Direct Microscopic Observation and Viability Determination of Campylobacter jejuni on Chicken Skin

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ABSTRACT

A method was developed to determine the survival of Campylobacter jejuni at specific sites on chicken skin, and this method was used to observe the survival of C. jejuni at various locations on the skin during storage. This method uses confocal scanning laser microscopy to visualize C. jejuni transformed with P_{gfp} plasmid (GFP-Campylobacter) and stained with 5-cyano-2,3-ditolyl tetrazolium chloride (CTC). The green fluorescence of dead C. jejuni cells and the red fluorescent CTC-formazan in viable Campylobacter cells were clearly visible on chicken skin. The GFP-Campylobacter remaining on the chicken skin surface after rinsing was mostly located in crevices, entrapped inside feather follicles with water, and entrapped in the surface water layer. Most viable cells were entrapped with water in the skin crevices and feather follicles. These sites provide a suitable microenvironment for GFP-Campylobacter to survive. The population of C. jejuni on chicken skin decreased by 1 log unit during storage at 25°C for 24 h. C. jejuni located in sites 20 to 30 μm beneath the chicken skin surface maintained viability during incubation at 25°C. C. jejuni on chicken skin stored at 4°C maintained constant numbers during a 72-h incubation with no significant changes in population feather follicles or crevices. Live and dead cells were initially retained with water on the skin and penetrated into the skin follicles and channels during storage. Microscopic observations of GFP-producing cells allowed the identification of survival niches for C. jejuni present on chicken skin.

Campylobacter jejuni subsp. jejuni (C. jejuni) was the leading cause of foodborne disease in the United States from 1996 to 2001 (7). Campylobacters are part of the normal flora in the intestinal tract of a large number of wild and domesticated animals, including food animals, chickens, and swine (3, 12). Campylobacters from a variety of sources on the farm colonize poultry and are carried into processing plants. Even in sanitary modern processing plants, campylobacters are present on poultry throughout processing operations. C. jejuni has been detected on raw chicken carcasses, and a high percentage of retail broiler chickens are contaminated with this pathogen (25).

Scalding, defeathering, evisceration, and giblet operations are the major transfer points of microorganisms (4). Levels of C. jejuni on carcass surfaces increase and decrease at various stages of processing (1). The chicken intestinal tract, ceca, and colon, which contain high levels of C. jejuni, may leak during processing, especially during defeathering, and result in adding C. jejuni contamination to the skin of the carcass (2). The skin of chicken is a major site of carcass contamination (13). C. jejuni is retained in a liquid film on the skin and becomes entrapped in skin ridges and crevices. In this way, chicken skin provides a microenvironment suitable for the survival of C. jejuni.

Lee et al. (12) found that C. jejuni could persist at high numbers on chicken skin stored at 4°C or grow rapidly on skin stored at room temperature in controlled atmosphere packaging. Blankenship and Craven (3) reported that C. jejuni survived well with spoilage microflora, including Pseudomonas sp. predominant in air-packaged products and Lactobacillus sp. in carbon dioxide–packaged products stored at low temperatures (3). In addition, the microorganisms can survive on chicken carcasses during refrigerated transport and frozen storage (18).

Determination of the effect of attachment to chicken skin on the viability of Campylobacter will aid in understanding its survival in this environment and its transmission to humans. Using a fluorescent antibody to identify Campylobacter cells has proven difficult because of low antibody specificity, which results in nonspecific fluorescent backgrounds (5). Bacteria with an intrinsic tag that can be detected in real time are useful for studying adherence (14). The green fluorescent protein (GFP) of Aequorea victoria, encoded by the gfp gene, fluoresces without added cofactors or substrates and can be expressed in a variety of bacterial species. Miller et al. (14) demonstrated that GFP in Campylobacter is stable and resistant to photobleaching. The intrinsic fluorescence and stability of GFP permit the nondestructive visualization of gfp-containing cells in complex microenvironments.

Microscopic observation of bacterial cell viability can be accomplished by staining with CTC (5-cyano-2,3-ditolyl tetrazolium chloride). CTC is a mononitrotetrazolium reduct dye that produces a fluorescent formazan (CTF) when chemically or biologically reduced. The CTF is deposited intracellularly. CTC was first used as a cellular reduct indicator of active respiration (i.e., electron transport) in cytosome-
ical experiments with Ehrlich ascites tumor cells (19). It has also been used for detecting viable but nonculturable \textit{C. jejuni} (6, 8, 22). CTC is the fluorescent redox dye often used in epifluorescent microscopy because CTC-formazan fluoresces primarily in the red region of the visible spectrum range, making it readily distinguishable from most background fluorescence, which typically emits in the blue or blue-green regions of the spectrum. The sensitivity of detection of fluorescent CTC-formazan facilitates the visualization of small bacteria and bacteria exhibiting low electron transport activity, both of which are important for the visualization of viable \textit{C. jejuni}.

The objective of this study was to develop a method to determine the survival of \textit{C. jejuni} at specific sites on chicken skin, including channels and ridges on the surface and below the surface in feather follicles. GFP-producing \textit{Campylobacter} sp. incubated with CTC were observed by confocal scanning laser microscopy. We used this technology to observe the survival of \textit{C. jejuni} located at various sites within the microtopography of the chicken skin during storage at different temperatures. This information will help explain how \textit{C. jejuni} survives on chicken skin and may be useful in the development of technologies for eliminating \textit{C. jejuni} from the skin.

**MATERIALS AND METHODS**

**Bacterial strain and plasmid.** GFP \textit{C. jejuni} RM 1221 (pWM1007) was provided by W. G. Miller, U.S. Department of Agriculture—Agricultural Research Service, Albany, Calif. The plasmid encoding GFP (pWM1007; Km; pMW10ΔlacZI(T1)y-P\textsubscript{C.gfp-T1}) had been mobilized into \textit{C. jejuni} strain RM 1221 from a chicken carcass isolate (14). Transformants were selected by plating on Cefex-campy agar (CCA) (20) supplemented with 200 \textmu g of kanamycin per ml (CCA-KM) and grown at 42°C for 48 h in a sealed bag with a mixture of 5\% \textsubscript{O} \textsubscript{2}, 10\% \textsubscript{CO} \textsubscript{2}, and 85\% \textsubscript{N} \textsubscript{2} (BOC Gases, Chattanooga, Tenn.). Colonies on CCA-KM were restreaked on fresh plates, and the expression of GFP-\textit{Campylobacter} was confirmed by observing bacterial smears with a Nikon Labophot epifluorescent microscope (Nikon, Inc., Garden City, N.Y.) equipped with a ×100 objective lens, a 100-W Hg lamp, and an XF19 filter cube (excitation filter = 455DF70; dichroic filter = 505DCLPETO2; and emission filter = 515EFLP; Omega Optical Inc., Brattleboro, Vt.) and a Kodak DC120 zoom digital camera (Eastman Kodak Company, Rochester, N.Y.). KM-resistant clones, which exhibited green fluorescence by microscopic observation, were stored at −70°C in 0.1\% phosphate-buff ered saline (PBS) with sterile 20\% glycerol.

**Culture preparation.** Stock cultures of GFP-\textit{Campylobacter} were resuscitated by streaking on CCA-KM (21) and incubated at 42°C for 48 h in a sealed bag containing of 5\% \textsubscript{O} \textsubscript{2}, 10\% \textsubscript{CO} \textsubscript{2}, and 85\% \textsubscript{N} \textsubscript{2}. Cultures were then transferred at least twice before using. For the attachment assay, a loopful of isolated colonies of GFP-\textit{Campylobacter} on CCA-KM agar was suspended in 2 ml of sterile deionized water (SDW). The optical density of the cell suspension was adjusted to about 0.4 to 0.5 using a spectrophotometer (Spectronic 21D, Milton Roy Co., Rochester, N.Y.) at 540 nm to yield 10\textsuperscript{8} to 10\textsuperscript{9} cells per ml.

**Chicken skin.** Freshly processed broiler carcasses were randomly collected immediately upon their exiting the chill tank in a commercial processing plant. Carcasses were individually bagged and stored in ice no more than 30 min before the breast skins were removed. Breast skins (6 by 6 cm) were removed from the same location on the breasts of broiler carcasses for each assay using a sterile scalpel and forceps. Chicken skin was aseptically stored at −20°C for no more than 1 week before using.

**Attachment assay.** Attachment was determined as described by Kim et al. (11). Frozen chicken skin was thawed by rinsing three times for 3 min each with 15 ml of 4°C SDW on an orbital shaker model no. 3520 (Lab Line Instrument Inc., Melrose Park, Ill.) at 100 rpm. Skin was then blotted dry in a laminar flow hood (SteriGard Hood, The Baker Company, Inc., Sanford, Maine). The outer skin surface was placed toward the inside of a sterile 2.5-cm-diameter bottomless centrifuge tube (Becton Dickinson, Sparks, Md.) and was kept in place with sterile rubber bands. Excess skin was trimmed, and remaining skin was covered with a piece of aluminum foil to prevent contamination and drying. The tube with its bottom covered by skin was placed upright, and a 2-ml suspension of 10\textsuperscript{8} to 10\textsuperscript{9} CFU/ml of GFP-\textit{Campylobacter} was added, thereby exposing the outer surface of skin to the bacteria. Attachment was allowed to take place at room temperature (21°C) while shaking on an orbital shaker at 70 rpm for 1 h. The cell suspension was then decanted, and unattached cells were removed by rinsing three times for 1 min each with 2 ml of SDW on an orbital shaker at 100 rpm. The inoculated skin was retrieved by aseptically cutting it from the 2.5-cm-diameter opening of the bottomless centrifuge tube. A negative control was prepared using the same procedure with 2 ml of SDW instead of the suspension of GFP-\textit{Campylobacter}. After exposure, the skin sample (2.5 cm in diameter, 5 cm\textsuperscript{2}) was placed in a sterile stomacher bag containing 5 ml of PBS and massaged in a stomacher (Stomach 80 Biomaster, Seward, UK) at high speed for 2 min. Aliquots of the skin wash were serially diluted, spread plated in duplicate on CCA-KM, incubated at 42°C for 48 h to enumerate GFP-\textit{Campylobacter} as well as on plate count agar (PCA) at 35°C for 24 h to detect natural microflora on skin. GFP-\textit{Campylobacter} cells that were not rinsed off the skin and that remained in the stomacher wash solution were defined as firmly attached cells. Three pieces of inoculated chicken skin and three controls were analyzed in each of three replications. Data from the chicken skin were not used if the uninoculated control had >500 CFU of total aerobic bacteria in 1 cm\textsuperscript{2}, because levels higher than this interfered with the microscopic observation of the inoculated \textit{C. jejuni}. Presumptive colonies of GFP-\textit{Campylobacter} were confirmed by observing their motility under a phase-contrast microscope (Lobophot, Nikon, Tokyo, Japan) and by a latex agglutination test (INDX-Campy, Integrated Diagnostics, Inc., Baltimore, Md.).

**Storage study.** After attachment, test tubes with chicken skin attached to the bottom were inverted and stored at 25 or 4°C in an ambient atmosphere. Chicken skin stored at 25°C was sampled at 0, 8, 16, and 24 h, and chicken skin stored at 4°C was sampled at 0, 24, 48, 72, and 96 h. The inoculated skin was retrieved by aseptically cutting it from the centrifuge tube around the opening where it was attached. Then, a 2.5-cm-diameter sample of the inoculated skin was placed in a sterile stomacher bag containing 5 ml of the enrichment broth medium BR Tween, which contained 28 g/liter of brucella broth (Acumedia, Baltimore, Md.), 1 g/liter of Tween 80 (Sigma Chemical Co., St. Louis, Mo.), 33 mg/liter of cefoperazone, FBP (0.5 g/liter ferrous sulfate, 0.2 g/liter sodium bisulfate, and 0.5 g/liter pyruvate), and Bolton’s \textit{Campylobacter} selective supplement (Dalynn Biologicals, Calgary, Alberta, Canada), which was used as recommended by the manufacturer. Skin samples with enrichment broth were massaged in a stomacher at high speed for 2 min. Aliquots of the skin wash were serially.
diluted, spread plated in duplicate on CCA-KM, and incubated at 42°C for 48 h to enumerate GFP-Campylobacter. Five milliliters of PBS was placed in a sterile stomacher bag with the uninoculated control, and the natural microflora on the skin were enumerated using PCA incubated at 35°C for 24 h. Three pieces of inoculated chicken skin and three uninoculated controls were analyzed at each sampling time in each replication. Data from chicken skin were not used if the uninoculated control initially had >500 CFU/cm² of total aerobic bacteria. Presumptive colonies of GFP-Campylobacter were confirmed as previously described.

CTC staining. CTC (Polysciences, Warrington, Pa.) was diluted with R2A broth (15, 16) to obtain a final concentration of 5 mM. One hundred fifty microliters of 5 mM CTC was applied to the GFP-Campylobacter-inoculated skin as well as to the control and then incubated in a dark chamber for 30 min at room temperature. CTC-stained skin was then rinsed with 15 ml of SDW three times (1 min each) on an orbital shaker at 100 rpm. A specimen measuring 1 by 1 cm was cut in the middle of the inoculated chicken skin for visual analysis.

Visualization of GFP-Campylobacter on chicken skin. Samples were placed in coverwell imaging chamber gaskets (Molecular Probes, Eugene, Ore.) of 2-mm thickness to prevent compression of the skin during microscopic observation. A drop of 50% glycerol in PBS was placed in the chamber as a mounting medium. The outer surface of the skin was placed upside down on mounting medium and then securely sealed within the chamber by placing a microscope slide against the gasket surface and pressing gently around the edges of the slide. At least 10 microscopic fields were randomly scanned in each specimen. The metabolic active and inactive GFP-Campylobacter cells were determined at different locations and depths under the surface.

Confocal scanning laser microscopy. A TCS NT SP2 Leica Microsystems (Heidelberg GmbH., Germany) confocal microscope equipped with a ×40 (numerical aperture = 1.25; Leica) and a ×100 oil immersion objective (numerical aperture = 1.3; Leica) was used for microscopic observation. GFP and CTC-formazan were excited using an Ar laser (excitation wavelength (λ) = 488 nm). Emitted light was collected through a triple dichroic mirror 488/568/633. The reflected light (wavelength = 483 to 495 nm) was assigned a gray color for chicken skin–reflected light; the emitted light (495 to 540 nm) was assigned a green color for the GFP image; and a 600- to 670-nm emission was assigned a red color for the CTC-formazan image. TCS NT software (version 1.6.551, Leica) and Adobe Photoshop version 6.0 (Adobe Systems Inc., San Jose, Calif.) were used to process images.

Validation and controls. To determine the ability of CTC to discriminate live cells from dead cells, GFP-Campylobacter was killed by overnight exposure to 30% (vol/vol) alcohol. One-milliliter cell suspensions of the untreated cells, the killed cells, and a mixture of the killed and untreated cells were stained with 150...Statistical analysis. All experiments were replicated three times. In experiments that used the enumeration of GFP-Campylobacter on chicken skin by confocal laser scanning microscopy, each replicate involved the observation of three pieces of skin. Data were analyzed with SAS software (Statistical Analysis Systems Institute, Cary, N.C.) using PROC analysis of variance. Significant differences between means were determined by the least significant difference test. Significance was determined using the 95% confidence level (P = 0.05).

RESULTS AND DISCUSSION

Location of Campylobacter on chicken skin. Both live and dead cells in a 48-h culture of GFP-Campylobacter exhibited green fluorescence (Fig. 1A). Miller et al. (14) found that >90% of the cells of this strain exhibited fluorescence after five subcultures. However, the stability of the fluorescence trait is a factor only when the transformants undergo multiple cell divisions, causing the proportion of the nonfluorescent subpopulation to increase with successive generations. Therefore, the effect of fluorescence instability was assumed to be minimal in this study.

The intracellular accumulation of CTC-formazan in viable GFP-Campylobacter was successfully observed as bright red fluorescent cells among the green fluorescent dead cells (Fig. 1B through 1D). Theoretically, viable cells could appear yellow because of an overlap of the green and red channels, but only a small number of yellow cells were observed. Most green fluorescent cells were not detected at the same location as red cells, an indication that CTC interfered with the detection of GFP. GFP-Campylobacter was detected in this study at 495 to 540 nm using a 488-nm Ar-Kr laser excitation. Severin et al. (17) showed that CTC-formazan exhibits broad excitation, from <400 to >550 nm, with an emission maximum at 630 nm (17). Therefore, emission by GFP-Campylobacter cells may be negated by CTC-formazan when both are in the same cell (either by the direct absorption of photons, emitted by GFP-Campylobacter, or by Förster energy transfer) (24). It is also possible that the green fluorescence of GFP-Campylobacter is degraded as a result of CTC-induced cell death. Because of this fluorescence exclusion phenomenon, total Campylobacter numbers were calculated by adding the number of CTC-stained cells (presumed to be viable) to the number of green fluorescent cells (presumed to be nonviable). This calculation was valid only when the background microflora were sufficiently low (we used only skin with <500 CFU/cm² total counts), since CTC staining is nonselective. After the rinsing procedure, 10⁸ to 10⁹ CFU/cm² of C. jejuni remained on chicken breast skin with no significant difference (P > 0.05) among replications (data not shown). Green fluorescent C. jejuni cells and CTC-formazan in viable Campylobacter were clearly visible on the chicken skin by confocal scanning laser microscopy. GFP-Campylobacter cells adhering to the skin were located primarily on rough areas of the chicken skin: in crevices or entrapped inside deep channels and feather follicles with water. Statistically significant differences (P < 0.05) were observed among the average of total cells, live cells (able to reduce CTC), and percent live cells at different locations.
FIGURE 1. Representative confocal scanning laser microscope images of live and dead GFP-producing C. jejuni. (A) Cells of GFP C. jejuni without 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) staining in which green color indicates both dead and live cells. (B) Cells of GFP C. jejuni stained with CTC in which red color indicates live cells (CTC reduction) and green color indicates dead cells. (C) Mixture of live GFP C. jejuni and dead GFP C. jejuni with CTC staining. (D) Dead GFP C. jejuni, killed by suspension in 30% alcohol overnight, stained with CTC. Scale bars represent 5 μm.

(Table 1). A greater amount of total Campylobacter cells (live plus dead) were located at the 0- to 10-μm depth in feather follicles (42 cells per microscopic field) than on the skin surface (28 cells per microscopic field) or at the top (0 to 10 μm) of channels (20 cells per microscopic field). There were no significant differences between the mean numbers of live cells or percentage of live cells at depths of 0 to 10 μm in feather follicles or channels and on the skin surface. However, at depths of 30 to 50 μm below the surface, the average of the total cells in feather follicles (14 cells per microscopic field) was significantly greater than that in channels at the same depth (3.4 cells per microscopic field) but was less than those found at depths of 0 to 10 μm. GFP-Campylobacter cells observed 30 to 50 μm deep in feather follicles had a greater chance of survival than those present on the skin surface or in the upper portion (0 to 10 μm) of the follicles (Table 1).

Kim et al. (11) observed Salmonella at locations on chicken skin similar to where we observed Campylobacter. They also observed Salmonella entrapped with water inside

**TABLE 1. Number of viable and total GFP-Campylobacter cells and percentage of viable cells at different locations on chicken skin as determined by direct microscopic observation**

<table>
<thead>
<tr>
<th>Type of cell</th>
<th>Surface (8.4–2.4 μm)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Crevice (17 ± 2.7 μm)</th>
<th>Crevise (49 ± 17 μm) 0–10 μm&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Crevise (49 ± 17 μm) 30–50 μm</th>
<th>Feather follicle (42 ± 23 μm) 0–10 μm</th>
<th>Feather follicle (42 ± 23 μm) 30–50 μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Live&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.3 AB&lt;sup&gt;e&lt;/sup&gt;</td>
<td>4.2.1 AB</td>
<td>4.1 AB</td>
<td>0.3 C</td>
<td>5.7 A</td>
<td>2.4 BC</td>
</tr>
<tr>
<td>Total</td>
<td>28 B</td>
<td>16 C</td>
<td>20 BC</td>
<td>3.4 D</td>
<td>42 A</td>
<td>14 C</td>
</tr>
<tr>
<td>% live&lt;sup&gt;f&lt;/sup&gt;</td>
<td>16 B</td>
<td>27 AB</td>
<td>25 AB</td>
<td>15 B</td>
<td>14 B</td>
<td>35 A</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values are the mean and standard deviation of three replications. Number of GFP-Campylobacter cells was determined per microscopic field (areas of 6.25 × 10<sup>2</sup> μm<sup>2</sup>) at the specified locations.

<sup>b</sup> Measurements are the distance and the mean and standard deviation of the distance from the uppermost position of the specimen calculated from three replications.

<sup>c</sup> Distance from the channel surface or feather follicle opening.

<sup>d</sup> Live cells represent cells able to reduce CTC; total cells are the total of green fluorescent cells and cells able to reduce CTC.

<sup>e</sup> Different letters in the same row indicate significant differences (P < 0.05) using the least significant difference test.

<sup>f</sup> % live cells = (numbers of live cells)/(number of total cells) × 100.
feather follicles at various depths. We were able to make a similar observation with Campylobacter, with the added feature of being able to differentiate between live and dead cells at these locations (Fig. 2). Kim et al. (9, 10) concluded that the physical structure of chicken skin plays a significant role in Salmonella attachment that was not influenced by viability or cell surface chemistry.

Water uptake by the poultry carcass during cleaning and immersion chilling may be a significant cause of microbial contamination (23). The microtopography of the tissue is altered by the swelling and exposure of deep channels and crevices. The water in these capillary-sized spaces is difficult to remove by conventional cleaning practices and can entrap bacteria, providing them an opportunity to penetrate the feather follicles. Skin swelling may not only trap bacteria already located in channels and crevices, rendering them less accessible to physical removal, but may also provide additional skin surface areas for contamination (23).

Greater total numbers of GFP-Campylobacter were observed at the initial 0 to 10 μm of the skin surface than at deeper sites (Table 1). However, the percentage of live cells in this location was generally lower than at deeper locations, where fewer total Campylobacter cells were observed. At the very bottom of most feather follicles and deep channels, we found both active and inactive cells (Fig. 2), indicating that these sites provide a suitable microenvironment for the survival of Campylobacter.

The average number of total aerobic bacteria (background microflora) on the uninoculated fresh chicken skin was 2.53 log CFU/cm² as determined by a plate count and 3.11 log CFU/cm² as determined by a direct cell count of CTC-stained samples (Table 2). Chicken skin containing...
>500 CFU (2.69 log/cm²) by a total aerobic plate count was not used in this experiment because the background microflora could not be distinguished from the CTC-stained Campylobacter. CTC-stained cells on uninoculated samples were initially observed primarily on the surface (0- to 10-μm depth); however, after storage at 25°C for 8 h and at 4°C for 48 h, they were also found at greater depths (20 to 30 μm). Plate counts indicated that total amounts of aerobic bacteria on chicken skin were as high as 7.29 log CFU/cm² after storage at 25°C for 16 h, but viable cells in these samples were too numerous to count via the direct microscopic method. The number of total aerobic bacteria increased by about 1 log CFU/cm² (plate count) and by 1.5 log CFU/cm² (direct microscopic count [DMC]) after storage at 4°C for 96 h. Most of these bacteria were located at the upper portion of the skin surface.

The average number of GFP-Campylobacter cells on chicken skin immediately after inoculation (0 h, Fig. 3) was 6.01 log CFU/cm², as determined by plating on CCA-KM, and 5.79 log cells per cm² of viable Campylobacter, as determined by DMC (this number was obtained by subtracting the DMC result for uninoculated samples shown in Table 2 from the inoculated DMC result). The average number of live cells determined by DMC was lower (P < 0.05) than that enumerated on CCA-KM, possibly because some labeled cells were missed due to a low amount of CTF deposition (16). It is also possible that sublethally injured cells were not able to reduce CTC. Data on the number of live GFP-Campylobacter and the total number of GFP-Campylobacter observed by direct microscopic observation could be obtained only up to 8 h for samples that were stored at 25°C and only up to 72 h for samples that
FIGURE 3. Survival of GFP-producing Campylobacter jejuni on the chicken skin during storage at 25 and 4°C. (A) Total number of cells per cm²; (B) number at 0 to 10 µm under the surface; and (C) number at 20 to 30 µm under the surface. Total (■), viable (▲), and inactive (○) C. jejuni were obtained by direct microscopic observation, and the total number of C. jejuni CFU (◇) was determined by plating on CCA-KM. This experiment was replicated three times, and mean numbers are presented. Statistical analysis indicated no significant changes in the direct microscopic count data over time (P = 0.05).

were stored at 4°C, after which time the natural microflora overgrew the inoculated Campylobacter (Fig. 3A).

Survival of Campylobacter on skin during storage. Campylobacter on chicken skin (as determined by plating on CCA-KM) decreased 1 log CFU/cm² (P > 0.05) during a 24-h incubation at 25°C (Fig. 3A). The total number of active GFP-Campylobacter on chicken skin and at the surface (25°C) as determined by direct viable counting decreased to a similar extent. The populations of active GFP-Campylobacter on the skin at 20- to 30-µm depths did not decrease during incubation at 25°C, indicating survival at these deeper attachment sites (Fig. 3C). Total numbers (in CFU) of C. jejuni on chicken skin stored at 4°C (Fig. 3A) remained relatively constant during the 72-h incubation. DMC data indicated that viable cells of GFP-C. jejuni maintained populations both on the surface and at 20- to 30-µm depths after an initial die-off during the first 24 h of incubation. Lee et al. (12) reported that C. jejuni was able to persist at high numbers or grow under normal packaging conditions on chicken skin stored at 4°C for 7 days and at ambient room temperatures for 3 days.

Kim et al. (9, 10) found that the attachment of Salmonella to poultry skin was primarily influenced by skin physical structure rather than by cell viability or cell surface structure. Our data demonstrate that Campylobacter maintains a population of live and inactive cells deep in skin openings over time. However, the majority of both live and inactive cells remained on the surface (0- to 10-µm depth) during storage at 4 and 25°C. The viability of GFP-Campylobacter at 20 to 30 µm stored at 25°C for 24 h (Fig. 3C) could not be determined because of the overgrowth of competing microflora, which interfered with direct microscopic observations; therefore, the 16- and 24-h microscopic counts are reported as inactive cells, even though the numbers increased. Data from this study support the view that live and dead campylobacters are initially retained with
Broiler carcass contamination with *Campylobacter* from feces during defeathering. *J. Food Prot.* 64:2063–2066.


