J. Microbiol. Biotechnol. (2007), **17**(2), 202–206

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Morphology and Adhesion of *Campylobacter jejuni* to Chicken Skin Under Varying Conditions

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Received: June 21, 2006
Accepted: September 2, 2006

Abstract The adhesion of *Campylobacter jejuni* to chicken skin, along with the associated morphological changes under aerobic conditions at 4, 25, and 37°C and microaerobic (O_2 5%, CO_2 10%, N_2 85%) conditions, were investigated using confocal laser scanning microscopy (CLSM), flow cytometry, and plate counting. The morphological change of *C. jejuni* from a spiral shape to a coccoid form or VBNC form (viable but nonculturable form) progressed rapidly under aerobic conditions at 25, 37, and 4°C. As regards adhesion, the *C. jejuni* cells were mostly located in the crevices and feather follicles of the chicken skin, where the cells in the feather follicles floated freely in the entrapped water, even after the skin was rinsed quite thoroughly. CLSM also revealed the penetration of some spiral-shaped *C. jejuni* cells into the chicken skin. Even after changing their shape at various temperatures, coccoid-form *C. jejuni* cells were still found in the crevices and feather follicles of the chicken skin.

Key words: Adhesion, *Campylobacter jejuni*, confocal microscopy, flow cytometry, morphological change, temperature

Bacterial food poisoning can be caused by bacteria multiplying in food during the distribution and storage process. *Campylobacter* spp. are a common and more frequent cause of food poisoning than salmonellosis, even in Western countries that have advanced hygiene standards. In the United States, 49% of all bacterial food poisoning is caused by *Campylobacter* spp. [14], and about 90% of *Campylobacter* spp. have been identified as *Campylobacter jejuni* [2].

Campylobacter spp. usually have a slim spiral or curved form, yet become coccoid under certain conditions, such as during prolonged culture or with temperature changes. These bacteria are Gram-negative, microaerobic, and kinetic, causing miscarriages and sterility in animals and diarrhea via intestinal tract inflammation in humans. The identified species include *C. jejuni*, *C. fetus*, *C. coli*, *C. sputorum*, and *C. concisus*, where *C. jejuni* is the major cause of bacterial diarrhea [17, 19].

Campylobacter spp. and *Salmonella* spp. are discharged with excreta, and ingested indirectly by drinking contaminated water or milk or by eating contaminated meat, such as chicken [8]. The contamination of chicken meat by *Campylobacter* spp. in slaughterhouses can occur via chicken excretion, chicken meat, cooling water, and the knives used to remove the viscera [16]. In several previous cases, the cooling water was identified as the major source of contamination [12, 16], and the infection rate by *C. jejuni* was 34.2%. Reports also exist of contamination by food-processing workers [16].

Under aerobic conditions, the viability of *Campylobacter* spp. is most stable at 4°C, rather than at 42°C, which is the optimal growth temperature. When *Campylobacter* spp. are exposed to aerobic conditions, the spiral forms of *C. jejuni* change to coccid forms, with only a few *C. jejuni* remaining in a spiral form [11], whereas 60–80% of the cells have been found to multiply under optimal microaerobic conditions (O_2 5%, CO_2 10%, N_2 85%) [18]. Furthermore, when the viable but nonculturable (VBNC) coccoid form of *C. jejuni* develops under aerobic conditions at 25 or

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37°C, and is subsequently maintained under microaerobic conditions, it can still revert to the spiral form and multiply [1]. When chickens [20] and young mice [7] ingested the VBNC form of *C. jejuni*, spiral *C. jejuni* were detected in their excretions. Moreover, Hazeleger *et al.* [6] examined VBNC *C. jejuni* and reported that the fatty acids formed were the same as those produced by the coccoid form under aerobic conditions at 25 and 37°C, yet also similar to those produced by the spiral form at 4°C.

Accordingly, this study examined the morphological changes of *C. jejuni* under aerobic conditions at different temperatures, along with the contamination of chicken skin by VBNC *C. jejuni* at various temperatures in order to develop ways of preventing poultry contamination by bacteria such as *Campylobacter* spp. The adhesion of the VBNC form of *Campylobacter* spp. to chicken skin and the exact sites of contamination were also examined at various temperatures.

MATERIALS AND METHODS

Bacterial Strain and Chicken

Campylobacter jejuni W1 was obtained from the United States Department of Agriculture (USDA). A Brucella broth (Difco, Detroit, MI, U.S.A.) containing an FBP mixture was placed in a 3.5-l anaerobic jar (Difco) holding a *Campylobacter* Microaerophilic System (Difco), including 5% oxygen and 10% carbon dioxide, and the bacteria cultured at 42°C for 48 h. The cells were then spread on an FBP-Brucella agar and stored at 4°C under microaerobic conditions. The cells were recultured every seventh day. The chicken meat used in the study was bought at a local market.

Medium

The FBP-Brucella broth was made by adding 0.9 mM ferrous sulfate (Sigma, St. Louis, MO, U.S.A.), 1.3 mM sodium metabisulfite (Sigma), and 2.3 mM sodium pyruvate (Sigma) to the Brucella broth. The broth was then autoclaved at 121°C for 15 min, cooled to 60°C, and 10 mg vancomycin, 5 mg trimethoprim lactate, 20,000 IU polymyxin B, and 50 mg cycloheximide (all from Sigma) were added per liter. For the liquid medium, 3% bovine calf serum (Hyclone, Logan, UT, U.S.A.) was added to the FBP-Brucella broth, whereas for the solid medium, 10% defibrinated sheep blood (Komed, Seoul, Korea) was added to the FBP-Brucella broth containing 2% agar [10, 18].

Viability and Morphology of *C. jejuni* at Different Temperatures Under Aerobic Conditions

The *Campylobacter jejuni* was cultured under microaerobic conditions (O_2 5%, CO_2 10%, N_2 85%) for 24 h at 42°C, and then incubated under aerobic conditions at 4, 25, and 37°C. The changes in the *C. jejuni* cells at each temperature were investigated using the plate count method until the bacteria stopped multiplying. To observe the changes in morphology, the solution used to culture the *C. jejuni* for 24 h was centrifuged for 4 min at 6,067 ×*g* at 4°C (VS-15000CFN; Vision, Kyunggi-do, Korea) and homogenized with a sterilized distilled solution. As a control, a similar solution without *C. jejuni* was used. The side scatter (SSC) and forward scatter (FSC) were then determined using flow cytometry (Ar ion laser, FACSCalibur; Becton Dickinson, San Diego, CA, U.S.A.) and compared directly. In addition, the *C. jejuni* cells were stained with 0.03% fluorescein isothiocyanate (FITC; Sigma) for 10 min, washed three times with sterilized distilled water, and optical sectioning used to observe the morphological changes based on confocal laser scanning microscopy (CLSM; MRC-1024 Kr/Ar ion laser; Bio-Rad, Oxford, U.K.).

Analysis of Chicken Skin Contaminated with *C. jejuni*

Campylobacter jejuni that had been cultured to 10^6 CFU ml^{-1} under microaerobic conditions, and *C. jejuni* that had been transformed into the coccoid form under aerobic conditions at 4, 25, and 37°C, were centrifuged for 4 min at 6,067 ×*g* at 4°C, stained with 0.03% FITC for 10 min, and recentrifuged three times with sterilized distilled water. A 1×1-cm^2 piece of skin was then cut from the chicken breast using a sterilized knife and pair of tweezers coated with Teflon. The skin was stained with 0.1% pyronin Y (Sigma) for 10 min, immersed in sterilized distilled water for 1 min three times, and air-dried on a slide glass. The stained skin was then inoculated with *C. jejuni* cultured at each temperature, stained with FITC, and fixed for 15 min [15]. Thereafter, the skin was washed with sterilized distilled water using a 5-ml pipette, and covered with a cover glass that was attached with adhesive tape. Finally, optical and vertical sectioning were used to evaluate the degree of contamination based on CLSM.

RESULTS

Morphological Changes of *C. jejuni* with Temperature Under Aerobic Conditions

Under aerobic conditions, the multiplication of *C. jejuni* cells stopped earlier in the temperature order of 25, 37, and 4°C (Fig. 1), which was consistent with the previously reported prolonged viability of *C. jejuni* in the spiral form at 4°C [8, 21]. The flow cytometry and CLSM also confirmed the transformation of *C. jejuni* from the spiral form to the coccoid form (VBNC, viable but nonculturable form) under aerobic conditions. In the flow cytometry histogram (Fig. 2), the FSC value represents the overall size, and the SSC value indicates the granularity of the

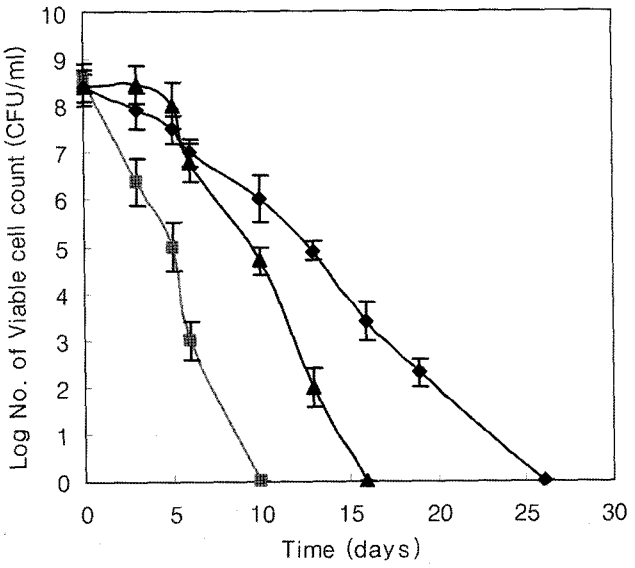

Fig. 1. Viability of *Campylobacter jejuni* under aerobic conditions at 4 (◆), 25 (■), and 37°C (▲).

cells. The initial *C. jejuni* cells were scattered in two spots, representing the coccoid and spiral forms. However, at 25 and 37°C, the *C. jejuni* cells were only concentrated in one spot, representing the coccoid form and VBNC state, demonstrating that *Campylobacter* spp. transform into cocci when they enter the VBNC state. Fig. 3 shows the changed shapes of *C. jejuni* at the different temperatures.

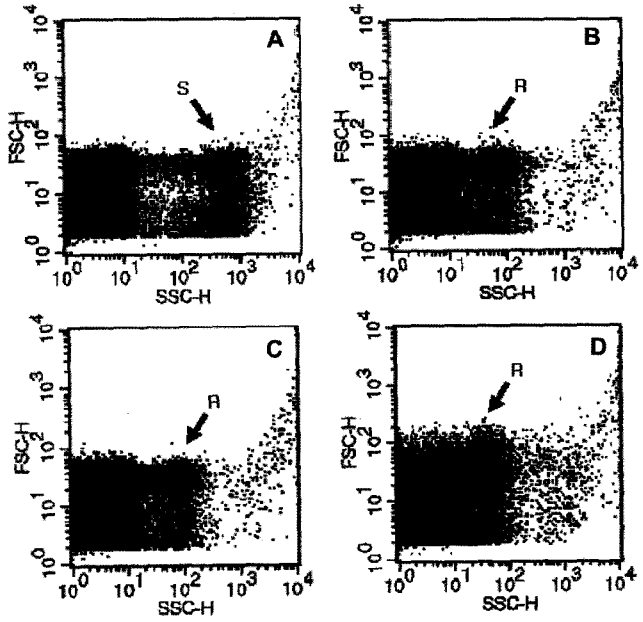

Fig. 2. Flow cytometry histograms showing morphological changes of *Campylobacter jejuni* under aerobic conditions at 4, 25, and 37°C: (**A**) initial *C. jejuni* and viable but nonculturable (VBNC) *C. jejuni* at (**B**) 4, (**C**) 25, and (**D**) 37°C after 10, 16, and 26 days, respectively.
S, Spiral form; R, Rod form; FSC (forward scatter): light scattered when particles pass in front of laser beam (0.5–10°), size, and refractive index; SSC (side scatter): light scattered by particles approximately 90° from incident beam, internal complexity (granularity).

Attachment Sites of *C. jejuni* on Chicken Skin

For the chicken skin contaminated with *C. jejuni*, the spiral and coccoid forms of *C. jejuni* were both found at 20–30 mm inside the crevices and feather follicles on the skin, rather than on the surface. The *Campylobacter jejuni* in the feather follicles floated freely in the surrounding liquid, even after the skin was rinsed (Fig. 4A). In addition, some of the spiral form were observed to penetrate the skin tissue (Fig. 4B).

Analysis of Chicken Skin Contamination by VBNC Form of *C. jejuni*

When the chicken skin was inoculated with a suspension of VBNC *C. jejuni* (*i.e.*, bacteria that did not multiply in a spread culture) and observed using CLSM, coccoid *C. jejuni* were observed in the crevices and feather follicles on the skin surface (Fig. 5). For the *C. jejuni* transformed into the coccoid VBNC form at 25 and 37°C, many *Campylobacter* cells were attached to the contaminated chicken skin, whereas only a few *Campylobacter* cells incubated at 4°C were found to be attached to the chicken skin. Therefore, this suggests that when *C. jejuni* is rapidly transformed into the VBNC form at 25 and 37°C, it still

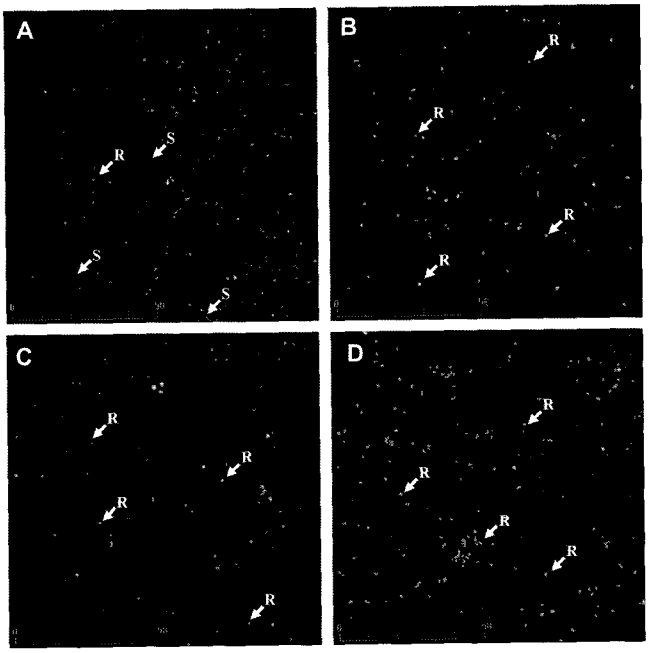

Fig. 3. CLSM images showing morphological changes of *Campylobacter jejuni* under aerobic conditions at 4, 25, and 37°C: (**A**) initial *C. jejuni* and viable but nonculturable (VBNC) *C. jejuni* at (**B**) 4, (**C**) 25, and (**D**) 37°C after 10, 16, and 26 days, respectively.
S, Spiral form; R, Rod form.

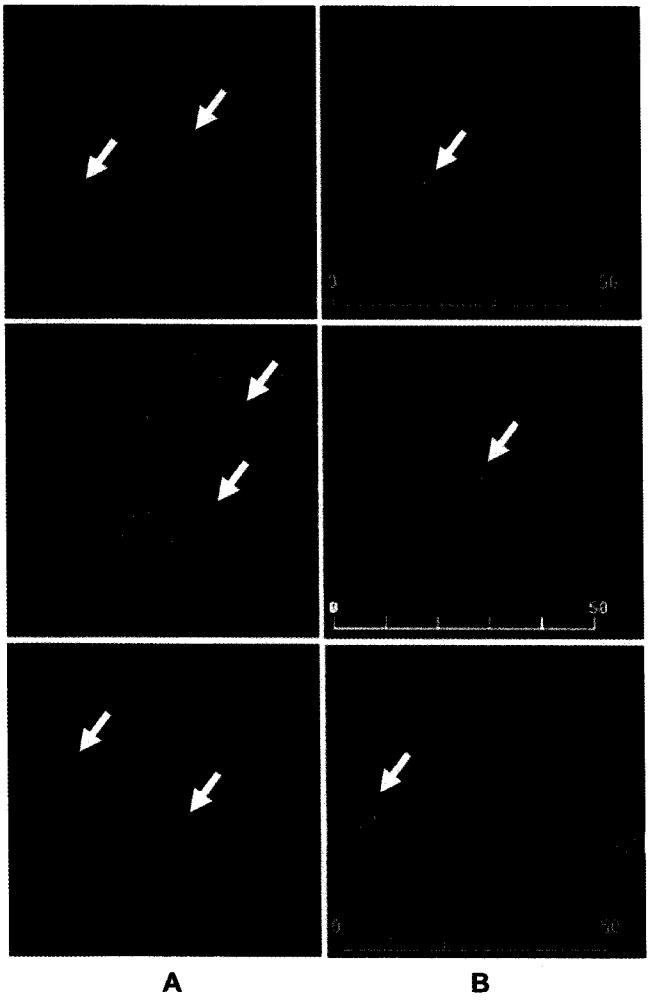

Fig. 4. CLSM images of *Campylobacter jejuni* (arrows) attached to chicken breast skin: (**A**) optical sectioning images and (**B**) vertical sectioning images.

retains its ability to adhere to skin. In contrast, when *C. jejuni* is incubated at 4°C, while its viability is maintained for a longer time, its adhesive ability is decreased.

DISCUSSION

Generally, *C. jejuni* becomes less viable under aerobic conditions. Nonetheless, the VBNC form of *C. jejuni* still poses a major risk, as Bovill and Mackey [1] reported that even when *C. jejuni* stopped multiplying under aerobic conditions at 25 and 37°C, it resumed multiplying when kept under microaerobic conditions, plus chicks [20] and young mice [7] that ingested the coccoid form of *C. jejuni* excreted the spiral form. Furthermore, based on the reports of Kim *et al.* [9] who located *Salmonella* spp. in the crevices and feather follicles on the skin, and Chung *et al.* [3] who noted the absence of competitive interaction in

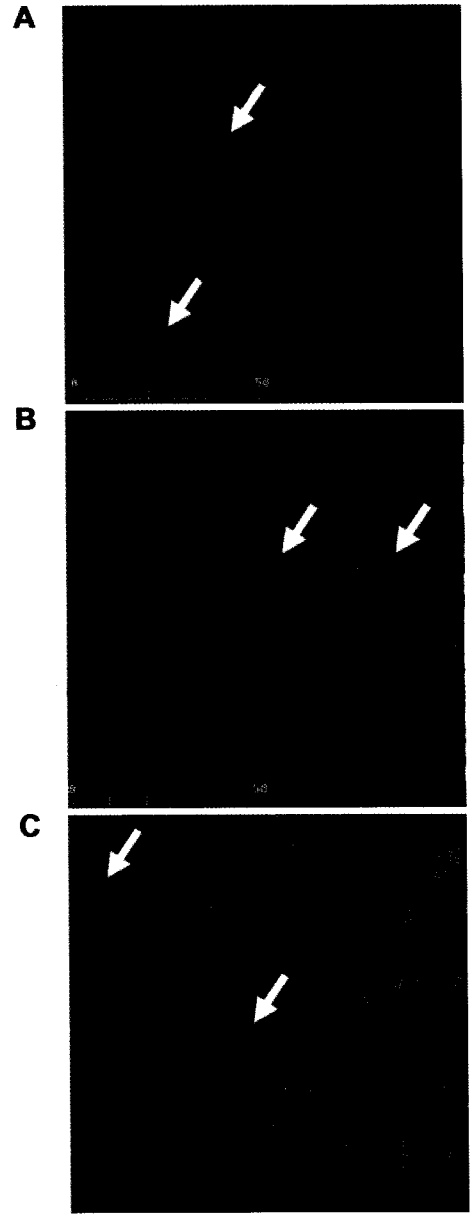

Fig. 5. CLSM images of *Campylobacter jejuni* (arrow) attached to chicken breast skin after culturing under aerobic conditions at (**A**) 4, (**B**) 25, and (**C**) 37°C.

lean or fat tissue on chicken skin contaminated with food-poisoning bacteria, it is likely that *Salmonella* spp. and *Campylobacter* spp. can coexist in contaminated chicken skin. The attachment of food-poisoning bacteria is believed to depend on both the characteristics of the bacteria and the substrate [4]. Thomas and McMeekin [22] demonstrated that bacteria attach to chicken muscle when the tissue absorbs moisture, while Lillard [13] stated that bacteria attach through their flagellar movement, and Dickson and Koohmaraie [5] reported that bacteria attach by utilizing the negative charges on the bacterial cell surface. Thus, it would seem to be more difficult to eliminate *C. jejuni*

contamination of chicken, as the bacteria attach in a different manner from other species of food-poisoning bacteria. However, the VBNC form of *C. jejuni* was demonstrated to attach to chicken skin under aerobic conditions at 4, 25, and 37°C. Consequently, the ability of VBNC *Campylobacter* spp. to adhere to the human gastrointestinal tract under microaerobic conditions is another crucial reason for preventing poultry contamination by bacteria such as *Campylobacter* spp.

Acknowledgments

This work was supported by a project (03-PJ1-PG1-CH11-0003) of the Health Technology Planning and Evaluation Board, Korean Ministry of Health and Welfare and by the Research Center for Bioresource and Health, ITEP.

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