Survival of *Campylobacter jejuni* in Biofilms Isolated from Chicken Houses

N. TRACHOO,1 J. F. FRANK,1* AND N. J. STERN2

1Center for Food Safety, Department of Food Science and Technology, University of Georgia, Athens, Georgia 30605-2106; and 2Richard B. Russell Agricultural Research Center, Poultry Microbiological Safety Research Unit, U.S. Department of Agriculture, P.O. Box 5677, Athens, Georgia 30613, USA

MS 01-299: Received 21 August 2001/Accepted 14 December 2001

ABSTRACT

*Campylobacter jejuni* is a thermophilic and microaerophilic enteric pathogen associated with poultry. Biofilms may be a source of *C. jejuni* in poultry house water systems since they can protect constituent microorganisms from environmental stress. In this study, the viability of *C. jejuni* in biofilms of gram-positive chicken house isolates (P1, Y1, and W1) and a *Pseudomonas* sp. was determined using a cultural method (modified brucella agar) and direct viable count (DVC). Two-day biofilms grown on polyvinyl chloride (PVC) coupons in R2A broth at 12 and 23°C were incubated with *C. jejuni* for a 6-h attachment period. Media were then refreshed every 24 h for 7 days to allow biofilm growth. Two-day biofilms of P1, Y1, and *Pseudomonas* sp. enhanced attachment (*P* < 0.01) of *C. jejuni* (4.74, 4.62, and 4.78 log cells/cm², respectively) compared to W1 and controls without preexisting biofilm (4.31 and 4.22 log cells/cm², respectively). On day 7, isolates P1 and Y1 and *Pseudomonas* biofilms covered 5.4, 7.0, and 21.5% of the surface, respectively, compared to 4.9% by W1. Viable *C. jejuni* on the surface decreased (*P* < 0.05) with time, with the greatest reduction occurring on surfaces without a preexisting biofilm. The number of viable *C. jejuni* determined by DVC was greater than that determined by the cultural method, indicating that *C. jejuni* may form a viable but nonculturable state within the biofilm. Both DVC and the cultural method indicate that biofilms enhance (*P* < 0.01) the survival of *C. jejuni* during incubation at 12 and 23°C over a 7-day period.

*Campylobacter* spp. are a major cause of foodborne illness in the United States and other countries and are often associated with poultry (9, 10, 20). According to a Minnesota study (20), 88% of poultry sampled from local supermarkets tested positive for *Campylobacter*. *Campylobacter* spp. often infect an entire poultry broiler flock. To control *Campylobacter* in poultry, additional information regarding its sources and how it survives in the environment and during poultry production is necessary.

*Campylobacter* has been isolated from wild animals, insects, river systems, water supplies, and groundwater (1, 6, 24, 34, 41). Pearson et al. (34) concluded that *Campylobacter jejuni* is part of the normal microflora of aquatic ecosystems because it is found in large numbers in the absence of lactose-fermenting coliforms, and it accumulates in biofilms. Somers et al. (40) reported that *C. jejuni* readily formed a biofilm of 10⁶ CFU/cm² on stainless steel within 2 days in brucella broth. Although since 1972 *C. jejuni* has become recognized as an important cause of diarrhea (33), little is known about its behavior as a constituent microorganism in biofilms. Such information is needed, since the physiology and nutritional requirements of planktonic and biofilm bacteria may differ (12, 26, 27).

In the United States, groundwater, often nonchlorinated, is distributed to chickens through polyvinyl chloride (PVC) pipes and nipple drinkers. Water may remain in the system pipes for extended periods, resulting in biofilm formation. Biofilms provide protection for pathogens from environmental stresses and antimicrobial agents (2, 14, 16, 27, 31, 38). Previous reports indicate that *C. jejuni* maintains its viability in biofilms in low-nutrient media and in normal atmospheric conditions for up to 1 week at 10°C, in spite of its susceptibility to oxygen and nutritional stress (8, 39). It is possible that *C. jejuni* can become incorporated into biofilms in chicken house water systems and infect chickens after biofilm detachment.

The objectives of this study were to determine the ability of *C. jejuni* to become integrated into and survive in preexisting biofilms in a low-nutrient growth medium under normal atmospheric conditions. The effects of temperature and type of biofilm producer on the attachment of *C. jejuni* to a PVC surface were also investigated.

MATERIALS AND METHODS

PVC plastic was obtained from Commercial Plastic (Atlanta, Ga.) and was cut into 1- by 4- by 0.16-cm coupons. Coupons were soaked with 2% Micro-90 (International Products, Burlington, N.J.) overnight and were then rinsed and steamed in deionized water for 30 min. Sample coupons from each batch were tested for sterility by incubation in 20 ml sterile tryptic soy broth (Difco Laboratories, Detroit, Mich.) at 23°C for 2 days and examination for turbidity.

**Biofilm producers and culture preparation.** Biofilm producers were collected by swabbing nipple drinkers in a commercial chicken house in northeastern Georgia. Colonies were isolated on R2A agar (37) (Difco) incubated at 23°C for 48 h. Three different biofilm cultures, P1, Y1, and W1, were isolated from swabs...
and characterized by the KOH gram test (36), the catalase test, the oxidase test, the assessment of cell and colony morphology, the motility with hanging drop method and the motility growth index medium (Difco), and the use of glucose and mannitol using Phenol Red Carbohydrate media (Difco). Stock cultures were stored frozen at −80°C in Microbank beads (Pro-Lab, Inc., Ontario, Canada). Stock cultures were activated by transferring onto R2A agar and incubating at 23°C for 2 to 5 days. This was followed by two additional transfers in a similar manner. 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 the Richard B. Russell Agriculture Research Center, U.S. Department of Agriculture (Athens, Ga.). On receipt, the Campylobacter strain was subcultured once on Brucella triphenyl tetrazolium chloride agar with reducing agents (BTTC-RA) before storing at −80°C on Microbank beads. BTTC-RA contained 43 g/liter brucella agar (Acumedia, Baltimore, Md.); ferrous bisulfate pyruvic acid stock solution added to provide 0.5 g/liter ferrous sulfate; 0.2 g/liter sodium bisulfite and 0.5 g/liter pyruvic acid (the stock solution was stored at −80°C); 50 mg/ml 2,3,5-triphenyl tetrazolium chloride (Sigma Chemical Co., St. Louis, Mo.); 5 mg/liter trimethoprim; 10 mg/liter vancomycin; 33 mg/liter cefoperazone; and 200 mg/liter cycloheximide (all antibiotics were from Sigma). Stock solutions of filtered sterile tetrazolium chloride and antibiotics were added to the agar before autoclaving. Before use, the stock culture of C. jejuni was activated by a series of three transfers on semisolid brucella-reducing (BR) agar at 42°C for 2 to 5 days. This was followed by two additional transfers in a similar manner. A fresh culture of each biofilm producer grown in R2A broth at 21°C for 2 to 4 days (depending on the culture growth rate) was diluted with 0.1% peptone water to obtain a concentration of 10⁸ CFU/ml C. jejuni was grown on a BR slant at 42°C in a microaerobic environment for 48 h. Growth on the slant was removed using peptone water to obtain a suspension of 10⁶ CFU/ml C. jejuni. Steamed PVC coupons were incubated with 10⁸ CFU/ml of each biofilm producer in R2A broth at 12 and 23°C for 2 days. The coupons were removed, rinsed twice with 15-ml sterile water using a steady stream from a plastic wash bottle, and placed into fresh R2A broth every 24 h. Two-day-old biofilms grown on PVC were then incubated with 10⁶ CFU/ml C. jejuni for a 6-h attachment period in R2A broth at 12 and 23°C. Negative (clean coupons) and positive (only C. jejuni) controls were included. After the 6-h attachment, coupons were rinsed and placed into a fresh R2A broth every 24 h for 7 days. Coupons were removed after 0, 4, and 7 days of incubation for analysis.

**Direct viable counts.** The direct viable count (DVC) method was based on that of Federighi et al. (13). After rinsing with sterile water, PVC sample coupons were incubated in nalidixic acid brucella broth for 24 h at 42°C in a microaerobic environment. The nalidixic acid brucella broth consisted of 28 g/liter brucella broth (Acumedia), 10 mg/liter nalidixic acid (Sigma), 33 mg/liter cefoperazone (Sigma), and Blaser-Wang Campylobacter-selective supplement (Oxoid, Hampshire, UK), used as described in the manufacturer’s instructions. The coupons were then removed from the broth, and C. jejuni was stained by the indirect fluorescence antibody technique (17). Rabbit anti-C. jejuni, the primary antibody (U.S. Biological, Swampscott, Mass.), and goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, Pa.) were used as secondary antibodies. Direct viable counts were performed by dilution and plating on R2A agar plates (23°C). Plates were incubated at 23°C for up to 14 days. Colonies were counted with the help of an automated colony counter (Omnion, Franklin Lakes, N.J.).

**Biofilm formation and attachment of C. jejuni.** A fresh culture of each biofilm producer grown in R2A broth at 21°C for 2 to 4 days (depending on the growth rate) was diluted with 0.1% peptone water to obtain a concentration of 10⁸ CFU/ml C. jejuni was grown on a BR slant at 42°C in a microaerobic environment for 48 h. Growth on the slant was removed using peptone water to obtain a suspension of 10⁶ CFU/ml C. jejuni. Steamed PVC coupons were incubated with 10⁸ CFU/ml of each biofilm producer in R2A broth at 12 and 23°C for 2 days. The coupons were removed, rinsed twice with 15-ml sterile water using a steady stream from a plastic wash bottle, and placed into fresh R2A broth every 24 h. Two-day-old biofilms grown on PVC were then incubated with 10⁶ CFU/ml C. jejuni for a 6-h attachment period in R2A broth at 12 and 23°C. Negative (clean coupons) and positive (only C. jejuni) controls were included. After the 6-h attachment, coupons were rinsed and placed into a fresh R2A broth every 24 h for 7 days. Coupons were removed after 0, 4, and 7 days of incubation for analysis.

**TABLE 1. Characteristics of gram-positive biofilm isolates from chicken house water systems.**

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Gram stain</th>
<th>Catalase test</th>
<th>Oxidase test</th>
<th>Cell shape</th>
<th>Rods</th>
<th>Rods/coecii</th>
<th>Rods</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>Circular, convex, pick</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Y1</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Circular, convex, yellow</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>W1</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>Circular, raised, white</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

* +, presence; −, undetectable.  
* Isolated from inside nipple drinkers in chicken houses.  
* KOH method was used to determine Gram stain reactions.  
* Rods appeared in young cultures, while cocci appeared in old cultures.
TABLE 2. Area covered by biofilms after 0, 4, and 7 days of incubation at 12 and 23°C in R2A broth

<table>
<thead>
<tr>
<th>Biofilm</th>
<th>12°C</th>
<th>23°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 4</td>
</tr>
<tr>
<td>P1</td>
<td>1.2 A</td>
<td>2.2 A</td>
</tr>
<tr>
<td>Y1</td>
<td>2.9 AB</td>
<td>4.5 A</td>
</tr>
<tr>
<td>W1</td>
<td>3.5 B</td>
<td>3.9 A</td>
</tr>
<tr>
<td>Pseudomonas sp.</td>
<td>8.9 C</td>
<td>9.0 B</td>
</tr>
</tbody>
</table>

a Means of four replications.
b Incubation time began when the 2-day-old biofilms were incubated with Campylobacter jejuni.
c Average values across temperature and day.
d Means in columns with no common letter differ at P < 0.05 (least significant difference test).

RESULTS AND DISCUSSION

**Biofilm producers.** Three gram-positive biofilm producers (P1, Y1, and W1) were isolated from chicken house nipple drinkers using R2A agar, a medium recommended for recovering microorganisms in water (3, 35, 37). The isolates are described in Table 1. At 23 and 12°C, P1 grew more slowly than Y1 and W1, as indicated by the time required to produce turbidity. Isolates P1 and Y1 were tentatively identified as Corynebacterium and Curtobacterium spp., respectively (22). W1 could not be tentatively identified. Other microorganisms, not culturable on this medium, were probably present in biofilms at the sampling sites (5). Microbial cells embedded in biofilm samples are often inactive cells and nonculturable (11, 14, 32). We selected isolates for this study on the basis of their ability to produce biofilm at 12 and 23°C. Pseudomonas sp. was included as an example of a gram-negative biofilm producer.

The area covered by biofilm on PVC coupons increased with time for all selected isolates (Table 2), but biofilms were produced at different rates. Pseudomonas sp. produced the most biofilm, which, after 7 days, covered 11.3 and 31.6% of the surface area of the PVC coupons at 12 and 23°C, respectively (Table 2). The area coverages of biofilms produced by P1, Y1, and W1 were not significantly different (P > 0.05). The area coverage was greater...
Fig. 1. Photomicrographs of biofilms of P1 (A), Y1 (B), W1 (C), and Pseudomonas sp. (D) incubated for 7 days at 23°C. Thick arrows indicate microcolonies of the biofilm producers. Biofilm producers were stained with SYTO Red 59, and C. jejuni (bright cells) were stained using indirect immunofluorescence. Photomicrographs E and F show C. jejuni cells that have survived within biofilms of W1 and Pseudomonas sp., respectively. Dotted arrows show elongated cells of C. jejuni, indicating viability. Thin arrows indicate dead C. jejuni attached to biofilm-free PVC surfaces (G) after incubation at 23°C for 7 days.

(P < 0.05) at 23°C than at 12°C. The 2-day biofilm formation of each biofilm producer was different at each temperature. Two-day (day 0 on Table 1) biofilms produced by W1 at 12°C were greater than those at 23°C, but eventually, biofilms of W1 at 23°C increased coverage to those produced at the lower temperature.

Photomicrographs (Fig. 1A through 1D) show typical biofilms of P1, Y1, W1, and Pseudomonas sp. Biofilms of P1 consisted mostly of individual cells and small microcolonies (Fig. 1A), whereas Y1 and W1 (Fig. 1B and 1C) produced biofilms with large microcolonies. The Pseudomonas sp. biofilm (Fig. 1D) was the most confluent.

Attachment of C. jejuni to biofilms. C. jejuni attached to biofilm-free PVC at a level of 4.3 log cells/cm². The number attached remained constant throughout the incubation period, whereas the viability of C. jejuni as determined by DVC decreased (Fig. 2). The type of biofilm producer—but not the temperature—affect the attachment (P > 0.05) of C. jejuni to the biofilm (Fig. 2). The better biofilm producers (P1, Y1, and Pseudomonas sp.) (Table 2) enhanced the attachment (P < 0.01) of C. jejuni (4.74, 4.62, and 4.78 log cells/cm², respectively) when compared to isolate W1 and to the control without biofilm (4.31 and 4.22 log cells/cm², respectively). It is likely that each biofilm producer exhibited physico-chemical characteristics such as surface hydrophobicity and charge that could influence the attachment of C. jejuni (15, 25).

Survival of C. jejuni in biofilms. After the 6-h attachment, 1.22 log cells/cm² (mean of both temperatures) of viable C. jejuni as determined by DVC were observed on...
the biofilm-free surface in the control, but the viability was reduced to 0.33 log cell/cm² after 7 days of incubation. The results obtained from plate counts confirm that the viability of *C. jejuni* decreased during incubation (Fig. 2). This could be due to oxidative stress. *C. jejuni* could not be recovered in samples with W1 and *Pseudomonas* biofilms incubated at 23°C after 4 days of incubation by direct plating on BTTC-RA agar, but it was present after enrichment (Table 3). This indicates either the recovery of injured cells or the presence of a low number of uninjured cells.

The decreased viability of *C. jejuni* in biofilms was directly related to incubation temperature and exposure time (length of time that *C. jejuni* was exposed to the atmospheric conditions in R2A broth). As indicated by the plate count data, the incubation temperature affected (*P* < 0.01) the survival of *C. jejuni*. *C. jejuni* in biofilms incubated at 12°C was detectable by direct plate count throughout 7 days of incubation, except in P1 at 12°C. However, *C. jejuni* in W1 and *Pseudomonas* biofilms became undetectable after 4 days at 23°C. DVC also indicated greater (*P* < 0.05) viability of *C. jejuni* in the biofilm-free control incubated at 12°C than in that incubated at 23°C. Thomas et al. (42) found increased survival of *C. jejuni* at low temperatures (as low as 5°C) in various water types (river water with or without sediment and deionized water). Similar observations have been reported by Hazeleger et al. (19).

In our biofilm environments, low temperature was less important in maintaining the viability of *C. jejuni* in the samples with extensive biofilm coverage (P1, Y1, *Pseudomonas* sp., or 7-day-old W1 biofilms) (Fig. 2).

Exposure time affected (*P* < 0.05) the level of viable *C. jejuni* in each biofilm differently. Viable *C. jejuni* within biofilms of P1, Y1, and W1 decreased (*P* < 0.05) with time, with the greatest reduction occurring in the biofilm-
free control (Fig. 2). Microscopic observation confirmed these data (Fig. 1G). After 7 days of incubation, it was rare to find elongated Campylobacter in P1, Y1, and W1 biofilms or in the biofilm-free control, possibly because there was little biofilm coverage on these surfaces. In contrast, the number of viable C. jejuni in Pseudomonas sp. biofilm as detected by DVC remained constant (P > 0.05), and large numbers of viable (elongated) cells of C. jejuni could be observed microscopically (Fig. 1F), indicating survival due to a protective biofilm.

The number of viable cells determined by the plate count method on day 0 was greater than that determined by DVC (Fig. 2). This may be due to the inactivation of some healthy C. jejuni cells by nalidixic acid (21), an effect that appears to be less significant after 4 days of incubation.

**Survival strategies of C. jejuni in biofilms.** Biofilm environments appear to protect C. jejuni from environmental stress. This may be partially due to its association with biofilm exopolymeric substance, since greater survival was observed in biofilms having greater surface coverage. C. jejuni is sensitive to atmospheric oxygen (18, 33). Since the interior of a biofilm has less dissolved oxygen than the exterior (44), C. jejuni integrated within a biofilm could be protected from oxygen inactivation. C. jejuni, being motile, may be able to migrate to an optimum environment by chemotaxis. Hazeleger et al. (19) reported that C. jejuni migrated through a semisolid gel to the most favorable concentration of oxygen when incubated aerobically at 20°C. Some of the Campylobacter cells in the Pseudomonas biofilm appeared to be embedded in the biofilm, as opposed to being on the surface. In addition, biofilms concentrate nutrients (27), which could possibly enhance the survival of C. jejuni in the low-nutrient R2A broth.

After 4 days of incubation, the number of viable C. jejuni in the biofilms as determined by DVC was greater than that determined by the cultural method, indicating that C. jejuni may form a viable but nonculturable (VBNC) state. Rollins and Colwell (39) reported that C. jejuni incubated in stream water at 4°C survived over 4 months and formed a VBNC state. The morphology of these cells was predominately coccoid (7, 29, 39). Cocoid C. jejuni cells were frequently observed in this study on biofilm-free controls (Fig. 1G). Cocoid forms of Campylobacter are associated with adaptation to environmental stresses, including oxidative stress and nutrient limitation (7, 18, 30). Cocoid C. jejuni cells have been reported to be metabolically inactive (4, 32) while retaining viability. In our study, the total number of C. jejuni cells attached on biofilms did not significantly increase during the 7-day incubation, even though culturability decreased. This indicates a mixed population of VBNC and dead C. jejuni. The significance of VBNC C. jejuni to the colonization of animals is uncertain and may differ with strain and animal species (23, 28, 43).

In conclusion, this study demonstrated that biofilms enhance the attachment and survival of C. jejuni. These results confirm the possibility that biofilms in water systems could potentially harbor C. jejuni in poultry farms.

**ACKNOWLEDGMENTS**

This research was supported by a grant from the U.S. Poultry and Egg Association and state and Hatch funds allocated to the Georgia Agricultural Research Station.

**REFERENCES**


**TABLE 3.** The presence of Campylobacter jejuni in preexisting biofilms and biofilm-free control after 0, 4, and 7 days of incubation at 12 and 23°C; samples were enriched in a modified bru- cella broth at 42°C for 48 to 72 h

<table>
<thead>
<tr>
<th>Day</th>
<th>Biofilm</th>
<th>Temperature</th>
<th>12°C, positive/total</th>
<th>23°C, positive/total</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>P1</td>
<td>12°C</td>
<td>3/4</td>
<td>4/4</td>
</tr>
<tr>
<td></td>
<td>Y1</td>
<td>23°C</td>
<td>4/4</td>
<td>4/4</td>
</tr>
<tr>
<td></td>
<td>W1</td>
<td>12°C</td>
<td>4/4</td>
<td>1/4</td>
</tr>
<tr>
<td></td>
<td>Pseudomonas sp.</td>
<td>12°C</td>
<td>3/4</td>
<td>1/4</td>
</tr>
<tr>
<td>7</td>
<td>P1</td>
<td>23°C</td>
<td>1/4</td>
<td>3/4</td>
</tr>
<tr>
<td></td>
<td>Y1</td>
<td>23°C</td>
<td>4/4</td>
<td>4/4</td>
</tr>
<tr>
<td></td>
<td>W1</td>
<td>23°C</td>
<td>3/4</td>
<td>1/4</td>
</tr>
<tr>
<td></td>
<td>Pseudomonas sp.</td>
<td>23°C</td>
<td>3/4</td>
<td>0/4</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>23°C</td>
<td>0/4</td>
<td>0/4</td>
</tr>
</tbody>
</table>

* Room temperature.


