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Campylobacter jejuni as a Primary Colonizing Biofilm Former

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Abstract: *Campylobacter jejuni* can be difficult in the environment and extremely fragile, therefore carry-over between flocks has been difficult to explain. The aim of the study was to determine if the survival of *C. jejuni* outside the host could be due to a capability to form biofilms. In these experiments, *C. jejuni* was cultured under conditions of starvation, temperature variations, different cell concentrations, after passage through a chick gastrointestinal tract or with a conditioning film. However, no evidence of attachment and biofilm formation was found outside of growth conditions. Since growth conditions usually do not occur outside the host, it may be concluded that *C. jejuni* is most likely not a primary biofilm colonizer outside the host. These studies indicate that *C. jejuni* may utilize a strategy other than primary biofilm formation to survive outside the host.

Key words: *Campylobacter jejuni*, biofilm, survival, conditioning film, virulence, motility

INTRODUCTION

Campylobacter jejuni colonizes the gastrointestinal tract of poultry as a commensal, but is pathogenic to humans (Lee and Newell, 2006). Poultry and poultry products are a primary source of infection to humans (Snelling *et al*., 2005). Controlling and reducing colonization is necessary in order to reduce the number of campylobacteriosis cases. Although, *C. jejuni* is a leading cause of food borne illness, it is extremely susceptible and dies quite quickly in the environment outside of the host (Obiri-Danso *et al*., 2001). Therefore, survival outside the host has been difficult to explain.

In order to survive inhospitable conditions, food borne pathogens including *Listeria monocytogenes, Salmonella, Shigella, Staphylococcus aureus, Escherichia coli* and *Enterobacter* have the ability to form biofilms (Bower and Daeschel, 1999; Stepanovic *et al*., 2004; Gunduz and Tuncel, 2006; Rode *et al*., 2007). Biofilms have been shown to protect bacteria from sanitizers, desiccation and antibiotics and can provide bacteria with increased access to nutrients and opportunities for genetic exchange. Since many food borne pathogens form biofilms and biofilms provide multiple advantages, it seems an appealing theory to explain survival of *Camp. jejuni* outside the host.

Biofilm formation is a 5-step process (Characklis and Cooksey, 1983). The first step is a conditioning film typically consisting of nutrients that can promote the adherence of bacteria to the surface. Once a conditioning film has been formed, some bacteria, termed primary colonizers, can attach to the surface via the conditioning film. Although, conditioning films may be helpful to bacterial attachment, they are not necessary. Controlling these initial stages in biofilm development can be crucial to preventing a potential long-term source of the organism.

Stress has been shown to be a factor that can promote biofilm formation (Ryu *et al*., 2004). Nutrient depletion has been shown to induce biofilm formation because starved cells may be attracted to conditioning films and are subsequently attached to surfaces via conditioning films (Dunne, 2002). Other factors that have been shown to be involved in biofilm formation are bacterial concentration, stress-adaptation, temperature and growth medium (Bagge *et al*., 2001; Ryu *et al*., 2004; Moltz and Martin, 2005). The objective of this research was to determine any ability of *C. jejuni* to attach to a surface under multiple conditions, including passage through the chick gastrointestinal tract.

MATERIALS AND METHODS

Bacterial strains and growth conditions: A total of 132 strains of *Campylobacter jejuni* were evaluated for the ability to form a biofilm. Isolates were collected from retail poultry carcasses (N = 50), pre-chill poultry carcasses (N = 15), post-chilled poultry carcasses (N = 15), retail turkey carcasses (N = 22) and turkey giblets (N = 10) using whole carcass rinse procedures according to the USDA recommended procedure (Ransom and Rose 1998). Additionally, isolates from human clinical patients (N = 19) exhibiting symptoms of campylobacteriosis also were used. All wild-type strains were characterized using Pulsed-Field Gel Electrophoresis (PFGE) and determined to be of non-clonal origin using a method previously described by our laboratory (Gilbert *et al*., 2008). In addition, the laboratory strain NCTC 11168 kindly donated by Dr. Qijing Zhang was utilized. To culture each strain of *C. jejuni*, a 10 µL loop of frozen culture was inoculated into 10 mL of Mueller-Hinton (MH) broth (Becton Dickinson, Franklion Lakes, NJ, USA) and incubated at 37°C for 48 h in microaerophilic conditions. For passage, a 10 µL loop of

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culture was inoculated into a tube of fresh MH broth. All cultures were passed twice prior to use. On the final passage, *C. jejuni* was incubated for 16 h to collect cells in the mid-exponential phase. *Pseudomonas aeruginosa* ATCC strain 10145 with known abilities to readily form a biofilm was used as a positive control (Moltz and Martin, 2005). *P. aeruginosa* also was cultured in MH broth as described for *C. jejuni*.

Microtiter plate biofilm assay: The microtiter plate assay for determining adherence and biofilm forming abilities was performed as previously described by Djordjevic *et al.* (2002) with modification. Briefly, cultures were collected by centrifugation at 8,000× g for 5 min. The pellet was washed twice in fresh medium and finally resuspended in fresh medium by vortexing. In each well of a 96-well microtiter plate (sterile, untreated for tissue culture), a 200 µL portion of each culture was transferred to a well. Each strain was replicated 8 times in one 96-well plate. Eight wells served as negative controls and eight wells with *P. aeruginosa* served as positive controls. The plates were incubated for 24, 48 or 72 h at 37°C under microaerophilic conditions. After incubation, the cultures were collected by pipette and samples were plated onto MH agar to determine culturability of bacteria. All 96-well plates then were washed three times with distilled water and tapped vigorously to remove remaining water. The plates were allowed to dry overnight. Wells were stained by filling each well with 200 µL of 0.7% crystal violet in water for 15 min. The plates then were rinsed and dried as described above. Stain was eluted by filling each well with 100% ethanol and biomass corresponding to biofilm formation was determined by reading plates spectrophotometrically with an absorbance of 595 nm.

Cell density dependent biofilm assay: *C. jejuni* cultures were prepared as described above. Starting with approximately 10^8 cfu mL^{-1}, 10-fold serial dilutions were made and each dilution was cultured in the wells of the 96-well plate. The initial concentration of the bacteria was determined by plating serial dilutions on MH agar. The 96-well plate assay was used to assess biofilm formation as described above.

Attachment via conditioning films: The 96-well plates were pre-conditioned with conditioning films by filling the wells of each plate with 200 µL of MH broth, *Campylobacter* enrichment broth (Med-Ox Diagnostics, Ottawa, Canada), R2A broth (Teknova, Hollister, CA, USA), biofilm broth (25% v/v tryp soy broth [Becton Dickinson], 25% v/v nutrient broth [Becton Dickinson], 25% v/v *Campylobacter* enrichment broth and 7% v/v glucose [Sigma Chemical Co., St. Louis, MO, USA]), 0.9% saline, fetal bovine serum (Sigma Chemical Co.) or 7% glucose in water. The plates were allowed to set overnight at room temperature. The plates then were rinsed 3 times with sterile distilled water and allowed to dry prior to use. Cultures were finally suspended in either 0.9% saline or MH broth as described above and 96-well plate assay was performed as described above. Effects of stresses on attachment and biofilm formation Cultures were prepared as described above but finally suspended in 0.9% saline solution to initiate a starvation stress response. The 96-well plate assay was used as described above to evaluate attachment and biofilm formation of cultures suspended in 0.9% saline. For temperature stress, cultures were suspended in MH broth, prepared and placed into 96-well plates as described above, but 96-well plates were incubated at 10, 23 or 32°C for 24 h.

Passage through chick gastrointestinal tract: *C. jejuni* NCTC strain 11168 was utilized in this study. A 10 µL loop of frozen culture was inoculated into 10 mL of Muller-Hinton (MH) broth and incubated at 42°C in microaerobic conditions for 48 h. The culture was passed by vortexing to re-suspend the cells and inoculating 10 µL loop of the suspended cells into fresh MH broth. The culture was passed twice before being used in challenges. Day-of-hatch broiler chicks were obtained from a local hatchery (Cobb-Vantress, Fayetteville, AR, USA) and placed in cages measuring approximately 15 ft^2. Birds had access to feed and water ad libitum for the entire experiment. The day after hatch, 20 chicks were dosed with 250 µL of bacteria suspended in Butterfield's phosphate diluent (BPD; 6.8% KH_2PO_4; 7.2 pH) using a 1 mL syringe and stainless steel cannula. On day 12 post-challenge, all chicks were humanely killed using CO_2 asphyxiation and ceca were collected and placed in individual sterile plastic bags. The contents of the ceca were squeezed into a sterile 15 mL centrifuge tube, weighed and re-suspended 1:10 in BPD. The suspensions were inoculated onto *Campylobacter* Line agar plates (Line, 2001) and incubated at 42°C for 48 h in microaerophilic conditions. Bacteria were collected from the plates by using a 10 µL loop and suspending the bacteria in fresh MH broth. Suspect *C. jejuni* colonies were confirmed using API Campy (BioMeriuex, Durham, NC, USA) and latex agglutination test (Panbio Inc., Columbia, MD, USA). Bacteria then were prepared by suspending in MH broth and used in the 96-well plate assay as described above. For negative controls, wells with MH broth or 0.9% saline were used and *P. aeruginosa* was used for a positive control.

Statistical analysis: The data were analyzed using Analysis of Variance (ANOVA) with the statistical software SAS (Statistical Analysis Systems Institute,

Cary, NC, USA). The Student's t-test was used to determine differences among means of optical density. Significant differences were reported at p = 0.05. All experiments used eight replicate samples within each trial and were repeated in three independent trials.

RESULTS

Of the 132 *C. jejuni* strains utilized in this study, none attached to the surface and formed biofilms on the surface of the 96-well plate after 24, 48 or 72 h (Fig. 1). The optical density of the wells with bacteria ranged from a mean of 0.059 to a mean of 0.084. The mean optical density of the wells with no bacteria was 0.082. The positive control, *P. aeruginosa*, readily formed a biofilm after only 24 h. The mean optical density of the wells with *P. aeruginosa* was 0.3953. Biofilm formation by *C. jejuni* was not cell density dependent. None of the bacterial concentrations attached or formed biofilms. However, pellicle formation was noted in all of the concentrations and the size of the pellicle was directly correlated to the amount of *Camp. jejuni* cultured in the well. These pellicles were formed in the center of the wells at the air-liquid interface and were not attached to the surface of the well.

Conditioning films composed of various nutrients failed to facilitate *C. jejuni* attachment to the surface whether *C. jejuni* was cultured in MH broth or in starvation conditions of 0.9% saline (Fig. 2a). There were no differences in the mean optical densities of *P. aeruginosa* cultured in MH broth in wells with conditioning films versus those wells without conditioning films (Fig. 2b). However, a statistically significant increase in biofilm formation occurred when *P. aeruginosa* was cultured in 0.9% saline in some wells with conditioning films versus culturing in 0.9% saline in wells without a conditioning film ($p<0.05$). This implies the conditioning film facilitated attachment of *P. aeruginosa* when cultured under starvation conditions. Stresses of temperature or starvation did not induce *C. jejuni* attachment and biofilm formation (data not shown). No culturable *C. jejuni* was detected after 24 h at 23°C, but culturable *C. jejuni* was detected even after 72 h at 10°C. Conversely, *P. aeruginosa* was able to form biofilms at all three temperatures and under starvation conditions.

After passing through the chick gastronintestinal tract, *C. jejuni* failed to attach and form a biofilm (Fig. 3). There was a statistically significant difference between the negative control well with just saline versus the wells with either MH broth or MH broth and *C. jejuni* ($p<0.05$). But there was no statistical difference between wells with *C. jejuni* and wells with MH broth.

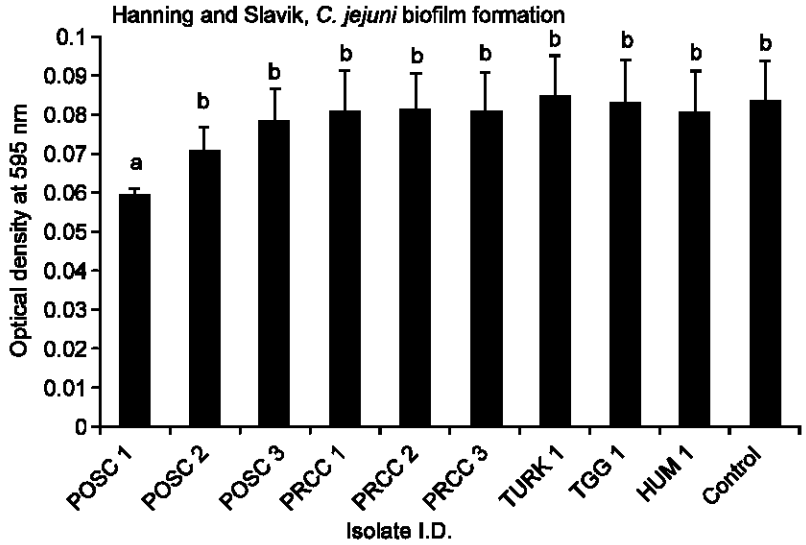

Fig. 1: Attachment and biofilm formation of select strains of *Campylobacter jejuni* measured using a 96-well plate assay. All strains of *C. jejuni* (n = 132) were cultured in Mueller-Hinton (MH) broth and passed twice prior to suspending in fresh medium. A 200 UL aliquot was incubated for 24, 48 or 72 h at 37°C. All plates then were washed, stained with 0.7% crystal violet and eluted with 100% ethanol. Optical density was read using a spectrophotometer at 595 nm. The results from 9 strains cultured at 37°C are shown. POSC (post-chilled chicken carcass), PRCC (pre-chilled chicken carcasses, TURK (turkey carcasses), TGG (turkey giblets), HUM (human clinical patients) and CONTROL (MH broth only). [ab]Columns with no common superscript differ significantly

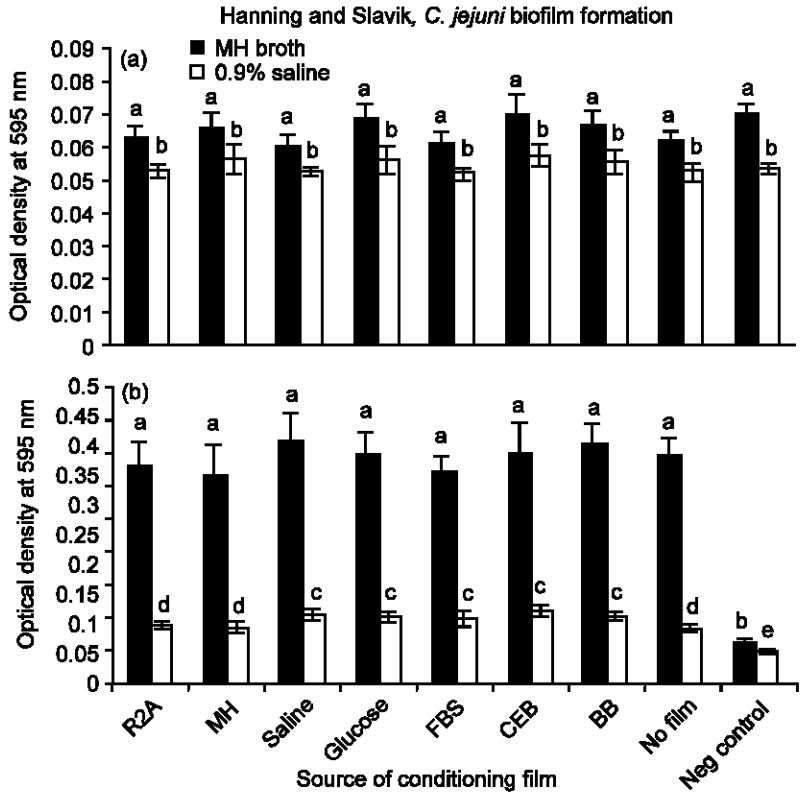

Fig. 2: The impact of conditioning films on *Campylobacter jejuni* and *Pseudomonas aeruginosa* attachment. *C. jejuni* NCTC strain 11168 (Panel A) and *P. aeruginosa* ATCC strain 10145 (Panel B) were cultured in Mueller-Hinton (MH) broth. Conditioning films were formed with 200 ul of R2A broth, Mueller-Hinton broth (MH), 0.9% saline, 7% glucose in water, fetal bovine serum (FBS), Campylobacter enrichment broth (CEB), biofilm broth (BB; 25% v/v tryp soy broth [Becton Dickinson], 25% v/v nutrient broth [Becton Dickinson], 25% v/v *Campylobacter* enrichment broth [Med-Ox Diagnostics] and 7% v/v glucose [Sigma Chemical Co.]). Plates with conditioning films were washed three times prior to use. Bacteria were suspended in fresh MH broth or 0.9% saline and a 200 µL aliquot was added to the pre-conditioned wells and incubated for 24, 48 or 72 h at 37°C. All plates then were washed, stained with 0.7% crystal violet and eluted with 100% ethanol. Optical density was read using a spectrophotometer at 595 nm. [abcde]Columns with no common superscript differ significantly

DISCUSSION

In this study, the 96-well plate assay was utilized to study *C. jejuni* attachment and biofilm formation under many conditions. Similar to our results, other researchers have reported that any type of biofilm formation by *C. jejuni* only occurred under growth conditions of *C. jejuni* (Joshua *et al*., 2006; Reeser *et al*., 2007). Although in these experiments no evidence of attachment to the surface was found, pellicle formation was observed under growth conditions. These pellicles are aggregates of bacterial cells that form at the air-liquid interface (Joshua *et al*., 2006). Some authors have suggested that these pellicles have biofilm like characteristics (Hall-Stoodley *et al*., 2004). In these experiments, pellicles were only formed under growth conditions. Since growth conditions usually do not occur outside the host, it is unknown what significance these pellicles may have and pellicles could even be laboratory artifacts.

Density dependent biofilm formation has been described in multiple enteric pathogens (Kendall and Sperandio, 2007). Bacteria can utilize quorum sensing signals to determine concentrations of other bacteria in the environment and respond accordingly. Research indicates that biofilm formation can be a cooperative effort and may be coordinated through cell-signaling when densities of cells are at desirable levels (Murray *et al*., 2007). *C. jejuni* does posses the genetic components for quorum sensing (Elvers and Park, 2002). However, no evidence of density dependent biofilm formation by *C. jejuni* was found in this research. Stress has been shown to be a factor in biofilm formation (Dunne, 2002). In some of these experiments the stress of non-optimal temperatures killed *C. jejuni*. The stress of starvation has been shown to induce biofilm formation in other bacteria including *L. monocytogenes* and *E. coli* (Dewanti and Wong, 1994; Helloin *et al*., 2003). Receptors for nutrients on the

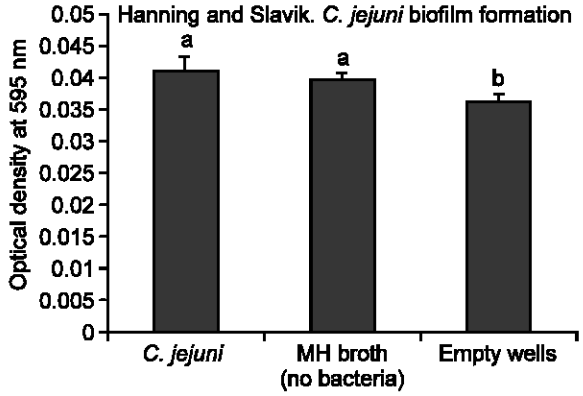

Fig. 3: The effect of passage through the chick gastrointestinal tract on *Campylobacter jejuni* attachment and biofilm formation. The day after hatch, chicks were orally challenged with *C. jejuni* strain 11168. After 12 days, chicks were humanely killed and cecal contents collected and cultured. Cells were collected, resuspended in fresh Mueller-Hinton broth, inoculated into a 96-well plate and incubated at 37°C for 24 h. For negative controls, sterile MH broth or 0.9% saline were used. All plates then were washed, stained with 0.7% crystal violet and eluted with 100% ethanol. [ab]Columns with no common superscript differ significantly

outside of the cell may be unbound under starvation conditions and these empty receptors may be available for attachment to a conditioning film (Kjelleberg and Hermansson, 1984). In addition, possible up-regulation of chemotaxis under starvation conditions might lead cells to conditioning films (Larsen *et al*., 2004). Conditioning films are also very valuable as a physical force that can entrap free-swimming cells. We created conditioning films using multiple media to determine if *C. jejuni* attachment would be facilitated by a conditioning film. Although, the film proved useful for *P. aeruginosa*, *C. jejuni* failed to attach to the conditioning film in the wells.

Biofilm formation is recognized as a virulence factor of some bacteria (Parsek and Singh, 2003) and passage through the chick gastrointestinal tract has been shown to enhance *C. jejuni* virulence (Sang *et al*., 1988). Since there was no correlation between *C. jejuni* biofilm formation and passage through the chick gastrointestinal tract, any role of virulence in *C. jejuni* biofilm formation from these experiments is unclear.

Explaining the environmental sources of *C. jejuni* and survival of this fragile bacterium outside the host has been difficult. The fact that *C. jejuni* is a leading cause of food borne gastroenteritis makes the problem even more perplexing. Once an explanation of how this organism survives outside the host is found, control measures can be implemented. From our experiments it is unclear what role, if any, biofilm formation has in the survival of *C. jejuni* outside the host. Since *C. jejuni* is a poor primary colonizer and does not utilize a conditioning film for attachment, it may be possible that *C. jejuni* is strictly a secondary colonizer of pre-established biofilms. Secondary colonization of biofilms has been described with *P. fragi* biofilms capturing and enhancing the biofilm formation of *L. monocytogenes* (Sasahara and Zottola, 1993). Therefore, secondary colonization by *C. jejuni* of poultry biofilms will be the focus of future research.

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