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Campylobacter jejuni biofilm cells become viable but non-culturable (VBNC) in low nutrient conditions at 4 °C more quickly than their planktonic counterparts

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ABSTRACT

Campylobacter jejuni remains the leading cause of foodborne disease in the developed world. In order to assess the ability of biofilm cells to enter and survive in a viable but non-culturable state, biofilm and planktonic cells of three strains of *C. jejuni* were incubated at 4 °C in phosphate buffered saline. Culturability was monitored by standard drop plating on Mueller Hinton agar and viability was measured using the LIVE/DEAD® *Bac*Light™ assay which assesses membrane integrity. Both biofilm and planktonic cells became non-culturable prior to becoming non-viable. Biofilm cells became non-culturable as early as 10 days for one strain, while planktonic cells became non-culturable after 30–40 days of treatment. Planktonic cells were still viable after 60 days of stress treatment. Biofilm cells showed significantly reduced viability by day 50 for the two clinical isolates and by day 60 for the poultry isolate. Of the media assessed for their ability to extend the culturability of the VBNC cells, Campylobacter Agar Base with the addition of Campylobacter Growth Supplement was most successful at prolonging culturability, but even with enrichment in Bolton broth, cells still remained viable and potentially infectious, longer than they were culturable.

1. Introduction

Campylobacter jejuni is currently considered the main cause of bacterial gastroenteritis in the developed world (Suzuki & Yamamoto, 2009; Whiley, van den Akker, Giglio, & Bentham, 2013). Most cases are associated with the consumption or handling of contaminated poultry and although poultry is considered the main reservoir, infections have also been linked to raw milk, untreated water, pets and farm animals (Whiley et al., 2013).

C. jejuni is a fastidious pathogen that can only grow at 30–45 °C in a microaerobic atmosphere, conditions found in the avian host. The pathogen also lacks many stress-response mechanisms commonly found in other Gram negative bacteria. Despite this sensitivity to stresses found outside the host, *C. jejuni* is prevalent in poultry houses and slaughter facilities (Cokal, Caner, Sen, Cetin, & Telli, 2011; Ellerbroek, Lienau, & Nather, 2010). Various hypotheses have been put forth to explain this conundrum, including the suggestion that *C. jejuni* survives in the environment by forming biofilms and by entering viable-but non-culturable (VBNC) state (Murphy, Carroll, & Jordan, 2006; Pitkanen, 2013).

C. jejuni can form mono-culture biofilms or establish in pre-existing biofilms of strong biofilm producers, such as *Pseudomonas* spp., *Flavobacterium* spp., *Corynebacterium* spp., *Staphylococcus* spp, or *Enterococcus* spp. (Ica et al., 2012; Teh, Flint, & French, 2010; Trachoo, Frank, & Stern, 2002). Such biofilms can develop in food processing environments, in drinking water systems, and also in water systems of poultry houses (Pitkanen, 2013; Sparks, 2009; Trachoo et al., 2002; Wingender & Flemming, 2011). *C. jejuni* cells in biofilms are very resistant to environmental stresses (Stoodley, Sauer, Davies, & Costerton, 2002) and many disinfectants (Alter & Scherer, 2006) and they can survive aerobic and low-temperature stress twice as long as planktonic cells (Joshua, Guthrie-Irons, Karlyshev, & Wren, 2006). *C. jejuni* cells can detach from biofilms. In food production environments, this leads to contamination of product; in water distribution systems, detached biofilm clusters may cause infection of humans or colonization of poultry (Lehtola et al., 2007; Schuster et al., 2005; Trachoo & Frank, 2002; Wingender & Flemming, 2011). Thus, *C. jejuni* in biofilms, pose a significant public health risk and are also considered an important contributor to the persistence and spread of *C. jejuni* in poultry houses and slaughter facilities.

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C. jejuni can become VBNC in response to various stressors, such as starvation, low temperature, and low pH (Cappelier & Federighi, 1998; Chaveerach, ter Huurne, Lipman, & van Knapen, 2003; Gangaiah, Kassem, Liu, & Rajashekara, 2009; Trevors, 2011). VBNC *C. jejuni* are more resistant to disinfection than actively growing cells (Davies, 2003), they can survive in the VBNC state for up to 7 months (Lazaro, Carcamo, Audicana, Perales, & Fernandez-Astorga, 1999), and they may not be detected by culture-based methods, even when an enrichment step is used to resuscitate injured cells (Baffone et al., 2006). Recent work shows that VBNC *C. jejuni* continue to express virulence genes and adhere to epithelial cells (Chaisowwong et al., 2012) substantiating the concern that these cells may remain infectious. Existence of VBNC *C. jejuni* was demonstrated for both planktonic cells and biofilm-associated cells (Buswell et al., 1998; Ica et al., 2012; Trachoo et al., 2002). Planktonic *C. jejuni* in the VBNC state can attach to surfaces, initiating biofilm formation (Duffy & Dykes, 2009).

Despite the public health concern of VBNC *C. jejuni*, there is limited quantitative information regarding the time course of VBNC development and the percentage of viable cells found in non-culturable populations of *C. jejuni* (Trevors, 2011). The objective of this study was to quantitatively assess and compare the development of VBNC *C. jejuni* in a planktonic and biofilm state using the LIVE/DEAD® *Bac*Light™ assay and culturing in media commonly used to detect and enumerate *C. jejuni*.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The sequenced strain, *C. jejuni* NCTC 11168 V1 was purchased from the ATCC and is representative of the original clinical isolate from a case of human enteritis in 1977 (Ahmed, Manning, Wassenaar, Cawthraw, & Newell, 2002). *C. jejuni* NCTC 11168 V26 (Carrillo et al., 2004), the laboratory passaged version of V1, was kindly donated by Dr. Brenda Allan from the Vaccine and Infectious Disease Organization (VIDO) in Saskatoon. *C. jejuni* 16-2R, a poultry isolate, was kindly donated by Dr. Joseph Odumeru, Laboratory Services Division, University of Guelph. All three strains were maintained at −80 °C in an ultra freezer (Thermo Electron). Cells from stock cultures were resuscitated on Mueller Hinton agar (MHA) by incubating at 42 °C under microaerobic conditions (5% 02, 10% C02 and 85% N2) for 24 h. Cells were then transferred onto fresh MHA and incubated at 37 °C under microaerobic conditions for 24 h prior to preparation of inocula.

2.2. Preparation of biofilm and planktonic cells

For each experiment, inocula were prepared fresh from frozen stock in order to avoid the transcriptional variation inherent in these bacteria. Bacteria were grown as a lawn on MHA for 24 h at 37 °C and then all cells were transferred from the subculture to 5 ml sterile phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, pH 7.4) using a polyester tipped sterile swab (Fisherbrand). Using a NovaSpec light spectrophotometer (Biochrom LTD, Cambridge, UK), the resulting suspension was standardized to an OD_{600} of 0.3 ± 0.015, which was equivalent to approximately 10^8 CFU/ml as confirmed by plate counting.

For development of biofilm cells, 0.1 g of glass fibre filters (Whatman GF/F, 2.1 cm diameter, 0.7 pore size) were placed in 250 ml glass bottles (Pyrex) which were then autoclaved at 121 °C for 20 min. Glass fibre filters provide extensive surface area for cell attachment and are amenable to the removal of cells (Kalmokoff et al. 2006). Bottles were cooled overnight and 20 ml of sterile Mueller Hinton broth (MHB) were added to each bottle. Each bottle was inoculated with 1.0 ml of standardized inoculum. Bottles were incubated microaerobically (5% O_2, 10% CO_2, 85% N) at 37 °C with gentle agitation (25 rpm) in an incubator shaker (New Brunswick Scientific, *Innova*™ 4430) for 24 h. Growth of planktonic cells followed the same procedure without the addition of glass fibre filters.

2.3. Harvesting and enumeration of biofilm and planktonic cells by standard plate counting

After 24 h of growth, bottles were removed from the incubator and placed on ice. For bottles with glass fibre filters, the broth containing planktonic cells was aseptically removed and discarded. The glass fibre filters were washed 3 times with 25 ml of cold PBS to remove any remaining planktonic cells. Biofilm cells were removed as described by Trachoo and Frank (2002): Filters were aseptically transferred to sterile 100 ml glass bottles containing 5 g of glass beads (SEPHEX, 450–600 nm) and 10 ml of PBS and vortexed vigorously for 2 min using a Fisher Scientific Analog Vortex Mixer set at the maximum speed of 10. The supernatant was then filtered through sterile stomacher bags to remove excess glass fibre and the removed biofilm cells were collected in sterile 15 ml tubes. Both the removed biofilm cells and planktonic cells (grown separately) were enumerated by drop plating on MHA, incubated microaerobically at 42 °C. Colonies were counted at 24 and 48 h and CFU/ml determined for each sample.

2.4. Induction of cells into the viable but non-culturable state

C. jejuni cells grown and harvested as described above were resuspended in 10 ml of PBS to give concentrations 10^8 CFU/ml for planktonic cells and 10^7 CFU/ml for biofilm cells. Samples were then incubated at 4 °C for up to 60 days in air without shaking. At specific time intervals (day 0, 10, 20, 30, 40, 50 and 60), 500 μl of each sample was stained with LIVE/DEAD® BacLight™ stain, imaged and biovolume values were obtained as described below. At the same time culturable counts for each sample were determined by drop plating on MHA as described above.

2.5. Extended culturability on alternative media

Once samples became non-culturable on Mueller Hinton agar, recovery by plating on supplemented agar and enrichment in Bolton broth was investigated. Supplemented agar was prepared using Campylobacter Agar Base (Oxoid CM0689) with the addition of Campylobacter Growth Supplement (Oxoid SR0232) which contains sodium pyruvate, sodium metabisulphite and ferrous sulphate. No selective supplements or antibiotics were added in order to prevent growth inhibition of injured cells. Plates were incubated microaerobically at 42 °C for 48 h prior to counting. For enrichment, 1 ml of cell suspension was added to 5 ml of sterile Bolton Broth (Oxoid CM0983) and incubated microaerobically at 37 °C with gentle agitation for 24 h. A 100 μl aliquot of this suspension was then plated on supplemented agar prepared as stated above and incubated at 42 °C for 48 h prior to counting.

2.6. Estimation of biovolumes using LIVE/DEAD® BacLight™ stain in conjunction with confocal scanning laser microscopy and PHLIP analysis

C. jejuni biofilm and planktonic cells grown as described above were stained with the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Molecular Probes, Invitrogen). The fluorescent dyes propidium iodide (PI) (20 mM in DMSO) and SYTO 9 (3.34 mM in DMSO) were mixed in a 2:1 ratio (PI:SYTO 9). Samples (500 μl) were incubated with 1.5 μl of dye mixture at 24 °C in the dark for 15 min. Samples