SHORT COMMUNICATION

Characteristics of biofilms associated with enhanced survival of *Campylobacter jejuni*

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Abstract

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The biofilm microflora isolated from a chicken house (Y1 and W1) and a meat plant (*Pseudomonas* sp.) enhanced survival of *C. jejuni* in a low nutrient environment. We employed direct microscopic observation techniques to accomplish this objective. Staining with cyanoditolyltetrazolium chloride CTC) indicated reduced oxygen tension in the biofilm environment, which could enhance the survival of *C. jejuni*. W1 biofilms with more CTC staining showed greater enhancement of *C. jejuni* survival than did *Pseudomonas* sp biofilm with more area coverage. This may indicate that reduced oxygen in biofilm of W1 plays important role in enhanced survival of *C. jejuni*. The *Pseudomonas* sp. biofilm exhibited the most extensive surface coverage and was thinner (approximately 1 μm) than Y1 and W1 biofilms. Reconstructed three-dimensional photomicrographs showed only one layer of cells in *Pseudomonas* sp. biofilm, while biofilms of W1 and Y1 were more complex. They consisted of different sizes of microcolonies with different thickness and void spaces in between. These morphological and physiological properties of the biofilms may be associated with increased survival of *C. jejuni* by providing improved nutrient entrapment and environmental stress protection, but no characteristic by itself could explain enhanced survival, which appears to be a complex phenomenon.

Key words : *Campylobacter jejuni*, CTC, biofilm

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ลักษณะของแผ่นชีวะและการรอดชีวิตของเชื้อแคมไพโลแบคเตอร์ เจจูไน

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จากการศึกษาถึงลักษณะของแผ่นชีวะที่อาจการส่งเสริมการรอดชีวิตของเชื้อแคมไพโลแบคเตอร์ เจจูไนในสภาวะจำกัดอาหารของเชื้อ Y1, W1 (แยกมาจากโรงเรือนเลี้ยงไก่) และ *Pseudomonas* sp. (แยกมาจากโรงงานผลิตผลิตภัณฑ์จากเนื้อสัตว์) โดยการใช้กล้องจุลทรรศน์ การย้อมสีแผ่นชีวะด้วย Cyanoditolyltetrazolium chloride แสดงถึงปริมาณออกซิเจนในแผ่นชีวะ ซึ่งออกซิเจนในแผ่นชีวะเป็นพิษต่อเชื้อแคมไพโลแบคเตอร์ เจจูไน และพบว่าแผ่นชีวะเชื้อ W1 ติดสีย้อมมากทั้งที่มีปริมาณแผ่นชีวะน้อยกว่าเชื้อ *Pseudomonas* sp. ซึ่งสอดคล้องกับผลการวิจัยครั้งก่อนที่พบว่าแผ่นชีวะ W1 ส่งเสริมการรอดชีวิตได้ดีกว่าเชื้อ *Pseudomonas* sp. เชื้อ *Pseudomonas* sp. สามารถสร้างแผ่นชีวะที่บาง (1 μm) แต่ขยายเป็นพื้นที่กว้างกว่า Y1 และ W1 ภาพถ่ายสามมิติด้วยกล้องจุลทรรศน์แบบคอนโฟคอลแสดงถึงการเรียงตัวของเซลล์แบบชั้นเดียวของแผ่นชีวะของเชื้อ *Pseudomonas* sp. ส่วนแผ่นชีวะของเชื้อ Y1 และ W1 มีความซับซ้อนกว่า คือมีการเรียงตัวของเซลล์มากกว่าหนึ่งชั้นและไม่สม่ำเสมอ นอกจากนี้ยังพบว่ามีการเกาะกลุ่มของแผ่นชีวะและช่องว่าง ลักษณะทางสัณฐานวิทยาและกายภาพของแผ่นชีวะหลายอย่างอาจจะเกี่ยวข้องกับการส่งเสริมการรอดชีวิตของเชื้อแคมไพโลแบคเตอร์ เจจูไน อย่างไรก็ดีลักษณะดังกล่าวเพียงอย่างหนึ่งอย่างใดอาจไม่สามารถนำมาอธิบายกลไลที่มีความซับซ้อนดังกล่าวได้

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Biofilms are an important reservoir of microbial contaminations in water systems (Mittelman, 1998). It is also difficult to eradicate biofilms from food processing environments (Lindsay *et al*., 1996, Wong, 1998). Biofilm bacteria are physiologically distinct from their planktonic counterparts. The presence of biofilm reduces the effectiveness of chemical sanitizers (Ren and Frank, 1993) and provides protection against environmental stress (Frank and Koffi, 1990; Mosteller and Bishop, 1993; Ronner and Wong, 1993).

Raw poultry and untreated water are major sources of Campylobacter (Bryan and Doyle, 1995, Butzler and Oosterom, 1991; Hingley, 1999). Pearson *et al*. (1993) reported that *C. jejuni* is a part of the normal flora of aquatic ecosystems because it is found in large numbers in the absence of lactose-fermenting coliforms and it accumulates in biofilms. Therefore, biofilm in water systems is a potential source of contamination in chicken houses. Buswell *et al*. (1998) observed extended survival of *Campylobacter* in the water system biofilms. In our previous study, biofilms enhanced survival of *C. jejuni* during incubation at 12ºC and 23ºC over a 7-day period.

The objective of this research was to investigate characteristics of biofilms which may enhance survival of *C. jejuni* in a low nutrient environment. To accomplish this objective we observed the structure of biofilms with different abilities to enhance survival of *C. jejuni*.

Materials and Methods

Substratum surfaces

Polyvinyl chloride plastic was obtained from Commercial Plastic (Atlanta, GA) and cut into 1 cm × 4 cm × 0.16 cm coupons. The coupons were cleaned by soaking with 2% Micro-90™ (International Products, Burlington, NJ) overnight, rinsed and steamed in deionized water for 30 min. Sample coupons from each batch were tested for sterility by incubating in Tryptic Soy Broth (Difco Laboratories, Detroit, MI) at 23ºC for 48 h.

Biofilm producers and culture preparation

Biofilm producers were collected by thoroughly swabbing nipple drinkers in a commercial chicken house. Colonies were isolated on R2A agar (Reasoner and Geldreich, 1985) (Difco) incubated at 23ºC for 48 h. Two different isolates, Y1 and W1, were used in this study. Stock cultures were stored frozen at -80ºC in Microbank™ beads (Pro-Lab, Inc., Ontario, Canada). Stock cultures were activated by transferring onto R2A agar and incubating at 23ºC for 48 h. This was followed by two additional transfers in a similar manner. *Pseudomonas*, a common biofilm producer, is often found in water distribution systems. A *Pseudomonas* sp. isolated from a meat processing plant was also included in this study. The *C. jejuni* strain was a chicken carcass isolate obtained from the Poultry Microbiological Safety Research Unit at Richard B. Russell Agriculture Research Center, U.S. Department of Agriculture (Athens, GA). On receipt, the *Campylobacter* strain was subcultured on semisolid brucella-reducing agar once before storing at -80ºC on Microbank™ beads. Prior to each use, the stock culture of *C. jejuni* was activated by a series of three transfers on semisolid brucella-reducing agar at 42ºC under microaerobic conditions (5% O2, 10% CO_2 and 85% N_2) using Oxoid CampyGen (Hampshire, England) for 48 h. Brucella-reducing medium contains brucella broth with 1.5 g/l agar and ferrous bisulfate pyruvic stock solution added to provide 0.5 g/l ferrous sulfate; 0.2 g/l sodium bisulfate and 0.5 g/l pyruvic acid (Sigma Chemical Co., St. Louis, Mo). The stock solution was stored at -80ºC.

Biofilm formation and attachment of *C. jejuni*

A fresh culture of each biofilm producer was diluted with 0.1% peptone water to obtain a concentration of 109 CFU/ml. *C. jejuni* was grown on brucella-reducing agar slant at 42ºC in a microaerobic environment for 48 h and diluted in peptone water before use to obtain 108 CFU/ml.

Sterile PVC coupons were incubated with 10^7 CFU/ml of each biofilm producer in R2A broth at 12ºC for 48 h. The coupons were removed, rinsed twice with 15-ml sterile water in a sterile Petri dish and placed into fresh R2A broth after 72 h for Y1 and W1 and 24 h for *Pseudomonas* sp. Two-day-old biofilms grown on PVC were incubated with 10^6 CFU/ml of *C. jejuni* for a 6-h attachment period in R2A broth at 12ºC. Negative (incubated with sterile medium) controls were included. After the 6-h attachment, coupons were rinsed and placed into a fresh R2A broth for an additional 7 days. Medium was refreshed every with 72 h for Y1 and W1 and 24 h for *Pseudomonas* sp.

Reducing activity biofilm determination

Reducing activity biofilm was determined based on the ability of the biofilm to 5-cyano-2, 3-ditolyl tetrazolium chloride (CTC). CTC has been previously used to probe the redox potential in tumor cells (Severin *et al*., 1985) and as an indicator for viability of *C. jejuni* (Federighi *et al*., 1998). In this study, 3 mM CTC (Polysciences, Warrington, PA) in R2A broth was applied to 4 and 7 day-old biofilms of Y1, W1 and *Pseudomonas* sp. and incubated for 30 min at 23ºC. The biofilms were then counterstained with acridine orange (Sigma) (0.1 g/L in 0.5 M acetate buffer; pH 3.5-4.0). Unincubated surfaces were stained to obtain background images, which were subtracted from images obtained from treatments. The coupons were then viewed using an epi-fluorescence microscope equipped with an FITC/Texas Red dual filter (Nikon Eclipes E600 and a TE-FM epifluorescence attachment, Nikon, Tokyo, Japan). Images (minimum of 10 per sample) were captured and analyzed for percent area coverage using ImageTool version 2.00 of the University of Texas Health Science Center at San Antonio. This experiment was repeated four times.

CSLM study

A Bio-Rad MTC-600 confocal scanning laser microscope (Bio-Rad Laboratories, Hemel Hempstead, UK) was employed to determine biofilm thickness, and to obtain optical sections. Biofilms were stained with Acridine Orange (Sigma) or SYTO Red 59™ (Molecular Probes, Inc. Eugene, OR) and examined under the CSLM by using a 60× oil immersion objective lens or a 40×

water immersion lens with 488 nm excitation wavelength for Acridine Orange or 568 nm excitation wavelength for SYTO Red 59™. Optical sections were reconstructed into a three-dimensional image by using computer software, Confocal Assistant version 4.02 (available at Bio-Rad Laboratories website, www.bio-rad.com).

Samples containing *C. jejuni* were stained using indirect fluorescence antibody technique (IFA) (Gerhardt *et al*., 1994). Rabbit anti-*C. jejuni*, a primary antibody, (US Biological, Swampscott, MA) and goat anti-rabbit IgG (H+L) with fluorescein isothiocyanate (FITC) conjugate, a secondary antibody, (KPL, Gaithersburg, MD) were diluted to 1:100 with PBS-BSA (0.01 M phosphate saline buffer, pH 7.2, containing 10 mg/ml bovine serum albumin). Ninety microliters of the primary antibody was dispensed onto each PVC coupon. The coupons were incubated in a humidified darkened chamber at 23ºC for 60 min with periodical gentle shaking and were then gently washed with sterile water for 10 s and air-dried. Ninety microliters of the secondary antibody was then placed on each coupon and the coupons were incubated, washed and dried as previously described. Finally, the coupons were stained with 30 µM SYTO Red 59™ (Molecular Probes, Inc.) for 30 min at 23ºC in a humidified darkened chamber, washed and dried as previously described. ProLong™ antifade (Molecular Probes, Inc.) was applied onto the stained PVC coupons prior to the examination by CLSM using a 60× oil immersion objective lens. Green fluorescence of FITC and red fluorescence of Sytored™ was observed at 488 nm and 568 nm excitation wavelengths, respectively.

Data analysis

Data was analyzed with SAS software (SAS Institute, Cary, NC) using PROC ANOVA. Significant differences between means were determined using Least Significant Difference (LSD) test at $P = 0.05$.

Results and Discussion

Two gram-positive biofilm producers (Y1 and W1) were isolated from chicken house nipple drinkers (Table 1) using R2A agar, a medium recommended for recovering microorganisms in water (Atlas, 1993, Percival *et al*., 1998, Reasoner and Geldreich, 1985). Cells of Y1 exhibited pleomorphic morphology being rods in the growth phase and coccoid in the stationary phase of growth. W1 was rod shaped and did not ferment glucose or manitol in Phenol Red Carbohydrate media, Y1 fermented glucose and manitol resulting in slight acid without gas. Y1 and W1 were motile. Isolate Y1 was identified as an irregular, nonsporing gram-positive rod, possibly in the genus Curtobacterium (Holt *et al*., 1994). W1 could not be tentatively identified. Other microorganisms, not culturable on this medium, and likely to be present in biofilms at the sampling sites (Berger

Table 1. Characteristics of Gram positive biofilm isolates from chicken house water systems.

Isolates[1]	Oxidase test	Catalase test	Gram stain[2]	Colony appearance	Utilization of glucose	Utilization of manitol	Motility test	Cell shape
Y1	-	+	+	Circular, convex, yellow	No gas, slight acid	No gas, slight acid	+	Rod/ Coccus[3]
W1	+	-	+	Circular, raised, white	-	-	+	Rod

[1] Isolated from inside nipple drinkers in chicken houses
[2] KOH method was used to determine Gram reaction
[3] Rods appeared in young cultures while cocci appeared in old cultures

et al., 1992). Minor populations of cells embedded in the biofilm samples are often inactive cells and nonculturable (Costerton *et al*., 1995, Fletcher, 1992, Mozes and Rouxhet, 1992). We selected isolates for this study based on their ability to produce biofilm at 12ºC (average local ground water) and 23ºC (average local room temperature). *Pseudomonas* sp. was included as an example of a gram negative biofilm producer, commonly found in water systems.

Pseudomonas sp. produced biofilm with the greatest ($P < 0.01$) area coverage (63%) compared to Y1 and W1 (6.71% and 11.85%, respectively) (Table 2). Coverage was greater than observed in a previous study (Trachoo *et al*., 2002) because biofilms were allowed to grow for a longer time before refreshing the media.

Biofilms contain metabolically active organisms which take up CTC and reduce it to orange or red fluorescent formazan. Reducing activity within the biofilm was determined by observing the reduction of CTC to water-insoluble formazan crystals (Rodriguez *et al*., 1992). Photomicrographs in Figure 1 show reducing activity of the biofilm as red areas. Although W1 biofilm covers almost six times less area than *Pseudomonas* sp., about half of its biofilm reduced the CTC (Table 2). Previous study demonstrated that W1 biofilm was associated with enhanced survival of *C. jejuni* equal to that of the *Pseudomonas* sp. at 12ºC even though it had significantly less area coverage (Trachoo *et al*., 2002). This suggests that reducing activity in biofilm is responsible for enhanced survival of *C. jejuni* in W1 and *Pseudomonas*. Biofilm producers in this study were aerobic microorganisms that metabolically depleted oxygen and reduced CTC to formazan. However, there was a report indicating that CTC can be chemically reduced in low-redox environment, for example in the presence of sodium dithionite (Rodriguez *et al*., 1992). The low nutrient medium, R2A, used in the study does not contain such constituents (Reasoner and Geldreich, 1985). In addition, the observed formazan was intracellular deposition not in the R2A medium. The reduction of CTC observed in this experiment is therefore associated with reduced oxygen tension that will benefit *C. jejuni*.

Y1 biofilm exhibited the least amount of reducing activity and had only 6.7% area coverage while it greatly enhanced survival of *C. jejuni* at 12º as demonstrated in our previous study (Trachoo *et al*., 2002). One of two phenomena could occur in biofilms that do not reduce CTC. These cells may be non-respiring cells (inactive) that cannot metabolically reduce CTC or the oxygen level may be sufficiently high to compete with CTC in electron transport system (ETS), resulting in less CTC reduction (Severin *et al*., 1985). Neither case enhances the survival of *C.*

Table 2. Area covered by biofilms after 7 days of incubation at 12ºC in R2A broth.

Biofilm	Percent area coverage[1]		
	Biofilm[2]	Active biofilm[3]	% Active biofilm
Y1	6.71[a]	0.37[a]	6.10[a]
W1	11.85[a]	5.15[b]	46.08[c]
Pseudomonas sp	63.05[b]	8.87[c]	14.90[b]

[1] Means of four replications. Images (minimum of 10 per sample) were captured and analyzed for percent area coverage by using ImageTool version 2.00 of the University of Texas Health Science Center at San Antonio.
[2] Biofilms stained with acridine orange
[3] Biofilms stained with cyanotolyltetrazolium chloride
[abc] Means in columns with no common superscript differ at $P < 0.05$ (LSD)

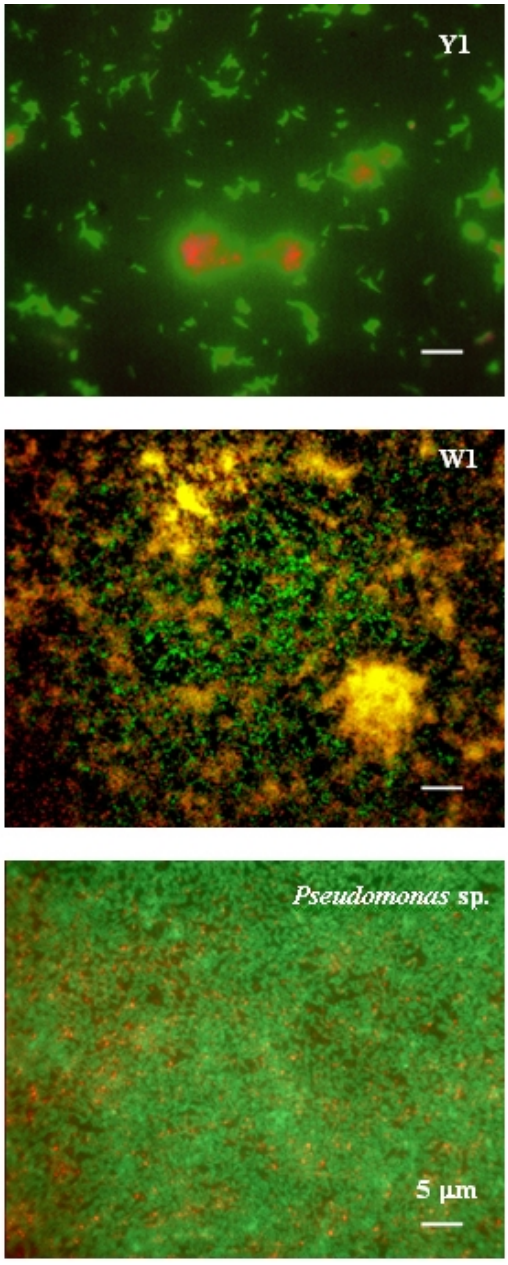

Figure 1. Images of 7 day-old Y1, W1 and *Pseudomonas* sp. biofilms stained with acridine orange and CTC. Cells uptake CTC and reduced it to red bright formazan are metabolically active and designated at active biofilms. Metabolically active cells are believed to associate with enhanced survival of *C. jejuni*.

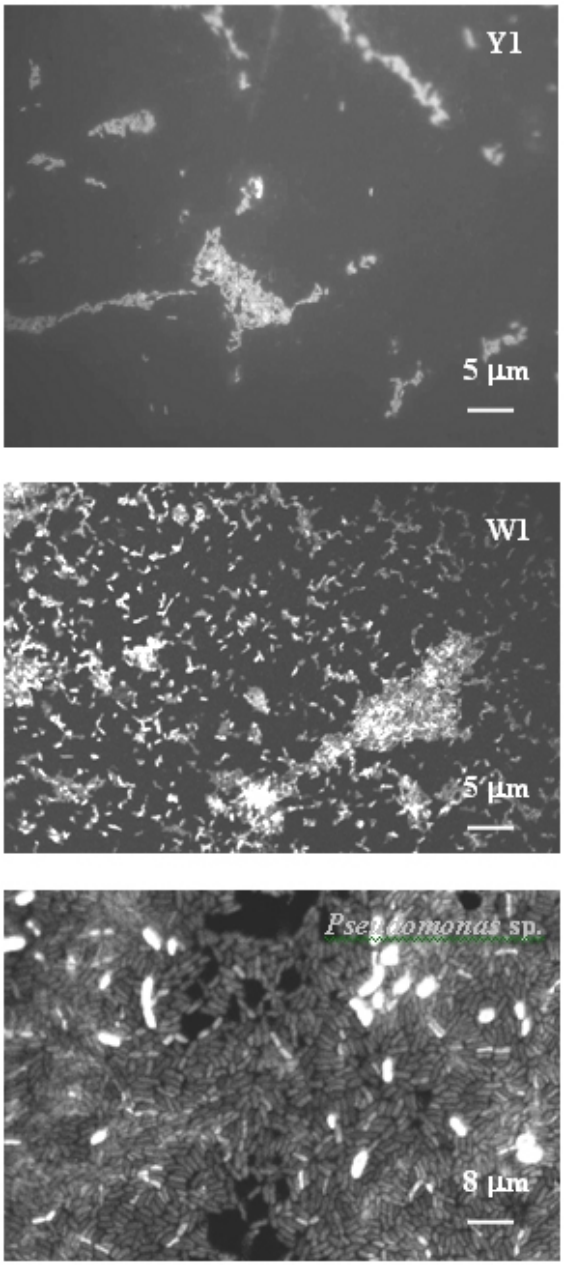

Figure 2. Typical biofilm of Y1, W1 and *Pseudomonas* sp. as stained with acridine orange.

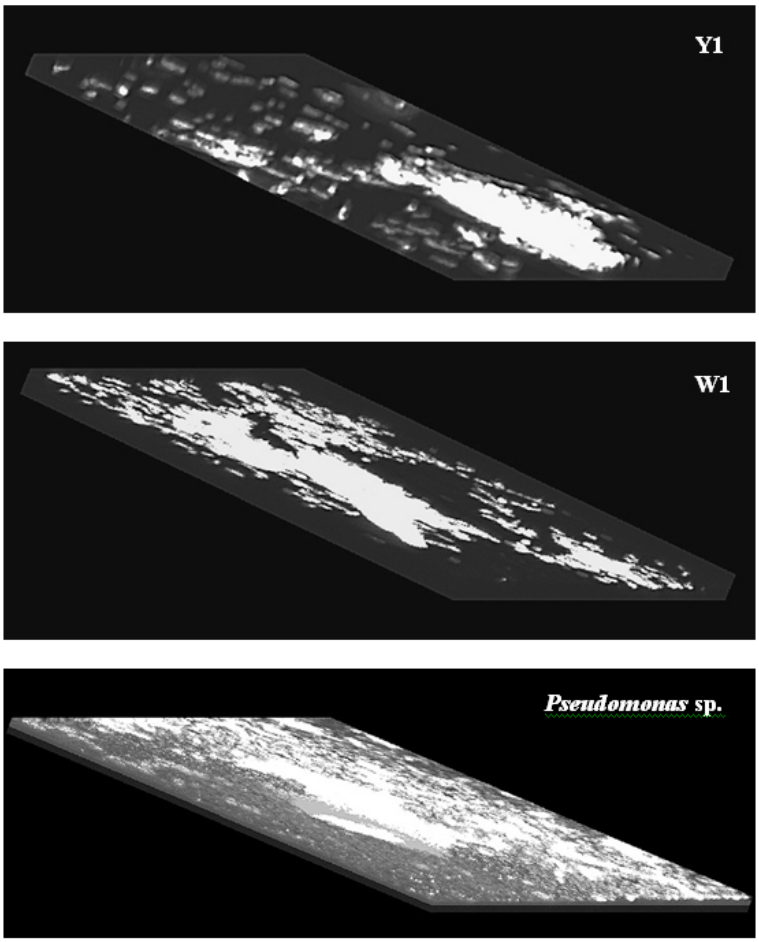

Figure 3. Reconstructed three-dimensional images from optical sections of Y1, W1 and *Pseudomonas* sp. biofilms stained with acridine orange. Optical sections were reconstructed into a three-dimensional image by using computer software, Confocal Assistant version 4.02.

jejuni, which is sensitive to oxygen. Thus, the improved survival of *C. jejuni* in Y1 biofilm was unlikely to be associates with reducing activity in this biofilm. This suggests that enhanced survival of *C. jejuni* in some biofilms is a complex phenomenon.

Thickness of 4- and 7-day-old biofilms was determined using the three dimensional scanning function of the confocal system. The thickness of each biofilm was similar at days 4 and 7. This indicates equilibrium between biofilm cell detachment and growth or a slow growth rate. Thickness of biofilm W1, Y1 and *Pseudomonas* sp. was 3 µm, 2 µm and 1 µm, respectively as determined by CSLM after rinsing samples to detach the loosely adherent cells. *Pseudomonas* sp. biofilm was primarily a single layer of cells (Figure 2) but they were closely packed and covered the most area of the three biofilms. Reconstructed three-dimensional images of biofilms (Figure 3) revealed similar results as seen in Figure 2 with the *Pseudomonas* sp. biofilm exhibiting high surface coverage with high cell density compared to Y1 and W1 biofilms. The extensive coverage of the *Pseudomonas* sp. biofilm was associated with high level of *C. jejuni* attachment as demonstrated in a previous study

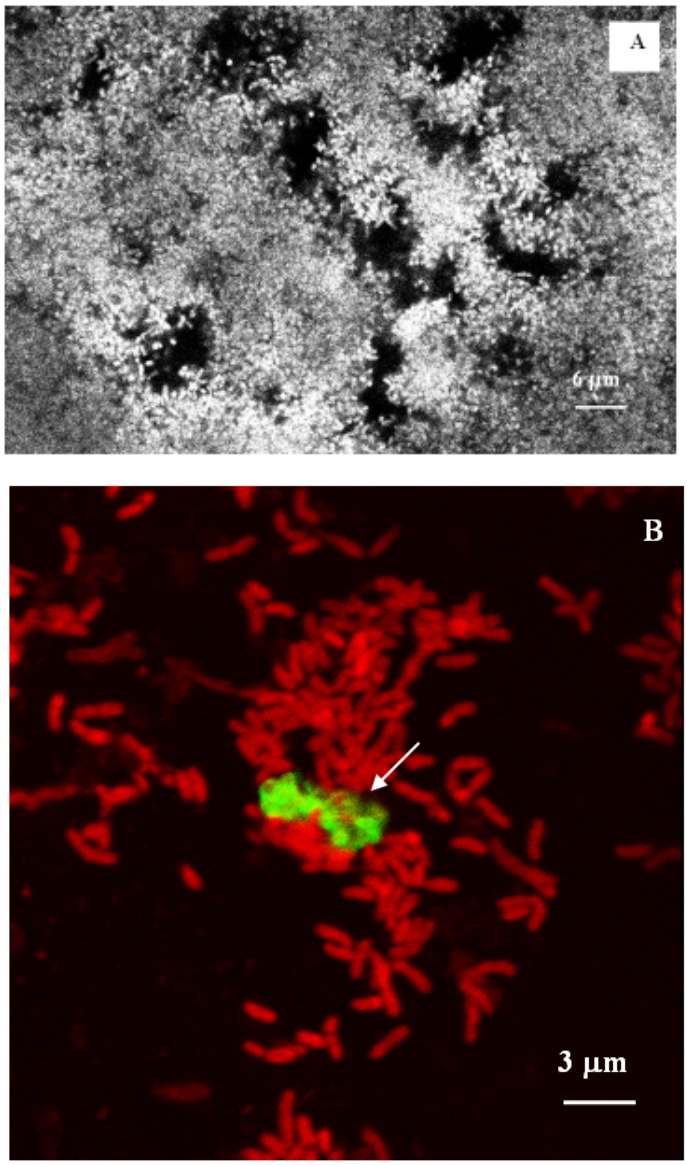

Figure 4. Typical 7 day-old W1 biofilm (A). The image was taken from a living biofilm stained with acridine orange. Optical sections were used to reconstruct this three dimensional image. *C. jejuni* attached underneath cells of W1 (arrow) (B).

(Trachoo *et al*., 2002). The lack of greater survival of *C. jejuni* in this biofilm as compared to the others may be due to its being primarily a single cell layer.

Figure 4A shows a reconstructed three-dimensional (XY-scan) image of a 7-day-old W1 biofilm. Void spaces and layers of cells can be observed. Figure 4B shows the attachment of *C. jejuni* on 7-day-old W1 biofilm. *C. jejuni* cells were stained with specific antibodies and W1 were stained with SYTO Red™ 59. In this image, part of *C. jejuni* is underneath the W1 cells (arrow). This might be evidence of how *C. jejuni* can possibly be protected by biofilms.

All the biofilms characterized in this study had previously been associated with enhanced

survival of *C. jejuni*. These biofilms exhibited different morphological and physiological characteristics, as determined by reducing activity, thickness, density of cells, and surface coverage. Even though this study was limited to three different biofilms, it is apparent from the results that survival of *C. jejuni* in these biofilms is a complex phenomenon, possibly associated with combination of biofilm properties, which may include but are limited to, reducing ability, thickness, and area coverage.

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