA*	*cfrA*	Cj0987c	Cj1097				
Unknown	Cj0055c	Cj0056c	Cj0069	Cj0569	Cj0814	Cj0815	Cj0816	Cj0859c	Cj1295	Cj1305c
	Cj1429c	*mloB*	Cj1679							

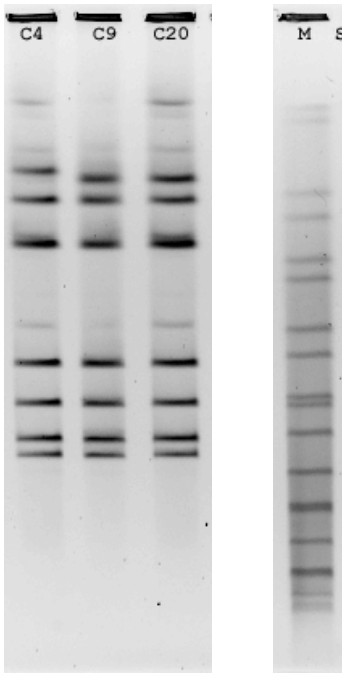
2.3.4 *Campylobacter* pulse field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) is a widely accepted method for the molecular typing of Gram-positive and Gram-negative bacteria (Chang and Chui, 1998). The PFGE was carried out on *C. jejuni* C4, C9 and C20 as an additional method to assess whether these strains are identical (Figure 2.9). Other *Campylobacter* strains were used as controls in the PFGE (data not shown). *C. jejuni* C4, C9 and C20 have similar banding patterns. This kind of PFGE banding pattern similarity would not be observed with strains of *Campylobacter* that were genetically different (PFGE results from other *Campylobacter* strains not shown). Additionally, a 40 gene multiplex PCR was performed on these 3 strains of *C. jejuni*, and they had exactly the same profile suggesting there are no differences between these strains (C. Carrillo, pers comm.).

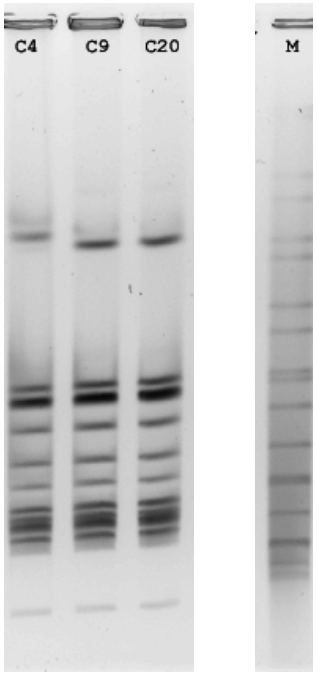
Figure 2.9. Pulse field gel electrophoresis of *C. jejuni* C4, C9 and C20.

Pulse field gel electrophoresis was used to type strain C4, C9 and C20 in order to determine whether or not they are identical. *SmaI* (panel A) and *KpnI* (panel B) were used in the restriction digestion step. (M) indicates the DNA marker.

A



B



2.4 Discussion

The objective of our study was to determine the incidence of *Campylobacter*-positive flocks at the farm level in Eastern Ontario and to assess if contamined-water could be a significant risk factor for *Campylobacter*-positive flocks.

Campylobacters are commonly isolated using culturing methods. This is achieved by plating serial dilutions of a sample onto a *Campylobacter* selective agar such as Karmali agar followed by incubation under microaerophilic conditions at 37°C or 42°C. Molecular methods, such as PCR, can also be used to detect *Campylobacters*. It takes at least 2 days to detect the presence of *Campylobacter* by culturing methods whereas it could take about 6 hours for the multiplex PCR to detect *Campylobacter* in an environmental sample. According to our results, culturing methods are more reliable in comparison to the multiplex PCR assay at detecting the presence of *Campylobacter* in a fecal or cecal sample because *Campylobacter* was recovered more often by culturing methods. Also, the multiplex PCR assay exhibited a 83.3% rate of false negative likely due to contaminants or insufficient *Campylobacter* DNA in the extracted sample and therefore the approach is not recommended to detect *Campylobacter* in fecal or cecal samples.

The incidence of *C. jejuni* positive flocks was found to be 16.7% in our study. A Quebec study showed a 40% prevalence of *C. jejuni* on poultry farms (Nadeau et al., 2002). Our finding was surprising because we had expected the incidence to be more similar to the Quebec study. The Quebec study had sampled over 2000 ceca on 57 farms (Nadeau et al., 2002) whereas we only collected 114 samples on 4 farms. Perhaps with a greater amount of farms and more frequent sampling, especially during the summer when the prevalence of *C.*

jejuni is known to peak in other studies (Miller et al., 2004; Nylen et al., 2002; Wedderkopp et al., 2001), the prevalence may increase.

C. jejuni can spread by contaminated water (discussed in Section 3.1). A Belgium study found that in some instances, broilers were colonized with the same *C. jejuni* genotype detected from the drinking water nipple and from water puddles (Messens et al., 2009). In our study, our results suggest that *C. jejuni* might have entered the flock of October 2008 by contaminated water. Indeed, the same *C. jejuni* strain was isolated from water, cecal and fecal samples as demonstrated by *flaA* typing, genomotyping and PFGE approaches.

The genomotyping approach was also used to characterize the genome of the *C. jejuni* isolates originating from the drinking water, denoted strain C9. The results show that *C. jejuni* C9 is genetically different compared to *C. jejuni* NCTC11168. The *modABC* genes in *C. jejuni* are responsible for uptake of molybdate and tungstate (Smart et al., 2009). The genomotyping results show that *modABC* genes were absent or divergent in *C. jejuni* C9. Since molybdate is an important co-factor in many enzymes (Smart et al., 2009), it is likely that *C. jejuni* C9 possesses divergent *modABC* genes, rather than these genes being absent. However, PCR and sequencing of that locus need to be performed in order to validate this speculation.

Bacteria require iron as a micronutrient for growth. *Campylobacter* acquires ferric iron by the use of several exogenous siderophores (Miller et al., 2009). Siderophores are small molecules that have a high affinity for iron. Ferri-enterobactin (a siderophore-iron complex) uptake requires both the outer-membrane receptor protein *cfrAB* (Holmes et al., 2005; Palyada et al., 2004; Xu et al., 2010) and the inner-membrane ABC transporter system *ceuBCDE* (Palyada et al., 2004). The *cfrAB* genes are essential for chick colonization of *C. jejuni* NCTC11168 (Palyada et al., 2004; Xu et al., 2010). The *cfrAB* genes were found to be

divergent/absent in C9. *C. jejuni* 81-176 also lack these genes (Hofreuter et al., 2006) but has the ability to colonize chicks (Naikare et al., 2006) suggesting that these genes are not essential for chick colonization in all strains of *C. jejuni*. In addition to siderophores, *C. jejuni* can also exploit host iron sources, such as haem compounds using *chuABCDZ* (Ridley et al., 2006) and transferrin using Cj0173c–Cj0178 (Miller et al., 2008). Finally, under anaerobic conditions similar to the gastrointestinal tract, iron is present under the form of ferrous ion and thus would be transported via the *feoB* protein (Naikare et al., 2006). All these genes were found to be present in *C. jejuni* C9 confirming a functional uptake systems in *C. jejuni* C9.

In conclusion, culture methods were found to be a reliable way to isolate *Campylobacter*. The incidence of *C. jejuni* positive flocks is 16.7% for Eastern Ontario. Identification of *C. jejuni* isolates from water, feces and cecal samples showed that *C. jejuni* entered the flock of October 2008 by contaminated water confirming that contaminated water could be a risk factor for *Campylobacter* positive flocks. Characterization of *C. jejuni* C9 reveals the presence of the iron metabolism/uptake system and possibly the absence/divergence of the molybdate and tungstate transport system.

CHAPTER 3. *ACANTHAMOEBA* AS AN ENVIRONMENTAL RESERVOIR OF *CAMPYLOBACTER JEJUNI* ON POULTRY FARMS

3.1 Introduction and research objectives

Many studies have been conducted with regards to understanding how *Campylobacter* is spreading and its mode of transmission in broiler chickens (reviewed in (Sahin et al., 2002)). Although the routes of transmission of *C. jejuni* are likely to be complex with many possible sources for a given poultry farm, there are more compelling evidence that horizontal transmission is the most probable source of poultry infection by *C. jejuni*, rather than vertical transmission (Sahin et al., 2002). For example, in an Icelandic study, researchers have taken eggs from *C. jejuni* positive grandfather flocks and found that progenies were never colonized by *C. jejuni* when reared in a biosecure facility (Callicott et al., 2006). However, when the progeny flocks were moved to a less biosecure facility, they became colonized with *C. jejuni*. In addition, the alleles found in the grandparent flock and the progeny flocks were not the same indicating the absence of vertical transmission of *C. jejuni* in broilers (Callicott et al., 2006).

Depending on the study performed and its location, horizontal transmission involves potential sources such as old litter (Kazwala et al., 1990), contaminated footwear and clothing of farmers (Kazwala et al., 1990), untreated drinking water (Kazwala et al., 1990), other farm animals (such as cattle, sheep, pigs (Ogden et al., 2009)), wild life species such as waterfowl (Van Dyke et al., 2010), or insects (such as flies (Sproston et al., 2010) and beetles (Templeton et al., 2006; Wales et al., 2010)). Broiler prevalence were found to peak in summer and autumn (July, August and September) and were found to be lowest in the colder months (January, February, March and April) in a Danish study (Wedderkopp et al., 2001).

In a study done in Iceland, researchers have found that higher broiler prevalence is related to higher ambient temperatures with an underlying assumption that higher temperatures promotes higher activities of flies in chicken barns (Guerin et al., 2008). Thus, warm ambient temperature has been described as a factor that might contribute to the seasonal variation of broiler prevalence. In short, the transmission of *C. jejuni* and how *C. jejuni* colonizes new poultry flocks is complex.

Amoeba species belonging to the genus *Acanthamoeba* are ubiquitous organisms that can be found free-living in water and soil and graze on bacteria as a food source (de Moraes and Alfieri, 2008). It is well documented that *Acanthamoeba* can sometimes harbor pathogenic bacteria like *Legionella* (Holden et al., 1984; Schuster and Visvesvara, 2004). Using microscopy and molecular methods, a study found that *Acanthamoeba* are amongst the diverse protozoa that live in the drinking water of poultry farms (Bare et al., 2009). *Acanthamoeba* have two forms: the trophozoite form which is the active feeding form resembling a cell and the cyst form resembling spores. Some recent studies have demonstrated that *Acanthamoeba* can take up and harbor *Campylobacter* (Axelsson-Olsson et al., 2005; Snelling et al., 2005). *C. jejuni* is a microaerophillic bacterium and cannot propagate planktonically under atmospheric oxygen conditions and below 31ºC (Hazeleger et al., 1998) which are conditions found in the drinking water of poultry houses (Snelling et al., 2008). *Campylobacter* was found to be able to persist for a longer period of time in co-culture with *Acanthamoeba* at lower temperatures ranging from 4ºC to 10ºC compared to planktonic *Campylobacter* in the same conditions (Axelsson-Olsson et al., 2005). At 37ºC, *Campylobacter* starts to grow in co-culture with *Acanthamoeba* (Axelsson-Olsson et al., 2007). Moreover, it has been shown that *Campylobacter* internalized within *Acanthamoeba* are able to colonize chickens (Snelling et al., 2008) suggesting that *Acanthamoeba* could act

as a significant source of *Campylobacter* infection. Furthermore, *Acanthamoeba* has also been found to increase the survival of *C. jejuni* under acidic conditions (Axelsson-Olsson et al., 2010b). Interestingly, at pH 4 to pH 5, there was an observed increase in *Campylobacter* motility as well as increase in adhesion/internalization of *Campylobacter* into *Acanthamoeba* (Axelsson-Olsson et al., 2010b). Since acidified water is used in poultry rearing practices, authors state that these findings could highlight the importance of protozoa as a potential epidemiological pathway of *Campylobacter* infection in broilers (Axelsson-Olsson et al., 2010b). Finally, internalized *Campylobacter* were also shown to be more resistant to free chlorine than planktonic organisms at the temperature at which reared broilers are maintained (25 ºC) (Snelling et al., 2005; Snelling et al., 2008). These properties may explain previous observations that *C. jejuni* colonization of broilers remain unaffected by chlorination of the broiler drinking water (Stern et al., 2002).

Campylobacter has been routinely identified in environmental surface water, drinking water and private wells (St-Pierre et al., 2009; Van Dyke et al., 2010). While *Acanthamoeba* has never been co-cultivated from the same water sample, *Acanthamoeba* and *Campylobacter* are likely to be commonly found together in water and thus could interact with one another.

Altogether, evidence from these studies suggests that *Acanthamoeba* species that live in the broiler water supplies could protect *Campylobacter* from the stressful environment of atmospheric oxygen and water chlorination treatments.

Acanthamoeba could be acting as an environmental reservoir protecting *Campylobacter* and allowing them to persist at a low level that is difficult to detect. This persistence could make *Acanthamoeba* a potential vector that allows *Campylobacter* to spread through water sources such as rivers and streams until a compatible host, such as

poultry, is encountered. The host drinks contaminated water and *Campylobacter* is able to colonize at a high level in its new host and shed anew in the feces. The minimum dose of *Campylobacter* required for colonization may be as low as 35 CFU via oral routes (Stern et al., 1988).

To determine whether *Acanthamoeba* could act as an environmental reservoir of *Campylobacter*, *Acanthamoeba* were isolated from the water sources of the poultry houses at the same time that *Campylobacter* isolation was performed (described in Chapter 2). *C. jejuni* NCTC11168 was used to bait and amplify *Acanthamoeba* before introducing them into axenic culture.

Previous co-culture studies have used type strains rather than environmental strains. The main goal of our study was to demonstrate that environmental strains of *Acanthamoeba* can actually internalize and amplify environmental strains of *Campylobacter*, isolated from the same poultry farm.

3.2 Methods

3.2.1 Sample collection and farms

Samples were collected as described in Section 2.2.1.

3.2.2 *Acanthamoeba* isolation and identification

3.2.2.1 Baiting and initial isolation

To isolate amoebae from water and filter samples, the same filtering apparatus was used as the step to isolate *Campylobacter* from water (see Chapter 2). The filtering apparatus consisting of a sterile funnel placed on top of a sterile 1 L Erlenmeyer side arm-flask was connected to vacuum by rubber tubing. A sterile 47 mm in diameter nitrocellulose filter with 5 μm pore size (Millipore) was placed on the funnel. The water/filter sample was poured into the funnel. The vaccuum was turned on to pass the water through the 5 μm filter. With sterile forceps, the filter was dropped in a Falcon tube containing 5 mL of Page's amoeba saline (PAS). The tube was vortexed for 10 sec to help dislodge cells from the filter into the solution. A volume of 500 μL was removed and directly plated on a Non-nutrient (amoeba saline) agar plate (NNA) (http://www.ccap.ac.uk/media/pdfrecipes.htm), in 5 replicates, with approximately 10^9 CFU of *Campylobacter jejuni* NCTC11168, as bacterial bait. The *C. jejuni* NCTC11168 bait was grown from an overnight culture in a bi-phasic MH, in a microaerophillic environment, and the CFU was determined by OD_{600} measurement and comparison to a standard curve. The NNA plates were parafilmed and incubated upright at room temperature for 3 to 4 weeks.

3.2.2.2 Amoeba colony purification, axenic culturing, and maintenance

To purify amoebae from the NNA plates, the plates were checked for amoebic cysts under light microscopy. Cloudy white areas on the NNA plate are usually indicative of the presence of amoebic cysts. An agar plug was taken where amoebic cysts were found. The agar plug was transferred to a new NNA plate containing a lawn of live *C. jejuni* NCTC11168. The carpet of bacteria allows amoebae to walk down the carpet while grazing over time. The plates were parafilmed and incubated at room temperature for 3 to 4 weeks. After this time, the plates were checked under light microscope for amoebic cysts that have ventured outside of the live bacterial carpet. These amoebic cysts are suitable for axenic culturing. To introduce the amoebae into axenic culture, the agar where these cysts were located was taken and deposited in an Nunc cell culture flask with 8 mL of Proteose peptone yeast extract glucose (PYG) media (Rowbotham, 1983). The Nunc cell culture flasks were incubated at room temperature in the dark for 3 to 4 weeks. Flasks were checked for cell proliferation under inverted microscope. For maintaining the axenic culture, 1 mL of the grown culture was inoculated into 9 mL of fresh PYG media in a new cell culture flask. Cultures were re-transferred this manner every 4-6 weeks.

3.2.2.3 Amoeba DNA extraction and genotyping

To extract amoebal DNA, about 1 mL of 1 month old amoebae cultured axenically in PYG was placed in a 2.0 mL microfuge tube. The amoebae were pelleted by centrifugation at 13,000 x g for 10 min. DNA was extracted using the UltraClean Soil DNA extraction kit (MO BIO) according to the manufacturer's instructions.

Amoebae were genotyped at the 18S rDNA locus similarly to the protocol in (Aguilera et al., 2006), using primers Euk1a and EukBr (Table 3.1). For the PCR master

mix, 0.2 mM dNTP's, 0.4 μM forward primer, 0.4 μM reverse primer, 1X PCR buffer minus Mg, 1.5 mM $MgCl_2$, 2.5 U Taq DNA polymerase enzyme, and 1 to 5 ng of genomic DNA were mixed in sterile water totalling 12.50 μL per reaction. The PCR reaction was run in a PTC-100 thermal cycler (MJ Research Inc.). The following profile was used to amplify the 18S rDNA genes: 95°C for 2 min (initial denaturation), then 35 cycles at 94°C for 45 s (denaturation), 50°C for 30 s (annealing), 72°C for 2 min (extension), then 72°C for 5 min (final extension). Completed PCRs were analysed by 0.8 % agarose gel electrophoresis. Sequencing (Big Dye Terminator) was done using primers Euk1a, EukBr. The sequences obtained were aligned by MAFFT (Katoh et al., 2009). From this alignment, PrimaClade (Gadberry et al., 2005) was used to design internal primers Euk1a471 and EukBr585 in order to sequence the entire length of the 18S rDNA gene. The sequences were assembled in the Seqman II module of Lasergene 8 (DNA Star). BLAST search of the full length 18S sequences were performed against GenBank.

3.2.2.4 Determination of *Acanthamoeba* ribotypes by phylogenetic analysis

Acanthamoeba identification, based on ribotypes, was done according to (Stothard et al., 1998). The data set from (Stothard et al., 1998) was downloaded from GenBank in FASTA format with the sequence data from this project added at the end of the file. The 18S sequences were aligned with MAFFT (Katoh et al., 2009). The alignment, in FASTA format, was loaded into BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html), ambiguously aligned positions were deleted and 5' and 3' ends of the alignment were trimmed. The edited alignment was then imported into MEGA v.4.0 (Tamura et al., 2007) where phylogenetic analysis was performed. The Neighbour Joining (NJ) method was used with the following parameters: 1000 bootstrap replicates as a test of phylogeny, gaps and

Table 3.1. List of all primers used in this chapter.

Name	Sequence (5' to 3')	Direction	Usage	Reference
Euk1a	CTGGTTGATCCTGCCAG	Forward	18S PCR/Sequencing	(Aguilera et al., 2006)
EukBr	TGATCCTTCTGCAGGTTCACCTAC	Reverse	18S PCR/Sequencing	(Aguilera et al., 2006)
Euk1a471	ACACGGGGAGGTAGTGACAA	Forward	18S Sequencing	This study
EukBr585	ATTTAGCAGGTTAAGGTCTCGTTC	Reverse	18S Sequencing	This study

missing data treated as complete deletion, p-distance nucleotide model, substitutions option set to "both transversions and transitions", homogenous pattern amongst lineages, and uniform rates amongst sites.

3.2.2.5 *Acanthamoeba* under the light microscope

Axenic cultures of *Acanthamoeba* 2-4 weeks old were checked under the light microscope (Nikon) for genus specific morphology such as double walled cysts and long spike shaped pseudopodia called acanthapodia. The wet mount was prepared by pipetting a drop of about 20 µL of the 2-4 weeks old *Acanthamoeba* culture on a glass slide followed by coverslip deposition on the slide.

3.2.3 Internalization of *C. jejuni* in *Acanthamoeba*

3.2.3.1 Gentamycin protection assay

To measure the internalization of *C jejuni* in *Acanthamoeba*, a gentamycin protection assay was used. *Acanthamoeba* from stock axenic culture was inoculated into 9 mL of PYG and allowed to multiply over 3 to 4 days in darkness at room temperature. Next, the PYG media was poured out and washed once with 3 mL of PAS. Next, 3 mL of PAS was added. The cells attached to the bottom of the cell culture flask were scraped using a sterile cell scraper. The suspended cells were transferred to a 50 mL Falcon tube. *Acanthamoeba* (*A. castellanii* Neff and *Acanthamoeba* sp. A18), in trophozoite form, were counted using a hemocytometer three times and the cell count was averaged to determine the cell concentration. *C. jejuni* (*C. jejuni* NCTC11168 and *C. jejuni* C9) was grown overnight at 37°C microaerophillically in a bi-phasic system and the bacteria concentration was determined by OD_{600} measurement and comparison to a standard curve. The *Acanthamoeba*

culture was diluted to 2.0 x 10^8 cells/mL and the bacterial culture was diluted to 2.0 x 10^9 CFU/mL. One mL of the *C. jejuni* culture at 2.0 x 10^9 CFU/mL was centrifuged at 13,000 x g for 5 min and the MH broth was removed and replaced with PAS media. In a sterile 24 well plate, 500 µL of amoebae and 500 µL of *C. jejuni* suspension were mixed, totalling 1 mL per well, giving a ratio of 1:10 *Acanthamoeba*:*Campylobacter* (same ratio used by (Snelling et al., 2008)). This was performed 3 times, representing 3 technical replicates. In separate 24 well plates, controls of *C. jejuni* alone and controls of amoebae alone were seeded with the addition of 500 µL of PAS in order to obtain a final volume of 1 mL per well.

All of the prepared 24 well plates were incubated at room temperature in the dark over 3 hours and/or 23 hours. After 3 hours and/or 23 hours, the co-culture in each well was washed once with PAS. Then, PAS containing gentamycin (at final concentration of 250 µg/mL per well) was added. The plate was manually shaken for a few seconds to homogenize. Incubation was continued for one more hour. The PAS containing gentamycin was removed and the co-culture wells were washed with 1 mL of fresh PAS. Next, 1 mL of fresh PAS was added to dislodge *Acanthamoeba* cells with internalized *Campylobacter*. This culture was transferred to a sterile 2 mL microfuge tube and was serially diluted up to 1:1000 in 1 mL of PAS. Then, 200 µL of the undiluted, 1:10 and 1:1000 dilutions were spread on MH agar and incubated under microaerophilic conditions at 37°C for 48-72 hours followed by colony counting.

The gentamycin protection assay was also done in PYG media following the same protocol as above.

A two tailed t-test assuming equal variance was performed to compare *C. jejuni* internalization between the 4 hours and 24 hours time point in PAS and PYG media.

3.2.3.2 Validation of the gentamycin assay by microscopy

To validate that the gentamycin protection assay was effective at eliminating external *Campylobacter* bound to *Acanthamoeba*, co-cultures of *Acanthamoeba* and *C. jejuni* were set up as described in section 3.2.3. Three wells of co-cultures were left to incubate at room temperature for 4 hours with no washes. Another three wells of co-culture were treated with gentamycin and washed according to section 3.2.3 but without serial dilution and plating on MH agar. Pictures of both co-cultures were taken with a camera mounted on an inverted microscope to determine whether the gentamycin treatment and the washes were effective at eliminating external *C. jejuni*.

To ease visualization of internalized bacteria, *C. jejuni* was stained with safranin. The stock safranin solution was prepared by dissolving 2.5g of safranin in 100 mL of 95% ethanol. To make the working safranin solution, 1 volume of the stock was added to 5 volumes of water followed by syringe filtration through 0.2µm filter. To stain *C. jejuni*, 500 µL of 4.0 x 10^9 CFU/mL of *C. jejuni* NCTC11168 grown in MH broth (bi-phasic) was centrifuged at 4,000 x g for 5 min in a 1.5 mL microfuge tube. The MH broth was removed and 500 µL of working safranin solution was added to the culture. The culture was washed three times with 1 mL of PAS. After these washes, 500 µL of the stained bacteria was added to 500 µL culture of *Acanthamoeba* in PAS in a 24 well plate in 2 replicates. In addition, to test whether safranin intrinsically stains the *Acanthamoeba* cells, an *Acanthamoeba* culture in PAS was washed once with 500 µL of diluted safranin solution (this solution was 3 times less concentrated than the working safranin solution) as a control for the dye.

3.2.4 Persistence assay

To test the persistence of *C. jejuni* in co-culture with *Acanthamoeba* compared to planktonic *C. jejuni*, the cultures of *C. jejuni* and *Acanthamoeba* were grown, diluted and mixed as described in Section 3.2.3. Two temperatures were tested: 4°C and 25°C (room temperature). Both PYG and PAS media were used to test at each temperature condition. Twenty four cultures of *C. jejuni* mixed with *Acanthamoeba* were prepared in 24 multi well cell culture plates. *Acanthamoeba* controls and *C. jejuni* alone controls were also prepared in 24 wells. The plates were covered with aluminum foil and left to incubate at either 4°C (placed in the fridge) or 25°C (placed on the bench).

C. jejuni viability was measured at day 1, 2, 3, 4, 7, 10, 17, and every 7 days from this point onward. To determine *Campylobacter* viability, the volume of one entire well from the 24 cell culture plate, for each condition and control, was spread on MH agar and then placed at 37°C under microaerophillic conditions for 3 days. *C. jejuni* growth was either deemed positive (a bacterial lawn was observed or multiple single colonies were observed) or negative (bacterial growth was absent). A two tailed t-test assuming equal variance was performed to compare *C. jejuni* survival between various conditions.

3.2.5 Growth assay

To measure the growth potential of *C. jejuni* in co-culture with *Acanthamoeba*, 1 mL of *Acanthamoeba* from stock axenic culture (Section 3.2.2), were inoculated into 9 mL of PYG and allowed to multiply over 3 to 4 days in darkness at room temperature. Next, the PYG media was replaced with 9 mL of fresh PYG and inoculated with a 1 ml suspension of *C. jejuni* at a concentration of 1.0×10^2 CFU/mL in PYG (as detemined by serial dilution on MH-agar plates). Three of such flasks were prepared. In addition, two control flasks were

prepared: *C. jejuni* alone (1 mL of *C. jejuni* added to 9 mL of PYG) and *Acanthamoeba* alone (1 mL of PYG added to 9 mL of *Acanthamoeba* culture). The cap of the flasks was loosely tightened. The flasks were wrapped in aluminum foil and placed in a 37°C incubator in aerobic conditions.

CFU counts were obtained at 24 hours, 48 hours and 72 hours. At each time point one cell co-culture flask was taken out of the incubator. The cells were directly scraped using a cell scraper and 1 mL of the co-culture was serially diluted up to $1:10^8$ dilution. The undiluted sample and selected diluted samples were plated in 3 replicates on MH agar plates and were incubated at 37°C microaerophillically for 3 days. The *C. jejuni* CFU/mL was determined by colony counting. This assay was also done using the PAS media.

3.3 Results

The objective of this study was to characterize the *Acanthamoeba* population in poultry drinking water and to demonstrate that environmental strains of *Acanthamoeba* can internalize and amplify environmental strains of *Campylobacter jejuni*, isolated from the same poultry farm.

3.3.1 *Acanthamoeba*: 18S sequences and phylogenetic analysis

A total of 9 amoeba strains were isolated from Farm 2, Farm 3 and Farm 4 and successfully established in axenic culture (Figure 3.1). Under the light microscope, the specific characteristics of the *Acanthamoeba* genus were observed. As described by Page (Page F.C., 1976), the isolated species were hyaline and produced many slender, tapering, flexible projections called acanthopodia. The cysts were found to be polyhedral or thickly biconvex. The endocyst was polygonal or stellate and the ectocyst was rippled. These morphological characteristics indicate that these amoebae belong to the *Acanthamoeba* genus. These characteristics are shown in Figure 3.2.

Amplification of the genomic DNA extracted from axenic cultures using primers Euk1a and EukBr generated an amplicon of approximately 2000 base pairs (not shown). BLAST analysis of the amplicon sequences indicated a 99% match with existing sequences of *Acanthamoeba*. The phylogenetic analysis of the obtained 18S sequences classified the isolated strains in the clade defined by the T4 ribotype (Figure 3.3).

Figure 3.1. Trophozoites and cysts of *Acanthamoeba* strains isolated from water on farms 2, 3 and 4.

Microphotographs of isolated *Acanthamoeba* strains were taken. Panel a = *Acanthamoeba* sp. A5, b = *Acanthamoeba* sp. A7, c = *Acanthamoeba* sp. A8, d = *Acanthamoeba* sp. A9, e = *Acanthamoeba* sp. A10, f = *Acanthamoeba* sp. A11, g = *Acanthamoeba* sp. A12, h = *Acanthamoeba* sp. A17, I = *Acanthamoeba* sp. A18, j = *Acanthamoeba castellanii* strain Neff. Scale bar = 25 μm.

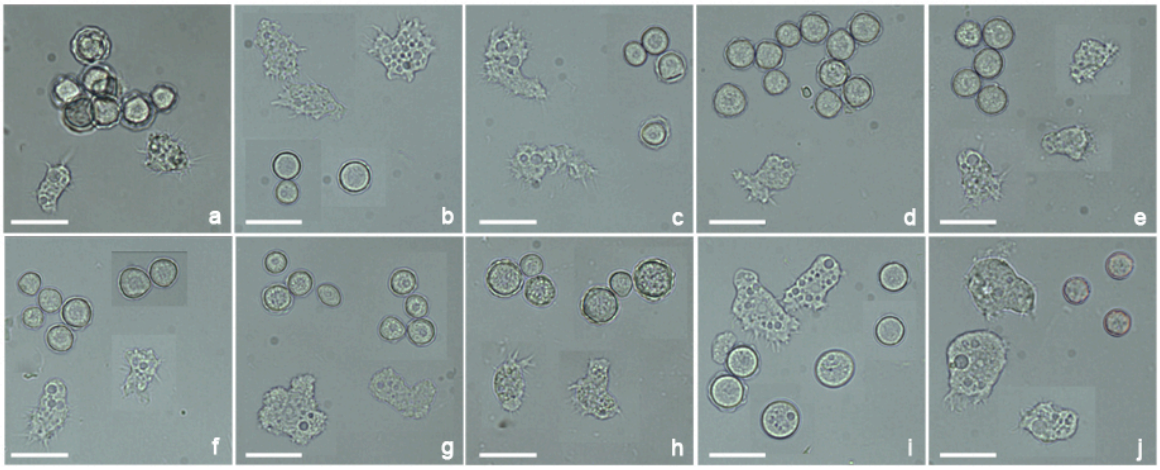

a
b
c
d
e
f
g
h
i
j

Figure 3.2. *Acanthamoeba* morphology.

Acanthamoeba sp. A5 was used as a model to illustrate *Acanthamoeba* genus specific morphology. *Acanthamoeba* cyst form (c) defined by polyhedral shape having double walls: a rippled ectocyst (ec) and a polygonal endocyst (en). *Acanthamoeba* trophozoite form (t) with long and slender tapering extensions called acanthapodia (a). Scale bar = 25 μm.

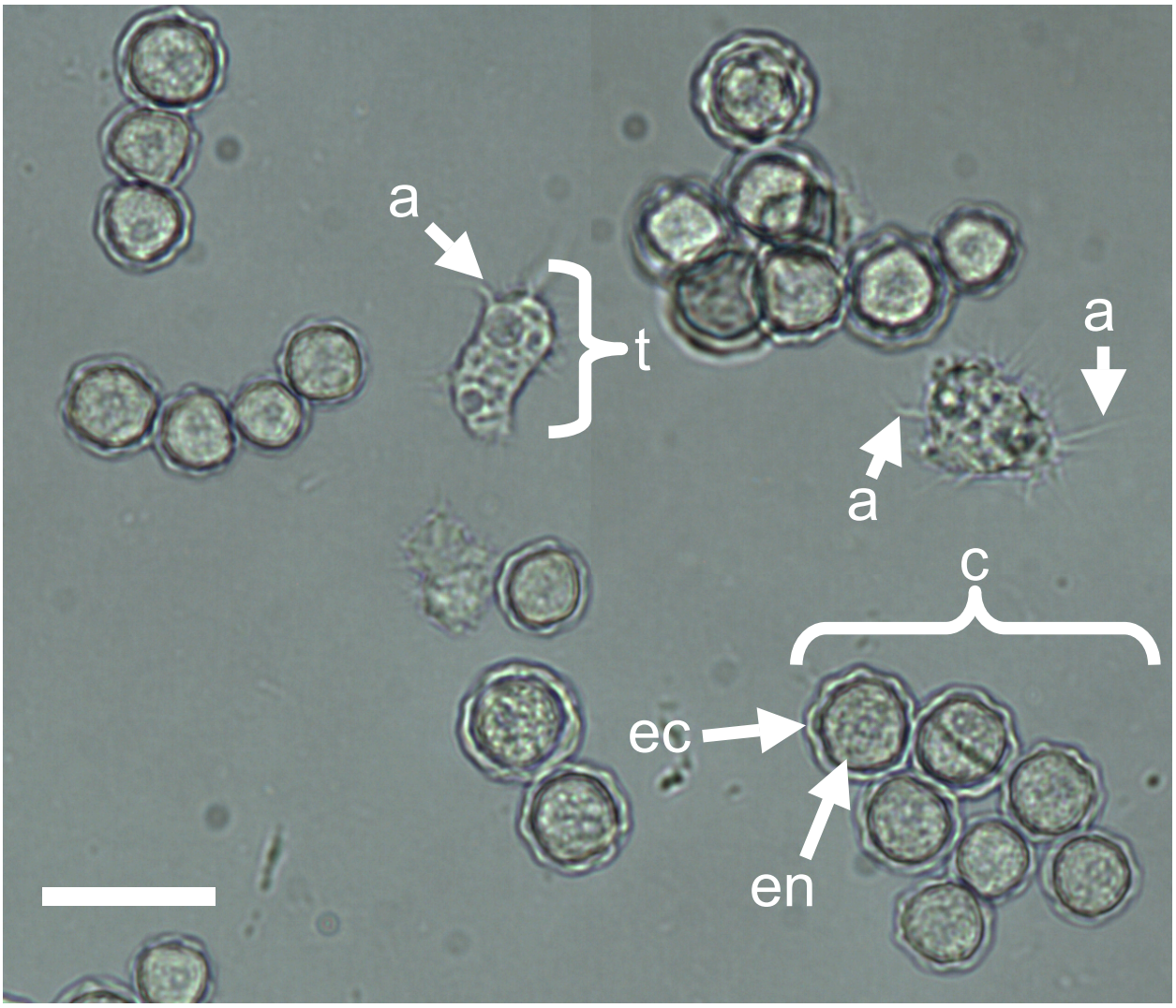
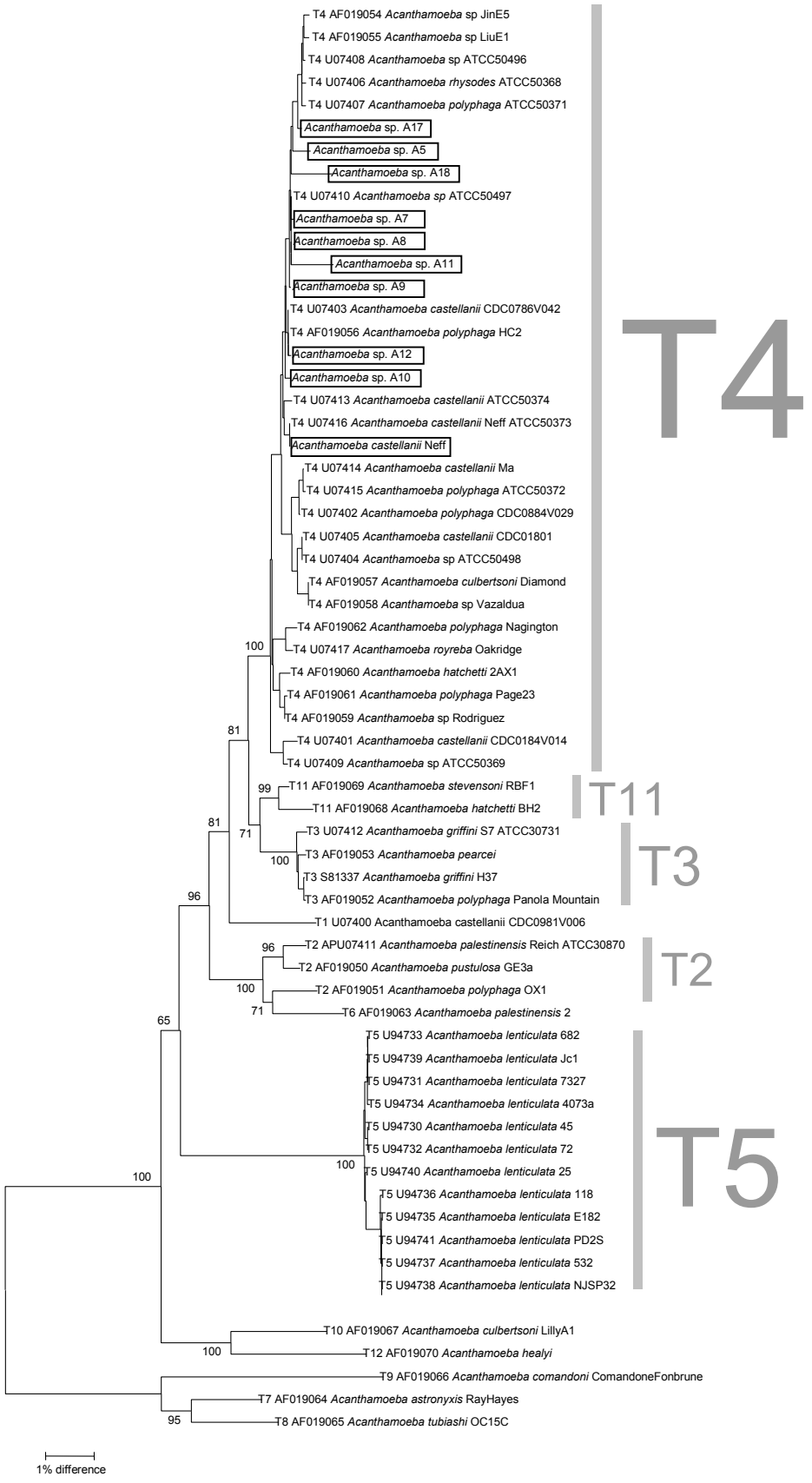

a
t
a
a
c
ec
en

Figure 3.3. Phylogenetic analysis of 18S sequences for determining *Acanthamoeba* ribotypes.

The neighbour joining method was used with 1000 bootstrap replicates. All positions containing large gaps and missing data were eliminated from the dataset. There were a total of 1853 positions in the final dataset. The numbers on the branches represent the bootstrap value which is a measure of certainty for a particular branching pattern. The boxed isolates on the phylogenetic tree are the strains isolated from farm water. In addition, we also sequenced the 18S rDNA from *A. castellanii* Neff strain.


T4 AF019054 Acanthamoeba sp JinE5
T4 AF019055 Acanthamoeba sp LiuE1
T4 U07408 Acanthamoeba sp ATCC50496
T4 U07406 Acanthamoeba rhysodes ATCC50368
T4 U07407 Acanthamoeba polyphaga ATCC50371
Acanthamoeba sp. A17
Acanthamoeba sp. A5
Acanthamoeba sp. A18
T4 U07410 Acanthamoeba sp ATCC50497
Acanthamoeba sp. A7
Acanthamoeba sp. A8
Acanthamoeba sp. A11
Acanthamoeba sp. A9
T4 U07403 Acanthamoeba castellanii CDC0786V042
T4 AF019056 Acanthamoeba polyphaga HC2
Acanthamoeba sp. A12
Acanthamoeba sp. A10
T4 U07413 Acanthamoeba castellanii ATCC50374
T4 U07416 Acanthamoeba castellanii Neff ATCC50373
Acanthamoeba castellanii Neff
T4 U07414 Acanthamoeba castellanii Ma
T4 U07415 Acanthamoeba polyphaga ATCC50372
T4 U07402 Acanthamoeba polyphaga CDC0884V029
T4 U07405 Acanthamoeba castellanii CDC01801
T4 U07404 Acanthamoeba sp ATCC50498
T4 AF019057 Acanthamoeba culbertsoni Diamond
T4 AF019058 Acanthamoeba sp Vazaldua
T4 AF019062 Acanthamoeba polyphaga Nagington
T4 U07417 Acanthamoeba royreba Oakridge
T4 AF019060 Acanthamoeba hatchetti 2AX1
T4 AF019061 Acanthamoeba polyphaga Page23
T4 AF019059 Acanthamoeba sp Rodriguez
T4 U07401 Acanthamoeba castellanii CDC0184V014
T4 U07409 Acanthamoeba sp ATCC50369
T11 AF019069 Acanthamoeba stevensoni RBF1
T11 AF019068 Acanthamoeba hatchetti BH2
T3 U07412 Acanthamoeba griffini S7 ATCC30731
T3 AF019053 Acanthamoeba pearcei
T3 S81337 Acanthamoeba griffini H37
T3 AF019052 Acanthamoeba polyphaga Panola Mountain
T1 U07400 Acanthamoeba castellanii CDC0981V006
T2 APU07411 Acanthamoeba palestinensis Reich ATCC30870
T2 AF019050 Acanthamoeba pustulosa GE3a
T2 AF019051 Acanthamoeba polyphaga OX1
T6 AF019063 Acanthamoeba palestinensis 2
T5 U94733 Acanthamoeba lenticulata 682
T5 U94739 Acanthamoeba lenticulata Jc1
T5 U94731 Acanthamoeba lenticulata 7327
T5 U94734 Acanthamoeba lenticulata 4073a
T5 U94730 Acanthamoeba lenticulata 45
T5 U94732 Acanthamoeba lenticulata 72
T5 U94740 Acanthamoeba lenticulata 25
T5 U94736 Acanthamoeba lenticulata 118
T5 U94735 Acanthamoeba lenticulata E182
T5 U94741 Acanthamoeba lenticulata PD2S
T5 U94737 Acanthamoeba lenticulata 532
T5 U94738 Acanthamoeba lenticulata NJSP32
T10 AF019067 Acanthamoeba culbertsoni LillyA1
T12 AF019070 Acanthamoeba healyi
T9 AF019066 Acanthamoeba comandoni ComandoneFonbrune
T7 AF019064 Acanthamoeba astronyxis RayHayes
T8 AF019065 Acanthamoeba tubiashi OC15C
100
81
99
81
71
100
96
96
100
71
65
100
100
100
95
T4
T11
T3
T2
T5
1% difference

3.3.2 Internalization of *C. jejuni* by gentamycin protection assay

The goal of this experiment was to visually demonstrate that the gentamycin protection assay is an effective method to eliminate external *C. jejuni* while retaining *Acanthamoeba* cells with internalized *C. jejuni*. Microphotographs showed that *C. jejuni* was internalized by *Acanthamoeba* when both species were co-cultured for 4 hours (Figure 3.4). Washes and gentamycin treatment yielded *Acanthamoeba* cells with internalized *C. jejuni* cells (Panel D in Figure 3.4).

The objective of the following experiment was to quantify the number of *C. jejuni* internalized by *Acanthamoeba*. Two interactions were tested: *C. jejuni* NCTC11168 co-cultured with *A. castellanii* Neff (model strains); and *C. jejuni* C9 co-cultured with *Acanthamoeba* sp. A18 (environmental strains). *C. jejuni* C9 and *Acanthamoeba* sp. A18 were both isolated from the same water sample from Farm 4 at the time when the flocks were *C. jejuni* positive. The coexistence of these two organisms in poultry drinking water is suggestive of their interactions at the farm level. *Acanthamoeba-Campylobacter* interactions were tested by monitoring the internalization level of *C. jejuni* using a gentamycin protection assay as described in the Materials and Methods section.

Internalization of *C. jejuni* was higher in model strains interactions compared to environmental strains interactions for both PAS and PYG media (Table 3.2). The planktonic counts for *C. jejuni* remain fairly constant after 4 hours and 24 hours for PYG media, but there is a 1 log reduction observed for the PAS media. The internalization of *C. jejuni* decreased after 24 hours of co-culture with *Acanthamoeba* in PAS media compared to 4 hours ($p < 0.05$). However, this decrease was not statistically significant for PYG. These patterns were observed for the model strains and the environmental strains interactions.

Figure 3.4. Gentamycin treatment is an effective method to eliminate external *C. jejuni* and retaining *Acanthamoeba* cells with internalized *C. jejuni*.

C. jejuni was added to cultures of *Acanthamoeba.* The co-culture before the gentamycin treatment and washes contained noticeable clumps of bacteria (panel A and C). Cells were retained without the clumps of bacteria after gentamycin treatment and the washes (panel B and D). In panels C and D, *C. jejuni* was stained red using the safranin dye. Safranin dye was added to a culture of *Acanthamoeba* alone (panel E) as a control demonstrating that the safranin dye does not intrinsically turn the cytoplasm of the cells red. Thus, the red clumps inside *Acanthamoeba* cells in panel D is in fact internalized *C. jejuni*.

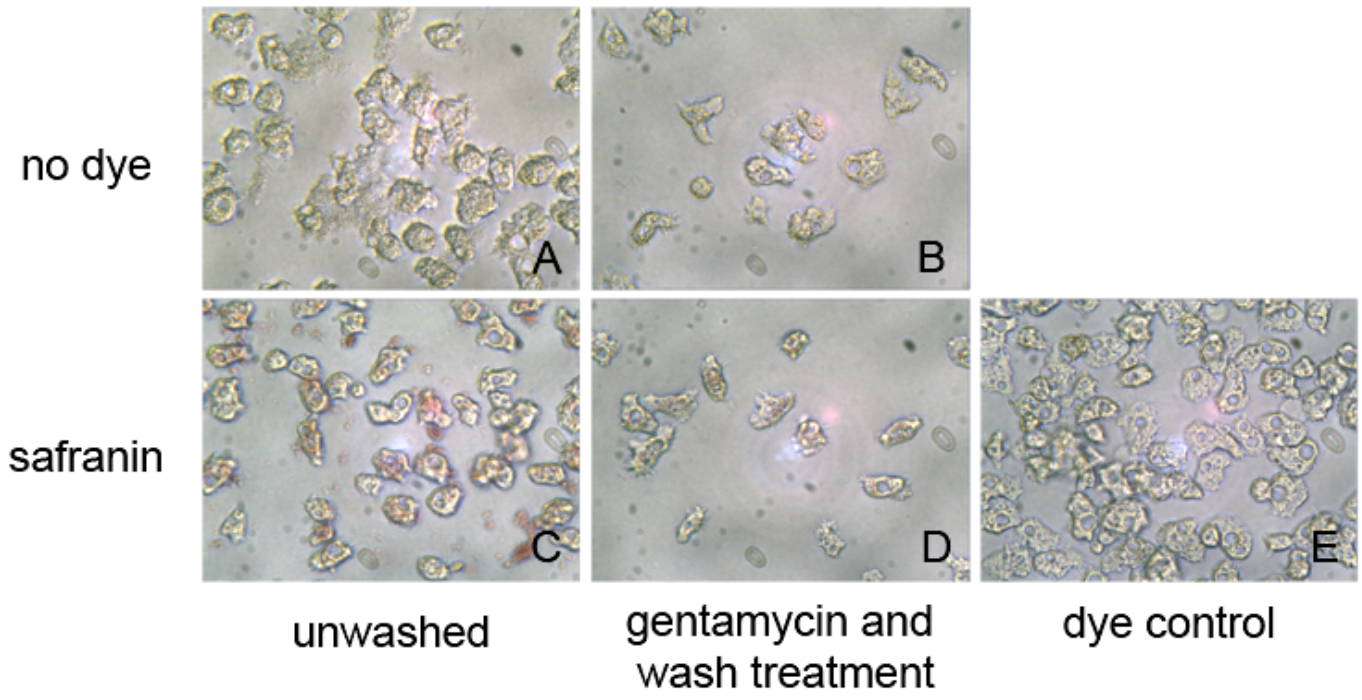

no dye
safranin
A
B
C
D
E
unwashed
gentamycin and
wash treatment
dye control

Table 3.2. *C. jejuni* is internalized by *Acanthamoeba* as measured by a gentamycin protection assay.

C. jejuni was added to cultures of *Acanthamoeba* followed by incubation, treatment with gentamycin and washing to eliminate external *C. jejuni*. Internal *C. jejuni* was measured as CFU/mL over time in PAS and PYG media.

PAS media

C. jejuni NCTC11168 and *A. castellanii* Neff co-cultured	4 hours	24 hours
Planktonic *C. jejuni* CFU/mL	$(3.15 \pm 0.74) \times 10^8$ (n=10)	$(1.87 \pm 0.40) \times 10^7$ (n=6)
Internalized bacterial CFU/mL	$(2.37 \pm 0.76) \times 10^6$ (n=6)	$(3.60 \pm 1.90) \times 10^1$ (n=4)

PAS media

C. jejuni C9 and *Acanthamoeba* sp. A18 co-cultured	4 hours	24 hours
Planktonic *C. jejuni* CFU/mL	$(2.70 \pm 0.66) \times 10^8$ (n=7)	$(3.48 \pm 1.67) \times 10^7$ (n=8)
Internalized bacterial CFU/mL	$(1.92 \pm 0.74) \times 10^3$ (n=5)	$(3.10 \pm 1.30) \times 10^1$ (n=5)

PYG media

C. jejuni NCTC11168 and *A. castellanii* Neff co-cultured	4 hours	24 hours
Planktonic *C. jejuni* CFU/mL	1.62×10^{10} (n=2)	2.82×10^{10} (n=2)
Internalized bacterial CFU/mL	2.23×10^5 (n=2)	6.04×10^4 (n=2)

PYG media

C. jejuni C9 and *Acanthamoeba* sp. A18 co-cultured	4 hours	24 hours
Planktonic *C. jejuni* CFU/mL	7.41×10^9 (n=2)	7.91×10^9 (n=2)
Internalized bacterial CFU/mL	1.15×10^4 (n=2)	3.79×10^3 (n=2)

3.3.3 *C. jejuni* persistence in co-culture with *Acanthamoeba*

The goal of the persistence assay was to compare the viability of planktonic *C. jejuni* and *C. jejuni* co-cultured with *Acanthamoeba* in PAS and PYG media at 4°C and 25°C over time (Table 3.3). *C. jejuni* remained viable for a longer period of time in both PYG and PAS media at 4°C planktonically or in a co-culture with *Acanthamoeba* than at 25°C. At 25°C, planktonic *C. jejuni* NCTC11168 and *C. jejuni* C9 were culturable after 3 to 4 days in both PAS and PYG media. In the co-culture with *Acanthamoeba*, *C. jejuni* NCTC11168 and *C. jejuni* C9 were culturable after 3.5 ± 0.5 days and 2 days respectively in PAS and 17 days in PYG. *C. jejuni* was found to be culturable up to 13 to 15 days longer in co-culture with *Acanthamoeba* in PYG media at 25°C than planktonic *C. jejuni*.

3.3.4 *C. jejuni* growth in co-culture with *Acanthamoeba*

C. jejuni growth was tested by inoculating a culture of *Acanthamoeba* in PYG media with *C. jejuni* NCTC11168. This culture was incubated at 37°C for 72 hours as previously published (Axelsson-Olsson et al., 2007). Growth of *C. jejuni* in a co-culture with *Acanthamoeba* in PAS media was also tested for comparison with PYG media. In addition, planktonic *C. jejuni* growth was determined in both PAS and PYG media under the same growth conditions. The results, expressed in CFU/mL, are summarized in Table 3.4.

Table 3.3. *C. jejuni* viability in a co-culture with *Acanthamoeba* in two media (PAS and PYG) and at two temperatures (4°C and 25°C).

C. jejuni was added to cultures of *Acanthamoeba*. These co-cultures were incubated over at 4°C and 25°C and *C. jejuni* viability was measured. Each data point is a measurement of *C. jejuni* viability in days. Two biological replicates and one technical replicate were performed for each condition.

	Temperature and Media			
	4°C		25°C	
Type of interaction	PAS	PYG	PAS	PYG
A. castellanii Neff & *C. jejuni* NCTC11168	34.5 ± 10.5	31 ± 7	3.5 ± 0.5	17
C. jejuni NCTC11168	36 ± 12	31 ± 7	4	4
Acanthamoeba sp. A18 & *C. jejuni* C9	17	17	2	17
C. jejuni C9	14 ± 7	17	3	3.5 ± 0.5

Table 3.4. *C. jejuni* growth in the presence of *Acanthamoeba* at 37°C over 72 hours in PAS and PYG media.

At the start of the experiment, *C. jejuni* was added to cultures of *Acanthamoeba*. Each data point is the average CFU/mL of six replicates with indicated standard error. The limit of detection of the assay is 2 CFU/mL. *C. jejuni* growth was detected in environmental and model strains in PYG media. *C. jejuni g*rowth was not detected in PAS media even in the presence of *Acanthamoeba*. Control experiments showed that planktonic *C. jejuni* did not grow at 37°C in PYG alone, under aerobic or microaerophillic conditions

	Time			
Type of interaction in PYG media	**0 h**	**24 h**	**48 h**	**72 h**
C. jejuni NCTC11168 & *A. castellanii* Neff	$(2.14 \pm 1.04) \times 10^{1}$	$(3.53 \pm 2.71) \times 10^{2}$	$(1.51 \pm 1.32) \times 10^{6}$	$(2.89 \pm 2.88) \times 10^{11}$
C. jejuni NCTC11168 alone	$(2.14 \pm 1.04) \times 10^{1}$	< 2	< 2	< 2
C. jejuni C9 & *Acanthamoeba* sp. A18	$(1.78 \pm 0.99) \times 10^{1}$	$8.83 \pm 1.67 \times 10^{1}$	$(1.57 \pm 1.56) \times 10^{6}$	$(7.76 \pm 4.48) \times 10^{8}$
C. jejuni C9 alone	$(1.78 \pm 0.99) \times 10^{1}$	< 2	< 2	< 2

	Time			
Type of interaction in PAS media	**0 h**	**24 h**	**48 h**	**72 h**
C. jejuni NCTC11168 & *A. castellanii* Neff	$(5.78 \pm 1.12) \times 10^{0}$	< 2	< 2	< 2
C. jejuni NCTC11168 alone	$(5.78 \pm 1.12) \times 10^{0}$	< 2	< 2	< 2
C. jejuni C9 & *Acanthamoeba* sp. A18	$(7.25 \pm 2.25) \times 10^{0}$	< 2	< 2	< 2
C. jejuni C9 alone	$(7.25 \pm 2.25) \times 10^{0}$	< 2	< 2	< 2

After 72 hours, *C. jejuni* growth reached up to 10^{11} CFU/mL for model strains and 10^{8} CFU/mL for environmental strains. *C. jejuni* did not grow and/or was under the detection limit of the assay when cultured planktonically in PYG media at 37°C under aerobic conditions. There was no *C. jejuni* growth in PAS media even in the presence of *Acanthamoeba*. In a control experiment, we observed that planktonic *C. jejuni* does not grow at 37°C in PYG even under microaerophillic conditions (data not shown).

C. jejuni growth was detected in co-culture with *Acanthamoeba* at 37°C under aerobic conditions in PYG media. This was observed for both environmental strains and model strains. The other conditions tested, namely PAS media in the presence and absence of *Acanthamoeba* and planktonic *C. jejuni* in PYG, were not suitable for the growth of *C. jejuni.*

3.4 Discussion

Protozoa were isolated from the water distribution systems of poultry houses whether the flocks were *Campylobacter* positive or not. The protozoa isolates were further characterized by morphological features and 18S rRNA ribosomal gene sequencing. Nine different *Acanthamoeba* strains were identified from the *Campylobacter*-positive poultry house, all belonging to the T4 ribotype. The coexistence of *Campylobacter* and Acanthamoeba in the water distribution system suggests a close relationship between these two organisms. Therefore, we examined the ability of the protozoa isolated on the farm to act as an environmental reservoir for *C. jejuni*. Previous studies on *Acanthamoeba-Campylobacter* interactions have been performed with type strains rather than environmental isolates (Axelsson-Olsson et al., 2005; Axelsson-Olsson et al., 2007; Axelsson-Olsson et al., 2010a; Snelling et al., 2005). The objective of our work was to demonstrate that environmental strains of *Acanthamoeba* can internalize and amplify environmental strains of *Campylobacter* isolated from the same water sample and similarly to the type laboratory strains. *C. jejuni* C9 and *Acanthamoeba* sp. A18 were isolated from the same water sample. As determined by *flaA* typing and genomotyping, *C. jejuni* C9 strain colonized the flock (Chapter 2). The importance of making these comparisons was to show further support for the hypothesis that amoebae can act as environmental reservoirs of *Campylobacter* and thus could be another source for *Campylobacter* colonization in poultry, in addition to other already known factors.

To characterize *Acanthamoeba-Campylobacter* interaction, we investigated the ability of *Acanthamoeba* to prolong *C. jejuni* survival at 25 and 4°C and under nutrient rich and nutrient poor conditions. PAS medium was used as the nutrient poor medium (PAS

consist of salts) and PYG medium was used as the nutrient rich medium (PYG is composed of proteose peptone, yeast extract and glucose). PAS and PYG media have been previously used by Snelling and Axelsson-Olson to study *Acanthamoeba-Campylobacter* interactions of type strains (Axelsson-Olsson et al., 2005; Axelsson-Olsson et al., 2007; Axelsson-Olsson et al., 2010a; Snelling et al., 2005; Snelling et al., 2008). Our study aimed to interconnect *Acanthamoeba-Campylobacter* interactions by the use of both media in all assays since it is reasonable to expect that *Acanthamoeba* will behave differently under nutrient poor and nutrient rich conditions. We speculate that PAS medium mimics the conditions encountered in the water distribution system of poultry houses while PYG medium mimics ditch water conditions where more bacterial prey is available to *Acanthamoeba*. Previous work showed that *C. jejuni* remains viable for a longer period of time in co-culture with *Acanthamoeba* compared to planktonic stage (Axelsson-Olsson et al., 2005; Axelsson-Olsson et al., 2010a; Snelling et al., 2005). We employed a persistence assay to measure the longetivity of *C. jejuni* in co-culture with *Acanthamoeba* in PYG and PAS media at two different temperatures (4°C and 25°C). Our results were in agreement with previous studies done in PYG media (Axelsson-Olsson et al., 2005; Axelsson-Olsson et al., 2010a). Type strains and environmental strains exhibited prolonged *C. jejuni* viability at 25°C in co-culture with *Acanthamoeba* as compared to mono-culture ($p < 0.05$), whereas no significant difference was observed in PYG medium at 4°C or in PAS medium at either temperature (Table 3.3). This suggests that the growth medium and the temperature are two important factors influencing the interaction between *Campylobacter* and *Acanthamoeba*.

In the current literature, *Acanthamoeba-Campylobacter* interactions have been tested by measuring the level of internalization by a disinfection assay, the viability over time and

the growth of *C. jejuni* in co-culture with *Acanthamoeba* (Axelsson-Olsson et al., 2005; Axelsson-Olsson et al., 2010a; Snelling et al., 2005).

In our study, we employed a gentamycin protection assay to measure the level of *C. jejuni* internalization in *Acanthamoeba* and viability of internalized *C. jejuni* over 24 hours. Internalized *C. jejuni* viability decreased by 5 and 2 logs for type strains and environmental strains respectively after 24 hours in PAS media (Table 3.2). Internalized *C. jejuni* viability decreased by 1 log for both environmental and model strains after 24 hours in PYG media. The decreased survivability of *C. jejuni* within *Acanthamoeba* in PAS medium as compared to PYG suggests that *Acanthamoeba* might metabolize *C. jejuni* as a food source under non nutrient conditions. Another possibility is that *C. jejuni* has entered a viable but non-culturable (VBNC) state while inside *Acanthamoeba*. Indeed, under stress conditions, such as nutrient poor aqueous environment, *Campylobacter* has been shown to persist up to 4 months in the VBNC state (Baffone et al., 2006).

Lastly, *Acanthamoeba-Campylobacter* interactions were investigated by a growth assay. Interestingly, *C. jejuni* grew and survived under aerobic conditions when co-cultured with *Acanthamoeba* at 37°C in PYG media (Axelsson-Olsson et al., 2007), while no growth was observed in absence of *Acanthamoeba*. In addition, our results show that *C. jejuni* does not grow in co-culture with *Acanthamoeba* in PAS media. Although the growth was measured as CFU/mL, it is not an accurate representation of the CFU/mL because first, the assay does not discriminate between internal and external *C. jejuni* and second, *C. jejuni* internalized in a single *Acanthamoeba* cell would only appear as one colony even if many *C. jejuni* cells have been internalized. In conclusion, the findings suggest that *C. jejuni* may only grow under aerobic conditions when two requirements are met. These are the presence of *Acanthamoeba* and a nutrient rich environment.

Although the growth of *Acanthamoeba* was not monitored, the growth of *Acanthamoeba* is expected to be minimal at 4°C. In addition, there are no available nutrients for *Acanthamoeba* growth in the PAS media. Taken together, along with the results of the persistence and growth assays, our data suggest that the growth of *Acanthamoeba* is necessary for the persistence and/or growth of *C. jejuni*. In nutrient poor conditions (such as PAS media), *Acanthamoeba* appears to resort to *C. jejuni* feeding as shown by the inability of *C. jejuni* to survive in co-culture with *Acanthamoeba* in PAS media. These observations suggest that *Acanthamoeba* might not enhance *Campylobacter* survival in drinking water (nutrient poor condition and relatively low temperature) but might contribute to the amplification of *Campylobacter* in the farm environment such as in soil and ditch water, which might in turn contaminate drinking water.

Our results demonstrate that farm isolates of *C. jejuni* have the capability of entering farm isolates of *Acanthamoeba* and surviving inside the protozoan. *Campylobacter* replication within *Acanthamoeba* is dependent on growth conditions. Our data suggest that *Acanthamoeba* might amplify *Campylobacter* in the farm environment (e.g. soil and ditch water), but might not play an important role in drinking water. Nevertheless, amplification of *Campylobacter* in the farm environment will undoubtedly contribute to the increase of the incidence of *Campylobacter*-positive flocks. Reduction of *Campylobacter* in environmental reservoirs could contribute to the prevention of flock contamination by this organism.

CHAPTER 4. TRANSCRIPTOME STUDIES OF THE INTERACTION OF *CAMPYLOBACTER JEJUNI* AND *ACANTHAMOEBA CASTELLANII*

4.1 Introduction and research objectives

In the environment, *C. jejuni* co-exists with amoeba, like *Acanthamoeba* spp. We and others have shown that *C. jejuni* has the capability of entering *Acanthamoeba* and of surviving inside the protozoan (Axelsson-Olsson et al., 2005; Axelsson-Olsson et al., 2010a; Axelsson-Olsson et al., 2010b; Snelling et al., 2005). Consequently, *Acanthamoeba* has the potential to act as a *Campylobacter* reservoir. To gain insights into the molecular mechanisms involved in *Acanthamoeba-Campylobacter* interactions, we characterized the transcriptomes of both organisms while in co-culture. In addition, we compared the transcriptomes of *Acanthamoeba* in axenic and co-cultures in order to decipher the precise response of the host to *Campylobacter* infection. The interactions of *Acanthamoeba* with other bacteria have been previously studied at a transcriptomic level using microarray approaches. Bruggermann et al. have characterized the transcriptome of *L. pneumophila* infecting *Acanthamoeba* (Bruggemann et al., 2006) while Feng et al. have characterized the transcriptome of *Salmonella* infecting *Acanthamoeba* (Feng et al., 2009). However, none of these studies have characterized the transcriptional response of *Acanthamoeba* to bacterial infection. This is likely because the genome of *Acanthamoeba* was not available when these studies were published. Therefore, it would not have been possible to construct an *Acanthamoeba* microarray. The draft of the *Acanthamoeba castellanii* genome is now available (http://www.hgsc.bcm.tmc.edu/microbial-detail.xsp?project_id=163). While microarrays are a popular approach for studying transcriptomes, their construction and validation are tedious and erroneous. With the advances of the next-generation sequencing

technologies, transcriptomes can now be studied by RNA sequencing (RNA-Seq) (Wang et al., 2009). This is an increasingly popular approach in many branches of biology. The main advantage of these next generation sequencing technologies to study host-pathogen interaction is that the transcriptome of both the host and the pathogen can be directly obtained *de novo*.

Previous studies have used RNA-Seq to characterize the transcriptome of *Helicobacter pylori* (Sharma et al., 2010), *Pseudomonas syringae* (Filiatrault et al., 2010), methicillin-resistant *Staphylococcus aureus* (Beaume et al., 2010), *Listeria monocytogenes* (Oliver et al., 2009), and *Burkholderia cenocepacia* (Yoder-Himes et al., 2009). These studies characterized the transcriptome of these bacteria under different growth conditions, or have compared the transcriptome of a pathogenic strain to its non-pathogenic counterpart. In general, these deep transcriptomic surveys revealed novel small RNA's (sRNAs), putative noncoding RNA (ncRNAs) genes, and identified differences in global gene expression patterns between strains and/or growth conditions. No studies, thus far, have looked at host-pathogen interaction simultanousy using RNA-Seq approaches. Also, no previous studies have looked at the transcriptome of *C. jejuni* nor the transcriptome of *Acanthamoeba* using RNA-Seq. The first objective of this study was to investigate the transcriptome of both *C. jejuni* and *Acanthamoeba* 3 hours post-infection, in nutrient poor medium and at room temperature mimicking natural aquatic systems. The second objective of this work was to test *C. jejuni* oxidative stress mutants, namely the catalase (*katA)* mutants and superoxide dismutase (*sodB)* mutants, in *Acanthamoeba-Campylobacter* interactions to highlight the importance of these genes for *C. jejuni* survival in amoebae and to cross validate the results of our RNA-Seq experiment.

In previous studies, *katA* was found to be essential for *C. jejuni* intramacrophage persistence and growth (Day et al., 2000) and *sodB* was deemed important for *Campylobacter* aerobic survival and chicken colonization (Purdy et al., 1999). This suggests that *Campylobacter* requires oxidative stress defence for interaction with eukaryotic cells. This transcriptomic study provided useful clues into some of the genetic mechanisms of how *Acanthamoeba* may act as a reservoir for *C. jejuni*.

4.2 Methods

4.2.1 The transcriptome of *C. jejuni* and *A. castellanii* in PAS media

4.2.1.1 Cell lysis

We used *C. jejuni* NCTC11168 and *A. castellanii* Neff in our transcriptomic studies. *C. jejuni* was internalized in *A. castellanii* by using the gentamycin protection assay procedure in PAS media (see Section 3.2.3) followed by total RNA extraction. *C. jejuni* co-cultured with *A. castellanii* (test condition) were prepared in 24 wells in a multi-well plate. Cultures of *A. castellanii* alone (control condition) were similarly prepared in a 24 wells plate. Six biological replicates of the test and control conditions were performed. After 3 hours of culture, both conditions were subjected to gentamycin treatment. Following gentamycin removal and washings with PAS medium, the samples were resuspended in 150 µL of PAS, combined in a sterile 50 mL Falcon tube, and mixed with 4 mL of RNA later. The addition of RNA later to *Acanthamoeba* samples before RNA extraction has been previously employed in another study (Grant et al., 2006) in order to preserve the transcriptome integrity. Finally, the solution was transferred and distributed evenly to several 2.0 mL RNAse free microfuge tubes. The tubes were centrifuged at 4,000 x g for 5 min and total RNA was extracted from the cell pellet using the QIAGEN RNeasy extraction kit according to the manufacturer's instructions. The QIAshredder columns were used in the homogenization step of the RNA extraction kit. The RNA was eluted in 100 µL of RNase free water.

4.2.1.2 DNase treatment and RNA quality and quantity

A volume of 0.5 µL of RNase inhibitor (RNase Out (40 U/ µL) from Invitrogen) was added to the RNA extracts followed by addition of 10 µL of 10X buffer (50 mM $MgCl_2$, 50 mM Tris-HCl pH7.5, 5 mM EDTA, 5 mM DTT) and 4 µL DNase RNase-free enzyme (Ambion). After a 30 min incubation period at 37°C, another 4 µL of DNase enzyme was added followed by 30 min incubation at 37°C. Next, the RNA was cleaned up using the QIAGEN RNeasy kit according to manufacturer's instructions. This DNase treatment was repeated one more time.

Two PCRs' were performed to confirm the absence of DNA using Fla4F/Fla625RU primers (see Table 2.1 in Chapter 2) for *C. jejuni* and JDP1/JDP2 primers for *Acanthamoeba*: JDP1 (5'- GGC CCA GAT CGT TTA CCG TGA A -3') and JDP2 (5'- TCT CAC AAG CTG CTA GGG GAG TCA -3') (Zhang et al., 2004). The PCR reaction was run in a PTC-100 thermal cycler (MJ Research Inc.) with the following cycling conditions for reactions with JDP1/JDP2 primers: 95°C for 7 min (initial denaturation), then 35 cycles at 95°C for 60 s (denaturation), 62°C for 45 s (annealing), 72°C for 45 s (extension), then 72°C for 5 min (final extension). The following cycling conditions were used for reactions with Fla4F/Fla625RU primers: 95°C for 2 min (initial denaturation), then 35 cycles at 94°C for 45s (denaturation), 55°C for 45 s (annealing), and 72°C for 1 min (extension); and 72°C for 5 min (final extension).

Completed PCRs were analysed by 0.8 % agarose gel electrophoresis. Absence of any bands in the RNA samples indicates there is no DNA contamination. Genomic DNA from *C. jejuni* NCTC11168 and *A. castellanii* Neff were used as positive controls in the PCR reactions. The quality and quantity of the RNA were checked using the BioRad Experion

Standard Sense RNA chip according to manufacturer's instructions. The A260/A280 and A260/A230 ratio was measured by Nanodrop. RNA samples were stored at -80°C.

4.2.1.3 Illumina sequencing and bioinformatics

Library construction, RNA-seq, and bioinformatics were done in collaboration with the Beijing Genome Institute (Shenzhen, China). To construct the library and perform RNA-Seq, RNA from three biological replicates of the control condition and RNA from three biological replicates of the test condition (see section 4.2.1.1) were combined separately. The mRNA was enriched following rRNA removal using the Ribominus kit (Invitrogen), and specifically designed probes. The Illumina HiSeq 2000 was used for RNA-Seq following the Illumina protocol. Two batches of reads were obtained after RNA-Seq.

The *Acanthamoeba* draft genome has not been annotated thus the reads obtained from the RNA-Seq experiment was considered as *de novo*. To predict the function and expression levels of these *de novo* genes, reads were assembled using SOAPdenovo (http://soap.genomics.org.cn/soapdenovo.html). The longest assembled sequences was called contigs. Then, contigs were linked into scaffolds. From the scaffolds, sequences called unigenes were obtained. Unigene expression levels are calculated using RPKM method (reads per kb per million reads) (Mortazavi et al., 2008). BLAST search was done on the unigene sequences and sequence orientations were determined according to the best hit in the database (Nr, cluster of orthologous group (COG), Kyoto Encyclopedia of Genes and Genomes (KEGG) and Swissprot). Protein function was predicted from annotation of the most similar protein in those databases. The KEGG pathway database records networks of molecular interactions in the cells, and variants of them specific to particular organisms (Kanehisa et al., 2010). Using the unigene sequences, pathway-based analysis was

performed to further understand genes' biological functions (Kanehisa et al., 2010). Gene ontology (GO) is an international standardized gene functional classification system. With Nr annotation (Nr is a Genbank database containing all non-redundant coding sequence translations), Blast2GO program (Conesa et al., 2005) was used to acquire GO annotation of the unigenes. Then, the WEGO software (Ye et al., 2006) was used to determine GO functional classification for all unigenes.

To determine the genes expressed in *C. jejuni* when internalized by *Acanthamoeba*, the reads from the co-culture condition were mapped to *C. jejuni* NCTC11168 reference genome using SOAP2 (Li et al., 2009). Gene expression levels were calculated using the RPKM method as described previously (Mortazavi et al., 2008).

4.2.2 Mutants competition assay

A mutant competition assay was performed to assess the contribution of *C. jejuni* oxidative stress defense system in the interaction with *Acanthamoeba*. The oxidative stress mutants of *C. jejuni* NCTC11168, namely Δ*sodB* and Δ*katA*, and as well as their complemented strains were tested (Palyada et al., 2009). The *Acanthamoeba* strain used was *A. castellanii* Neff.

Briefly, *C. jejuni* NCTC11168 wild type, mutants and their complements, as well as *A. castellanii* Neff were grown up and diluted as described in Section 3.2.3. Only the PAS media was tested. Since it is a competition assay, 250 µL of the wild type *C. jejuni* and 250 µL of either the mutant or its complement were mixed with 500 µL of *A. castellanii* totalling a volume of 1 mL per well. In each well, there were 1.0×10^8 cells of *A. castellanii,* 5.0×10^8 CFU of *C. jejuni* wild type and 5.0×10^8 CFU of *C. jejuni* mutant or its complement. The cultures were incubated at room temperature for 3 hours and the gentamycin treatment

was carried out as described in Section 3.2.3. Cultures of *C. jejuni* mutants alone, *C. jejuni* complemented mutants alone and *C. jejuni* wild type alone were also prepared to determine the viability of these strains for the duration of the experiment.

Next, 100 μL aliquots of the undiluted samples and 1:100 diluted samples were plated on MH agar and on MH agar with chloramphenicol or kanamycin (to select for the mutant or complemented strain respectively). Cultures of *C. jejuni* mutants alone, *C. jejuni* complemented mutants alone and *C. jejuni* wild type alone were diluted up to $1:10^7$ in PAS media and plated on MH agar. Plates were incubated at 37°C under microaerophillic conditions for 3 days.

Colonies were counted. The number of wild type *C. jejuni* was determined by subtracting the number of colonies on antibiotic plates from the MH agar plate without antibiotics. The competitive index was determined. The competitive index (CI) is the ratio of mutant or complemented strain to wild-type bacteria recovered (output ratio averaged across all replicates) divided by the ratio of mutant or complemented strain to wild-type bacteria inoculated (input ratio averaged across all replicates). To determine the p-value, a two sample t-test assuming equal variance was used. For the t-test, the CI determined by the experiment for each mutant/complement was compared to a CI of 1.00.

4.3 Results

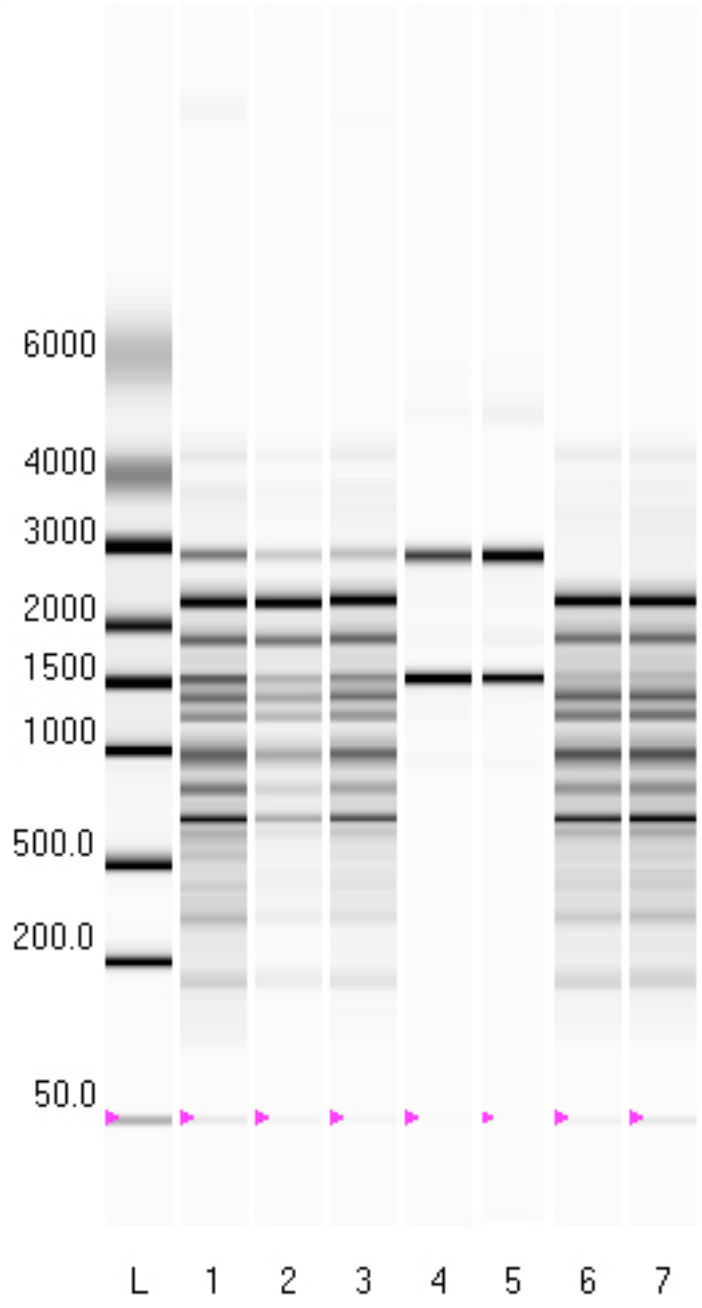
4.3.1 The transcriptome of *C. jejuni* and *A. castellanii* in PAS media

The objective of this study was to characterize the transcriptomes of *C. jejuni* and *A. castellanii* while interacting together. RNA was isolated from a culture of *A. castellanii* with internalized *C. jejuni*, and a culture of axenic *A. castellanii* for comparison purpose. The RNA yield from each biological replicate ranged from approximately 10 µg to 20 µg of total RNA. Quality of the RNA samples was assessed using the BioRad Experion system (see Figure 4.1). Interestingly, in addition to the classical ribosomal RNA bands expected (16S, 18S, 23S, and 28S), analysis of RNA extracts from the samples of *A. castellanii* alone or with internalized *C. jejuni* revealed additional bands. *Acanthamoeba* possesses many species of ribosomal RNA such as the 23S, 16S, 5S mitochondrial RNA's, 26S RNA, 5S RNA, and 5.8S RNA (Bullerwell et al., 2003; D'Alessio et al., 1981; Grant et al., 2006; MacKay and Doolittle, 1981), in addition to possible ribosomal RNA originating from intracellular organisms (Thomas et al., 2009). Noticeably, in the co-culture samples, there were 2 distinct bands at around 3000 bp and 1500 bp which corresponded to *C. jejuni* ribosomal RNA because the *A. castellanii* alone samples were lacking these bands (Figure 4.1).

After confirming that the total RNA samples were of good quality, library construction and RNA-Seq were done in collaboration with BGI. The RNA-Seq yielded 13,870,154 reads for the *C. jejuni-A. castellanii* co-culture sample and 13,500,862 reads for the *A. castallanii* alone sample.

Figure 4.1. RNA quality of samples.

Post DNase treated RNA samples were loaded on a BioRad Experion RNA StdSens chip to determine RNA quality. The RNA ladder is represented by lane L. Lanes 1, 2, and 3 were loaded with RNA from *C. jejuni* co-cultured with *A. castellanii*. Lanes 4 and 5 were loaded with RNA from planktonic *C. jejuni.* Lanes 6 and 7 were loaded with RNA from cultures of *A. castellanii* alone.


6000
4000
3000
2000
1500
1000
500.0
200.0
50.0
L
1
2
3
4
5
6
7

From the co-culture reads, a total of 69,703 reads (0.50% of the total reads) were mapped to *C. jejuni* genes. Nine hundred eighty six (986) genes out of 1654 *C. jejuni* genes (59.6%) were detected. Transcript levels can be quantified by calculating the reads per kilobase of exon model per million mapped reads (RPKM) (Mortazavi et al., 2008). The RPKM measure reflects the molar concentration of a transcript in the analyzed sample by normalizing for RNA length and for the total read number in the measurement (Mortazavi et al., 2008). This facilitates comparison of transcript levels both within and between samples (Mortazavi et al., 2008). The RPKM of all gene transcripts detected for *C. jejuni* is shown in Figure 4.2 arranged according to their chromosomal location. Cj0170 had the highest RPKM value indicating that Cj0170 is the most highly expressed gene. Functional categories were assigned to genes detected. The percentage of genes for each functional category is shown in Figure 4.3. The sum of RPKM values of genes belonging to a particular functional category is a good indication of the genes that play a role in *C. jejuni* interaction with *A. castellanii*. In this case, the sum of RPKM values majorly represented (RPKM=1.24 x 10^8 or 64%) genes with unknown functions although only 32% of unknown genes were detected.

Oxidative stress defense in *C. jejuni* is important for its interaction with eukaryotic cells and its survival in aerobic conditions. These oxidative stress defense genes include *sodB* (superoxide dismutase), *katA* (catalase), *ahpC* (alkyl-hydroxyperoxidase), *cft* (ferritin), *fdxA* (ferredoxin) and *perR* (peroxide sensing regulator). Table 4.1 lists all the detected oxidative stress genes belonging to *C. jejuni* and their respective RPKM values. As far as oxidative stress defense, *fdxA* is the most highly expressed followed by *sodB*, *ahpC*, *cft*, and finally *katA*. The *perR* gene transcript was not detected. Previous reports have shown that *ahpC* and *sodB* are required for *C. jejuni* survival in aerobic conditions (Baillon et al., 1999; Purdy et al., 1999). It was also observed that *C. jejuni katA* and *sodB* mutants have a reduced

Figure 4.2. The RPKM of all gene transcripts detected in *C. jejuni* in co-culture with *A. castellanii* arranged according to their chromosomal location.

The RPKM values are plotted as a log scale ranging from 10 (most inner circle) to 10^5 (outer circle). The red line represents the Cj0170 gene which has the highest RPKM value.

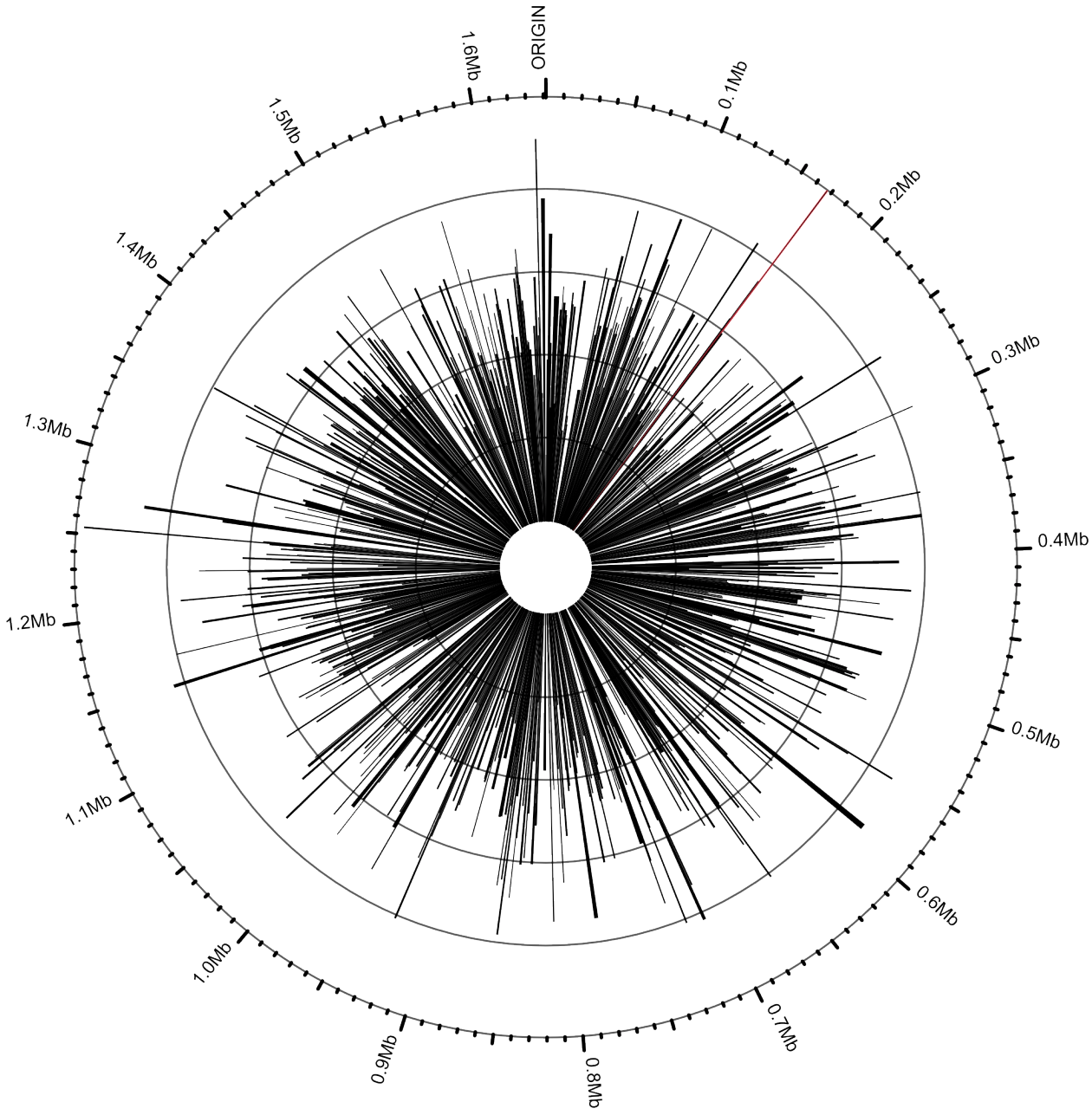
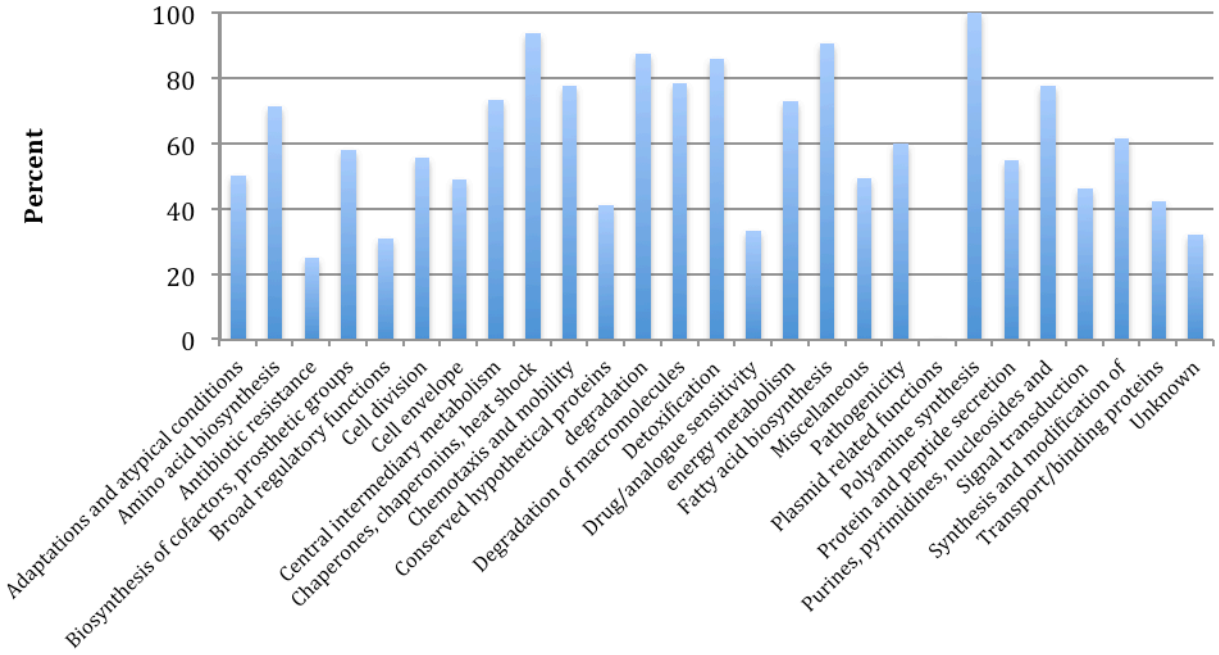

ORIGIN
0.1Mb
0.2Mb
0.3Mb
0.4Mb
0.5Mb
0.6Mb
0.7Mb
0.8Mb
0.9Mb
1.0Mb
1.1Mb
1.2Mb
1.3Mb
1.4Mb
1.5Mb
1.6Mb

Figure 4.3. Percentage of *C. jejuni* genes detected based on their functional category (panel A) and the sum of RPKM of the genes in that functional category (panel B).

A


Percent
100
80
60
40
20
0
Adaptations and atypical conditions
Amino acid biosynthesis
Antibiotic resistance
Biosynthesis of cofactors, prosthetic groups
Broad regulatory functions
Cell division
Cell envelope
Central intermediary metabolism
Chaperones, chaperonins, heat shock
Chemotaxis and mobility
Conserved hypothetical proteins
degradation
Degradation of macromolecules
Detoxification
Drug/analogue sensitivity
energy metabolism
Fatty acid biosynthesis
Miscellaneous
Pathogenicity
Plasmid related functions
Polyamine synthesis
Protein and peptide secretion
Purines, pyrimidines, nucleosides and
Signal transduction
Synthesis and modification of
Transport/binding proteins
Unknown

B

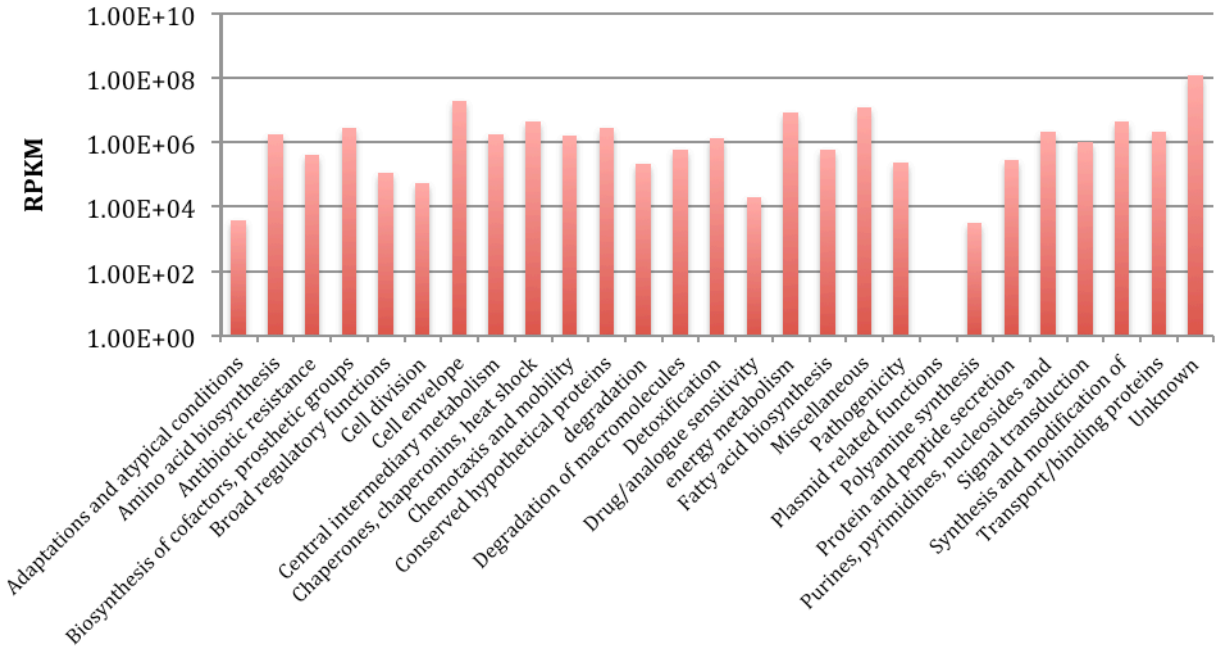

RPKM
1.00E+10
1.00E+08
1.00E+06
1.00E+04
1.00E+02
1.00E+00
Adaptations and atypical conditions
Amino acid biosynthesis
Antibiotic resistance
Biosynthesis of cofactors, prosthetic groups
Broad regulatory functions
Cell division
Cell envelope
Central intermediary metabolism
Chaperones, chaperonins, heat shock
Chemotaxis and mobility
Conserved hypothetical proteins
degradation
Degradation of macromolecules
Detoxification
Drug/analogue sensitivity
energy metabolism
Fatty acid biosynthesis
Miscellaneous
Pathogenicity
Plasmid related functions
Polyamine synthesis
Protein and peptide secretion
Purines, pyrimidines, nucleosides and
Signal transduction
Synthesis and modification of
Transport/binding proteins
Unknown

Table 4.1 *C. jejuni* oxidative stress defence genes and their respective RPKM.

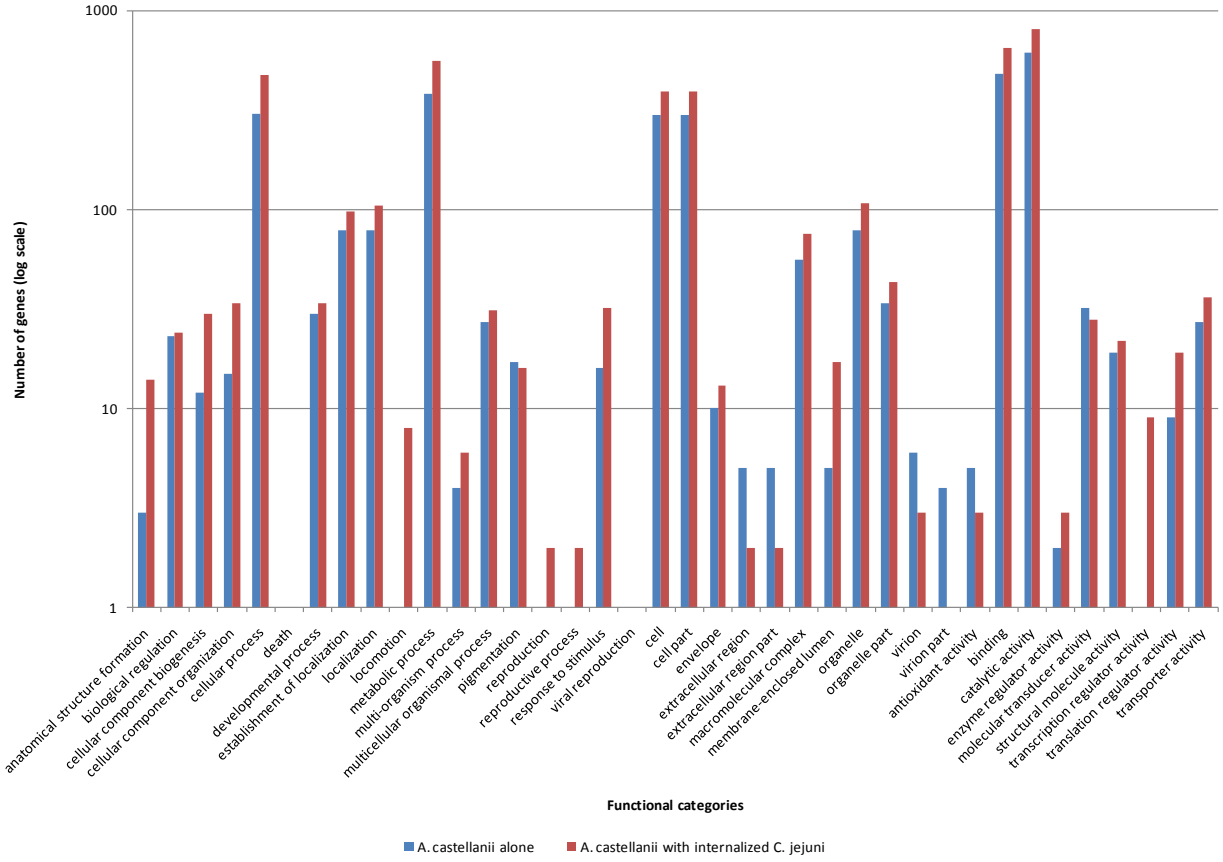
Gene name	RPKM
katA	191
ahpC	2836
sodB	4544
cft	427
fdxA	14951
perR	not detected

ability to survive within macrophages and INT407 cells, respectively (Day et al., 2000; Purdy et al., 1999). *C. jejuni cft* mutants were found to be more sensitive to H_2O_2 (Wai et al., 1996). An *fdxA*-deficient mutant showed reduction in aerotolerance, but its resistance to H_2O_2 was unaffected (van Vliet et al., 2001). The oxidative stress defense mechanisms in *C. jejuni* are regulated by the peroxide-sensing regulator, *perR* (van Vliet et al., 1999). The *perR* protein is a repressor so its inactivation results in constitutive expression of *katA* and *ahpC*, and leads to enhanced resistance to peroxide stress (van Vliet et al., 1999).

Campylobacter require iron as a micronutrient for growth (discussed in Chapter 2). Only some of the iron metabolism genes were detected: *cfrA*, *Cj0175c*, *feoB*, and *fur*. Their RPKM values were 14, 4096, 23, and 121 respectively. *Campylobacter* uses the flagellum for motility. The genes *flaABC* (flagellar structural proteins), *fliD* (flagellar capping protein), *flgG* (flagellar basal body rod protein) were detected with RPKM values of 17301, 2003, 1071, 1926, 9202 respectively. The genes related to cell adhesion and invasion *peb1A* and *cadF* were detected with RPKM values of 1490 and 374. Chaperone proteins, *GroEL*, *GroES* and *DnaK* were highly expressed with RPKM values of 11369, 11158 and 10185 respectively. Genes related to metabolism such as *frdA* (fumerate reductase flavoprotein subunit), *aspA* (aspartate ammonia-lyase), *metK* (S-adenosylmethionine synthase), *acnB* (aconitate hydratase), *sdhA* (succinate dehydrogenase flavoprotein subunit) were detected with RPKM values of 8397, 7290, 5082, 4298, and 4149 respectively.

The draft genome of *A. castellanii* Neff is available online but not annotated. RPKM values for d*e novo* RNA transcripts (called unigenes) were calculated and some of these unigenes were found to match existing sequences by BLAST. These matches were classified according to their functional categories (Figure 4.4). In this broad overview of genes, differences were detected in *A. castellanii* with or without internalized *C. jejuni* in almost

Figure 4.4. ***A. castellanii*** **Neff genes detected, based on gene ontology (GO) functional category classifications, while in co-culture with** ***C. jejuni*** **and in culture without** ***C. jejuni*****.**


1000
100
10
1
Number of genes (log scale)
anatomical structure formation
biological regulation
cellular component biogenesis
cellular component organization
cellular process
death
developmental process
establishment of localization
localization
locomotion
metabolic process
multi-organism process
multicellular organismal process
pigmentation
reproduction
reproductive process
response to stimulus
viral reproduction
cell
cell part
envelope
extracellular region
extracellular region part
macromolecular complex
membrane-enclosed lumen
organelle
organelle part
virion
virion part
antioxidant activity
binding
catalytic activity
enzyme regulator activity
molecular transducer activity
structural molecule activity
transcription regulator activity
translation regulator activity
transporter activity
Functional categories
A. castellanii alone
A. castellanii with internalized C. jejuni

every functional category. For example, more unigenes were detected in the functional category of cellular component biogenesis, locomotion, metabolic process, reproduction, reproductive process, membrane enclosed lumen, transcription regulator activity when *A. castellanii* contained internalized *C. jejuni*. When *A. castellanii* was alone, more unigenes related to extracellular region, virion and antioxidant activity were detected compared to *A. castellanii* with internalized *C. jejuni*. Many of the unigenes had no match with any of the databases and thus are unknown. Over 17,000 unigenes and 15,000 unigenes were detected in the *Acanthamoeba* with *C. jejuni* and *Acanthamoeba* alone sample respectively. Most of the known unigenes with high RPKM values appear to be related to ribosomal proteins and translation elongation factors. There are also many unigenes with high RPKM values which are unknown.

KEGG pathway analysis was also performed on the unigenes. The KEGG pathway analysis allows the mapping of unigenes to a collection of manually drawn pathway maps representing our knowledge on the molecular interaction and reaction networks. In this analysis, the unigenes are assumed to be homologues, and this allows us to elucidate higher level systemic functions with a dataset of differentially expressed genes (ie. Fatty acid metabolism, citric acid cycle, phagocytosis, DNA replication, RNA degradation, etc).

Eukaryotic cells uptake materials from their environment by endocytosis. Phagocytosis is the engulfment of large particles like whole micro-organisms and is a form of endocytosis. *Acanthamoeba*, like many other unicellular amoebae, feed on bacteria so we looked closely at some of the differences in the phagocytosis pathway. Initially, when bacteria are engulfed in small membrane enclosed sacks called vesicles, certain proteins are delivered to the vesicles forming endosomes. Through maturation steps (early to late

endosomes), these eventually fuse with lysosomes where antimicrobial molecules and ROS generation assist in killing bacteria (Cosson and Soldati, 2008).

Small GTPases of the Rab family control endosomal biogenesis, fusion and maturation (Zerial and McBride, 2001). Rab5 facilitates membrane docking of molecules in the early endosome and plays a role in the regulation of early endosome trafficking in the cell (Stenmark et al., 1994). Rab5 is found at the plasma membrane and in coated vesicles, where its activity may regulate the fusion of these vesicles with early endosomes (Bucci et al., 1992). After engulfment by phagocytosis, the phagosome containing bacteria is fused with a lysosome where the bacteria are killed by reactive oxygen species (ROS). ROS killing is mediated by the enzyme NADPH oxidase recruited to the membrane of the phagosome to catalyze NADPH and oxygen to superoxide and hydrogen peroxide (Sheppard et al., 2005). The gp91 protein is a membrane bound subunit of NADPH oxidase (Sheppard et al., 2005). When *Acanthamoeba* was co-cultured with *C. jejuni*, rab5 and gp91 were expressed whereas these genes were not detected when *Acanthamoeba* was alone in PAS medium.

SNARE proteins are a critical component of the membrane fusion machinery in cells. Syntaxin 7 (stx7) is a SNARE protein that allows late endosome fusion with lysosomes (Mullock et al., 2000). The stx7 transcript was detected when *Acanthamoeba* was alone but not found by the analysis when *Acanthamoeba* was co-cultured with *C. jejuni*.

The genome size of *A. castellanii* Neff is estimated to be 45 megabases (Mb). We have sequenced 1215 Mb for the *A. castellanii* alone sample covering the genome 27X in terms of nucleotides. The length of all scaffolds in our RNA-Seq experiment was determined to be 5.9 Mb. Thus, the percent genome coverage by our RNA-Seq experiment is 13%. Our sequencing results were compared to the draft genome of *A. castellanii* at

Baylor College of Medicine (BCM) (Table 4.2). BCM has sequenced nearly the entire genome of *A. castellanii* with a length of scaffolds stretching to 47 Mb with 2.0 Mb of gaps.

4.3.2 *C. jejuni* oxidative stress mutants and *Acanthamoeba*

To assess whether oxidative stress defence in *C. jejuni* is essential for the interaction with *Acanthamoeba*, two oxidative stress mutants were tested in their ability to survive in *Acanthamoeba* using a competition assay. The two *C. jejuni* oxidative stress mutants were the catalase (Δ*katA*) and superoxide dismutase (Δs*odB*) mutants. The mutants, their complements, and the wild type strain were found to survive similarly for 4 hours at 25°C (room temperature) in PAS media (Table 4.3). The purpose of the competition assay was to determine if these mutant were affected in their ability to internalize/survive in *Acanthamoeba*. The wild type *C. jejuni* strain was found to out-compete the *katA* ($p < 10^{-12}$) and *sodB* ($p < 10^{-17}$) mutants and the *sodB* complemented mutant ($p < 10^{-10}$) (Figure 4.5). There was no statistical difference in the CI value for the *katA* complemented mutant ($p = 0.33$). Moreover, both complemented strains of *katA* and *sodB* showed a higher CI value compared to their respective mutants indicating a full or partial restoration of the wild-type phenotype.

Table 4.2 Sequencing of *A. castellanii* Neff from our study compared to the study at Baylor College of Medicine.

	Baylor College of Medicine	This study
Total number of reads	11.23 Million	12.15 Million
Coverage by nucleotides (X)	25X	27X
Number of contigs	54947	124840
Number of scaffolds	18936	22049
Length of all scaffolds	47.0 Mb	5.9 Mb

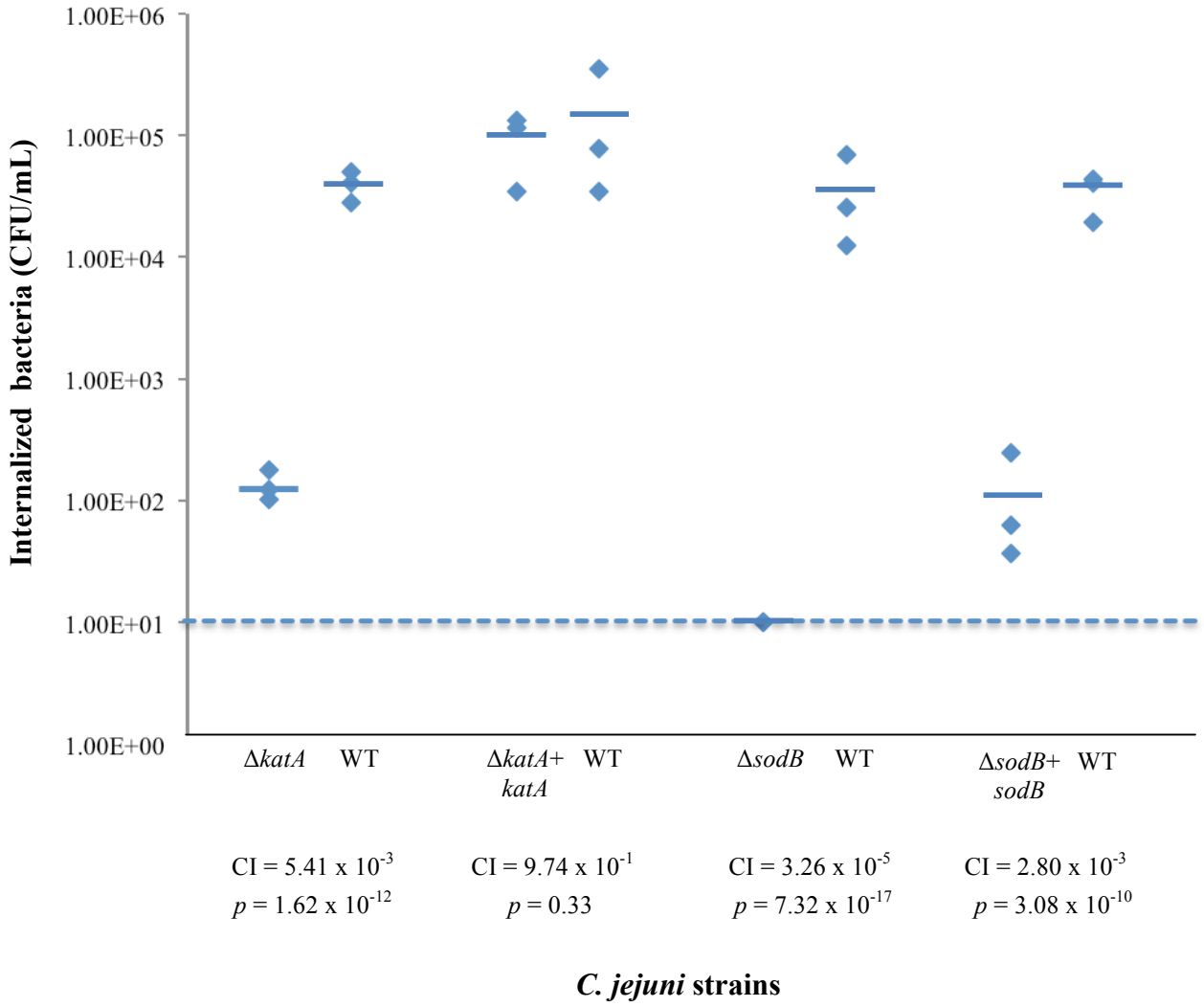
Table 4.3. *C. jejuni* NCTC11168 wild type, mutants and complements planktonic counts at the start of the experiment and after 4 hours in PAS media at room temperature.

C. jejuni NCTC11168 wild type, mutants and complements were tested in their ability to survive under normal atmospheric oxygen conditions at room temperature at the start of the experiment and after 4 hours, without *Acanthamoeba*. Three biological replicates were performed and each biological replicate contained 3 technical replicates. The values represent measurements of CFU/mL of *C. jejuni*. The values shown in the table were averaged across biological replicates. The mutants, their complements, and the wild type strain were found to survive similarly for 4 hours at room temperature in PAS media.

	Time	
Strain	**0 hours**	**4 hours**
katA mutant	$(2.92 \pm 0.32) \times 10^8$	$(3.24 \pm 0.39) \times 10^8$
katA complement	$(2.88 \pm 0.21) \times 10^8$	$(3.57 \pm 0.95) \times 10^8$
sodB mutant	$(4.47 \pm 1.09) \times 10^8$	$(3.58 \pm 1.36) \times 10^8$
sodB complement	$(5.50 \pm 0.04) \times 10^8$	$(4.50 \pm 0.33) \times 10^8$
wild type	$(4.64 \pm 0.72) \times 10^8$	$(4.83 \pm 0.92) \times 10^8$

Figure 4.5. Internalized *C. jejuni* mutants or complements in a competition assay with *C. jejuni* wild type in a co-culture with *A. castellanii.*

C. jejuni NCTC11168 wild type, mutants, complemented mutants and *A. castellanii* Neff were used in these experiments. The wild type and the mutant or complement bacterial strains were mixed at a ratio of 1:1 and then co-cultured with *Acanthamoeba* in PAS medium at room temperature for 4 hours. The mutants and complemented mutants were selected using antibiotic containing MH-agar plates. Three biological replicates were performed and each biological replicate contained 3 technical replicates. The line represents the average of the data across three biological replicates. The competitive index (CI) was calculated from the 3 biological averages of internalized bacteria. The p value of the competitive index was determined by using two sample t-test assuming equal variance. The t-test was used to determine whether the obtained CI value was different from a CI value of 1.00. A CI with a p value of less than 0.05 was considered as statistically significant. The detection limit of the assay is 10 CFU/mL and is marked by the dashed line.


Internalized bacteria (CFU/mL)
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
ΔkatA WT
ΔkatA+ katA WT
ΔsodB WT
ΔsodB+ sodB WT
CI = 5.41 x 10-3
p = 1.62 x 10-12
CI = 9.74 x 10-1
p = 0.33
CI = 3.26 x 10-5
p = 7.32 x 10-17
CI = 2.80 x 10-3
p = 3.08 x 10-10
C. jejuni strains

4.4 Discussion

The objective of this study was to uncover the transcriptome of *C. jejuni* and *A. castellanii* interacting together. Although only 59.6% of the *C. jejuni* genes were detected by RNA-Seq, it is reasonable to expect that these detected genes were the most highly expressed genes while *C. jejuni* was internalized in *A. castellanii*.

C. jejuni oxidative stress defence genes were detected (Table 4.2). Oxidative stress defence in *C. jejuni* plays an important role in its survival in the environment and interaction with host cells (see section 4.3.1). This is the first study to look at the role of oxidative stress defence of *C. jejuni* in protozoa interaction. We speculate that oxidative stress defence in *C. jejuni* permits interaction with *A. castellanii* and perhaps with other protozoa.

C. jejuni possesses the peroxide-sensing regulator, *perR*, to regulate oxidative stress defence genes (van Vliet et al., 1999). Inactivation of *perR* results in constitutive expression of *katA* and *ahpC*, and enhanced resistance to peroxide stress (van Vliet et al., 1999). *PerR* senses hydrogen peroxide by Fe^{2+} oxidation in its regulatory binding site, which leads to Fe^{2+} release and subsequent de-repression of *perR* regulated genes (Palyada et al., 2009). When *C. jejuni* is in co-culture with *Acanthamoeba*, the *perR* transcript was not detected and only some of the iron metabolism genes were detected with low RPKM values. It is of note that fur transcript was detected with a low RPKM of 121. The *fur* protein is the transcription factor regulating iron metabolism genes in *C. jejuni*. When the *fur* protein binds its co-repressor Fe^{2+}, *fur* binds to a consensus sequence in the promoter region of iron acquisition genes, repressing their transcription (Palyada et al., 2009). The down play of iron metabolism and acquisition genes in *C. jejuni* suggests that iron is present. Flagellar genes were detected with high RPKM values suggesting that *C. jejuni* may require the flagellum in

the first 3 hours of interaction with *Acanthamoeba* even when *C. jejuni* has been internalized. The flagellum was the first *C. jejuni* determinant found to be involved in adherence and invasion (Grant et al., 1993). Other genes related to cell adhesion and invasion, such as *peb1A* and *cadF* were detected. A *C. jejuni peb1A* mutant showed 50- to 100-fold less adherence to HeLa cells and 15-fold less invasion of INT 407 intestinal epithelial cell culture and also was affected in intestinal colonization in the BALB/c mouse colonization model following oral challenge (Pei et al., 1998). The binding of *C. jejuni* to fibronectin (a component of the extracellular matrix) is mediated by an outer-membrane protein called *cadF*. It was shown that *C. jejuni* 81-176 (a highly invasion strain of *C. jejuni*) utilizes *cadF* as an adhesin for host cells by competitive binding assays using wild-type and *cadF* mutant of *C. jejuni* isolate (Monteville et al., 2003). Also, *cadF* mutants do not colonize newly hatched chickens (Ziprin et al., 1999). The *peb1A* transcript was detected with an RPKM value of 1490 while the *cadF* transcript was detected with an RPKM of 374 suggesting that *peb1A* may play a more important role than *cadF* in the interaction with *Acanthamoeba*. *C. jejuni* synthesizes a set of proteins collectively referred to as *Campylobacter* invasion antigens (Cia proteins) during coculture with epithelial cells that help *C. jejuni* to internalize into the mammalian cells (Konkel et al., 1999). While *C. jejuni* was in co-culture with *Acanthamoeba*, the Cia transcripts were not detected suggesting that Cia proteins may not be essential for internalization in *Acanthamoeba* but only in mammalian cells. The chaperonin genes were detected with high RPKM values suggesting that *C. jejuni* is undergoing stress during the interaction with *A. castellanii*. The genes related to metabolism were detected indicating that *C. jejuni* is still metabolically active during the first 3 hours of internalization in *Acanthamoeba*. The colony counts of *C. jejuni* drastically decreases after 24 hours of co-culture with *Acanthamoeba* in PAS (see Chapter 3). Although we did not

perform RNA-Seq on a 24 hours co-culture sample, it would be interesting to see if there are changes in expression in *C. jejuni*'s general metabolism genes. If the metabolism genes decrease in expression as compared to 3 hours of co-culture, it might suggest that *C. jejuni* is going into a dormancy state (i.e. VBNC state) or that *C. jejuni* is dying thus explaining a lower colony count after 24 hours of co-culture with *Acanthamoeba*.

The gene Cj0170 was found to be most highly expressed while *C. jejuni* was internalized in *A. castellanii*. No information is known about Cj0170. Interestingly, Cj0170 has a 73.8% amino acid identity with Cj1325 which is the second most highly expressed gene. According to xBASE (http://www.xbase.ac.uk/campydb/), Cj1325 is a putative methyltransferase and is part of the O-linked glycosylation locus (Cj1293 - Cj1342). The gene Cj1725 is the third most highly expressed gene and it is classified as a putative periplasmic protein in xBASE. When the genes were placed into functional categories, the unknown genes were found to be most highly expressed according to the sum of their RPKM values (Figure 4.3). It is possible that many of these genes with unknown functions could play a role in *C. jejuni* interaction with protozoa and thus would be an interesting avenue to explore for future studies.

The genes in the *A. castellanii* transcriptome were classified into functional categories to compare differences between *A. castellanii* with or without *C. jejuni* (Figure 4.4). In general, there were noticeable differences between *A. castellanii* with and without *C. jejuni* in terms of functional classification. By only comparing the differences in the number of genes detected in both conditions, it is difficult to assign statistical significance to these differences and thus we can only speculate that *Acanthamoeba* is preying in *C. jejuni* explaining, for example, an upregulation of the genes related to locomotion, response to stimuli, and transcription regulators in *Acanthamoeba* with internalized *C. jejuni*.

The unigene (annotated as unigene11692 found in scaffold18885) with the highest RPKM value in the *Acanthamoeba* with internalized *C. jejuni* is of unknown origin. The sequence matching unigene11692 was also found in the *Acanthamoeba* alone sample (in scaffold7911) but it was not assigned as a unigene in that data set. Unigene11692 was also not mapped to any homologues in the KEGG pathway analysis. Our data set will be much more valuable once annotation of the *A. castellanii* genome is available so we can map the sequences and extract the list of genes with their respective RPKM values for comparison.

We chose to closely look at phagocytosis (by KEGG pathway analysis) because this process is first initiated when *Acanthamoeba* encounters bacteria such as *C. jejuni*. In this discussion we also assumed that the level of expression of a transcript is proportional to the level of protein expression for a given gene. Following phagocytosis, certain proteins are delivered to the vesicles forming endosomes, and through maturation steps (early to late endosomes), these eventually fuse with lysosomes where antimicrobial molecules and ROS generation assist in killing bacteria (ie. Capthepsin G kills *S. aureus* and beta-hexoaminidase kills mycobacteria) (Cosson and Soldati, 2008). The GTPase Rab5 was detected when *Acanthamoeba* was co-cultured with *C. jejuni*. Rab5 is a protein found at the membrane mediating vesicle fusion to early endosomes (see section 4.3.1). Rab5 expression is expected because when *C. jejuni* is internalized by phagocytosis, creating a vesicle with *C. jejuni* inside, *Acanthamoeba* would express Rab5 to fuse these vesicles to early endosomes. *Acanthamoeba* rab proteins have not been studied previously but this normally happens in eukaryotic cells such as *Dictyostelium* and macrophages (Cosson and Soldati, 2008). The expression of gp91 (a membrane bound subunit of NADPH oxidase responsible for generating ROS) was also not surprising because *Acanthamoeba* would have to recruit NADPH oxidase to vesicle membranes to produce ROS to kill *C. jejuni* and ultimately

metabolize them as food. These results would be consistent with the findings of the internalization assay (Chapter 3) where we saw a decrease in viability of *C. jejuni* after 24 hours of co-cultured with *Acanthamoeba* in PAS medium. ROS production in *Acanthamoeba* would also explain the upregulation of *C. jejuni* oxidative defence genes in order to help counteract the ROS damage inside *Acanthamoeba* vesicles. This is also consistent with the observation that chaperonin genes were upregulated in *C. jejuni* perhaps to assist with counteracting the ROS stress on proteins and the requirement of *sodB* and *katA* to survive inside *Acanthamoeba*. Interestingly, stx7 was not detected when *Acanthamoeba* was co-cultred with *C. jejuni* after 3 hours. Stx7 is a protein responsible for the fusion of late endosomes with lysosomes. Absence of stx7 transcript may suggest that the late endosomes containing *C. jejuni* may not fuse with *Acanthamoeba*'s lysosomes. Perhaps *C. jejuni* is able to partially subvert the normal fate of early endosomes in *Acanthamoeba* by somehow preventing the early endosomes from fusing with lysosomes. Afterall, microscopy observations indicate that *C. jejuni* does reside in vesicles once ingested by *Acanthamoeba* (Axelsson-Olsson et al., 2005). *C. jejuni* may be able to survive using temporarily by this mechanism. However, over long periods of time, the ROS generated from *Acanthamoeba*'s NADPH oxidase may be able to eventually overcome the subversion/defence mechanisms of *C. jejuni*. This would explain the drastic decrease of *C. jejuni* viability in co-culture with *Acanthamoeba* in PAS media after 24 hours demonstrated by the internalization protection assay (Chapter 3). However, *C. jejuni* was found to survive for longer time in PYG medium, and even grow in PYG medium in the presence of *Acanthamoeba* (Chapter 3), suggesting that environmental conditions could change how *Acanthamoeba* behaves thus may increase the effectiveness of these defense mechanisms in *C. jejuni*. It would certainly be interesting to perform the RNA-Seq experiment under PYG medium to assess these differences in

vesicle trafficking in *Acanthamoeba*. Transcriptomics provide possible clues but more experiments (ie. fluorescence microscopy and *Acanthamoeba* knockout mutants) would have to be performed to confirm the exact role of rab5, gp91, and stx7 in *Acanthamoeba-Campylobacter* interactions with regards to vesicle trafficking. If *C. jejuni* possesses a subversion mechanism for amoeba, perhaps one of the unknown proteins, such as the putative periplasmic protein Cj1725, serves as an effector molecule making it a key player in the prevention of vesicles fusion with lysosomes in the host amoeba. It is also clear from the transcriptomic data that *Acanthamoeba-Campylobacter* interactions are not of a friendly or symbiotic nature.

The main purpose of this study was to uncover some genetic clues which may play a role in *Acanthamoeba-Campylobacter* interactions. It is difficult, at this point, to speculate how these genes could be important. However, we have determined that Cj0170, Cj1325 and Cj1725, and that oxidative stress defence genes in *C. jejuni* could be important for its interaction with *A. castellanii* and perhaps other protozoa. The detection of flagellar genes and metabolism genes suggest that *C. jejuni* is motile and metabolically active during the first 3 hours of internalization in *Acanthamoeba*. The transcriptomic data for *A. castellanii* alone and with internalized *C. jejuni* is now available for comparison once *A. castellanii* genes could be mapped to its genome. However, for the time being, it appears that there is a difference in the transcriptome of *A. castellanii* in the presence of *C. jejuni*. There are likely many important differences but for now, we have highlighted some differences in phagocytosis/endosome maturation pathway that warrants further investigation in future studies. We also note that unigene11692 may be somehow important in the *A. castellanii* host while interacting with *C. jejuni*.

CHAPTER 5. MODEL OF *ACANTHAMOEBA-CAMPYLOBACTER* INTERACTIONS

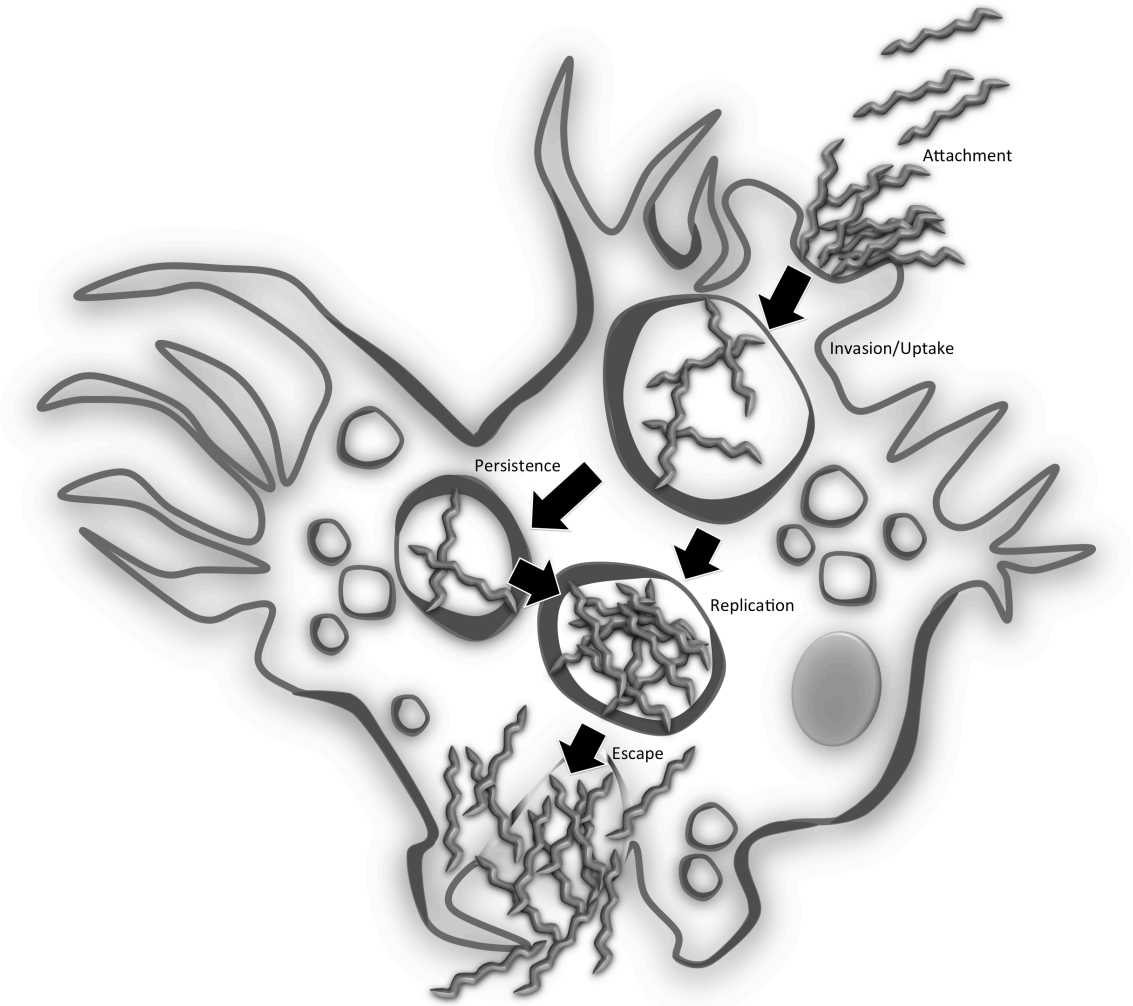
The information acquired through this study and previous studies could be summarized in a model (Figure 5.1). *C. jejuni* encounters *Acanthamoeba* in aquatic environments such was water or soil. The *C. jejuni* cells will invade or be uptaken by *Acanthamoeba* by phagocytosis as observed previously (Axelsson-Olsson et al., 2005), and in Figure 3.4 in Chapter 3. We speculate that oxidative stress defence genes and Cj0170, Cj1325, Cj1725, possibly other unknown genes, flagellar proteins in *C. jejuni* are required for this interaction to occur based on our transcriptomic studies (Chapter 4). However, only the oxidative stress defence genes, namely *katA* and *sodB*, were confirmed to be essential in the interaction with *Acanthamoeba*, by a mutant competition assay (Chapter 4). Once inside *Acanthamoeba*, *C. jejuni* resides in a vesicle (Axelsson-Olsson et al., 2005). Differences in the transcriptome of *Acanthamoeba* with internalized *C. jejuni* as compared to *Acanthamoeba* alone shows differences in the transcription of genes related to vesicle trafficking when *C. jejuni* is inside *Acanthamoeba* (Chapter 4). Transcriptomic data suggests a possible vesicle subversion mechanism in *C. jejuni* which may work by preventing fusion of the late endosomes to lysosomes.

When *Acanthamoeba*'s food source is high (in high nutrient media like PYG or in ditch water) and the temperature is 37°C (such as warm weather which heats up a small body of water), *C. jejuni* grows from inside *Acanthamoeba* (see Chapter 3; Axelsson-Olsson et al., 2005). *C. jejuni* is killed by *Acanthamoeba* when *Acanthamoeba* remains in a low nutrient environment (such as PAS media) for more than 3 days (Chapter 3) but *C. jejuni* could potentially persist with no growth (Chapter 3) and be protected from predation and biocide treatment (Snelling et al., 2005) during this interim period. In the former case scenario,

when *C. jejuni* growth has overwhelmed the *Acanthamoeba* cell, *C. jejuni* will burst out of the *Acanthamoeba* cell (Axelsson-Olsson et al., 2005) and escape into the environment and invade into another host. A favourable host (such as chickens) could get colonized by *C. jejuni* if it ingests trophozoite *Acanthamoeba* containing internalized *C. jejuni* (Snelling et al., 2008).

Figure 5.1. A model of *Acanthamoeba-Campylobacter* interactions.

The interactions could be described in several steps. First *C. jejuni* invades or is uptaken inside by *Acanthamoeba*. *C. jejuni* could persist (when *Acanthamoeba* is in a low nutrient environment) but grows if the conditions are favourable (high food source for *Acanthamoeba* and temperatures of 37°C). Eventually, *C. jejuni* bursts out of the *Acanthamoeba* cell and finds a new host.


Attachment
Invasion/Uptake
Persistence
Replication
Escape

REFERENCES

1. Abd, H., Johansson, T., Golovliov, I., Sandstrom, G., and Forsman, M. (2003). Survival and growth of *Francisella tularensis* in *Acanthamoeba castellanii*. Appl. Environ. Microbiol. *69,* 600-606.
2. Abd, H., Weintraub, A., and Sandstrom, G. (2005). Intracellular survival and replication of *Vibrio cholerae* O139 in aquatic free-living amoebae. Environ. Microbiol. *7,* 1003-1008.
3. Adekambi, T., Reynaud-Gaubert, M., Greub, G., Gevaudan, M.J., La Scola, B., Raoult, D., and Drancourt, M. (2004). Amoebal coculture of "*Mycobacterium massiliense*" sp. nov. from the sputum of a patient with hemoptoic pneumonia. J. Clin. Microbiol. *42,* 5493-5501.
4. Adeleke, A.A., Fields, B.S., Benson, R.F., Daneshvar, M.I., Pruckler, J.M., Ratcliff, R.M., Harrison, T.G., Weyant, R.S., Birtles, R.J., Raoult, D., and Halablab, M.A. (2001). *Legionella drozanskii* sp. nov., *Legionella rowbothamii* sp. nov. and *Legionella fallonii* sp. nov.: three unusual new *Legionella* species. Int. J. Syst. Evol. Microbiol. *51,* 1151-1160.
5. Aguilera, A., Gomez, F., Lospitao, E., and Amils, R. (2006). A molecular approach to the characterization of the eukaryotic communities of an extreme acidic environment: methods for DNA extraction and denaturing gradient gel electrophoresis analysis. Syst. Appl. Microbiol. *29,* 593-605.
6. Alsam, S., Jeong, S.R., Sissons, J., Dudley, R., Kim, K.S., and Khan, N.A. (2006). *Escherichia coli* interactions with *Acanthamoeba*: a symbiosis with environmental and clinical implications. J. Med. Microbiol. *55,* 689-694.
7. Arsenault, J., Letellier, A., Quessy, S., and Boulianne, M. (2007). Prevalence and risk factors for *Salmonella* and *Campylobacter* spp. carcass contamination in broiler chickens slaughtered in Quebec, Canada. J. Food Prot. *70,* 1820-1828.
8. Axelsson-Olsson, D., Ellstrom, P., Waldenstrom, J., Haemig, P.D., Brudin, L., and Olsen, B. (2007). *Acanthamoeba-Campylobacter* coculture as a novel method for enrichment of *Campylobacter* species. Appl. Environ. Microbiol. *73,* 6864-6869.
9. Axelsson-Olsson, D., Olofsson, J., Svensson, L., Griekspoor, P., Waldenstrom, J., Ellstrom, P., and Olsen, B. (2010a). Amoebae and algae can prolong the survival of *Campylobacter* species in co-culture. Exp. Parasitol. *126,* 59-64.
10. Axelsson-Olsson, D., Svensson, L., Olofsson, J., Salomon, P., Waldenstrom, J., Ellstrom, P., and Olsen, B. (2010b). Increase in acid tolerance of *Campylobacter jejuni* through coincubation with amoebae. Appl. Environ. Microbiol. *76,* 4194-4200.
11. Axelsson-Olsson, D., Waldenstrom, J., Broman, T., Olsen, B., and Holmberg, M. (2005). Protozoan *Acanthamoeba polyphaga* as a potential reservoir for *Campylobacter jejuni*. Appl. Environ. Microbiol. *71,* 987-992.
12. Baffone, W., Casaroli, A., Citterio, B., Pierfelici, L., Campana, R., Vittoria, E., Guaglianone, E., and Donelli, G. (2006). *Campylobacter jejuni* loss of culturability in aqueous microcosms and ability to resuscitate in a mouse model. Int. J. Food Microbiol. *107,* 83-91.
13. Baillon, M.L., van Vliet, A.H., Ketley, J.M., Constantinidou, C., and Penn, C.W. (1999). An iron-regulated alkyl hydroperoxide reductase (AhpC) confers

aerotolerance and oxidative stress resistance to the microaerophilic pathogen *Campylobacter jejuni*. J. Bacteriol. *181,* 4798-4804.

14. Bare, J., Sabbe, K., Van Wichelen, J., van Gremberghe, I., D'hondt, S., and Houf, K. (2009). Diversity and habitat specificity of free-living protozoa in commercial poultry houses. Appl. Environ. Microbiol. *75,* 1417-1426.
15. Barker, J., Humphrey, T.J., and Brown, M.W. (1999). Survival of *Escherichia coli* O157 in a soil protozoan: implications for disease. FEMS Microbiol. Lett. *173,* 291-295.
16. Beaume, M., Hernandez, D., Farinelli, L., Deluen, C., Linder, P., Gaspin, C., Romby, P., Schrenzel, J., and Francois, P. (2010). Cartography of methicillin-resistant *S. aureus* transcripts: detection, orientation and temporal expression during growth phase and stress conditions. PLoS One *5,* e10725.
17. Berk, S.G., Gunderson, J.H., Newsome, A.L., Farone, A.L., Hayes, B.J., Redding, K.S., Uddin, N., Williams, E.L., Johnson, R.A., Farsian, M.*, et al.* (2006). Occurrence of infected amoebae in cooling towers compared with natural aquatic environments: implications for emerging pathogens. Environ. Sci. Technol. *40,* 7440-7444.
18. Berk, S.G., Ting, R.S., Turner, G.W., and Ashburn, R.J. (1998). Production of respirable vesicles containing live *Legionella pneumophila* cells by two *Acanthamoeba* spp. Appl. Environ. Microbiol. *64,* 279-286.
19. Blatt, S.P., Parkinson, M.D., Pace, E., Hoffman, P., Dolan, D., Lauderdale, P., Zajac, R.A., and Melcher, G.P. (1993). Nosocomial Legionnaires' disease: aspiration as a primary mode of disease acquisition. Am. J. Med. *95,* 16-22.
20. Bogliun, G., Beghi, E., and Guillain-Barre Syndrome Registry Study Group. (2002). Validity of hospital discharge diagnoses for public health surveillance of the Guillain-Barre syndrome. Neurol. Sci. *23,* 113-117.
21. Bohaychuk, V.M., Gensler, G.E., King, R.K., Manninen, K.I., Sorensen, O., Wu, J.T., Stiles, M.E., and McMullen, L.M. (2006). Occurrence of pathogens in raw and ready-to-eat meat and poultry products collected from the retail marketplace in Edmonton, Alberta, Canada. J. Food Prot. *69,* 2176-2182.
22. Boyanova, L., Gergova, G., Spassova, Z., Koumanova, R., Yaneva, P., Mitov, I., Derejian, S., and Krastev, Z. (2004). *Campylobacter* infection in 682 bulgarian patients with acute enterocolitis, inflammatory bowel disease, and other chronic intestinal diseases. Diagn. Microbiol. Infect. Dis. *49,* 71-74.
23. Bruggemann, H., Hagman, A., Jules, M., Sismeiro, O., Dillies, M.A., Gouyette, C., Kunst, F., Steinert, M., Heuner, K., Coppee, J.Y., and Buchrieser, C. (2006). Virulence strategies for infecting phagocytes deduced from the in vivo transcriptional program of *Legionella pneumophila*. Cell. Microbiol. *8,* 1228-1240.
24. Bucci, C., Parton, R.G., Mather, I.H., Stunnenberg, H., Simons, K., Hoflack, B., and Zerial, M. (1992). The small GTPase rab5 functions as a regulatory factor in the early endocytic pathway. Cell *70,* 715-728.
25. Bullerwell, C.E., Schnare, M.N., and Gray, M.W. (2003). Discovery and characterization of *Acanthamoeba castellanii* mitochondrial 5S rRNA. RNA *9,* 287-292.
26. Callicott, K.A., Friethriksdottir, V., Reiersen, J., Lowman, R., Bisaillon, J.R., Gunnarsson, E., Berndtson, E., Hiett, K.L., Needleman, D.S., and Stern, N.J. (2006). Lack of evidence for vertical transmission of *Campylobacter* spp. in chickens. Appl. Environ. Microbiol. *72,* 5794-5798.

27. Callicott, K.A., Hargardottir, H., Georgsson, F., Reiersen, J., Frigriksdottir, V., Gunnarsson, E., Michel, P., Bisaillon, J.R., Kristinsson, K.G., Briem, H., *et al.* (2008). Broiler *Campylobacter* contamination and human campylobacteriosis in Iceland. Appl. Environ. Microbiol. *74,* 6483-6494.
28. Chang, N., and Chui, L. (1998). A standardized protocol for the rapid preparation of bacterial DNA for pulsed-field gel electrophoresis. Diagn. Microbiol. Infect. Dis. *31,* 275-279.
29. Cirillo, J.D., Falkow, S., Tompkins, L.S., and Bermudez, L.E. (1997). Interaction of *Mycobacterium avium* with environmental amoebae enhances virulence. Infect. Immun. *65,* 3759-3767.
30. Conesa, A., Gotz, S., Garcia-Gomez, J.M., Terol, J., Talon, M., and Robles, M. (2005). Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics *21,* 3674-3676.
31. Cosson, P., and Soldati, T. (2008). Eat, kill or die: when amoeba meets bacteria. Curr. Opin. Microbiol. *11,* 271-276.
32. D'Alessio, J.M., Harris, G.H., Perna, P.J., and Paule, M.R. (1981). Ribosomal ribonucleic acid repeat unit of *Acanthamoeba castellanii*: cloning and restriction endonuclease map. Biochemistry *20,* 3822-3827.
33. Day, W.A.,Jr, Sajecki, J.L., Pitts, T.M., and Joens, L.A. (2000). Role of catalase in *Campylobacter jejuni* intracellular survival. Infect. Immun. *68,* 6337-6345.
34. de Moraes, J., and Alfieri, S.C. (2008). Growth, encystment and survival of *Acanthamoeba castellanii* grazing on different bacteria. FEMS Microbiol. Ecol. *66,* 221-229.
35. Deckert, A., Valdivieso-Garcia, A., Reid-Smith, R., Tamblyn, S., Seliske, P., Irwin, R., Dewey, C., Boerlin, P., and McEwen, S.A. (2010). Prevalence and antimicrobial resistance in *Campylobacter* spp. isolated from retail chicken in two health units in Ontario. J. Food Prot. *73,* 1317-1324.
36. El-Etr, S.H., Margolis, J.J., Monack, D., Robison, R.A., Cohen, M., Moore, E., and Rasley, A. (2009). *Francisella tularensis* type A strains cause the rapid encystment of *Acanthamoeba castellanii* and survive in amoebal cysts for three weeks postinfection. Appl. Environ. Microbiol. *75,* 7488-7500.
37. Esposito, L., Seydel, A., Aiello, R., Sorrentino, G., Cendron, L., Zanotti, G., and Zagari, A. (2008). The crystal structure of the superoxide dismutase from *Helicobacter pylori* reveals a structured C-terminal extension. Biochim. Biophys. Acta *1784,* 1601-1606.
38. Evstigneeva, A., Raoult, D., Karpachevskiy, L., and La Scola, B. (2009). Amoeba co-culture of soil specimens recovered 33 different bacteria, including four new species and *Streptococcus pneumoniae*. Microbiology *155,* 657-664.
39. Feng, Y., Hsiao, Y.H., Chen, H.L., Chu, C., Tang, P., and Chiu, C.H. (2009). Apoptosis-like cell death induced by *Salmonella* in *Acanthamoeba rhysodes*. Genomics *94,* 132-137.
40. Fenstersheib, M.D., Miller, M., Diggins, C., Liska, S., Detwiler, L., Werner, S.B., Lindquist, D., Thacker, W.L., and Benson, R.F. (1990). Outbreak of Pontiac fever due to *Legionella anisa*. Lancet *336,* 35-37.
41. Fields, B.S., Barbaree, J.M., Sanden, G.N., and Morrill, W.E. (1990). Virulence of a *Legionella anisa* strain associated with Pontiac fever: an evaluation using protozoan, cell culture, and guinea pig models. Infect. Immun. *58,* 3139-3142.

42. Filiatrault, M.J., Stodghill, P.V., Bronstein, P.A., Moll, S., Lindeberg, M., Grills, G., Schweitzer, P., Wang, W., Schroth, G.P., Luo, S.*, et al.* (2010). Transcriptome analysis of *Pseudomonas syringae* identifies new genes, noncoding RNAs, and antisense activity. J. Bacteriol. *192,* 2359-2372.
43. Fraser, D.W., Tsai, T.R., Orenstein, W., Parkin, W.E., Beecham, H.J., Sharrar, R.G., Harris, J., Mallison, G.F., Martin, S.M., McDade, J.E., Shepard, C.C., and Brachman, P.S. (1977). Legionnaires' disease: description of an epidemic of pneumonia. N. Engl. J. Med. *297,* 1189-1197.
44. Friedman, C.R., Neimann, J., Wegener, H.C., and Tauxe, R.V. (2000). Epidemiology of *C. jejuni* infections in the United States and other industrialized nations. Nachamkin, and Blaser, M. J. eds., (Washington: American Society for Microbiology) pp. 121-138.
45. Gadberry, M.D., Malcomber, S.T., Doust, A.N., and Kellogg, E.A. (2005). Primaclade--a flexible tool to find conserved PCR primers across multiple species. Bioinformatics *21,* 1263-1264.
46. Galanis, E. (2007). *Campylobacter* and bacterial gastroenteritis. Cmaj *177,* 570-571.
47. Gao, L.Y., Harb, O.S., and Abu Kwaik, Y. (1997). Utilization of similar mechanisms by *Legionella pneumophila* to parasitize two evolutionarily distant host cells, mammalian macrophages and protozoa. Infect. Immun. *65,* 4738-4746.
48. Gaze, W.H., Burroughs, N., Gallagher, M.P., and Wellington, E.M. (2003). Interactions between *Salmonella typhimurium* and *Acanthamoeba polyphaga*, and observation of a new mode of intracellular growth within contractile vacuoles. Microb. Ecol. *46,* 358-369.
49. Grant, C.C., Konkel, M.E., Cieplak, W.,Jr, and Tompkins, L.S. (1993). Role of flagella in adherence, internalization, and translocation of *Campylobacter jejuni* in nonpolarized and polarized epithelial cell cultures. Infect. Immun. *61,* 1764-1771.
50. Grant, S., Grant, W.D., Cowan, D.A., Jones, B.E., Ma, Y., Ventosa, A., and Heaphy, S. (2006). Identification of eukaryotic open reading frames in metagenomic cDNA libraries made from environmental samples. Appl. Environ. Microbiol. *72,* 135-143.
51. Guerin, M.T., Martin, S.W., Reiersen, J., Berke, O., McEwen, S.A., Fridriksdottir, V., Bisaillon, J.R., Lowman, R., and Campy-on-Ice Consortium. (2008). Temperature-related risk factors associated with the colonization of broiler-chicken flocks with *Campylobacter* spp. in Iceland, 2001-2004. Prev. Vet. Med. *86,* 14-29.
52. Hazeleger, W.C., Wouters, J.A., Rombouts, F.M., and Abee, T. (1998). Physiological activity of *Campylobacter jejuni* far below the minimal growth temperature. Appl. Environ. Microbiol. *64,* 3917-3922.
53. Hofreuter, D., Tsai, J., Watson, R.O., Novik, V., Altman, B., Benitez, M., Clark, C., Perbost, C., Jarvie, T., Du, L., and Galan, J.E. (2006). Unique features of a highly pathogenic *Campylobacter jejuni* strain. Infect. Immun. *74,* 4694-4707.
54. Holden, E.P., Winkler, H.H., Wood, D.O., and Leinbach, E.D. (1984). Intracellular growth of *Legionella pneumophila* within *Acanthamoeba castellanii* Neff. Infect. Immun. *45,* 18-24.
55. Holmes, K., Mulholland, F., Pearson, B.M., Pin, C., McNicholl-Kennedy, J., Ketley, J.M., and Wells, J.M. (2005). *Campylobacter jejuni* gene expression in response to iron limitation and the role of Fur. Microbiology *151,* 243-257.

56. Huws, S.A., Smith, A.W., Enright, M.C., Wood, P.J., and Brown, M.R. (2006). Amoebae promote persistence of epidemic strains of MRSA. Environ. Microbiol. *8,* 1130-1133.
57. Jeon, K.W., and Lorch, I.J. (1967). Unusual intra-cellular bacterial infection in large, free-living amoebae. Exp. Cell Res. *48,* 236-240.
58. Jeong, H.J., and Yu, H.S. (2005). The role of domestic tap water in *Acanthamoeba* contamination in contact lens storage cases in Korea. Korean J. Parasitol. *43,* 47-50.
59. Jeong, H.J., Jang, E.S., Han, B.I., Lee, K.H., Ock, M.S., Kong, H.H., Chung, D.I., Seol, S.Y., Cho, D.T., and Yu, H.S. (2007). *Acanthamoeba*: could it be an environmental host of *Shigella*? Exp. Parasitol. *115,* 181-186.
60. Jolley, K.A., Chan, M.S., and Maiden, M.C. (2004). mlstdbNet - distributed multi-locus sequence typing (MLST) databases. BMC Bioinformatics *5,* 86.
61. Jules, M., and Buchrieser, C. (2007). *Legionella pneumophila* adaptation to intracellular life and the host response: clues from genomics and transcriptomics. FEBS Lett. *581,* 2829-2838.
62. Kanehisa, M., Goto, S., Furumichi, M., Tanabe, M., and Hirakawa, M. (2010). KEGG for representation and analysis of molecular networks involving diseases and drugs. Nucleic Acids Res. *38,* D355-60.
63. Katoh, K., Asimenos, G., and Toh, H. (2009). Multiple alignment of DNA sequences with MAFFT. Methods Mol. Biol. *537,* 39-64.
64. Kazwala, R.R., Collins, J.D., Hannan, J., Crinion, R.A., and O'Mahony, H. (1990). Factors responsible for the introduction and spread of *Campylobacter jejuni* infection in commercial poultry production. Vet. Rec. *126,* 305-306.
65. Keener, K.M., Bashor, M.P., Curtis, P.A., Sheldon, B.W., and Kathariou, S. (2004). Comprehensive review of *Campylobacter* and poultry processing. Comprehensive Reviews in Food Science and Food Safety *3,* 105-116.
66. Khan, N.A. (2006). Acanthamoeba: biology and increasing importance in human health. FEMS Microbiol. Rev. *30,* 564-595.
67. Kilvington, S., and Price, J. (1990a). Survival of *Legionella pneumophila* within cysts of *Acanthamoeba polyphaga* following chlorine exposure. J. Appl. Bacteriol. *68,* 519-525.
68. Kilvington, S., and Price, J. (1990b). Survival of *Legionella pneumophila* within cysts of *Acanthamoeba polyphaga* following chlorine exposure. J. Appl. Bacteriol. *68,* 519-525.
69. Kim, C.C., Joyce, E.A., Chan, K., and Falkow, S. (2002). Improved analytical methods for microarray-based genome-composition analysis. Genome Biol. *3,* RESEARCH0065.
70. King, C.H., Shotts, E.B.,Jr, Wooley, R.E., and Porter, K.G. (1988). Survival of coliforms and bacterial pathogens within protozoa during chlorination. Appl. Environ. Microbiol. *54,* 3023-3033.
71. Konkel, M.E., Kim, B.J., Rivera-Amill, V., and Garvis, S.G. (1999). Bacterial secreted proteins are required for the internaliztion of *Campylobacter jejuni* into cultured mammalian cells. Mol. Microbiol. *32,* 691-701.
72. Kuiper, M.W. (2006). Occurrence of *Legionella pneumophila* and *Hartmannella vermiformis* in fresh water environments and their interactions in biofilms. PhD thesis.

73. La Scola, B., and Raoult, D. (2001). Survival of *Coxiella burnetii* within free-living amoeba *Acanthamoeba castellanii*. Clin. Microbiol. Infect. *7,* 75-79.
74. Lahiri, R., and Krahenbuhl, J.L. (2008). The role of free-living pathogenic amoeba in the transmission of leprosy: a proof of principle. Lepr. Rev. *79,* 401-409.
75. Lamothe, J., Thyssen, S., and Valvano, M.A. (2004). *Burkholderia cepacia* complex isolates survive intracellularly without replication within acidic vacuoles of *Acanthamoeba polyphaga*. Cell. Microbiol. *6,* 1127-1138.
76. Landers, P., Kerr, K.G., Rowbotham, T.J., Tipper, J.L., Keig, P.M., Ingham, E., and Denton, M. (2000). Survival and growth of *Burkholderia cepacia* within the free-living amoeba *Acanthamoeba polyphaga*. Eur. J. Clin. Microbiol. Infect. Dis. *19,* 121-123.
77. Li, R., Yu, C., Li, Y., Lam, T.W., Yiu, S.M., Kristiansen, K., and Wang, J. (2009). SOAP2: an improved ultrafast tool for short read alignment. Bioinformatics *25,* 1966-1967.
78. Linton, D., Lawson, A.J., Owen, R.J., and Stanley, J. (1997). PCR detection, identification to species level, and fingerprinting of *Campylobacter jejuni* and *Campylobacter coli* direct from diarrheic samples. J. Clin. Microbiol. *35,* 2568-2572.
79. Long, A.D., Mangalam, H.J., Chan, B.Y., Tolleri, L., Hatfield, G.W., and Baldi, P. (2001). Improved statistical inference from DNA microarray data using analysis of variance and a Bayesian statistical framework. Analysis of global gene expression in *Escherichia coli* K12. J. Biol. Chem. *276,* 19937-19944.
80. Ly, T.M., and Muller, H.E. (1989). Heat tolerance of free living and of intracellular Listeria. Zentralbl. Hyg. Umweltmed. *189,* 272-276.
81. Ly, T.M., and Muller, H.E. (1990). Ingested *Listeria monocytogenes* survive and multiply in protozoa. J. Med. Microbiol. *33,* 51-54.
82. MacKay, R.M., and Doolittle, W.F. (1981). Nucleotide sequences of *Acanthamoeba castellanii* 5S and 5.8S ribosomal ribonucleic acids: phylogenetic and comparative structural analyses. Nucleic Acids Res. *9,* 3321-3334.
83. Mahoney, F.J., Hoge, C.W., Farley, T.A., Barbaree, J.M., Breiman, R.F., Benson, R.F., and McFarland, L.M. (1992). Communitywide outbreak of Legionnaires' disease associated with a grocery store mist machine. J. Infect. Dis. *165,* 736-739.
84. Markoula, S., Giannopoulos, S., Sarmas, I., Tzavidi, S., Kyritsis, A.P., and Lagos, G. (2007). Guillain-Barre syndrome in northwest Greece. Acta Neurol. Scand. *115,* 167-173.
85. Medeiros, D.T., Sattar, S.A., Farber, J.M., and Carrillo, C.D. (2008). Occurrence of *Campylobacter* spp. in raw and ready-to-eat foods and in a Canadian food service operation. J. Food Prot. *71,* 2087-2093.
86. Messens, W., Herman, L., De Zutter, L., and Heyndrickx, M. (2009). Multiple typing for the epidemiological study of contamination of broilers with thermotolerant *Campylobacter*. Vet. Microbiol. *138,* 120-131.
87. Michel, R., Burghardt, H., and Bergmann, H. (1995). *Acanthamoeba*, naturally intracellularly infected with *Pseudomonas aeruginosa*, after their isolation from a microbiologically contaminated drinking water system in a hospital. Zentralbl. Hyg. Umweltmed. *196,* 532-544.
88. Miller, C.E., Rock, J.D., Ridley, K.A., Williams, P.H., and Ketley, J.M. (2008). Utilization of lactoferrin-bound and transferrin-bound iron by *Campylobacter jejuni*. J. Bacteriol. *190,* 1900-1911.

89. Miller, C.E., Williams, P.H., and Ketley, J.M. (2009). Pumping iron: mechanisms for iron uptake by *Campylobacter*. Microbiology *155,* 3157-3165.
90. Miller, G., Dunn, G.M., Smith-Palmer, A., Ogden, I.D., and Strachan, N.J. (2004). Human campylobacteriosis in Scotland: seasonality, regional trends and bursts of infection. Epidemiol. Infect. *132,* 585-593.
91. Monteville, M.R., Yoon, J.E., and Konkel, M.E. (2003). Maximal adherence and invasion of INT 407 cells by C*ampylobacter jejuni* requires the CadF outermembrane protein and microfilament reorganization. Microbiology *149,* 153-165.
92. Mortazavi, A., Williams, B.A., McCue, K., Schaeffer, L., and Wold, B. (2008). Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nat. Methods *5,* 621-628.
93. Mullock, B.M., Smith, C.W., Ihrke, G., Bright, N.A., Lindsay, M., Parkinson, E.J., Brooks, D.A., Parton, R.G., James, D.E., Luzio, J.P., and Piper, R.C. (2000). Syntaxin 7 is localized to late endosome compartments, associates with Vamp 8, and is required for late endosome-lysosome fusion. Mol. Biol. Cell *11,* 3137-3153.
94. Mura, M., Bull, T.J., Evans, H., Sidi-Boumedine, K., McMinn, L., Rhodes, G., Pickup, R., and Hermon-Taylor, J. (2006). Replication and long-term persistence of bovine and human strains of *Mycobacterium avium* subsp. paratuberculosis within *Acanthamoeba polyphaga*. Appl. Environ. Microbiol. *72,* 854-859.
95. Nachamkin, I. (2002). Chronic effects of *Campylobacter* infection. Microbes Infect. *4,* 399-403.
96. Nadeau, E., Messier, S., and Quessy, S. (2002). Prevalence and comparison of genetic profiles of *Campylobacter* strains isolated from poultry and sporadic cases of campylobacteriosis in humans. J. Food Prot. *65,* 73-78.
97. Naikare, H., Palyada, K., Panciera, R., Marlow, D., and Stintzi, A. (2006). Major role for FeoB in *Campylobacter jejuni* ferrous iron acquisition, gut colonization, and intracellular survival. Infect. Immun. *74,* 5433-5444.
98. Newell, D.G., and Fearnley, C. (2003). Sources of *Campylobacter* colonization in broiler chickens. Appl. Environ. Microbiol. *69,* 4343-4351.
99. Nikul'shin, S.V., Onatskaia, T.G., Lukanina, L.M., and Bondarenko, A.I. (1992). Associations of the soil amoeba *Hartmannella rhysodes* with the bacterial causative agents of plague and pseudotuberculosis in an experiment. Zh. Mikrobiol. Epidemiol. Immunobiol. *(9-10),* 2-5.
100. Nylen, G., Dunstan, F., Palmer, S.R., Andersson, Y., Bager, F., Cowden, J., Feierl, G., Galloway, Y., Kapperud, G., Megraud, F*., et al.* (2002). The seasonal distribution of campylobacter infection in nine European countries and New Zealand. Epidemiol. Infect. *128,* 383-390.
101. Ogden, I.D., Dallas, J.F., MacRae, M., Rotariu, O., Reay, K.W., Leitch, M., Thomson, A.P., Sheppard, S.K., Maiden, M., Forbes, K.J., and Strachan, N.J. (2009). *Campylobacter* excreted into the environment by animal sources: prevalence, concentration shed, and host association. Foodborne Pathog. Dis. *6,* 1161-1170.
102. Oliver, H.F., Orsi, R.H., Ponnala, L., Keich, U., Wang, W., Sun, Q., Cartinhour, S.W., Filiatrault, M.J., Wiedmann, M., and Boor, K.J. (2009). Deep RNA sequencing of *L. monocytogenes* reveals overlapping and extensive stationary phase and sigma B-dependent transcriptomes, including multiple highly transcribed noncoding RNAs. BMC Genomics *10,* 641.

103. Page F.C. (1976). An illustrated key to freshwater and soil amoebae. Published by Freshwater Biological Association.
104. Palyada, K., Sun, Y.Q., Flint, A., Butcher, J., Naikare, H., and Stintzi, A. (2009). Characterization of the oxidative stress stimulon and PerR regulon of *Campylobacter jejuni*. BMC Genomics *10,* 481.
105. Palyada, K., Threadgill, D., and Stintzi, A. (2004). Iron acquisition and regulation in *Campylobacter jejuni*. J. Bacteriol. *186,* 4714-4729.
106. Paszko-Kolva, C., Yamamoto, H., Shahamat, M., Sawyer, T.K., Morris, G., and Colwell, R.R. (1991). Isolation of amoebae and *Pseudomonas* and *Legionella* spp. from eyewash stations. Appl. Environ. Microbiol. *57,* 163-167.
107. Pedersen, K. (1982). Factors regulating microbial biofilm development in a system with slowly flowing seawater. Appl. Environ. Microbiol. *44,* 1196-1204.
108. Pei, Z., Burucoa, C., Grignon, B., Baqar, S., Huang, X.Z., Kopecko, D.J., Bourgeois, A.L., Fauchere, J.L., and Blaser, M.J. (1998). Mutation in the peb1A locus of *Campylobacter jejuni* reduces interactions with epithelial cells and intestinal colonization of mice. Infect. Immun. *66,* 938-943.
109. Persson, S., and Olsen, K.E. (2005). Multiplex PCR for identification of *Campylobacter coli* and *Campylobacter jejuni* from pure cultures and directly on stool samples. J. Med. Microbiol. *54,* 1043-1047.
110. Purdy, D., Cawthraw, S., Dickinson, J.H., Newell, D.G., and Park, S.F. (1999). Generation of a superoxide dismutase (SOD)-deficient mutant of *Campylobacter coli*: evidence for the significance of SOD in *Campylobacter survival* and colonization. Appl. Environ. Microbiol. *65,* 2540-2546.
111. Rahman, M., Abd, H., Romling, U., Sandstrom, G., and Mollby, R. (2008). *Aeromonas-Acanthamoeba* interaction and early shift to a viable but nonculturable state of *Aeromonas* by *Acanthamoeba*. J. Appl. Microbiol. *104,* 1449-1457.
112. Ribot, E.M., Fitzgerald, C., Kubota, K., Swaminathan, B., and Barrett, T.J. (2001). Rapid pulsed-field gel electrophoresis protocol for subtyping of *Campylobacter jejuni*. J. Clin. Microbiol. *39,* 1889-1894.
113. Ridley, K.A., Rock, J.D., Li, Y., and Ketley, J.M. (2006). Heme utilization in *Campylobacter jejuni*. J. Bacteriol. *188,* 7862-7875.
114. Rodriguez-Zaragoza, S. (1994). Ecology of free-living amoebae. Crit. Rev. Microbiol. *20,* 225-241.
115. Rohr, U., Weber, S., Michel, R., Selenka, F., and Wilhelm, M. (1998). Comparison of free-living amoebae in hot water systems of hospitals with isolates from moist sanitary areas by identifying genera and determining temperature tolerance. Appl. Environ. Microbiol. *64,* 1822-1824.
116. Rowbotham, T.J. (1980). Preliminary report on the pathogenicity of *Legionella pneumophila* for freshwater and soil amoebae. J. Clin. Pathol. *33,* 1179-1183.
117. Rowbotham, T.J. (1983). Isolation of *Legionella pneumophila* from clinical specimens via amoebae, and the interaction of those and other isolates with amoebae. J. Clin. Pathol. *36,* 978-986.
118. Rowbotham, T.J. (1986). Current views on the relationships between amoebae, legionellae and man. Isr. J. Med. Sci. *22,* 678-689.
119. Saeed, A., Abd, H., Edvinsson, B., and Sandstrom, G. (2009). *Acanthamoeba castellanii* an environmental host for *Shigella dysenteriae* and *Shigella sonnei*. Arch. Microbiol. *191,* 83-88.

120. Sahin, O., Morishita, T.Y., and Zhang, Q. (2002). *Campylobacter* colonization in poultry: sources of infection and modes of transmission. Anim. Health. Res. Rev. *3,* 95-105.
121. Scaglia, M., Strosselli, M., Grazioli, V., Gatti, S., Bernuzzi, A.M., and de Jonckheere, J.F. (1983). Isolation and identification of pathogenic *Naegleria australiensis* (Amoebida, Vahlkampfiidae) from a spa in northern Italy. Appl. Environ. Microbiol. *46,* 1282-1285.
122. Schuster, C.J., Ellis, A.G., Robertson, W.J., Charron, D.F., Aramini, J.J., Marshall, B.J., and Medeiros, D.T. (2005). Infectious disease outbreaks related to drinking water in Canada, 1974-2001. Can. J. Public Health *96,* 254-258.
123. Schuster, F.L., and Visvesvara, G.S. (2004). Opportunistic amoebae: challenges in prophylaxis and treatment. Drug Resist Updat *7,* 41-51.
124. Sharma, C.M., Hoffmann, S., Darfeuille, F., Reignier, J., Findeiss, S., Sittka, A., Chabas, S., Reiche, K., Hackermuller, J., Reinhardt, R., Stadler, P.F., and Vogel, J. (2010). The primary transcriptome of the major human pathogen *Helicobacter pylori*. Nature *464,* 250-255.
125. Sheppard, F.R., Kelher, M.R., Moore, E.E., McLaughlin, N.J., Banerjee, A., and Silliman, C.C. (2005). Structural organization of the neutrophil NADPH oxidase: phosphorylation and translocation during priming and activation. J. Leukoc. Biol. *78,* 1025-1042.
126. Shoff, M.E., Rogerson, A., Kessler, K., Schatz, S., and Seal, D.V. (2008). Prevalence of *Acanthamoeba* and other naked amoebae in South Florida domestic water. J. Water. Health. *6,* 99-104.
127. Singh, T., and Coogan, M.M. (2005). Isolation of pathogenic *Legionella* species and legionella-laden amoebae in dental unit waterlines. J. Hosp. Infect. *61,* 257-262.
128. Skriwan, C., Fajardo, M., Hagele, S., Horn, M., Wagner, M., Michel, R., Krohne, G., Schleicher, M., Hacker, J., and Steinert, M. (2002). Various bacterial pathogens and symbionts infect the amoeba *Dictyostelium discoideum*. Int. J. Med. Microbiol. *291,* 615-624.
129. Smart, J.P., Cliff, M.J., and Kelly, D.J. (2009). A role for tungsten in the biology of *Campylobacter jejuni*: tungstate stimulates formate dehydrogenase activity and is transported via an ultra-high affinity ABC system distinct from the molybdate transporter. Mol. Microbiol. *74,* 742-757.
130. Snelling, W.J., McKenna, J.P., Lecky, D.M., and Dooley, J.S. (2005). Survival of *Campylobacter jejuni* in waterborne protozoa. Appl. Environ. Microbiol. *71,* 5560-5571.
131. Snelling, W.J., Stern, N.J., Lowery, C.J., Moore, J.E., Gibbons, E., Baker, C., and Dooley, J.S. (2008). Colonization of broilers by *Campylobacter jejuni* internalized within *Acanthamoeba castellanii*. Arch. Microbiol. *189,* 175-179.
132. Sproston, E.L., Ogden, I.D., Macrae, M., Forbes, K.J., Dallas, J.F., Sheppard, S.K., Cody, A., Colles, F., Wilson, M.J., and Strachan, N.J. (2010). Multi-locus sequence types of *Campylobacter* carried by flies and slugs acquired from local ruminant faeces. J. Appl. Microbiol.
133. St-Pierre, K., Lévesque, S., Frost, E., Carrier, N., Arbeit, R.D., and Michaud, S. (2009). Thermotolerant coliforms are not a good surrogate for *Campylobacter* spp. in environmental water. Appl. Environ. Microbiol *75,* 6736-6744.

134. Steinberg, K.M., and Levin, B.R. (2007). Grazing protozoa and the evolution of the *Escherichia coli* O157:H7 Shiga toxin-encoding prophage. Proc. Biol. Sci. *274,* 1921-1929.
135. Steinert, M., Birkness, K., White, E., Fields, B., and Quinn, F. (1998). *Mycobacterium avium* bacilli grow saprozoically in coculture with *Acanthamoeba polyphaga* and survive within cyst walls. Appl. Environ. Microbiol. *64,* 2256-2261.
136. Stenmark, H., Parton, R.G., Steele-Mortimer, O., Lutcke, A., Gruenberg, J., and Zerial, M. (1994). Inhibition of rab5 GTPase activity stimulates membrane fusion in endocytosis. EMBO J. *13,* 1287-1296.
137. Stern, N.J., Bailey, J.S., Blankenship, L.C., Cox, N.A., and McHan, F. (1988). Colonization characteristics of *Campylobacter jejuni* in chick ceca. Avian Dis. *32,* 330-334.
138. Stern, N.J., Robach, M.C., Cox, N.A., and Musgrove, M.T. (2002). Effect of drinking water chlorination on *Campylobacter* spp. colonization of broilers. Avian Dis. *46,* 401-404.
139. Stothard, D.R., Schroeder-Diedrich, J.M., Awwad, M.H., Gast, R.J., Ledee, D.R., Rodriguez-Zaragoza, S., Dean, C.L., Fuerst, P.A., and Byers, T.J. (1998). The evolutionary history of the genus *Acanthamoeba* and the identification of eight new 18S rRNA gene sequence types. J. Eukaryot. Microbiol. *45,* 45-54.
140. Strahl, E.D., Gillaspy, G.E., and Falkinham, J.O.,3rd. (2001). Fluorescent acid-fast microscopy for measuring phagocytosis of *Mycobacterium avium*, *Mycobacterium intracellulare*, and *Mycobacterium scrofulaceum* by *Tetrahymena pyriformis* and their intracellular growth. Appl. Environ. Microbiol. *67,* 4432-4439.
141. Tamura, K., Dudley, J., Nei, M., and Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. *24,* 1596-1599.
142. Templeton, J.M., De Jong, A.J., Blackall, P.J., and Miflin, J.K. (2006). Survival of *Campylobacter* spp. in darkling beetles (*Alphitobius diaperinus*) and their larvae in Australia. Appl. Environ. Microbiol. *72,* 7909-7911.
143. Thom, S., Warhurst, D., and Drasar, B.S. (1992). Association of *Vibrio cholerae* with fresh water amoebae. J. Med. Microbiol. *36,* 303-306.
144. Thomas, V., and McDonnell, G. (2007). Relationship between mycobacteria and amoebae: ecological and epidemiological concerns. Lett. Appl. Microbiol. *45,* 349-357.
145. Thomas, V., McDonnell, G., Denyer, S.P., and Maillard, J.Y. (2009). Free-living amoebae and their intracellular pathogenic microorganisms: risks for water quality. FEMS Microbiol. Rev.
146. Van Dyke, M.I., Morton, V.K., McLellan, N.L., and Huck, P.M. (2010). The occurrence of *Campylobacter* in river water and waterfowl within a watershed in southern Ontario, Canada. J. Appl. Microbiol.
147. van Vliet, A.H., Baillon, M.A., Penn, C.W., and Ketley, J.M. (2001). The iron-induced ferredoxin FdxA of *Campylobacter jejuni* is involved in aerotolerance. FEMS Microbiol. Lett. *196,* 189-193.
148. van Vliet, A.H., Baillon, M.L., Penn, C.W., and Ketley, J.M. (1999). *Campylobacter jejuni* contains two fur homologs: characterization of iron-responsive regulation of peroxide stress defense genes by the PerR repressor. J. Bacteriol. *181,* 6371-6376.

149. Vesaluoma, M., Kalso, S., Jokipii, L., Warhurst, D., Ponka, A., and Tervo, T. (1995). Microbiological quality in Finnish public swimming pools and whirlpools with special reference to free living amoebae: a risk factor for contact lens wearers? Br. J. Ophthalmol. *79,* 178-181.
150. Visvesvara, G.S., Moura, H., and Schuster, F.L. (2007). Pathogenic and opportunistic free-living amoebae: *Acanthamoeba* spp., *Balamuthia mandrillaris*, *Naegleria fowleri*, and *Sappinia diploidea*. FEMS Immunol. Med. Microbiol. *50,* 1-26.
151. Wai, S.N., Nakayama, K., Umene, K., Moriya, T., and Amako, K. (1996). Construction of a ferritin-deficient mutant of *Campylobacter jejuni*: contribution of ferritin to iron storage and protection against oxidative stress. Mol. Microbiol. *20,* 1127-1134.
152. Wales, A.D., Carrique-Mas, J.J., Rankin, M., Bell, B., Thind, B.B., and Davies, R.H. (2010). Review of the carriage of zoonotic bacteria by arthropods, with special reference to *Salmonella* in mites, flies and litter beetles. Zoonoses Public. Health. *57,* 299-314.
153. Wang, Z., Gerstein, M., and Snyder, M. (2009). RNA-Seq: a revolutionary tool for transcriptomics. Nat. Rev. Genet. *10,* 57-63.
154. Wedderkopp, A., Gradel, K.O., Jorgensen, J.C., and Madsen, M. (2001). Pre-harvest surveillance of *Campylobacter* and *Salmonella* in Danish broiler flocks: a 2-year study. Int. J. Food Microbiol. *68,* 53-59.
155. Whan, L., Grant, I.R., and Rowe, M.T. (2006). Interaction between *Mycobacterium avium* subsp. *paratuberculosis* and environmental protozoa. BMC Microbiol. *6,* 63.
156. Winer, J.B. (2008). Guillain-Barre syndrome. BMJ *337,* a671.
157. Winiecka-Krusnell, J., Wreiber, K., von Euler, A., Engstrand, L., and Linder, E. (2002). Free-living amoebae promote growth and survival of *Helicobacter pylori*. Scand. J. Infect. Dis. *34,* 253-256.
158. Wisessombat, S., Kittiniyom, K., Srimanote, P., Wonglumsom, W., and Voravuthikunchai, S.P. (2009). A novel method and simple apparatus for the detection of thermophilic *Campylobacter* spp. in chicken meat products. J. Microbiol. Methods *76,* 169-173.
159. Xu, F., Zeng, X., Haigh, R.D., Ketley, J.M., and Lin, J. (2010). Identification and characterization of a new ferric enterobactin receptor, CfrB, in *Campylobacter*. J. Bacteriol. *192,* 4425-4435.
160. Ye, J., Fang, L., Zheng, H., Zhang, Y., Chen, J., Zhang, Z., Wang, J., Li, S., Li, R., Bolund, L., and Wang, J. (2006). WEGO: a web tool for plotting GO annotations. Nucleic Acids Res. *34,* W293-7.
161. Yoder-Himes, D.R., Chain, P.S., Zhu, Y., Wurtzel, O., Rubin, E.M., Tiedje, J.M., and Sorek, R. (2009). Mapping the *Burkholderia cenocepacia* niche response via high-throughput sequencing. Proc. Natl. Acad. Sci. U. S. A. *106,* 3976-3981.
162. Yuki, N. (2001). Infectious origins of, and molecular mimicry in, Guillain-Barre and Fisher syndromes. Lancet Infect. Dis. *1,* 29-37.
163. Zerial, M., and McBride, H. (2001). Rab proteins as membrane organizers. Nat. Rev. Mol. Cell Biol. *2,* 107-117.
164. Zhang, Y., Sun, X., Wang, Z., Li, R., Luo, S., Jin, X., Deng, S., and Chen, W. (2004). Identification of 18S Ribosomal DNA Genotype of *Acanthamoeba* from Patients with Keratitis in North China. Investigative Ophthalmology & Visual Science *45,* 1904-1907.

165. Ziprin, R.L., Young, C.R., Stanker, L.H., Hume, M.E., and Konkel, M.E. (1999). The absence of cecal colonization of chicks by a mutant of *Campylobacter jejuni* not expressing bacterial fibronectin-binding protein. Avian Dis. *43,* 586-589.

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