RECOVERY AND TRANSCRIPTOME ANALYSIS OF *CAMPYLOBACTER JEJUNI* ASSOCIATED WITH PROCESSED POULTRY

by

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ABSTRACT

Three studies were conducted to determine how *Campylobacter* spp. are capable of surviving on raw refrigerated poultry products and potentially cause human illness despite stresses it may encounter. The first study investigated the impact of carcass rinse and chicken exudate (weep) sampling on the recovery of *Campylobacter* spp. subtypes. It is important that sampling procedures provide subtype information indicative of human exposure. Our hypothesis was that sampling exudate allowed for the recovery of subtypes that may have been injured and non-culturable during the time of rinse. Three farms were sampled, collecting fecal samples and subsequent pre-chill rinse, post-chill rinse, 2-day exudate and 6-day exudate samples. Results suggest the same subtypes are not recovered by the two sampling procedures. This finding is important because different *C. jejuni* subtypes have been shown to have different virulence potentials. The second study investigated changes in transcriptome of *C. jejuni* in response to exposure to chicken exudate using expression microarrays. RNA was isolated from *C. jejuni* not exposed to exudate, after 15 minutes exposure and 1 hour exposure at 42°C under microaerobic conditions. Several stress response genes including, *clpB*, *grpE*, *groES*, and *ahpC,* were identified as differentially expressed. In addition genes such as *acnB*, *ppi* and *spoT,* which are possibly involved in the regulation of other genes and possible virulence

factors, were identified as differentially expressed. In the third study three *C. jejuni* isolates possessing mutations at genes identified as differentially expressed in the previous study (*ahpC, clpB, spoT*) were used to determine if expression of these genes influenced survival of *C. jejuni* in raw refrigerated poultry products. Both chicken skin and chicken exudate were used as substrates, and the levels of wild-type *C. jejuni* 11168 as well as the *ΔahpC,ΔclpB*, and *ΔspoT* mutants were recorded at 0, 16, 24, 48, and 96 hours post inoculation. Results from the skin model suggest little difference in survival of the wild-type and the mutants, however, the chicken exudate model indicated that lack of expression of *ahpC* and *spoT* has an adverse effect on the survival of *C. jejuni* in raw refrigerated poultry products.

INDEX WORDS: *Campylobacter*, poultry skin, chicken exudate, microbial recovery, expression microarray

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Bachelors of Science, University of Florida, 2000

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2007

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August 2007

DEDICATION

I dedicate this work to my mother Alise Balams, whose constant love, support and guidance have helped me stay on this path. I would not be here without the lessons she taught me. I hope she sees this as proof that she is an exceptional mother and never doubts that.

ACKNOWLEDGEMENTS

I would like to first acknowledge the help of my major-professor Dr. Joseph Frank who allowed me to pursue my PhD in his lab and helped me to hone both my laboratory skills and analytical skills. He was always available when I needed guidance during my program of study. I would also like to acknowledge the help and patience of my committee. Dr. Berrang and Dr. Harrison both provided me with a theoretical and practical foundation in food microbiology since my Masters degree. Dr. Meinersmann helped me develop a better understanding adaptation, physiology, and molecular techniques. I would also like to thank Dr. Jake Mulligan for serving on my committee at short notice.

I would also like to acknowledge the contributions of Dr. Kelli Hiett. Though not on my committee she trained me in molecular techniques, pushed me to be a better communicator, and helped me become a more confident person. The skills she's taught me have not only helped me become a better scientist but a better person as well.

I would also like to acknowledge all of the help and assistance of people in both of the labs I worked in as well as those outside of them; Revis Chemilewski, Marie Maier, Robin Kuntz, Johnna Garrish, and Deann Akins. I would also like to thank all of my friends for being patient with my throughout my program; Cam, Latoya, Bruce, and everyone else.

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INTRODUCTION

Campylobacter spp. are Gram-negative, non-sporeforming, spiral rod shaped bacteria. They are currently the leading cause of bacterial foodborne illness. Campylobacteriosis, the disease caused by *Campylobacter* spp., usually cause mild and selflimiting nausea, abdominal pain and diarrhea. However, those infected may develop secondary sequelae such as Guillain-Barre syndrome (GBS) an autoimmune disease of the peripheral nervous system.

The Economic Research Service estimates that the annual economic cost due to foodborne *Campylobacter* spp. infections to be 0.7 to $4.3 billion, and cases associated with GBS to be 0.1 to $1.3 billion. These estimates are based on medical costs due to infection, productivity loss due to missed work, and the value of premature deaths. Therefore, reducing *Campylobacter* spp. infections would not only improve public health but also prevent up to $5.6 billion in costs annually.

Campylobacter spp. infections are linked to several sources, including contaminated water, unpasteurized milk, cattle, pigs, and domestic pets. Perhaps the most commonly associated source is the consumption and handling of contaminated poultry. For this reason, a reduction in poultry associated *Campylobacter* spp. would potentially reduce human exposure and subsequent illness. A reduction could be accomplished by better understanding the physiology of *Campylobacter* spp. and directing mitigation strategies towards survival mechanisms that have been identified.

The purpose of this research is to first better understand the diversity of *Campylobacter* spp. subtypes humans are potentially exposed to, secondly identify differentially expressed genes when *Campylobacter jejuni* is exposed to chicken exudate,

and finally determine if a sample of these genes are involved in the survival of *Campylobacter jejuni* when it is associated with poultry under refrigerated conditions. The results of these experiments will potentially lead to future investigations which may aid in better understanding *Campylobacter* spp. physiology and developing mitigation strategies specific to it.

LITERATURE REVIEW

History and General Characteristics

In 1886 Theodor Escherich observed and described a nonculturable spiral shaped organism isolated from the stool of infants with diarrhea. Kist *(46)* suggested these organisms were most likely *Campylobacter* due to their typical morphology, association with enteritis in neonates, infants and kittens, and the failure to grow on solid media despite microscopic observation. In 1963 Sebald and Veron *(72)* created the genus *Campylobacter* based on their low GC content, microaerobic growth requirements, and their inability to ferment sugars.

Campylobacter are slender, spiral curved rods 0.2-0.5 μm wide and 0.5-5 μm long. They are Gram negative and non-sporeforming. They have a single polar flagellum at one or both end which gives them their characteristic corkscrew motility *(68)*.

Campylobacter spp. are chemo-organotrophs which do not metabolize simple carbohydrates. They derive their energy from amino acids or tricarboxylic acid cycle intermediates. The substrate most readily used is species dependent. Westfall et. al *(86)* showed *C. jejuni* utilized glutamine more readily than glutamate or formate while the reverse was true for *C. coli*. Also Hoffman and Goodman *(35)* showed formate and other substrates are more rapidly metabolized because they are utilized in the periplasmic space and transport across the cytoplasmic membrane is not required.

Optimal growth of *Campylobacter* spp. are obtained in a micoaerobic atmosphere (5% oxygen, 10% carbon dioxide, 85% nitrogen) *(43)*. The optimum temperature for growth is between 42 and 45°C and the optimum pH between 6.5 and 7.5 *(26)*. In addition to sensitivity to oxygen, *Campylobacter* is also more sensitive to heat, cold and disinfectants than other bacterial pathogens such as E. coli O157:H7. Koidis and Doyle *(47)* found D-values for *C. jejuni* at 49 and 57°C were approximately 20min and 0.8min respectively. *Campylobacter* is capable of surviving refrigerated temperatures in food *(47)*. Also, oxygen consumption, catalase activity, ATP generation, chemotaxis and protein synthesis are all observed in *Campylobacter* at 4°C *(32)*. However, *Campylobacter* may be sublethally injured during refrigeration at 4°C or during freezing at -20°C *(65)*.

Despite *Campylobacter*'s sensitivity to environmental stress, campylobacteriosis is one of the leading causes of bacterial gastroenteritis in the world. In 2003, *Campylobacter* spp. accounted for 33.4% of the laboratory diagnosed cases of foodborne bacterial gastroenteritis reported by Foodnet *(19)*.

Clinical Features and Pathogenesis

The typical incubation period for *Campylobacter* infections can be as few as 24 hours and as long as 10 days *(75)*. The predominate clinical manifestations are bloody diarrhea, acute abdominal cramping and fever *(11)*. Colonoscopy and biopsy also revealed diffuse inflammatory colitis *(13)*.

Despite the high incidence of campylobacteriosis and knowledge of its clinical features, little is known about the mechanism of its pathogenesis. Johnson and Lior *(40)*

first described a heat labile protein toxin which caused Chinese hamster ovary (CHO), Hep-2, and vero cells to slowly distend and die. This toxin is now known as cytolethal distending toxin (CDT). In regards to the inflammatory aspect of the disease Hickey et al. *(33)* demonstrated that CDT induced the release of interleukin-8(IL-8), a proinflammatory cytokine and mediator for local inflammatory response. *C. jejuni* has also been shown to induce secretion of proinflammatory chemokine from human epithelial cells *(36)*.

The role of CDT in diarrheal disease is still not completely understood. Mechanisms known to cause diarrhea such as the intracellular accumulation of cyclic AMP was found not to be a significant attribute of the toxin. Pickett *(61)* suggests that it may be due to blocking the development of mature epithelial cells needed for absorptive function. This idea is supported by results from Whitehouse et al. *(87)* who showed that *C. jejuni* CDT caused cells to become locked in the G2 phase of the cell cycle.

Poultry Associated *Campylobacter*

Pre-harvest

Handling and consumption of poultry or poultry related products are considered to be a primary source for *Campylobacter* induced disease in humans *(15)*, *(41)*, *(45)*. In poultry *Campylobacter* is a commensal organism and colonizes the mucus overlying the epithelial cells primarily in the ceca and small intestine, but may also be recovered from various places in the gastrointestinal tract and from the liver and spleen *(4)*.

Several sources of potential *Campylobacter* contamination of poultry have been considered, including vertical transmission *(23)*, contaminated water, the rearing environment, pests, other farm animals, and also farm personnel *(42)*.

Vertical transmission, transfer from hen to chick, is a controversial theory on the mode of transmission. *Campylobacter* has been isolated from various segments of the reproductive tract of chickens *(16)*. Genotyping has shown organisms from the oviduct can also be found in the feces, suggesting some of these organisms may ascend from the cloaca. Allen and Griffiths *(2)* showed *Campylobacter jejuni* could permeate an intact egg shell by immersing eggs in a suspension of luminescent *Campylobacter*. However, the bacteria are restricted to the inner shell and not the actual egg contents. Subtyping data from Bull et al. *(17)* refutes the theory of vertical transmission. Despite colonization of parent flocks, 30% of broiler flocks were not colonized during their lifetime. Also different subtypes of *Campylobacter* were isolated from parent and progeny for the 4 out of 6 positive flocks. Those that were the same may have been due to the close geographic proximity of the breeder and broiler houses (*17*).

Drinkers and water lines in broiler houses have been reported to be positive for *Campylobacter*, with the percent of positive samples as high as 88% *(6)*. However, several studies have shown that this contamination usually occurs after colonization of the flock rather than precedes it *(28)*, *(53)*, *(42)*. Nevertheless, Trachoo et al. *(83)* demonstrated that *C. jejuni* under water-related environmental stress, goes into a viable but non-culturable state, especially in the presence of poultry house derived biofilms. The survival of such forms has been reported to be as long as 4 months *(67)*. Thus it may

be possible flocks to be colonized from *Campylobacter* spp. left in a biofilm from a previous flock.

In general, clean dry litter and fresh feed are not considered to be sources of colonization due to the low water activity being lethal to *Campylobacter (6)*. Also disinfection and cleaning between flocks is usually adequate in preventing contamination of subsequent flocks. Shreeve et al. *(74)* showed only 15% of sequential flocks in 100 houses had evidence of genetically identical strains. The area surrounding the poultry houses may be a more probable source of colonizing *Campylobacter*. Areas outside of poultry houses which do not receive routine cleaning and disinfection between flocks may however be a source of colonization. Bull et al. *(17)* found subtypes of *Campylobacter* isolated from puddles prior to chick placement to be indistinguishable from isolates found in flocks towards the end of production.

Flies, mice, and other pests have also been examined as a potential colonization source. Stern et al *(77)* found 25% of insects caught outside of poultry houses to be *Campylobacter*-positive four weeks prior to detection of *Campylobacter* in flocks. Berndtson et al. *(6)* showed that flies can act as carriers of *C. jejuni* for up to 2 days after infection. Thus flies may be capable of spreading *Campylobacter* spp. from one flock to another in the same house or in adjacent houses. In a later study *(7)* flies caught in houses with *Campylobacter*-negative flocks were never positive, but one out of three flies was positive in a house with a positive flock. Mouse rinses and intestinal samples have also been found to be *Campylobacter*-positive *(7)*, *(77)*.

Campylobacter spp. has also been shown to be present and possibly transferred by workers as well as equipment. In a survey by Shreeve et al. *(74)* in poultry houses where

bird movement was restricted, the first birds colonized were those closest to doors without hygiene barriers used by the staff. Bull et al. *(17)* found flocks that were negative at the farm were partly colonized with *Campylobacter* at slaughter with a subtype indistinguishable from that found on transport crates. *Campylobacter* spp. are capable of surviving on feces on transport crates as long as 48 hours *(8)*. Rambau et al. *(63)* also found truck beds and pallets positive for *Campylobacter* spp.

Once a bird is colonized it takes little time for the infection to spread throughout a flock. Shanker et al. *(73)* showed that the spread from an infected bird to susceptible birds occurs within 72 hours. In flocks of 20,000 birds this spread is logorithimic in nature *(57)*.

Post-harvest

The high number of positive birds persists into the first stages of processing. *Campylobacter* spp. are not only isolated from carcasses but also from the air in the hanging area, picker room, and evisceration room *(6)*. Son et al. *(76)* reported that pre-scald and pre-chill carcasses had the highest contamination rate of *Campylobacter* amongst in plant sites sampled, at 92% and 100% respectively. Izat et al. *(38)* described a similar decrease after scalding followed by an increase before entering the chiller. In the study, *Campylobacter* levels decreased significantly ($\geq$1.84 log cfu p-value $\leq$ 0.05) after scalding, however this was followed by a significant increase in *Campylobacter* levels after the picking procedure.

Campylobacter spp. are thought to contaminate carcasses by initially being retained in a liquid film on the skin from which they migrate and then become entrapped in ridges and crevices *(52),(82)*. The scalding process opens feather follicles to aid in

picking and the follicles remain open throughout processing until immersion chilling at which time any organisms taken up during water uptake are retained due to the feather follicles closing *(82)*. Thus contamination level is directly related to bacterial levels in the processing water *(55)*. Water samples from bird washers and chlorinated water from chillers have been found to be *Campylobacte*r-positive *(6)*. Hiett et al. *(34)* isolated *Campylobacter* spp. more frequently from pre- and postscald water than for pre- and postchill water.

Cools et al. *(22)* sampled several surfaces that come in contact with poultry carcasses during deboning and portioning, including converyer belts, cutting boards, worker gloves and knives. All of the surfaces sampled were negative prior to the start of processing, suggesting cleaning and sanitizing regimens in the plant sampled were effective. However, the study demonstrated cross-contamination does occur. After a batch of carcasses that were 80% contaminated with *C. jejuni* the surfaces were contaminated within 30 min. Although the second batch of carcasses were not initially contaminated with *C. jejuni,* cross-contamination from the surfaces gave rise to contamination of 40% of the breasts produced.

Several in-plant mitigation steps have been shown to significantly reduce the level of *Campylobacter* spp. on poultry carcasses. Lie et al. *(51)* looked at the effect of high temperature inside-outside bird washers on survival of *C. jejuni* attached to carcasses. When sprayed with tap water *C. jejuni* was reduced by 0.5, 1.28 and 1.43 log cfu/carcass for the 20, 55, and 60°C spray treatments, respectively. When sprayed with a chlorine solution (50 ppm) C. jejuni was reduced by 1.21, 1.81, and 2.16 log cfu/carcass for the 20, 55, and 60°C spray treatments, respectively. Chlorination of spray significantly

increased the reduction of *C. jejuni* ($p<0.05$), however, in the case of the chlorine treatments temperature did not significantly affect the reduction.

Other mitigation steps focus on preventing contamination from fecal or cecal sources. Jeffrey et al. *(39)* found that the odds of having a positive skin sample were 35 times greater for carcasses with positive intestines than for carcasses with negative intestines. Berrang et al. *(9)* tested the effect of placing vinegar on the cloaca prior to defeathering, a processing step known to increase levels of *Campylobacter* spp. on the carcass. The use of vinegar resulted in a significantly lesser increase in *Campylobacter* levels. Prespraying chicken carcasses with alkaline electrolyzed water significantly lowered cecal material attachment scores (3.77) compared to tap water (4.07) and 10% TSP (4.08) upon treatment of the dorsal area *(44)*.

Despite mitigation steps, up to 98% of retail broilers have been reported as being positive for *Campylobacter* spp. *(78)*. Thus it is likely that many consumers purchase contaminated products. How consumers handle products will affect the probability of cross-contamination and infection. In regards to holding temperatures, Solow et al. (2003) reports that the highest level of viability of both *C.jejuni* and *C. coli* was maintained at 4°C. At room temperature (25°C) viable counts declined 1 to 2 log CFU/cm^2 over 48 hours and at -20°C viable counts decreased 2 to 3 log CFU/cm^2. In contrast to these results, Lee et al. *(50)* reported *C. jejuni* cells could actually replicate at room temperature and under refrigeration at 4°C. Davis and Conner *(24)* reported that *Campylobacter* spp. survived significantly better on skin samples than on meat samples. This increased survival may be due to the topology of the skin as suggested by Chantarapanont et al. *(21)* who reported that most viable *C. jejuni* were entrapped within

water in skin crevices and feather follicles. Luber et al. *(54)* quantified *Campylobacter* cross-contamination during the handling of contaminated fresh chicken parts in kitchens. Transfer rates for *Campylobacter* from unwashed kitchen utensils or hands to ready-to-eat foods such as fried sausage, cucumber slices, and bread were 27.5, 10.3 and 2.9%, respectively.

Stress Response of *Campylobacter*

During poultry production, processing, and in retail products, *Campylobacter* encounters several environmental stresses, including ambient oxygen, temperature extremes, and nutrient limitation. Despite these stresses *Campylobacter* spp. survive long enough to infect consumers even though it is more fragile than other foodborne pathogens and lacks several well characterized stress response systems such as RpoS, SoxRS, RpoH, and CspA *(58)*. However several studies have researched other stress defense mechanisms in *Campylobacter* spp.

Ambient oxygen levels is one of the major environmental stresses encountered by *Campylobacter*. Although oxygen is required as a terminal respiratory electron acceptor the generation of reactive oxygen species such as superoxide and hydrogen peroxide can be detrimental to cells. There are three major proteins involved in the inactivation of reactive oxygen species . Superoxide dismutase (SodB) acts by removing superoxide anions by their dismutaion into hydrogen peroxide and oxygen *(62)*:

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

The enzyme catalase (KatA) protects against peroxide stress by degrading peroxide into water and oxygen *(30)*. There is also an iron-regulated alkyl hydroxide reductase (AhpC)

which has the ability to destroy toxic hydroperoxide intermediates as well as repair damaged molecules that have been peroxidized *(3)*. Both KatA and AhpC expression are regulated by a PerR homolog described by van Vilet et al. *(84)*. Mutation of the PerR gene in *C. jejuni* resulted in high levels of AhpC and KatA and a subsequent hyperresistance to peroxide stress.

HtrA, a protease found in *E. coli* required for growth and degredation of proteins, also plays a role in combating environmental stress. Bronsted et al. *(14)* constructed a *C. jejuni HtrA* mutant to determine the role of HtrA in stress tolerance. Both the mutant and wildtype showed similar sensitivities to hydrogen peroxide, cumene hydroperoxide and paraquat. However, less mutant cell colonies were formed when grown at 44°C and at an oxygen concentration of 17-20%. These findings indicate a role in heat and oxygen tolerance.

Iron plays a critical role in oxidative stress due to the Fenton reaction. Superoxide does not damage DNA directly, it is thought to do so by producing hydroxyl radicals *(25)*. In the Fenton reaction iron is reduced by O_2^- and then subsequently oxidized by hydrogen peroxide generating a hydroxyl radical which can attack DNA *(20)*:

$$O_2^- + Fe^{3+} \rightarrow O_2 + Fe^{2+}$$

$$H_2O_2 + Fe^{2+} \rightarrow OH\cdot + OH- + Fe^{3+}$$

Guerry et al. *(31)* described two genes from *C. coli*, but also found in *C. jejuni* strains, involved in iron utilization. One gene encodes an iron-repressible outer

membrane protein with significant sequence similarity to siderophore receptors of *Bordetella bronchiseptica* and *V. cholera*. The other encodes a TonB like protein which is required for utilization of hemin, ferichrome and enterochelin

vanVilet et al *(85)* found a gene encoding a putative ferredoxin (fdxA) upstream of ahpC. It was shown that this gene was induced approximately 3 fold by iron. fdxA mutants were equally susceptible to H_2O_2 stress as wildtype strains, however they possessed significantly less aerotolerance, dying ≥ 2 hours earlier than wild-type strains.

One protection method *Campylobacter* spp. have against iron induced oxidative stress is the Dps protein. In *E. coli* Dps protein binds non-specifically to DNA forming stable complexes protecting it from oxidative stress. Dps also exhibits an iron binding property which may protect against oxidative stress. Ishikawa et al *(37)* purified the Dps protein in *C. jejuni* and showed that it has iron binding capabilities, but did not bind to DNA. Dps deficient mutants generated during this study were significantly inhibited by H_2O_2 in comparison to the wild-type parent strain.

vanVilet et al. *(84)* identified a *PerR* homolog in *C. jejuni*. *PerR* is responsible for regulation of *AhpC* and *KatA* in *B. subtilis*. Mutation of the PerR gene in *C. jejuni* resulted in high levels of KatA and AhpC expression and a subsequent hyperresistance to peroxide stress.

As previously stated, *Campylobacter* spp. are regarded as heat sensitive. However, a thermal stress response has been characterized in *C. jejuni*. There was a preferential synthesis of 24 proteins by *C. jejuni* immediately following heat shock including the heat shock proteins GroELS, DnaK, and ClpB *(48)*.

The 60kDa GroEL, in conjuction with the 10 kDa GroES protein, prevents misfolding and aggregation of partially denatured proteins through an ATP-depedent process *(81)*.

In regards to cold stress, *C. jejuni* generally survives better at 4°C than at 25°C *(12)*. Lazaro et al *(49)* demonstrated oxygen consumption, protein synthesis, motility and survival of *C. jejuni* at 4°C. Though *C. jejuni* lacks the major cold shock protein CspA, which acts as an RNA chaperone *(59)*, Moen et al. *(56)* found a number of genes involved in energy metabolism were upregulated at 5°C when compared with 25°C.

In addition to these regulatory systems *C. jejuni* also mounts a global stress response known as the stringent stress response *(29)*. The stringent stress response is typically activated by nutrient deprivation, which results in an abundance of uncharged tRNA molecules at the ribosomal receptor site. This causes the ribosome to stall and ribosomal bound SpoT to catalyze synthesis of guanosine pentophosphate (pppgpp) which is then hydrolyzed to guanosine tetraphosphate, which is thought to bind to RNA polymerase and alter gene expression by affecting promoter specificity, transcription initiation, and elongation *(18)*. Gaynor et al. *(29)* showed via SpoT deletions that the stringent stress response is involved capnophilic growth, aerotolerance, adherence and invasion, and intracellular survival in two epithelial cell culture models.

Microarray Technology

A microarray is a set of genes deposited on a glass slide or other substrate which is used in a hybridization reaction similar to a large scale dot-blot *(88)*. Microarrays can be used to identify genes present in a cellular sample as well as genes expressed by hybridizing cDNA from reverse transcribed sample RNA. However, it should be noted that microarrays are limited to investigating pre-determined genes of interest which are

printed on the slide. Also with regards to expression, some genes may be constitutively expressed and are regulated at the translational or post-translational level, thus this type of expression would not be directly detected by microarrays.

The use of glass microscope slides for printing DNA microarrays began with the Brown group in the mid 1990s *(71)*. Glass is used as a microarray substrate because of its low fluorescence, low cost, high heat resistance and rigidity. For DNA immobilization/spotting to occur glass, slides must be cleaned and coated *(1)*. Slides are coated either adsorptively, as with poly-l-lysine or covalently, as with functionalized saline. The most common non-covalent method to bind nucleic acids to slides utilized is poly-lysine slides *(71)*. In this case the polyanionic DNA interacts with the polycationic surface via coulombic attraction. The DNA is then locked into place by ultraviolet radiation or baking. Though non-covalent mechanisms are predominantly used, covalent binding has several advantages. The DNA is capable of being oriented a certain way, increasing probe availability for hybridization, allowing less non-specific binding, and more stringent washes can be used *(5)*. An example of a covalent binding is the use of amino modified DNA to bind to epoxide activated glass slides. The epoxides react to form amino alcohols via nucleophilic displacement *(1)*.

Before sample cDNA is hybridized to slides it is fluorescently labeled using either direct or indirect labeling. Direct labeling of cDNA is accomplished by a reverse transcriptase reaction with mRNA as a template, dye conjugated nucleotides, and oligo(dT)n as primers *(10)*. One problem with direct labeling is dye-conjugated nucleotides are incorporated less efficiently than unmodified DNTPs because their incorporation leads to chain termination due to steric problems *(89)*.

Also Cy5 nucleotides are incorporated less efficiently than Cy3 substituted nucleotides, which creates the need for dye swapping, thus doubling the amount of work. Indirect labeling incorporates amino-allyl dUTP into the cDNA and the primary amine groups are subsequently conjugated to succinimidyl ester of Cy3 *(64)*. Indirect labeling makes dye:nucleotide ratios higher, however the intensity of fluorescent signal is decreased and the method takes longer and requires more steps *(64)*.

In regards to experimental design of expression, microarray experiments must be replicated since up to 1/3 of cDNA chips fail or are badly distorted. If the aim of the study is to find large differences (more than 2 fold change) between two conditions, then a design with 3 samples per group is adequate *(66)*.

Once data is collected it must be normalized to remove the effects of systematic technical differences between chips. It is commonly observed that under-expressed genes are upregulated in the red channel and moderately expressed genes are upregulated in the green channel. This is due to quenching, a phenomenon where dye molecules adjacent to each other reabsorb light from each other, thus diminishing the signal. Also the amount of re-absorption changes with concentration differently for the two dyes *(66)*. The most common normalization procedure for dual-channel arrays is local regression *(27)*. It removes the curvilinear tendencies of data and centers the red-to-green ratios throughout the entire intensity range, unlike median normalization which does not correct for bias in lower expression ranges *(70)*.

Once data is normalized it can then be subjected to statistical testing. However, it should be noted that when doing statistical test each gene's expression cannot be considered an independent testable hypothesis.

Thus you must test several hypotheses simultaneously. For this reason one must consider the likely probability of Type I error. Type I error is essentially a false positive, rejecting the null hypothesis when it is in fact true. The probability of Type I error is given by the formula $P(\text{Type I error}) = 1-(1-\alpha)^m$,

where α is the significance level and m is the number of simultaneous test. For example if 100 test were performed at a significance level of 0.05, the probability of type I error would be calculated as: $0.994 = 1-(1-0.05)^{100}$. Thus for a microarray experiment in which thousands of genes are tested this can have serious consequences. One remedy to this problem is to use the Bonferronni correction factor. Essentially, if m simultaneous tests are performed, each p-value is compared to $\alpha' = \alpha/m$ instead of α *(60)*.

Microarray Experiments on *Campylobacter* spp.

Several microarray studies have been conducted on *C. jejuni*. Most of which examine changes in expression due to stresses it may encounter in food products, during poultry colonization, or human infection. Stintzi *(79)* investigated changes in the expression profile of *C. jejuni* after experiencing a temperature up-shift from 37 to 42°C. This temperature change was used to model a change in host from human (37°C) to avian (42°C). It was reported that 20% of *C. jejuni* genes were identified as significantly up-regulated or down-regulated over a 50 minute period after the temperature increase. Initially a number of genes encoding ribosomal proteins were down-regulated followed by an up-regulation of chaperones, chaperonins and heat shock proteins. This pattern of expression change suggests an initial stop in growth upon experiencing temperature stress, allowing the bacteria to redirect its energy towards adaptation and survival.

Moen et al. *(56)* also looked at changes in expression of *C. jejuni* with respect to temperature stress. However, they used a multifactor approach to examine the combined effects of temperatures (5 and 25°C) and oxygen tension (anaerobic, microaerobic, and aerobic). Microarray results showed response to oxygen was greatest at higher temperatures which were also the lowest survival conditions.

Stintzi et al. *(80)* investigated gene expression of *C. jejuni* during in vivo growth using a rabbit illeal loop model. Among the genes up-regulated were *cmeABC,* which encodes for a multidrug efflux pump and contributes to bile salt resistance, *clpB*, which encodes an ATP-dependent protease, and *sodB*, which encodes the oxidative stress response protein superoxide dismutase. Additionally there were variations in expression between the individual rabbits, particularly genes encoding proteins in cell envelope composition. These changes reflect the hyperosmotic, oxygen limited, and nutrient poor nature of the intestinal environment.

Gaynor et al *(29)* investigated the effects of mutating the stringent response gene *spoT* on the survival and virulence of *C. jejuni*. Several operons involved in redox balance, metabolism, and energy production and conversion were down regulated in *spoT* mutants, while genes encoding proteins involved in protein folding, degradation and repair were up-regulated in *spoT* mutants.

Sampathkumar et al. *(69)* investigated differences in gene expression between sessile and planktonic *C. jejuni*. The study indicated immobilized cells undergo a shift in cellular priorities away from motility, protein synthesis, and metabolic activities towards iron uptake, oxidative stress defense and membrane transport.

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CHAPTER 1

COMPARISON OF POULTRY EXUDATE AND CARCASS RINSE SAMPLING METHODS FOR THE RECOVERY OF *CAMPYLOBACTER* SPP. SUBTYPES IN THE UNITED STATES[1]

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Abstract

The carcass rinse procedure is a method commonly used for the detection of *Campylobacter* spp. on processed poultry products. Alternatively, carcass exudate (weep or drip), a viscous fluid comprised of blood and water that leaks into packaging, can also be sampled. It is unknown however if these methods preferentially recover different *Campylobacter* spp. subtypes. If there is a difference in subtypes recovered, the *Campylobacter* spp. subtypes from carcass rinse analysis may not be indicative of consumer exposure, as the exudate is the fluid to which consumers are potentially exposed to dues to kitchen cross-contamination. Experiments were conducted to determine if there are differences in recovery of *Campylobacter* spp. subtypes between the two methodologies. The experiment was performed in triplicate using three flocks located on different farms. For each flock 50 fecal samples were obtained on the farm, 25 carcass rinses during pre-chill processing, 25 carcass rinses during post-chill processing, and 50 samples from exudates from carcasses stored at 4°C (25 after 2-day storage and 25 after 6 day storage). Each sample type was cultured for *Campylobacter* spp. Isolates recovered from positive samples were subtyped using *fla*A SVR (flagellin A-short variable region) DNA sequence typing and compared for relatedness. The data demonstrated that multiple subtypes were present in a flock, and that subtypes present in a flock during production were also present on the final processed product. Subtypes recovered by the two recovery methodologies were similar. Combining the totals from all 3 flocks a total of 14 subtypes were recovered from post-chill carcass rinses and 12 subtypes recovered from 6-day exudate samples.

Introduction

Campylobacter is a Gram-negative, microaerophilic foodborne pathogen. In the United States there are an estimated 2.1 to 2.4 million cases of *Campylobacter* spp. related gastroenteritis a year *(27)*. The most common symptoms of campylobacteriosis include nausea, abdominal cramps, and bloody diarrhea. Most cases are mild and self-limiting. However, *Campylobacter* infections have also been associated with the subsequent development of reactive arthritis and Guillain-Barré Syndrome, an autoimmune disease which attacks the peripheral nervous system *(2, 22)*.

Handling and consumption of poultry or poultry related products are primary sources for *Campylobacter* spp. induced disease in humans *(4)*,*(13)*,*(14)*. *Campylobacter* spp. has been cultured from as many as 75% of the live broilers sampled and from as much as 80% of commercially sold processed poultry meat samples *(11)*, *(12)*,*(23)*. A commonly accepted methodology employed for the detection of *Campylobacter* spp. on processed poultry products is a carcass rinse procedure. This procedure involves placing the whole processed carcass in a bag with a 400 ml of sterile buffered peptone water, and vigorously shaking the carcass to dislodge organisms for subsequent collection and cultural analysis *(3)*. This method is beneficial because it can recover organisms from both the external or internal surface and is non-destructive to the sample. An alternative to the carcass rinse procedure is sampling of the carcass exudate (weep or drip), a viscous fluid comprised of blood and water that leaks into commercial packages containing the broiler carcasses.

Recent analyses comparing recovery rates of *Campylobacter* spp. from rinse samples and from exudate samples demonstrated no significant difference in the ability of the two methods to recover numbers of *Campylobacter* spp. *(18)*.

While the two sampling methodologies may be comparable in recovery efficiency, it remains to be determined if there is a preferential selection for *Campylobacter* spp. subtypes between the two methods. It may well be that conditions and stresses associated with the two distinct environments leads to the preferential survival of specific *Campylobacter* spp. subtypes within each environment. If so, the recovery of *Campylobacter* spp. subtypes from carcass rinses may not be indicative consumer exposure. In an effort to further investigate this, *Campylobacter* spp. recovered from the feces of three shedding chicken flocks, the pre-chill area (post-evisceration) of the three processed flocks, carcass rinse samples from the three processed flocks, carcass exudates samples after 2 and 6 days storage, from the three flocks were cultured for *Campylobacter* spp. Recovered isolates were subtyped using *flaA* SVR (short variable region) DNA sequence analysis and compared for relatedness.

Materials and Methods

Broiler Flocks: For each trial the participating producer, located in Georgia, provided access to four rearing houses, each located on a different farm. Each broiler flock investigated was comprised of ca. 20,000 birds involving an "all-in, all-out" stocking policy with a rearing period of six to eight weeks. The pine shaving litter upon which the birds were raised was replaced annually.

Sample Collection:

Fecal samples (production): Three trials were conducted. During the first trial 2 rearing houses of the 4 sampled were used. For the 2nd and 3rd trial only one house of the four sampled was used. The first trial was conducted in May while the second and third trials were conducted in November the following year. Fecal samples were collected one week prior to slaughter of each flock to ensure the flock was *Campylobacter* spp. positive. For the first trial 25 fresh fecal samples were collected while 50 fresh fecal samples were collected for the second and third trial. During the first trial 2 colonies were picked for further analysis from each sample type, while only one was chosen in trials 2 and 3. However, in cases where there were negative samples more colonies were picked from positive samples. Fresh fecal samples were individually placed in sterile specimen cups, placed in ice for transport to the laboratory, and processed the same day of collection. Fecal samples were weighed and diluted 1:3 (w/v) with Difco buffered peptone water (BPW; Becton Dickinson, Sparks, MD). Serial dilutions were prepared and plated onto Campy-Cefex agar and incubated at 42°C for 36-48 hours in a microaerobic atmosphere (5% O_2, 10% CO_2, 85% N_2) (Stern et al, 1992). Following incubation, presumptive *Campylobacter* spp. colonies were confirmed by observation of typical cellular morphology using phase contrast microscopy and with a commercial latex agglutination kit (Integrated Diagnostics, Inc., Baltimore, MD).

Carcass Rinse Samples (processing): In all 3 trials, flocks sampled were the first flock processed of the day. Additionally, flocks were tracked by trailer number to confirm they were the same flocks sampled at the farm level. For each flock investigated, 25 pre-chill samples and 25 post-chill samples (carcass rinses) were collected as previously described

(8). Briefly, carcasses were collected either prior to entry of the initial chill tank or upon exit from the final chill tank. Each carcass was placed into a clean bag with 100 ml of sterile PBS and vigorously shaken for 1 min. Rinsate was transferred to a sterile specimen cup, placed in ice, transported to the laboratory, and processed the same day within 1 hour. A 0.1 ml aliquot was plated onto duplicate Campy-Cefex agar plates and incubated as described for fecal samples. Confirmation procedures for recovered carcass rinse isolates were identical to those used for fecal isolates.

Exudate samples: For each flock investigated, twenty-five fully processed carcasses (collected post-chill) were individually placed into sterile cryobags. Excess air was evacuated, bags were securely tied, and placed in ice chests. Ice chests containing the carcasses were maintained in a cold room (4°C) for the duration of the sampling period. Exudate samples were obtained on day two and day six. For each sampling, a sterile syringe and needle were used to aseptically aspirate exudate from bags containing the carcasses. A 0.1 ml aliquot was plated onto duplicate Campy-Cefex agar plates and incubated as described for fecal samples. Confirmation procedures for recovered exudate isolates were identical to those used for fecal isolates.

Molecular Subtype Analysis:

Isolated colonies of *Campylobacter* spp. were re-suspended in 300 μL of sterile H_2O and placed at 100°C for 10 minutes. Ten microliters of each boiled cell suspension was used as template for *flaA* SVR PCR with the following primers: FLA242FU: $^{5'}$CTA TGG ATG AGC AAT TWA AAA T$^{3'}$ and FLA625RU: $^{5'}$CAA GWC CTG TTC CWA CTG AAG$^{3'}$ *(16)*. A 35-cycle reaction was used with 1 min denaturing at 96°C, 1 min annealing at 52°C, and a 1 min extension at 72°C.

The resulting product was approximately 425bp. Alternatively, the FlaA4F primer $^{3'}$GGA TTT CGT ATT AAC ACA AAT GGT GC$^{5'}$ was used in conjunction with the FLA625RU primer to produce an amplicon approximately 621 bp in size (*(19)*,*(6)*). Thermocycling conditions remained as above.

Sequence data was generated using either the FLA242FU primer or the FLA625RU primer with the Big-Dye Dye-Terminator Cycle Sequencing Kit (ABI-PE, Foster City, CA). Data was assembled with Sequencher 4.2 (GeneCodes Corp., Ann Arbor, MI) and aligned using Clustal W algorithm in Megaline (Lasergene). Aligned sequences were compared and dendrograms generated using the UPGMA algorithm or neighbor joining with HKY85 distance measurements in PAUP*4.0b 1(Phylogentic Analysis Using Parsimony) *(26)*.

Results and Discussion

Incidence of Positive Samples

For all 3 trials, all fecal samples were positive for *Campylobacter* spp. For the second and third trial all pre-chill samples were positive for *Campylobacter* spp., 23/25 (92%) samples were positive during the first trial. There was a decrease in the number of *Campylobacter* spp. positive carcasses for post-chill samples, for both the second and third trial 11/25 (44%) positive samples were identified and 22/25 (88%) positive post-chill samples were identified during the first trial. This reduction in *Campylobacter* spp. positive samples is consistent with other studies which have demonstrated lethality to microorganisms such as *Campylobacter* spp. and *Salmonella* spp. during chlorinated chilling *(1)*, *(24)*. This reduction in *Campylobacter* spp. prevalence was found in the 2nd and 3rd trial where 6/25 (24%) and 7/25 (28%) positive 6-day exudate samples were

found, respectively. However, for the first trial 19/25(96%) 6-day exudate samples were positive for *Campylobacter* spp. This difference in reduction over time may be due to the seasonality of *Campylobacter* spp, as reported by Meldrum et al. *(17)*, as trial 1 was conducted in May, while trials 2 and 3 were conducted in November. Therefore if there was a higher number of *Campylobacter* spp. per carcass in the first trial a similar reduction would not be reflected when comparing incidence of positive carcasses.

Subtype Recovery and Analysis

For the 3 trials a total of 34 unique *Campylobacter* spp. *flaA* SVR subtypes were recovered (Table 1.1). There was no overlap in subtype recovery between the trials. This is in contrast to results of Hiett et al. *(10)* who isolated closely related clones at various producer locations in the U.S. and Wittwer et al. *(29)* who isolated *Campylobacter* spp. with identical or nearly identical genetic profiles from different farms in different locations in Switzerland .

For all 3 trials multiple *Campylobacter* spp. subtypes were recovered from fecal samples for each flock. These results are in agreement with those of Bull et al. *(5)*. Additionally there were generally one or two predominant subtypes for each flock, a finding similar to that of vanWorth et al. *(28)*. The observation of 1-2 predominant subtypes within a flock may be the result of differing colonization potentials for different isolates. Alternatively recovery of a few predominant subtypes may be due to sampling bias since fecal samples were plated directly on Campy-Cefex and there may be strain-dependent differences in susceptibility to the selective agents in the media.

In the second trial, two predominant subtypes, A2 and D2 were recovered from feces. These *flaA* SVR types were recovered in 19/43 (42%) and 22/43 (49%) of the fecal samples respectively. Both subtypes were present at later sampling points as well, with the exception of the 6-day exudate sample from which only subtype D2 was recovered. This may be due to the subtype being better suited for that particular niche, particularly that of refrigerated chicken exudate. Newell et al. *(21)* observed that some *Campylobacter* spp. subtypes survived poultry processing better than others. However, to accurately determine if there is a difference in the ability of the two distinct subtypes to survive the harsh processing environment further investigations are needed. In addition to comparing survival rates of these two subtypes in inoculated birds the current study should be repeated selecting more isolates for subtyping to reduce sampling error. If there is indeed a difference in survival rate, comprehensive genotypic comparison of the two subtypes might further elucidate survival mechanisms of poultry-associated *Campylobacter* spp.

In all 3 trials additional subtypes were recovered during processing that were not recovered at the farm level. This observation could suggest that carcasses may have been contaminated during processing as previously described *(10)*,*(28)*. This observation is somewhat unexpected given all flocks sampled were the 1st flocks processed of the day. Sanitation regimens should prevent cross-contamination from the previous day. Also if some strains did persist despite sanitation efforts one would expect the 2nd and 3rd trials would have some subtypes in common given they were processed in the same plant but 1 day apart.

The lack of cross-contamination between the 2nd and 3rd trial suggest sanitation regimens do work. An alternative to processing plant contamination, is that these subtypes were present at the farm but at a low level.

The combined data from the three trials indicates that there is no difference in the number of subtypes of C*ampylobacter* spp. recovered using two different methods. A total of 14 subtypes were recovered from post-chill carcass rinses and 12 subtypes recovered from 6-day exudate samples (Table 1.1). However, the two sampling methods recovered different subtypes. For example, in the first trial subtype J1 was recovered from 6-day exudate samples but not from post-chill rinse samples. Subtype J1 accounted for 28% (11/39) of the *Campylobacter* spp. recovered from 6-day exudate samples. This percentage is reasonable and not likely due to contamination as subtype J1 was on of the predominant subtypes at the farm level accounting for 24% (8/33) of the fecal isolates. This presence, absence, and reappearance of this subtype at a similar percentage suggest that recovery from 6-day exudate sample is not simply due to a dilution of rinse samples. Furthermore it is likely that this subtype was present during other sampling periods but recovering from injury.

In the 3rd trial subtypes C3 and N3 were recovered from 6-day exudate samples and not from post-chill rinse samples or any other sample point. In both cases these subtypes account for only 4% (1/24) of the isolates, which might suggest contamination during storage. However, if this were the case, 6-day exudate samples from trial 2 would likely be contaminated as they were stored in the same refrigerated are at the same time. Recovering different subtypes by the two sampling methods is in contrast to results of Stern et al. *(25)* who reported that both carcass rinse and exudate sampling recovered the

predominant subtype present as well as less common subtypes present on the carcass. One major difference in the two studies is Stern et al. *(25)* collected both the exudate and rinse samples at the same time, i.e. 24-30 hours post processing. Therefore, any changes in subtype population due to isolates recovering from injury over time would not be seen. Additionally this difference in results may also be due to this study being conducted in the United States while the investigation of Stern et al. *(25)* was conducted in Iceland. There are differences in industry size and possibly the level homogeneity of subtypes.

For all 3 trials the predominant subtype recovered from post-chill rinse samples was also the predominant subtype recovered from 6-day exudates samples. However, there were additional subtypes recovered from the 6-day exudates samples not present in the post-chill samples or at a different level. Due to the small number of isolates belonging to these additional subtypes, the smaller sample size they were collected from, and the length of storage time required to collect them, their presence has little practical significance for a poultry processor. However, the observation that different subtypes are recovered using different methodologies may important from an epidemiological or risk assessment standpoint as several studies have demonstrated not all *Campylobacter jejuni* strains have the same virulence potential. Fauchere et al. *(9)* reported *C. jejuni* isolated from patients with fever and diarrhea adhered to cultured cells with greater potential than those isolated from asymptomatic patients. Newell et al. *(20)* found environmental isolates were much less invasive for HeLa cells than clinical isolates. More recently Malik-Kale et al. *(15)* found that isolates that were indistinguishable by various typing methods differed in their in vitro and in vivo virulence due to a difference in a single residue of the FlgR protein. Champion et al. *(7)* showed that different flagellar

glycoforms were expressed in different host environments which could also alter virulence potential. Simmons et al. (unpublished data) found gene expression is changed in *C. jejuni* in response to exposure to chicken exudate. Thus mutation and gene expression may alter virulence potential.

Thus when investigating foodborne illness outbreaks, it may be best to sample in a way that best mimic both temporal exposure (time to consumer as related to mutation rate) as well as the environment that will influence its expression profile just prior to exposure.

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Table 1.1: Distribution of *Campylobacter* subtypes at different stages of poultry production and processing as determined by *flaA* typing

Number of isolates belonging to subtype for given sample type					
Trial 1 subtype	Fecal	Pre-Chill	Post-chill	2-day Exudate	6-day Exudate
A1	8	2	7	4	1
B1	0	3	0	4	1
C1	0	6	8	8	11
D1	0	0	1	0	0
E1	0	0	0	0	3
F1	0	1	0	0	0
G1	8	24	16	16	13
H1	6	1	3	5	0
I1	0	0	0	0	0
J1	8	0	0	0	11
K1	0	0	0	1	0
L1	0	0	0	0	1
M1	3	0	0	0	0
Trial 2 subtype	Fecal	Pre-Chill	Post-chill	2-day Exudate	6-day Exudate
A2	19	14	7	18	0
B2	2	0	0	0	0
C2	0	0	1	0	0
D2	22	11	15	7	24
E2	1	0	0	0	0
F2	0	0	1	0	0
G2	1	0	0	0	0
Trial 3 subtype	Fecal	Pre-Chill	Post-chill	2-day Exudate	6-day Exudate
A3	0	0	4	1	4
B3	26	22	18	22	18
C3	0	0	0	0	1
D3	0	0	1	0	0
E3	0	0	0	1	0
F3	1	0	0	0	0
G3	0	0	1	0	0
H3	1	0	0	0	0
I3	14	2	1	1	0
J3	1	0	0	0	0
K3	4	0	0	0	0
L3	0	0	0	0	0
M3	0	1	0	0	0
N3	0	0	0	0	1

CHAPTER 2

GENE EXPRESSION OF *CAMPYLOBACTER JEJUNI* IN RESPONSE TO EXPOSURE TO CHICKEN EXUDATE[2]

[2] Simmons,M., K.L. Hiett, A. Stintzi, B. Seal, and J.F. Frank. To be submitted to Applied and Environmental Microbiology.

Abstract

Campylobacter spp. are one of the most common causes of foodborne bacterial gastroenteritis. Handling and consumption of raw poultry products are considered to be a major source of *Campylobacter* induced disease in humans. There is a high incidence of *Campylobacter*-positive poultry carcasses, even though *Campylobacter* spp. are more fragile than other foodborne pathogens and lack various stress response systems. The purpose of this study was to determine if *C. jejuni* genes are differentially expressed upon exposure to chicken exudate. Two genome sequenced strains of *C. jejuni,* NCTC11168 and RM1221, were grown to mid-exponential phase at 42°C in a microaerobic chamber in Mueller-Hinton biphasic cultures. RNA was extracted from cells under these conditions or upon exposure to 20%(v/v in MH broth) chicken exudate for 15 minutes or 1 hour. The RNA was reverse transcribed using random hexamers in the presence of amino-allyl dUTP. cDNAs were labeled with monoreactive dyes Cy3 and Cy5, and hybridized to a whole genome microarray of 11168 and RM1221 unique genes. In total 214 different genes were identified as differentially expressed in at least one of the 4 strain/exposure time combinations, with 106 down-regulated and 108 up-regulated.

The majority of the genes (194) were identified as differentially expressed in the first 15 minutes as opposed to the 56 identified as differentially expressed after one hour exposure to exudate, suggesting rapid changes upon exposure to exudate and following equilibrium. Several genes previously identified as stress response genes including, *clpB*, *grpE*, *groES*, and *ahpC,* were identified as differentially expressed. Also genes such as *acnB*, *ppi* and *spoT,* which are possibly involved in the regulation of other genes and possible virulence factors, were identified as differentially expressed.

Overall this data provides insight into the changes *C. jejuni* must make to its transcriptome to survive in chicken exudate.

Introduction

Campylobacter spp. are one of the most common causes of foodborne bacterial gastroenteritis around the world. In 2003, campylobacters accounted for 33.4% of the laboratory diagnosed cases of foodborne bacterial gastroenteritis reported by Foodnet *(10)*. The most common symptoms of campylobacteriosis are nausea, abdominal cramps, and bloody diarrhea. In most cases the infection is mild and self-limiting. *Campylobacter* infections have also been associated with reactive arthritis development of Guillain-Barré syndrome, an autoimmune disease which attacks the nervous system (Peterson 1994).

Handling and consumption of poultry or poultry related products are considered primary sources for *Campylobacter* induced disease in humans *(8)*, *(21)*, *(22)*. *Campylobacter* has been cultured from as many as 75% of the live broiler population and from as much as 80% of commercially sold processed poultry meat samples *(19)*, *(20)*, *(28)*.

Despite this high incidence *Campylobacter* is more fragile than other foodborne pathogens in terms of tolerance to oxidative, heat and cold stresses. *Campylobacter* lacks several well characterized stress response systems such as RpoS, SoxRS, RpoH, and CspA *(24)*.

Birk et al. *(4)* compared the survival of *C. jejuni* in chicken exudate, a viscous fluid comprised of blood and water that leaks into the packages of broiler carcasses, and conventional media. They showed that *C. jejuni* survived longer at both refrigerated (4 and 10°C) and at 48°C in chicken exudate than in conventional media even when supplemented with blood. Also Birk et al. *(5)* demonstrated that the medium around the bacteria is a major contributing factor for survival of the pathogen at freezing

temperatures. Thus it may be possible that exposure to chicken exudate alters the gene expression of *C. jejuni* allowing it to survive longer under environmental stress. The purpose of this study was to determine what genes are differentially expressed when *C. jejuni* is exposed to chicken exudate using cDNA microarrays. This study should provide information as to why *C. jejuni* is able to survive and cause illness when it is associated with refrigerated poultry products in spite of its lack of stress response systems. We chose to focus this study solely on the influence of exudate fluid on gene expression so as not to confound the results with the effects of reduced temperature or elevated oxygen.

Materials and Methods

Preparation of Exudate

Fresh post-chill carcasses were obtained from local poultry processors. They were placed in individual bags and stored at 4°C. Exudate was obtained from each carcass and pooled together to eliminate bird to bird variation. To eliminate large particles the exudate was centrifuged at 10,000 rpm for 10 minutes. The resulting supernatant was then filter sterilized using a 0.2-µm filter. To check for sterility the exudate was plated on LB agar and MH agar, incubated at 37 and 42°C under both aerobic and microaerobic conditions. Also to ensure exudate did not confound the results by non-specific hybridization to the array, DNA isolated from chicken blood was hybridized to the microarray. The sterile exudate was stored at -80°C and allowed to thaw at 4°C before use, however it was warmed to 42°C prior to exposure to ensure differential expression was due to exposure to exudate and not a shift in temperature.

Array construction and validation

A library of 1633 oligonucleotide probes, 70 bp each, specific for *C. jejuni* isolate 11168(GS) was purchased from MWG-Biotech (High Point, NC). Furthermore, ORFs determined unique to RM1221 relative to 11168(GS) were used to design an additional 313 oligonucleotide probes (70 bp) specific for the genome sequenced isolate RM1221 (MWG-Biotech). Sixteen Arabidopsis oligonucleotides (70 bp) were included in the library as controls. Oligonucleotides were spotted in triplicate onto UltraGAPS amino-silane coated slides. Slides were subsequently baked at 80°C to fix the DNA. The quality of the microarray was evaluated using Cy3 and Cy5 labeled 11168(GS) and RM1221 genomic DNA for hybridization.

The data generated was validated by real time quantitative reverse-transcription PCR (RT-PCR) by using an ABI Prism 7700 DNA analyzer (Applied Biosystems, Foster City, Calif.) and Quantitech SYBR green RT-PCR analysis. Nine ORFs exhibiting low, moderate, or high expression (as identified by microarray analysis), were selected for comparative real-time PCR analysis (Fig 2.1). The gene expression levels obtained by real-time quantitative RT-PCR analysis were normalized to that of the 16s ribosomal gene, since it was found to be invariant under different exposures to chicken exudate. Quantitative values were obtained by using the comparative threshold cycle ($\Delta\Delta C_T$) method recommended by Applied Biosystems. The C_T value corresponds to the PCR cycle at which the first detectable change in fluorescence occurs associated with exponential increase in PCR products. The relative expression was determined 3 times for both the control 11168 (no exposure to exudate) and the experimental (15 minute

exposure to exudate) RNA samples and were expressed as the fold difference in quantity of cDNA molecules present after 15 minutes exposure to exudate relative to no exposure. The resulting expression ratio was log transformed and plotted against the average log ratio values obtained by microarray analysis. A concordance level of $r = 0.882$ was observed between microarray and RT-PCR data indicating the microarray produced accurate fold change differences with sufficient sensitivity to identify differentially expressed transcripts.

Experimental Design

Two strains of *C. jejuni*, 11168 (GS) and RM1221, were grown to early exponential phase in biphasic Meuller Hinton cultures at 42°C under microaerobic conditions (5%O_2, 15%CO_2, 80%N_2) in a miniMACs workstation (Microbiology International) which was allowed to equilibrate over a 24 hour period. Once the cultures reached early exponential phase (12 hours) treated samples were supplememted with chicken exudate (to total 25% of the media already present). After 15 minutes and 1 hour exposure to the exudate a stop solution was added to the cultures. Cultures were then placed on ice, pelleted at 4°C (5 minutes 8000 x g) and resuspended in TE buffer (50mM Tris-Cl [pH8], 1mM EDTA). Total RNA was then extracted using a hot phenol-chloroform procedure. Total RNA was reverse-transcribed using random hexamers in the presence of amino-allyl-dUTP, followed by labeling with succinimidyl ester monoreactive dyes (Cy3 or Cy5). The level of gene expression was monitored by competitive hybridization to the microarray with cDNA obtained from bacteria not exposed to exudate and exposed for 15minutes or 1 hour.

Each hybridization experiment was repeated 3 times for both strains using total RNA isolated from 3 independent experiments.

Data Acquisition and Analysis

Slides were scanned with a ScannArray Express 1.0 scanner (Packard Bioscience). Spot intensities were quantified using the ScanArray software. Only genes that met all three of the following criteria were selected as differentially expressed. First, genes must have been associated with signal intensity more than 3 times the background at least in one of the channels . Secondly, a change in relative expression level > 1.5 fold was required. Third, a Student t-test was applied to the data, and genes with a p-value< 0.01 were selected (*29*).

Results and Discussion

In total, 214 different genes were identified as differentially expressed in at least one of the 4 strain/exposure time combinations, with 106 down-regulated and 108 up-regulated. However, the majority of the genes which were differentially expressed encode for proteins of unknown function, highlighting the limited understanding of *C. jejuni* physiology. Thus the discussion of the results will focus on genes of known function. For strain 11168(GS), 66 genes were identified as differentially expressed after 15 minutes of exposure to exudate, with 26 being up-regulated and 40 being down-regulated (Table 2.1). In contrast almost twice as many genes (128) were identified as differentially expressed for strain RM1221 after 15 minutes exposure to exudate, with 73 being up-regulated 55 being down-regulated (Table 2.3). The difference in the number of differentially expressed genes between strains may be associated with their different phenotypes. The genome sequenced 11168 strain is a poor colonizer, less virulent, and

less motile, than the clinical isolate it was derived from *(14)*, while strain RM1221 is a good colonizer of chicks (unpublished data). Fewer genes being differentially expressed may be indicative of less ability to adapt to different environmental conditions.

For both 11168(GS) and RM1221, fewer genes were identified as differentially expressed after 1 hour exposure to exudate. For 11168(GS) only 11 genes were differentially expressed, 9 genes were up-regulated and 2 down-regulated (Table 2.2). For RM1221, 45 genes were identified as differentially expressed, 16 genes were up-regulated and 29 down-regulated (Table 2.4). This decrease in the number of genes differentially expressed over time post-treatment is in agreement with the results of Stintzi *(29)* who found the majority of changes in gene expression occurred within the first 5 minutes after *C. jejuni* was subjected to a temperature upshift.

Figure 2.2 provides a summary of the number of genes identified as differentially expressed by 11168(GS) after 15 minutes exposure to exudate grouped by functional categories. The majority of the genes up-regulated, excluding those that encode proteins of unknown function, belonged to 2 categories, cell wall/membrane/envelope biogenesis and post-translational modification/protein turnover/chaperones. Each category contains 4 genes. Changes in the cell membrane may be due to changes in solute concentration and a need to adjust to a different osmotic pressure, or possibly due to the need to incorporate different transport proteins due to a change in the molecules in the surrounding media. *pldA,* which encodes a membrane associated phospholipase A, was up-regulated 1.9 fold. This up-regulation could lead to an increase in virulence as phospholipase A is associated with the lysis of erythrocytes by a number of bacterial pathogens *(17)*.

However, *peb1A* was down-regulated 1.98-fold upon exposure to exudate, and it has been shown to play a role in epithelial cell interactions and intestinal colonization of mice *(25)*.

The genes belonging to the post-translational modification/protein turnover/chaperones category include *clpB*, which encodes the ATP binding subunit of an ATP-dependent protease and *cj1365c*, a putative secreted serine protease. These proteases may function in preventing the accumulation of misfolded proteins created by an environmental stress, thus they may contribute to increased survival. ClpB protein is involved in the prevention of aggregation and the refolding of misfolded proteins. Goloubinoff et al. *(16)* described a sequential mechanism by which this is accomplished: (i) ClpB binds to protein aggregates, ATP induces structural changes in ClpB which (ii) increase hydrophobic exposure of the aggregate and (iii) allow DnaK-DnaJ-GrpE to mediate dissociation and refolding of solubilized peptides into native proteins.

Most of the genes down-regulated belong to the category energy-production and conversion. This down-regulation may be a survival strategy to conserve energy since the TCA cycle, or other metabolic pathway intermediates, may be already provided by the chicken exudate. *oorA, oorC,* and *oorD* were all down-regulated after 15 minutes of exposure to chicken exudate. OOR is an oxoglutarate: acceptor ooxidoreductase that catalyzes oxidative decarboxylation of 2-oxoglutarate to form succinyl coenzyme A, a major intermediate of the tricarboxylic acid (TCA) cycle. This enzyme, in addition to pyruvate is generally associated with anaerobic metabolism and found in obligate anaerobes. These genes' products may function similarly in *C. jejuni* when present in the avian or mammalian gut, which are essentially anaerobic environments.

However, evidence provided by Stintzi et al. *(30)* does not suggest this, as oorA and oorD were down regulated in rabbits in comparison to in vitro.

Related to anaerobic growth, *napD* was up-regulated. NapD is suggested to be involved in the maturation of NapA. NapA is a molybdoprotein nitrate reductase. Reyes et al. *(27)* showed that mutation of the *napD* gene in *Rhodobacter sphaeroides* resulted in the loss of 95% of nitrate reductase activity.

In addition to metabolic genes which were down regulated, were genes related to possible virulence factors and oxidative stress such as *ppi* and *acnB,* respectively. A gene encoding a peptidyl-prolyl cis-trans isomerase (ppi) was down-regulated almost 2-fold in 11168 after 15 minutes exposure to chicken exudate. Because proline has a five member ring it imposes rotational constraints on the peptide introducing a bend in the peptide chain. Proline residues also have a relatively high intrinsic probability of having the cis rather trans isomer of the preceeding peptide bond *(7)*. The enzyme ppi catalyzes the cis-trans conversion. Ppiase activity has been characterized as a virulence factor in several pathogens. In *Helicobacter pylori,* it induces apoptosis of gastric epithelial cells *(2)*. In *Legionella pneumophila* ppiase activity is involved in entry of host cells and intracellular replication *(13)*. In *Streptococcus pneumoniae* a ppi contributed to colonization of the upper airways *(18)*.

acnB, which encodes an aconitase, was down regulated in both strains after 15 minutes exposure to chicken exudate. Aconitases are monomeric iron-sulphur proteins that catalyze the interconversion of citrate to isocitrate in the citric acid cycle *(6)*. AcnB is synthesized during exponential growth of *E. coli* and is not as stable as AcnA which is a stress induced stationary phase enzyme *(11)*. Oxidative stress has been shown to cause

a loss of enzymatic activity as a result of iron-sulphur cluster disassembly. This inactivation is thought to mediate post-transcriptional regulation because the resulting apo-protein binds to specific mRNAs *(33)*, *(34)*. Depending on the binding location, transcription stability can be enhanced or translation can be inhibited. Tang et al *(35)* showed that *Salmonella acnB* mutants possessed less flagella and motility and indicated that AcnB may mediate flagella synthesis under certain conditions. In the current study the flagellar *flaA* gene was concurrently down-regulated in strain RM1221 after 15 minutes exposure to chicken exudate.

Figure 2.3 provides a summary of the number of genes identified as differentially expressed by RM1221 after 15 minutes exposure to exudate grouped by functional categories. The majority of the genes up-regulated, excluding those that encode proteins of unknown function, belong to the category translation/ribosomal structure/and biogenesis, including several genes encoding ribosomal proteins (*rpsS, rpsM, rpsC, rpiV, rpiX*) as well as *infA*, which encoded translation initiation IF-1, and *tsf*, which encodes elongation factor Ts. This up-regulation suggests an increase in overall protein synthesis. This could be indicative of a change in growth rate or differences in growth phase. However, growth curves were generated to ensure the growth phase was the same for both strains and throughout the experiment. It should also be noted that *rpoB*, which encodes DNA directed RNA polymerase beta chain, was also identified as up-regulated. The majority of the genes down-regulated belonged to the category amino acid transport and metabolism. This down regulation may be due to less need for means to transport amino acids into the cell due to a higher concentration outside the cell due to the addition of exudate, or less need to synthesize amino acids which were previously unavailable.

In regards to genes which may increase survival, one gene of interest is *spoT* which was up-regulated 1.7-fold. *spoT* encodes a putative guanosine-3'-5' bis(diphosphate) 3' pyrophosphohydrolase and has been shown to regulate a stringent stress response in *C. jejuni (15)*. The stringent stress response is typically activated by nutrient deprivation, which results in an abundance of uncharged tRNA molecules at the ribosomal receptor site. This causes the ribosome to stall and ribosomal bound SpoT to catalyze synthesis of guanosine pentophosphate (pppgpp). Pppgpp is then hydrolyzed to guanosine tetraphosphate, which is thought to bind to RNA polymerase and alter gene expression by affecting promoter specificity, transcription initiation, and elongation *(9)*. Gaynor et al. *(15)* showed via *spoT* deletions that the stringent stress response is involved capnophilic growth, aerotolerance, adherence and invasion, and intracellular survival in two epithelial cell culture models.

Other genes that could contribute to enhanced survival that were identified up-regulated are *grpE* and *groES*, which encode for heat-shock proteins. These genes were also identified as upregulated by Stintzi *(29)* when *C. jejuni* experienced a temperature upshift from 37 to 42°C. However, the oxidative stress gene *ahpC*, which encodes for alkyl hydroperoxide reductase, was down-regulated 1.7-fold. This down regulation may be associated with an abundance of iron in the exudate. van Vilet et al *(36)* showed *ahpC* expression is regulated by a PerR homolog which prevents the expression of *ahpC* in the presence of higher iron concentrations.

Another gene that was up-regulated that may be of interest is *wlaI*, which was up-regulated 1.9-fold. *wlaI* is involved in a mechanism of general protein glycosylation in *C. jejuni (32)*. This is interesting because it suggests that the importance of protein

modifications that cannot be seen at the expression level. It was once thought that post-translational modification was a phenomenon unique to eukaryotes. However, several studies demonstrated the ability of prokaryotes to glycosylate proteins (*(12)*, *(26)*, *(3)*). In *Pseudomonas aeruginosa,* different glycoforms appear to be expressed in different hosts and environments *(1)*. Logan et al. *(23)* showed that flagellin of *Campylobacter* spp. were post-translationally modified by glycosylation and the modification contributed to serospecificity. Szymanski et al. *(31)* demonstrated that *C. jejuni* mutants with disrupted glycosylation have a reduced capacity for attachment and invasion of human epithelial tissue derived cell lines, as well as impaired colonization in both mice and chicken intestinal tracts.

In summary, changes in gene expression upon exposure to exudate appear to be strain dependent for the 2 strains tested. Also the majority of the changes occur within the first 15 minutes and are no longer differentially expressed after 1 hour. The RM1221 expression profile provides evidence that genes encoding stress response proteins and the genes regulating stringent response system are upregulated in response to exudate exposure, suggesting possible survival mechanisms for poultry carcass associated *C. jejuni*. In addition to genes involved in possible survival mechanisms being identified as being differentially expressed, genes that encode for possible virulence factor regulators were also identified as differentially expressed. The reason for this may be due to *C. jejuni* responding to small cellular components present in the exudate. This change in expression in response to host cells would be in agreement with Gaynor et al. *(15)* who found similar changes in *spoT* expression in host cell-associated cells. However, it is unkown if virulence upon exposure to exudate truly changes, thus cell adhesion/invasion

assays as well as in vivo colonization needs to be investigated. If there truly is a difference due to exposure to exudate, it would be benefical to determine the exact component that induces such a change in expression. Upon identification a quenching agent for this component should be investigated, as it could potentially be added to packagaing as an additional mitigation step.

This study did not consider how gene expression would be affected by the combined effect of exudate, reduced temperature, and elevated oxygen levels. Therefore further, more complex, experiments are needed to determine how *C. jejuni* persists in refrigerated poultry products and subsequently cause illness. Furthermore, it would be useful to determine the effects of individual exudate components on the gene expression profile of *C. jejuni*.

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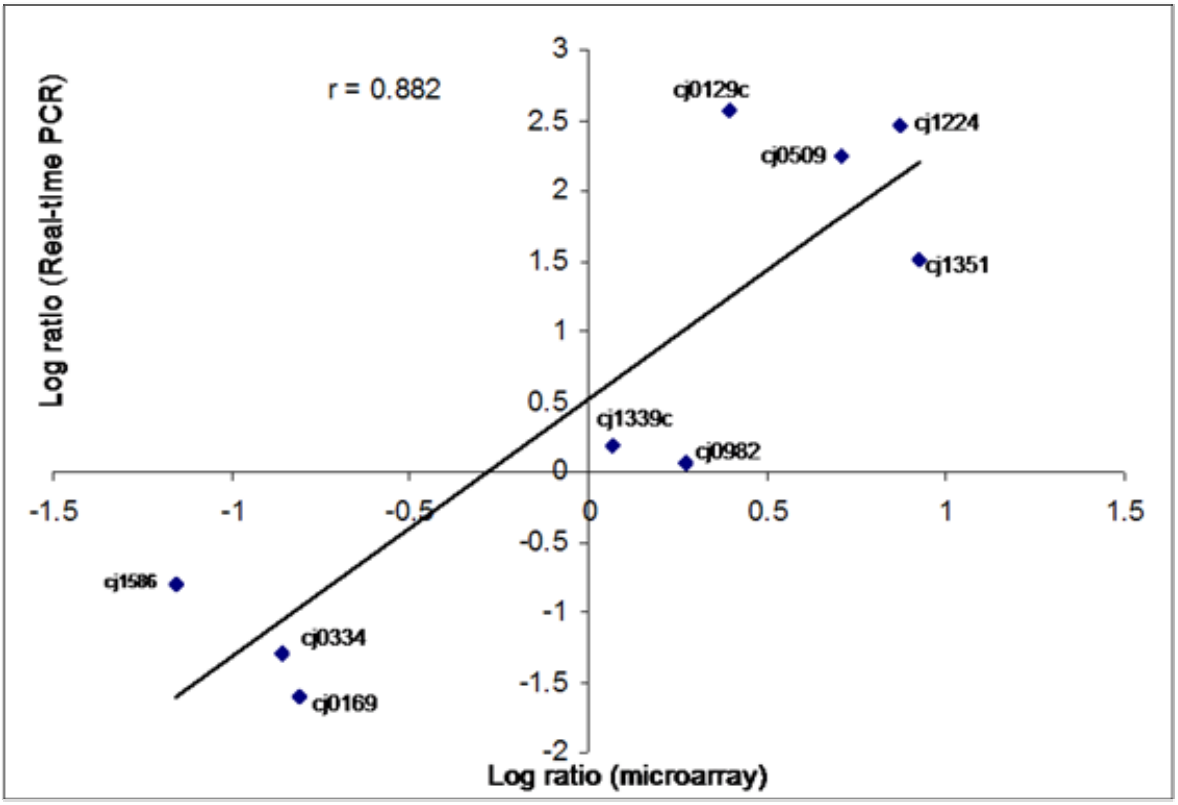

Figure 2.1: Comparison of expression measurements by microarray and RT-PCR assays. The fold change in gene expression in response to 15 minutes exposure to exudate were log transformed (in base 2). The real-time RT-PCR $\log_2$ values were plotted against the microarray data $\log_2$ values.

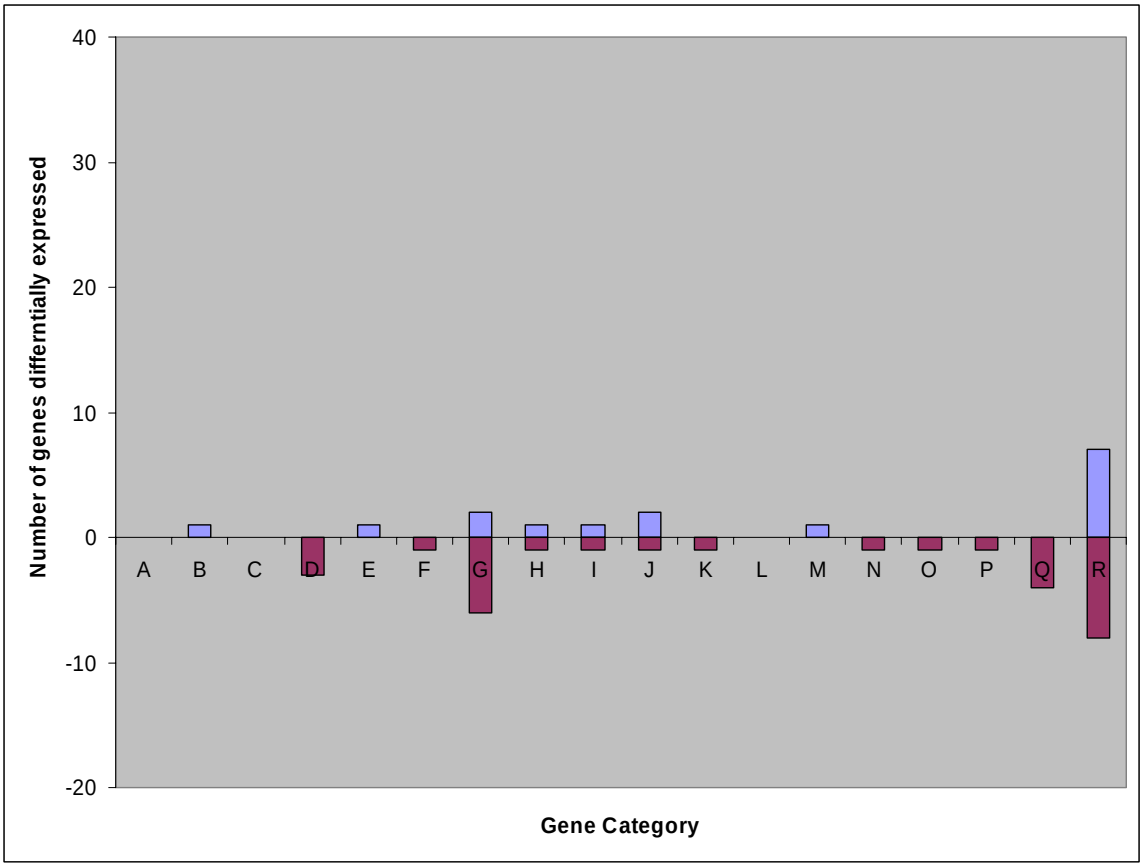

Figure 2.2: Differentially expressed genes in *Campylobacter jejuni* 11168 (GS) 15 minutes post exudate exposure grouped by functional category according to NCBI database. This strain is a poor colonizer of chicks. Columns: A, Amino acid transport and metabolism; B, Carbohydrate transport and metabolism; C Cell cycle control, cell division, chromosome partitioning; D, Cell wall/membrane/envelope biogenesis; E Cell motility; F Coenzyme transport and metabolism; G, Energy production and conversion; H, Post-translational modification, protein turnover, chaperones; I, Replication, repair, recombination; J, Inorganic ion transport and metabolism; K, transcription; L, Lipid transport and metabolism; M, Translation, ribosomal structure and biogenesis; N, Intracellular trafficking, secretion, vesicular transport; O, Signal transduction; P, Nucleotide transport and metabolism; Q, Other; R, Unknown.

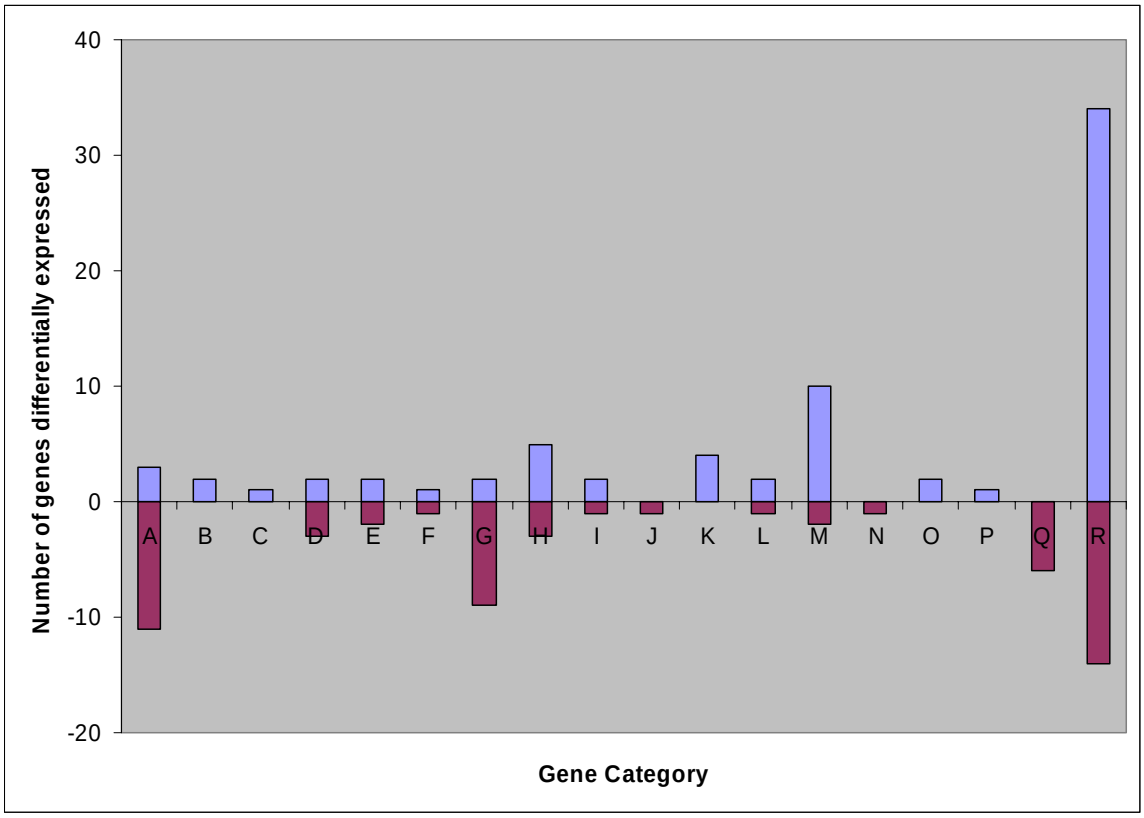

Figure 2.3: Differentially expressed genes in *Campylobacter jejuni* RM1221 15 minutes post exudate exposure grouped by functional category according to NCBI database. This strain is a good colonizer of chicks. Columns: A, Amino acid transport and metabolism; B, Carbohydrate transport and metabolism; C Cell cycle control, cell division, chromosome partitioning; D, Cell wall/membrane/envelope biogenesis; E Cell motility; F Coenzyme transport and metabolism; G, Energy production and conversion; H, Post-translational modification, protein turnover, chaperones; I, Replication, repair, recombination; J, Inorganic ion transport and metabolism; K, transcription; L, Lipid transport and metabolism; M, Translation, ribosomal structure and biogenesis; N, Intracellular trafficking, secretion, vesicular transport; O, Signal transduction; P, Nucleotide transport and metabolism; Q, Other; R, Unknown.

Table 2.1: Genes of known function identified as differentially expressed in *C. jejuni* 11168 after 15 min exposure to exudate

Gene ID	Mean	Std.Dev.	p-value	fold change	up/down	Description
cj1365c	0.63	0.34	0.0062	1.55	up	putative secreted serine protease
cj1719c	-0.75	0.46	0.0048	1.69	down	leuA 2-isopropyl-malate synthase
cj1032	0.65	0.21	0.0001	1.57	up	putative membrane fusion component of efflux pump
cj0935c	-1.30	0.47	0.0003	2.47	down	putative transmembrane transport system
cj1549c	-0.73	0.48	0.0069	1.66	down	putative restriction enzyme type R protein
cj1351	0.93	0.65	0.0049	1.90	up	pldA phospholipase A
cj0017c	0.92	0.44	0.0014	1.90	up	putative ATP/GTP binding protein
cj0805	0.67	0.36	0.0011	1.59	up	putative zn protease
cj1492c	0.86	0.16	0.0000	1.82	up	putative 2-component sensor
cj1682c	-0.82	0.53	0.0065	1.76	down	gltA citrate synthase
cj0785	0.66	0.21	0.0001	1.58	up	napD possible napD protein homolog
cj0686	0.68	0.14	0.0000	1.60	up	gcpE 4hodroxy-3-methylbut-2-en-1-yl diphosphat synthase
cj0076c	-2.04	1.29	0.0029	4.11	down	lctP L-lactate permease
cj1491c	0.62	0.26	0.0002	1.54	up	putative 2 component regulator
cj1224	0.87	0.33	0.0001	1.83	up	putative iron binding protein
cj0509c	0.71	0.18	0.0000	1.63	up	clpB ATP dependent clp protease ATP binding subunit
cj0252	0.65	0.14	0.0000	1.57	up	moaC molybdenum cofactor biosynthesis portein C
cj0183	0.86	0.52	0.0023	1.81	up	putative integral membrane protein with hemolysin domain
cj1502c	-1.24	0.72	0.0081	2.36	down	putP sodium/proline symporter
cj0369c	0.68	0.10	0.0000	1.60	up	ferredoxin domain-containing integral membrane protein
cj1586	-1.15	0.09	0.0021	2.22	down	putative bacterial hemoglobin
cj0074c	-1.61	1.19	0.0065	3.05	down	putative iron-sulfur protein
cj0536	-0.61	0.33	0.0012	1.53	down	oorA 2-oxoglutarate ferrodoxin oxidoreductase
cj0334	-0.86	0.58	0.0043	1.81	down	ahpC alkyl hydroperoxide reductase
cj0688	-0.61	0.48	0.0088	1.52	down	pta putative phosphate acetyl transferase
cj0037c	-1.45	0.52	0.0001	2.74	down	putative cytochrome C
cj0393c	-0.81	0.38	0.0013	1.76	down	putative oxidoreductase
cj0367c	0.65	0.20	0.0020	1.57	up	putative membrane fusion component of efflux pump
cj0778	-0.67	0.31	0.0005	1.59	down	peb2 major antigenic peptide
cj1519	-0.65	0.29	0.0026	1.57	down	moeA2 putative molybdopterin biosynthesis protein

cj0903c	-1.70	0.86	0.0047	3.25	down	putative amino acid transport protein
cj0699c	-0.69	0.06	0.0000	1.61	down	glnA glutamine synthetase
cj0484	-0.81	0.26	0.0022	1.76	down	transmembrane transport protein
cj1378	0.62	0.19	0.0005	1.54	up	selA selenocysteine synthase
cj0835c	-0.98	0.44	0.0029	1.98	down	aconitate hydrotase
cj0538	-0.72	0.26	0.0012	1.64	down	oorC indolepyruvate ferredoxin oxidoreductase
cj0920c	-1.08	0.27	0.0039	2.12	down	putative ABC-type amino acid transporter permease protein
cj0833c	-0.64	0.18	0.0014	1.56	down	oxidoreductase
cj0535	-0.66	0.33	0.0043	1.58	down	oorD subunit of 2-oxoglutarate: acceptor oxidoreductase
cj1451	0.60	0.17	0.0003	1.52	up	dut putative dUTPase
cj0169	-0.81	0.40	0.0045	1.75	down	sodB superoxide dismutase (Fe)
cj1530	-0.65	0.28	0.0067	1.57	down	putative ATP/GTP binding protein
cj0921c	-0.99	0.39	0.0017	1.98	down	peb1A probable ABC-type amino acid transporter periplasmic solute binding protein
cj1171c	-0.97	0.37	0.0013	1.95	down	ppi peptidyl-prolyl cis-trans isomerase
cj0168c	-2.17	0.32	0.0071	4.50	down	putative periplasmic protein

Table 2.1 continued

Table 2.2: Genes of known function identified as differentially expressed in *C. jejuni* 11168 after 1 hour exposure to exudate

Gene ID	Mean	Std.Dev.	p-value	Fold change	up/down	Description
cj0562	-0.85	0.37	0.0024	1.80	down	dnaB replicative DNA helicase
cj0548	0.71	0.41	0.0009	1.63	up	fliD flagellar hook associated protein
cj0478	0.63	0.46	0.0037	1.54	up	rpoB DNA-directed RNA polymerase beta chain
cj1181c	0.80	0.65	0.0061	1.74	up	tsf elongation factor Ts
cj1692c	1.01	0.74	0.0060	2.02	up	rplF 50S ribosomal protein L6
cj0671	1.05	0.85	0.0100	2.07	up	dcuB anaerobic C4-dicarboxylate transporter
cj1375	0.88	0.71	0.0099	1.84	up	putative efflux protein

Table 2.3: Genes of known function identified as differentially expressed in *C. jejuni* RM1221 after 15 min exposure to exudate

Gene ID	Mean	Std.dev.	p-value	fold change	up/down	Description
cj1019c	-0.62	0.15	0.0001	1.54	Down	livJ branched-chain amino-acid ABC transport system periplasmic binding protein
cj0093	0.69	0.10	0.0000	1.61	Up	putative periplasmic protein
cj0018c	1.04	0.41	0.0005	2.06	Up	small hydrophobic protein
cj0919c	-0.77	0.43	0.0033	1.71	Down	putative ABC-type amino acid transporter permease protein
cj1549c	-0.73	0.47	0.0016	1.66	Down	putative type1restriction enzyme R protein
cj1227c	0.61	0.29	0.0034	1.53	Up	putative 2-component regulator
cj0572	0.83	0.20	0.0002	1.78	Up	ribA bifunctional 3,4-dihydroxy-2-butanone 4-phosphate synthase/GTP cyclohydrolase II
cj1492c	1.20	0.35	0.0004	2.30	up	putative 2-component sensor
cj1593	0.71	0.20	0.0004	1.64	up	rpsK 30S ribosomal protein S11
cj1272c	0.76	0.32	0.0062	1.69	up	spoT putative guanosine-3',5'-bis(diphosphate) 3'-pyrophosphohydrolase
cj0779	-0.89	0.27	0.0005	1.85	down	tpx probable thiol peroxidase
cj0845c	-0.83	0.15	0.0000	1.78	down	gltX glutamyl tRNA synthetase
cj0769c	-0.74	0.19	0.0002	1.67	down	flagellar basal body P-ring biosynthesis protein
cj1231	0.70	0.19	0.0003	1.62	up	kefB putative glutathione-regulated potassium-efflux system protein
cj1377c	0.67	0.15	0.0001	1.59	up	putative ferredoxin
cj0562	-1.04	0.52	0.0044	2.06	down	dnaB replicative dna helicase
cj0203	-0.67	0.19	0.0003	1.60	down	putative transmembrane transport protein
cj1111c	-0.70	0.27	0.0042	1.63	down	putative integral membrane protein
cj1095	-0.65	0.22	0.0002	1.57	down	apolipoprotein N-acyltransferase
cj1489c	-0.67	0.27	0.0019	1.59	down	ccoO cb-type cytochrome C oxidase subunit II
cj1071	0.60	0.13	0.0001	1.51	up	ssb single strand DNA binding protein
cj0509c	1.54	0.17	0.0000	2.91	up	clpB ATP-dependent CLP protease ATP-binding subunit
cj0880c	-0.89	0.33	0.0012	1.85	down	hypothetical protein
cj1703c	0.59	0.38	0.0068	1.50	up	rpsS 30S ribosomal protein S19
cj0982c	-0.60	0.11	0.0000	1.51	down	putative amino-acid transporter periplasmic solute-binding protein
cj0764c	-0.60	0.07	0.0000	1.51	down	speA arginine decarboxylase
cj0909	-0.77	0.35	0.0028	1.71	down	putative periplasmic protein
cj0956c	-0.74	0.22	0.0067	1.67	down	thdF tRNA modification GTPase
cj1339c	-0.90	0.26	0.0004	1.86	down	flaA

cj1018c	-0.86	0.36	0.0020	1.82	down	livK branched-chain amino-acid ABC transport system periplasmic binding protein
cj1502c	-1.16	0.27	0.0001	2.23	down	putP sodium/proline symporter
cj1220	0.60	0.29	0.0014	1.52	up	groES co-chaperonin GroES
cj0369c	0.67	0.38	0.0033	1.59	up	ferredoxin domain-containing integral membrane protein
cj1382c	-1.03	0.16	0.0000	2.04	down	fldA flavodoxin
cj1186c	-0.77	0.09	0.0000	1.70	down	petA putative ubiquinol-cytochrome C reductase iron-sulfur subunit
cj1591	0.93	0.43	0.0013	1.90	up	rpmJ 50S ribosomal protein L36
cj0962	0.66	0.22	0.0024	1.58	up	putative acetyl transferase
cj0715	-0.69	0.33	0.0035	1.61	down	transthyretin-like periplasmic protein
cj1349c	0.71	0.18	0.0002	1.63	up	possible fibronectin/fibrinogen-binding protein
cj0758	1.32	0.17	0.0000	2.49	up	grpE heat shock protein
cj0334	-0.78	0.47	0.0093	1.72	down	ahpC alkyl hydroperoxide reductase
cj0393c	-0.83	0.37	0.0028	1.78	down	putative oxidoreductase
cj0367c	1.00	0.18	0.0000	2.01	up	putative membrane fusion component of efflux system
cj0810	-0.95	0.29	0.0005	1.94	down	nadE putative NH(3)-dependent NAD(+) synthetase
cj0903c	-2.11	0.66	0.0002	4.31	down	putative amino-acid transport protein
cj0931c	-0.95	0.44	0.0085	1.93	down	argH argininosuccinate lyase
cj0508	-0.98	0.19	0.0000	1.98	down	pbpA penicillin-binding protein
cj1592	0.90	0.45	0.0045	1.86	up	rpsM 30S ribosomal protein S13
cj0346	0.81	0.45	0.0072	1.75	up	trpD anthranilate synthase component II
cj1625c	-1.31	0.27	0.0001	2.48	down	sdaC serine transporter
cj0386	0.89	0.17	0.0003	1.85	up	GTP-binding protein EngA
cj1378	1.42	0.48	0.0008	2.67	up	selA selenocysteine synthase
cj1229	0.89	0.30	0.0008	1.85	up	cbpA putative curved-DNA binding protein
cj0835c	-0.91	0.31	0.0003	1.88	down	acnB aconitate hydratase
cj0922c	-1.48	0.26	0.0000	2.79	down	pebC ABC-type amino-acid transporter ATP-binding protein
cj0920c	-1.22	0.70	0.0037	2.33	down	putative ABC-type amino-acid transporter permease protein
cj1299	0.74	0.48	0.0064	1.67	up	acpP2 putative acyl carrier protein
cj1416c	-0.60	0.27	0.0073	1.51	down	putative sugar nucleotidyltransferase
cj0833c	-0.72	0.21	0.0004	1.64	down	oxidoreductase
cj0535	-0.61	0.18	0.0017	1.53	down	oorD subunit of 2-oxoglutarate:acceptor oxidoreductase
cj0601c	0.73	0.23	0.0022	1.66	up	putative sodium-dependent transmembrane transport protein
cj0003	0.66	0.29	0.0026	1.58	up	gyrB DNA gyrase subunit B

cj1280c	0.66	0.23	0.0008	1.59	up	putative ribosomal pseudouridine synthase
cj1590	1.02	0.39	0.0015	2.03	up	infA translation initiation factor IF-1
cj0147c	-0.69	0.34	0.0045	1.61	down	trxA thioredoxin
cj0441	-0.80	0.16	0.0001	1.74	down	acpP acyl carrier protein
cj0780	-0.61	0.16	0.0002	1.52	down	napA periplasmic nitrate reductase
cj1511c	-0.68	0.14	0.0000	1.60	down	fdhA putative formate dehydrogenase large subunit (Selenocysteine containing)
cj0533	-0.68	0.15	0.0001	1.61	down	sucC succinyl-coA synthetase beta c
cj1701c	0.70	0.33	0.0014	1.63	up	rpsC 30S ribosomal protein
cj0548	0.88	0.38	0.0024	1.84	up	fliD flagellar hook protein
cj1702c	0.70	0.33	0.0035	1.62	up	rplV 50S ribosomal protein L22
cj1696c	0.68	0.13	0.0000	1.60	up	rplX 50S ribosomal protein L24
cj0825	1.10	0.50	0.0079	2.14	up	putative processing peptide
cj0339	0.63	0.24	0.0013	1.55	up	putative transmembrane transport protein
cj0478	0.62	0.23	0.0013	1.53	up	rpoB DNA-directed RNA polymerase beta chain
cj1000	0.64	0.34	0.0056	1.56	up	putative transcriptional regulator (lysR family)
cj1181c	0.66	0.37	0.0032	1.58	up	tsf elongation factor Ts
cj1123c	0.95	0.40	0.0007	1.94	up	wlaI putative transferase
cj0118	0.69	0.28	0.0018	1.61	up	Predicted ATPase of the PP-loop superfamily implicated in cell cycle control
cj1375	0.70	0.13	0.0003	1.62	up	putative efflux protein
cj1109	0.79	0.37	0.0037	1.72	up	aat putative leucyl/phenylalanyl-tRNA--protein transferase

Table 2.3 continued

Table 2.4: Genes of known function identified as differentially expressed in *C. jejuni* RM1221 after 1 hour exposure to exudate

Gene ID	Mean	Std.Dev.	p-value	fold change	up/down	Description
cj1564	0.83	0.38	0.0032	1.77	up	putative methyl-accepting chemotaxis signal transduction protein
cj0309c	0.60	0.23	0.0000	1.52	up	putatuve efflux protein
cj1254	0.77	0.47	0.0024	1.71	up	hypothetical protein G:T/U mismatch-specific DNA glycosylase
cj0865	1.76	0.09	0.0008	3.38	up	dsbB putative disufide oxidoreductase
cj1206c	-0.89	0.53	0.0010	1.85	down	ftsY putative signal recognition particle protein
cj1184c	0.59	0.20	0.0000	1.50	up	petC putative ubiquinol-cytochrome C reductase cytochrome C subunit
cj0007	-0.62	0.34	0.0006	1.54	down	gltB glutamate synthase (NADPH) large subunit
cj1284	-0.93	0.10	0.0038	1.90	down	ktrA putative K+ uptake protein
cj0005c	-0.88	0.06	0.0016	1.84	down	putative molybdenum containing oxidoreductase
cj1620c	-0.65	0.49	0.0040	1.57	down	mutY A/G-specific adenine glycosylase
cj0076c	-0.71	0.48	0.0020	1.64	down	L-lactate permease
cj0394c	-0.59	0.39	0.0019	1.51	down	Putative transcriptional regulator, homolog of Bvg accessory factor
cj1170c	0.64	0.21	0.0000	1.56	up	outter membrane protein
cj0596	-0.62	0.37	0.0009	1.54	down	peb4\cbf2 peptidyl-prolyl cis-trans isomerase
cj0822	-0.62	0.27	0.0025	1.54	down	dfp phosphopantothenoylcysteine synthase/decarboxylase
cj0783	-0.75	0.18	0.0000	1.68	down	napB periplasmic nitrate reductase small subunit (cytochrome C-type protein)
cj0688	-0.74	0.11	0.0074	1.67	down	pta putative phophate acetyl transferase
cj0037c	-0.87	0.15	0.0100	1.83	down	putative cytochrome C
cj0393c	-1.15	0.06	0.0009	2.22	down	putative oxidoreductase
cj1616	-0.96	0.48	0.0008	1.94	down	chuC putative haemin uptake system ATP-binding protein
cj1086c	-0.81	0.53	0.0019	1.75	down	Integral membrane protein CcmA involved in cell shape determination
cj0835c	-0.93	0.04	0.0007	1.90	down	acnB aconitate hydrotase
cj1002c	-0.62	0.22	0.0003	1.53	down	hypothetical protein Phosphohistidine phosphatase SixA
cj0532	-0.77	0.04	0.0008	1.70	down	mdh malate dehydrogenase
cj0639c	-1.31	0.60	0.0002	2.47	down	adk adenylate kinase
cj0304c	-0.60	0.23	0.0001	1.51	down	bioC putative biotin synthesis protein
cj1122c	-0.62	0.08	0.0058	1.53	down	wlaJ putative integral membrane proteins
cj0408	0.64	0.30	0.0005	1.56	up	frdC fumarate reductase cytochrome B subunit
cj0960c	1.17	0.23	0.0003	2.25	up	rnpA putative ribonuclease P protein component

CHAPTER 3

THE EFFECT OF MUTATION OF *AHPC*, *CLPB*, AND *SPOT* ON THE SURVIVAL *CAMPYLOBACTER JEJUNI* ON RAW REFRIGERATED POULTRY[3]

[3] Simmons, M., K.L. Hiett, A. Stintzi, J.F. Frank. To be submitted to Poultry Science.

Abstract

Previous expression microarray experiments demonstrated that *ahpC*, *clpB*, and *spoT* were differentially expressed in *C. jejuni* when exposed to chicken exudate. The purpose of this experiment was to investigate the effect of mutating these genes on the survival of *C. jejuni* stored at 4°C under ambient atmospheric conditions on chicken skin samples as well as a liquid chicken exudate model. The skin model showed similar declines in the level of *C. jejuni* for the wild-type, *clpB* mutant, and *ahpC* mutant which declined 1.36, 1.19 and 1.25 log cfu, respectively. The level of the *ahpC* mutant declined the most on skin after 96 hours (1.87 log cfu). In the exudate model after 48 hours there was a 0.91 log cfu decrease for the *clpB* mutant, 1.28 log cfu decrease for the wild-type, 1.8 log cfu decrease for the *ahpC* mutant, and a 4.68 log cfu decrease for the *spoT* mutant. After 96 hours both the *ahpC* mutant and *spoT* mutant decreased to non-detectable levels in the chicken exudate while the wild-type and *clpB* mutant decreased 3.83 and 4.24 log cfu, respectively. The study demonstrated that chicken exudate is a more reliable model than chicken skin due to less variation and experimental constraints and suggests the *ahpC* and *spoT* expression are likely to have an effect on survival of *C. jejuni* in raw refrigerated poultry products.

Introduction

Campylobacter spp. are one of the most common causes of foodborne bacterial gastroenteritis around the world. In 2003, campylobacters accounted for 33.4% of the laboratory diagnosed cases of foodborne bacterial gastroenteritis reported by Foodnet *(4)*. Handling and consumption of poultry or poultry related products are considered to be a primary source for *Campylobacter* induced disease in humans *(3)*, *(10)*, *(11)*. During poultry production, processing, and in retail products *Campylobacter* encounters several environmental stresses, including elevated oxygen, temperature extremes, and nutrient limitation. Despite these stresses, *Campylobacter* spp. survive long enough to infect consumers even though it is more fragile than other foodborne pathogens and lacks several well characterized stress response systems such as RpoS, SoxRS, RpoH, and CspA *(13)*. In a previous study by Simmons et al (2007 in preparation), several genes were found to be up-regulated or down-regulated in *C. jejuni* 11168 upon exposure to chicken exudate. Among the genes up-regulated were *clpB* and *spoT*. The oxidative stress protein *ahpC,* however was down regulated upon exposure to exudate.

clpB encodes for a protein that is involved in the prevention of aggregation and the refolding of misfolded proteins. Goloubinoff et al. *(9)* described a sequential mechanism by which this is accomplished: (i) ClpB binds to protein aggregates, ATP induces structural changes in ClpB which (ii) increase hydrophobic exposure of the aggregate and (iii) allow DnaK-DnaJ-GrpE to mediate dissociation and refolding of solubilized peptides into native proteins. *spoT* encodes a putative guanosine-3'-5' bis(diphosphate) 3' pyrophosphohydrolase and has been shown to regulate a stringent stress response. Gaynor et al. *(8)* showed via SpoT deletions that the stringent stress

response is involved capnophilic growth, aerotolerance, adherence and invasion, and intracellular survival in two epithelial cell culture models. *ahpC* encodes for alkyl hydroxide reductase which has the ability to destroy toxic hydroperoxide intermediates as well as repair damaged molecules that have been peroxidized *(1)*.

Each of these genes appear to play a role in stress response and survival of *C. jejuni* however, it is unknown what effect these genes have on survival of C. jejuni on chicken skin or in chicken exudate. The objective of this experiment is to determine the effect of insertional mutagenesis of *clpB*, *spoT*, and *ahpC* on the survival of *C. jejuni* 11168 on both chicken skin and a exudate model.

Materials and Methods

Preparation of chicken skin

Broiler carcasses were randomly collected immediately after exiting the chill tank of a commercial processing plant in Athens, Ga. Carcasses were individually bagged and stored on ice during transport to the laboratory. Within 30 min skin samples were removed from the carcass. Skin samples were obtained by cutting 6.45cm^2 from various areas on fresh broiler carcasses, excluding areas close to the neck and the evisceration opening. Pieces were rinsed twice in sterile phosphate buffered saline (PBS) and were then stored in individual Petri dishes. To check the level of background microflora, representative skin samples from each carcass were stomached in PBS and serial dilutions were plated on PCA and Campy-Cefex agar. Samples from carcasses with greater than 1 log cfu/cm^2 or *Campylobacter* spp. present were not used in the study. The pieces were stored frozen at -80°C until inoculation.

At the time of initial inoculation, skin pieces were thawed by rinsing samples 3 times in 10 ml ice-cold PBS on an orbital shaker for 1 minute.

Preparation of chicken exudate

Fresh post-chill carcasses were obtained from local poultry processors. They were placed in individual bags, and allowed to drip at 4°C. Exudate was obtained from each carcass and pooled together to eliminate bird to bird variation. The exudate was centrifuged at 10,000 rpm for 10 min To eliminate large particles. The resulting supernatant was then sterilized using a 0.2-μm filter. The exudate was plated on LB agar and MH agar, incubated at 37 and 42°C under both aerobic and microaerobic conditions to check for sterility. Sterile exudate was stored at -80°C and allowed to thaw at 4°C before use.

Bacterial strains, preparation of inoculum and inoculation

Wild-type *C. jejuni* NCTC11168, NCTC 11168Δ*ahpC*, NCTC 11168Δ*clpB*, and NCTC 11168Δ*spoT* were obtained from Dr. Alain Stintzi. *C. jejuni* 11168 and the insertional mutants were grown to early exponential phase (16 hours) in Mueller-Hinton biphasic cultures at 37°C under microaerobic conditions. Optical density (600nm) of cell suspensions were adjusted to a value of 0.1. Both skin samples and 10ml preparation of chicken exudate were inoculated with 50 μl of the suspension. Inoculated samples were then stored at 4°C.

Microbial Analysis

Counts on chicken skin were determined by placing the skin sample in a stomacher bag with 10 ml of PBS, stomached for 1 minute, and serially diluted.

The dilutions were then plated on Campy-Cefex agar and incubated at 37°C for 48 hours under microaerobic conditions. The exudate samples were also serially diluted and plated on Mueller-Hinton plates.

Experimental Design

Survival at 4°C was monitored over time by sampling at 0, 12, 24, 48, and 96 hours. Three pieces of skin were used for each time point for both 11168 and the insertional mutants. Similarly, 3 tubes were used for each time point for both 11168 and the insertional mutant. The experiment was repeated 3 times.

Results and Discussion

Survival on chicken skin under refrigeration (4°C) and ambient atmosphere generally resulted in similar decreases over the 96 hour period between the different strains (Fig 3.1). All of the strains experience a large decrease upon inoculation (~ 3 log reduction). Over the remaining time period (0.25 – 96 hours) all of the strains show at least a 1 log reduction. At 16 and 96 hours there is approximately a 1 log difference between the wild-type strain and the *spoT* mutant. However, all other differences between the wild-type and mutants are less than 1 log. This suggests *spoT* expression may affect the survival of *C. jejuni* on chicken skin at 4°C.

Other studies have demonstrated little change in level of *Campylobacter* spp. present on chicken skin when stored at 4°C. Chantarapanont et al. *(5)* reported no significant change in counts of *C. jejuni* (green fluorescent protein reporter strain) was seen when cells were inoculated on non-sterile chicken skin and stored at 4°C for 72 hours.

In a study by Bhaduri and Cottrell *(2)* a maximum decline of 0.63 log cfu/g of *C. jejuni* on chicken skin was observed after 7 days storage at 4°C under ambient atmosphere. Lee et al. *(12)* reported little to no change in level of *C. jejuni* inoculated onto UV-irradiated raw chicken breasts and even increased counts after 7 days at 4°C. These reports suggest that the skin offers a protective environment to *Campylobacter* spp. from stresses it may encounter. The present study suggest this protection is extended to both *ahpC* and *clpB* mutants as there is little difference in change in level between them and the wild-type. The lack of difference in survival between the *ahpC* and the wild-type is in contrast to results of Purdy et al *(14)* who demonstrated a 7 log reduction within 24 hours for an *sodB*, a gene encoding for another oxidative stress response protein, deficient *C. coli* mutant inoculated onto chicken skin stored at 25°C. The greater increase in reduction is most likely due to a higher storage temperature.

Nevertheless, any variation in reduction may be due to variations in chicken skin samples such as the number of feather follicles, fat level, and background microflora. Also due to the destructive nature of the sampling procedure each time point measurement was taken from a different piece of skin. The number of feather follicles present per skin piece may influence counts. Chantarapanont et al. *(5)* reported *C. jejuni* was present in feather follicles and crevices. However, a later study *(6)* suggested the inability of chemical sanitizers to effectively eliminate *C. jejuni* on chicken skin is not the result of protection by location in feather follicles, crevices or other depressions in the skin. These cells located in feather follicles may be less likely to be dislodged and removed during stomaching resulting in lower counts.

The level of fat present may influence counts as well as oxidation may result in production of fatty acids lethal to *C. jejuni*. A study by Thormar et al. *(16)* demonstrated fatty acids were inhibitory to *C. jejuni*. Also, background microflora may inhibit the survival of *C. jejuni*. Davis and Conner *(7)* demonstrated that *Pseudomonas aeruginosa* inhibited survival of *C. jejuni* on poultry products. The present study attempted to minimize these sources of variation by randomly assigning skin samples to different treatment groups. Perhaps a better approach would have been to only use UV-treated skin samples from a given location on a carcass with a controlled number of feather follicle, and fat level. However, given the importance of simulating a real life situation as closely as possible this route was not chosen.

The chicken exudate survival model showed greater differences in survival over time (Fig 3.2). After 48 hours there was a 1.04 log cfu reduction in *C. jejuni* levels for the *clpB* mutant, 1.12 log cfu reduction for the wild-type, 2.34 log cfu reduction for the *ahpC* mutant and a 4.66 log cfu reduction for the *spoT* mutant. After 96 hours both the *ahpC* and *spoT* mutants decreased to non-detectable levels, while the wild-type and *clpB* mutants decreased 3.66 and 3.27, log cfu respectively. These results suggest that expression of *ahpC* and *spoT* both play a role in survival in chicken exudate at 4°C, while expression of *clpB* has less of an effect. These results are not in direct agreement with those obtained from microaray experiment in which both *spoT* and *clpB* were upregulated and *ahpC* was down regulated when *C. jejuni* was exposed to chicken exudate. These results would suggest that there should be decreased survival in chicken exudates when *clpB* and *spoT* are no longer expressed and little change in survival from *ahpC* no longer being expressed. However, these differences are most likely due to the fact that the

microarray experiments were conducted at 42°C while the present study was conducted at 4°C. The *spoT* mutant may have had the greatest decrease in level in the shortest time period because it potentially regulates the expression of several response genes. Stintzi et al. *(15)* reported decreased colonization of *spoT* mutants, and Gaynor et al. *(8)* reported *spoT* mutants as deficient for growth and survival in low CO_2 high O_2 environments, impaired rifampicin resistance, and a stationary phase defect. Since the level of the *clpB* mutant present in exudate samples decreased at a rate similar to the wild-type, there may have been little difference because its function is to prevent the aggregation of misfolded proteins. Misfolding of proteins is associated more with higher temperatures. Similarly, the effect of *ahpC* mutation may be more apparent at low temperatures than at 42°C because gas solubility decreases as temperature increases.

In summary, the present study was conducted to determine if genes identified as differentially expressed in microarray experiments affect survival of *C. jejuni* on raw refrigerated poultry products. Although results from experiments using skin indicate only *spoT* expression affects survival, the survival in chicken exudate was influenced by the expression of both *ahpC* and *spoT*. The exudate model is a good model not only because of less variation than chicken skin, but also because exudate serves as a vector in cross-contamination events leading to consumer exposure to *C. jejuni*. Therefore, if mitigation strategies can be developed that decrease survival of *C. jejuni* in chicken exudates there is likely to be a decline in human exposure.

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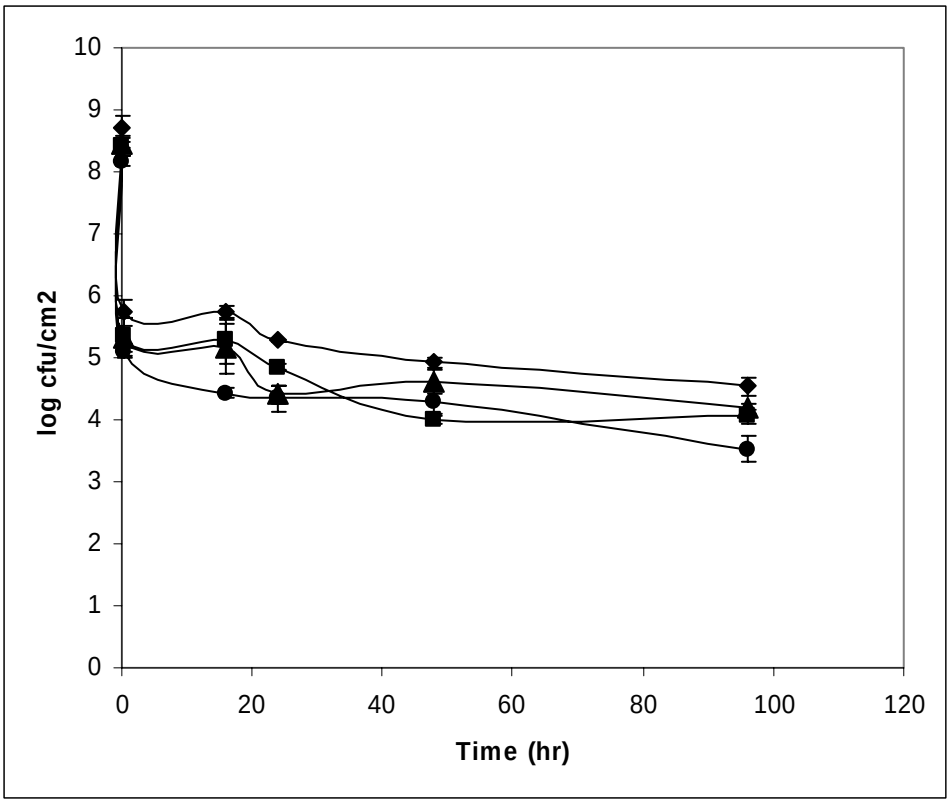

Figure 3.1: Survival of *C. jejuni* on chicken skin at refrigerated temperature (4°C). (♦) wild-type; (■) *ahpC* mutant; (▲) *clpB* mutant; (●) *spoT* mutant.

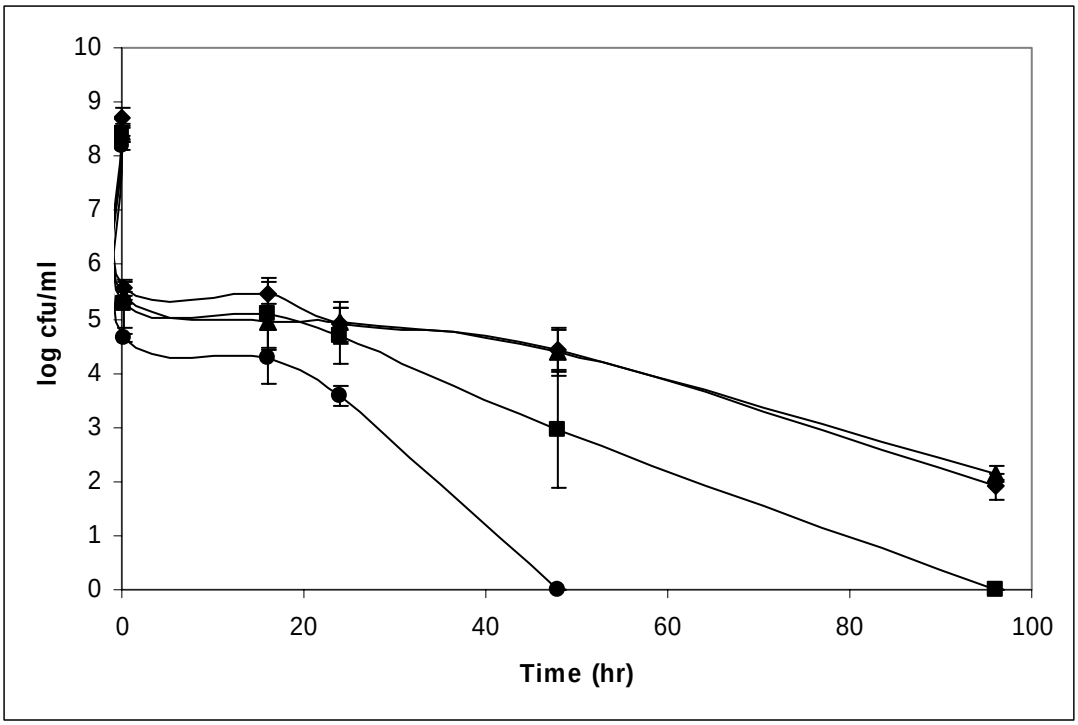

Figure 3.2: Survival of C. jejuni in chicken exudate at refrigerated temperature (4°C). (♦) wild-type; (■) *ahpC* mutant; (▲) *clpB* mutant; (●) *spoT** mutant.**spoT* mutant below detectable limit of 1log cfu/ml after 48 hours.

CONCLUSIONS

Sampling chicken exudates as opposed to whole carcass rinses does appear to influence subtype recovery. The subtype predominant in the post-chill rinse samples was also the predominant subtype in chicken exudates after 6 days of cold storage. However, there were additional subtypes recovered in the exudates samples not present in the post-chill rinse samples or present at a lower level. Due to the low number of isolates belonging to the subtypes unique to exudates samples, they may not be of practical significance to poultry processors. However, comparisons of these isolates to those found in the post-chill rinse samples may provide information on genotypes associated with prolonged survival or greater virulence potential. Such information could be used to test poultry flocks for *Campyobacter* spp. that pose a greater threat to human health, just as cattle and flocks are tested for *E. coli* O:157H:7 and *Salmonella typhimurium* DT104.

The results from the expression microarray experiment provide information on the physiology of *C. jejuni* when it is associated with poultry exudates. By better understanding the physiology it may be possible to develop mitigation strategies that specifically target survival mechanisms *Campylobacter* spp. use during processing as well as in retail packages.

The third experiment investigated genes potentially involved in survival of Campylobacter at 4°C on both chicken skin and in chicken exudates. Similar future investigations may help elucidate survival mechanisms. This information can be used to formulate better recovery media and isolation procedures.

The 3 experiments will hopefully serve as a basis for future investigations which will help us better understand Campylobacter spp. A better understanding of the organism will potentially help develop better strategies for reducing poultry associated Campylobacter spp. and potentially reduce human exposure to the organism and improve public health.