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Expression of Flagellin Proteins of *Campylobacter jejuni* within Microaerobic and Aerobic Exposures

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Abstract *Campylobacter*, one of the emerging foodborne pathogens, is highly adaptable to the external environments by changing its morphology. In the present study, a question of whether the whole-cell antibody would still be effective for its detection even though the morphology of *C. jejuni* was changed was examined. When microaerophilic *C. jejuni* was exposed to aerobic conditions for 48 h, its morphological change was detected by confocal laser scanning microscope: Its morphology was confirmed as a spiral-bacilli form in microaerobic condition, however, as a coccoid form with a little spiral-bacilli form, when exposed to aerobic conditions. Also, the expressions of the whole-cell proteins of *C. jejuni*, and the suppression or induction of newly synthesized proteins in both aerobic and microaerobic conditions were analyzed by two dimensional gel electrophoresis. Additionally, immunoblotting assay with the whole cell antibody for the proteins expressed under the two conditions was performed. It was confirmed that the commercial whole-cell antibody of *C. jejuni* raised in rabbit was reactive. When analyzed with MALDI-TOF MS, the expressed proteins were confirmed as flagellins. Therefore, even though the morphology changed in aerobic condition, these flagellins were expressed and worked as the eitope proteins, thus making it possible to utilize for the development of an immunosensor for real-time detection of any kind of *C. jejuni* cell.

Key words: *C. jejuni*, aerobic stress, morphology, flagellin

Campylobacteriosis is increasing worldwide, therefore, *Campylobacter* is one of the interesting foodborne pathogens [9, 10, 23, 29]. *Campylobacter* has characteristics of being microaerophilic and thermotolerant. The size of its genome is 1.6–1.7 Mbp with 30% GC ratio and encodes about 1,654 proteins [18, 20, 22]. It has generally been known that it induces food poisoning with a low infective dose of 20–500 cells, which is different from other foodborne pathogens. In cases of contamination of foodborne pathogens in food, it is always affected by stresses such as low pH, acidity, heat, cold, dryness, low water activity, chemical preservatives, antibiotics, and microbial competition [1, 5]. However, such stresses induce the growth of new genotypes against these stresses and create the pathogens with adaptable modification. As a result, it can survive in such environments, and its virulence is increased with a low infective dose and hazard is also increased [2, 11, 30]. When the foodborne pathogen is stressed, a complicated protective mechanism is induced: If it adapts to one stress, it will acquire cross-protection resistant to another stress [32]. Therefore, the contaminated food is highly hazardous due to increment of virulence and stress-adapted and multidrug resistant mutant. Consequently, the foodborne pathogen of adaptive modification is difficult to control. There are some concerns on countermeasure about these changes that the spiral-bacilli form of *Campylobacter* was changed by external stress, and that morphology of viable but nonculturable (VBNC) cell was changed into coccoid form by external stress [7, 21, 27]. Furthermore, among many foodborne pathogens, *Campylobacter*, *Salmonella*, *E. coli*, *Listeria monocytogens*, *Vibrio parahaemolyticus*, *H. pylori*, and *Enterococcus faecalis* have characteristics changed to VBNC cell by external stress [12, 16, 17, 28]. Therefore, foodborne pathogens in food exist in the sublethal condition or is changed by environmental stress due to deficiency of oxygen and nutrition. In this study, when

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C. jejuni was affected by external stress, it was changed into the coccoid form, which madd the conventional detection difficult. However, the change of morphology was confirmed with a confocal laser scanning microscope, and whole-cell proteins were compared through two-dimensional gel electrophoresis and continually expressed proteins were analyzed by MALDI-TOF MS. The results described are expected to contribute to the development of real-time detection by means of an immunosensor for any kind of cell with immunofluorescence.

MATERIALS AND METHODS

Strain and Culture Conditions

Strain for the experiment was *C. jejuni* ATCC43429. *C. jejuni* incubated in Brucella blood agar has been inoculated into FBP-Brucella broth [supplemented with Brucella broth: 0.9 mM ferrous sulfate, 1.3 mM sodium metabisulfite, 2.3 mM sodium pyruvate] (Sigma, St. Louis, U.S.A.) with the additional 3% bovine calf serum (Hyclone, Utah, U.S.A.) and incubated in a microaerobic chamber (Bug box, Ruskin Tech. Co., U.K.) for 48 h at 37°C [25].

Morphological Change in Aerobic Conditions

C. jejuni was incubated in FBP-Brucella broth with additional 3% bovine calf serum in microaerobic condition for 48 h at 37°C or aerobic conditions for 48 h at 37°C, following microaerobic preincubation. The cells from 1ml of culture solution were harvested, washed twice with PBS (pH 7.0), and resuspended in PBS. It was stained with 0.025% acridine orange, and then morphology was confirmed on the slide glass by confocal laser scanning microscope (OLYMPUS 41, NY, U.S.A.) [19, 24].

Two-Dimensional Gel Electrophoresis [6]

Campylobacter was incubated in a microaerobic chamber for two days at 37°C and then reincubated for 2 days in aerobic condition. After centrifugation of the culture solution for 5 min at 10,000 rpm and 4°C, the cell pellet was washed twice with PBS (pH 7.0). The pellet was resuspended in the sample buffer [Tris·HCl 100 mM (pH 7.0), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM EDTA] and the cell suspension sonicated (pulse on 1 sec, pulse off 1 sec) six times in an ice box. After the lysis buffer [2 M thiourea, 7 M urea, 1 mM PMSF, 1 mM EDTA, 4% {3-[(3-cholamidopropyl)-dimethylammonio] propane sulfonate} (CHAPS), 100 mM dithiothreitol (DTT), 0.5% of immobilized pH gradient (IPG) buffer, pH3.0–pH10] was added, this solution was vigorously agitated for 20 min at room temperature, and the mixture was finally centrifuged (20 min at 15,000 rpm and 4°C). After the supernatant was treated with acetone, precipitated proteins were quantified by the Bradford method. Proteins were added into the rehydration buffer [8 M urea, 1 mM PMSF, 1mM EDTA, 2% CHAPS, 0.5% IPG buffer, 10 mM DTT], and its mixture was applied on to the immobilized pH gradient (pH 3–10 Immobilin dry strip 13 cmIPG) strips with the Ettan IPGphor™ (Amersham Bioscience, NJ, U.S.A.): the following conditions were employed for the 1st dimension analysis: instrument temperature, 20°C; maximum 0.05 mA/strip; rehydration step 1 at 30 V for 12 h; step 2 at 150 V for 1 h; step 3 at 300 V for 1 h; step 4 to 600 V for 4 h; step 5 at 5,000 V for 14 h. The second-dimensional SDS-PAGE was done, using 12.5% separating polyacrylamide gel without any stacking gel. IPG strips were equilibrated prior to electrophoresis at room temperature for 15 min in SDS equilibration buffer [10 ml; 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 0.0025% bromophenol blue]. IPG strips were then sealed on top of SDS-PAGE gels with agarose and run at 10°C by using the SDS electrophoresis buffer. Following the completion of SDS-PAGE, the gels were visualized by silver staining, and image analysis using a melanie 4 viewer (ExPASy Molecular Biology Server).

Immunoblotting [4]

Primary antibody used for immunoblotting was rabbit anti-*Campylobacter jejuni* (Fitzgerald, MA, U.S.A.), and whole specificity was the whole cells of *C. jejuni*. 2D polyacrylamide gels were electrophoretically transferred from gels to polyvinylidene fluoride membrane (PVDF). Transfer was performed for 1 h at 12 V. PVDF were blocked with 5% skim milk in TBS (0.02 M Tris base, 0.14 M NaCl, 2.7 mM KCl, pH adjusted to 7.4) and were incubated overnight. After incubation for 2 h with primary antibody, PVDF was washed three times for 5 min with TBST containing 0.05% Tween 20. PVDF were then incubated for 1 h with anti-rabbit IgG conjugated to HRP (Zymed, SA, U.S.A.). The membrane was washed three times for 5 min with TBST, and reaction products were visualized on autoradiographic film by chemiluminescence, using the ECL immunoblotting detection system (Amersham Bioscience, NJ, U.S.A.).

MALDI-TOF MS [26]

The selected protein spots were cut from the gel and they were then destained with 100 µl of destaining solution (30 mM potassium ferricyanide, 100 mM sodium thiosulfate) with shaking for 5 min. After removal of solution, the gel spots were incubated with 200 mM ammonium bicarbonate for 20 min. The gel pieces were dried in a speed vacuum concentrator for 5 min. The pieces were rehydrated with 20 µl of 50 mM ammonium bicarbonate, containing 0.2 µg modified trypsin, for 30 min on ice. After removal of the solution, 30 µl of 50 mM ammonium bicarbonate was added, and the sample was digested overnight at 37°C. After removal of residual trypsin, peptides were desalted, using a C18 nanoscale column: The peptides were eluted with 0.8 µl of matrix solution (70% acetonitrile, 0.1%

TFA, 10 mg/ml α-cyano-4-hydroxycinnamic acid). The eluted peptides were spotted onto a stainless steel target plate. Masses of the peptides were determined by using MALDI-TOF mass spectrometery (Micromass, MA, U.S.A.). Calibration was carried out by using the internal mass of trypsin autodigestion product (m/z 2211.105). Peptide masses were matched with the theoretical peptides of all human proteins in the NCBI database, using Mascot software and Profound software.

RESULTS AND DISCUSSION

Morphological Change *of Campylobacter jejuni* under Aerobic Exposure

Campylobacter jejuni was exposed to aerobic conditions for 48 h, and morphological changes were analyzed, using a confocal laser scanning microscope (Fig. 1). It has been reported that motile spiral-bacilli form of *Campylobacter* is converted to the nonmotile coccoid form (VBNC cell) by external environmental stresses. When *C. jejuni* was in aerobic condition and microaerobic condition, the typical spiral-bacilli form of *Campylobacter* was shown, however only some cells were in the spiral-bacilli form under aerobic condition, and most of them were changed into the coccoid form by oxidative stress from high atmospheric oxygen content.

Analysis of the Expressed Proteins by Two-Dimensional Gel Electrophoresis

Following the exposure of *C. jejuni* to aerobic condition for 48 h at 37°C, the whole-cell protein were analyzed by two-dimensional gel electrophoresis (Fig. 2). Morphological change of *C. jejuni* under aerobic conditions indicated that most of them were changed into the coccoid form (Fig. 1). When the proteins of *C. jejuni* at aerobic exposure were compared with microaerobic exposure, there seemed to be many changes such as continuous expression, and suppression

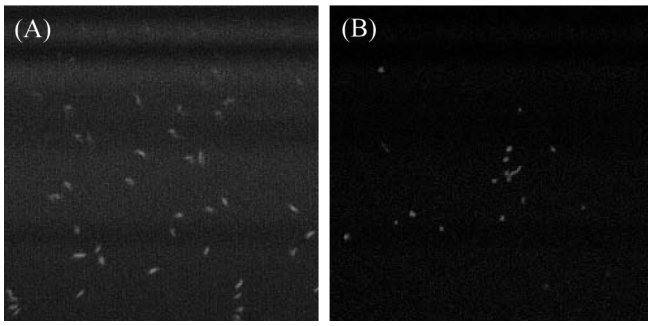

Fig. 1. Different morphologies of *C. jejuni* in aerobic and microaerobic exposures.
(A) Microaerobic condition, (B) Aerobic condition following microaerobic condition preincubation.

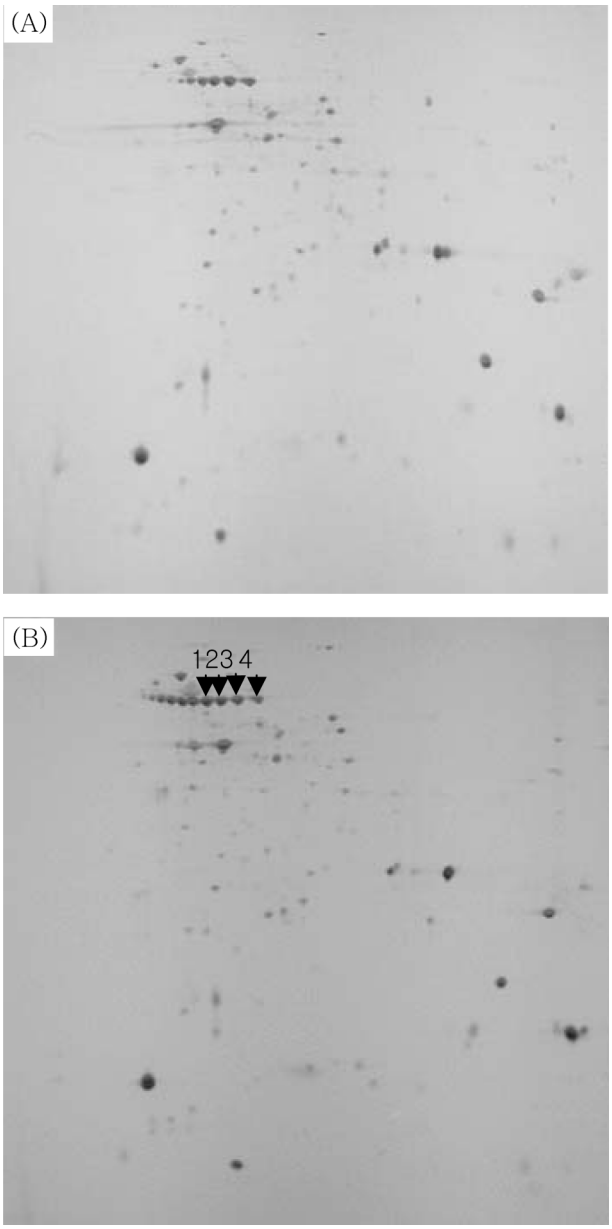

Fig. 2. Comparison of total expressed proteins on the 2-D gel electrophoresis of *C. jejuni* ATCC 43429 exposed to aerobic condition for 2 days.
(A) Microaerobic condition, (B) Aerobic condition following microaerobic condition preincubation (→: major maintained spot).

or induction of a newly synthesized protein. When *Campylobacter* was exposed to external stress like aerobic conditions, some operons seemed to work defensively as in other microorganisms. For example, *Campylobacter* was confirmed as increasing protein expressions and some suppressions [3, 14, 15, 31]. Although the major defense ability of *Campylobacter* against oxidative stress by superoxide dismutase (SOD), catalase, and alkylhydroperoxide reductase would be more expressed, when it was exposed to one

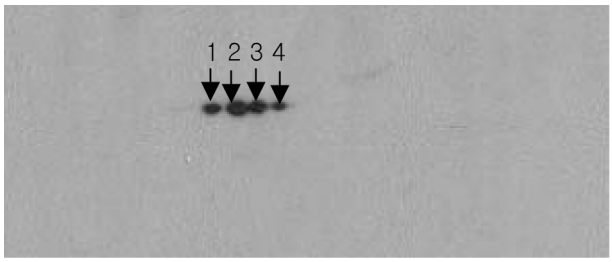

Fig. 3. Immunoblotting of *C. jejuni* on the two-dimentional electrophoresis gel.

stress, many other genes seemed to be expressed for cross-protection besides some target genes [32].

Flagellin Detection by Immunoblotting and MALDI-TOF MS

To confirm whether the expressed proteins on the 2-D gels were the epitope protein when *C. jejuni* was incubated under microaerobic conditions and aerobic conditions, immunoblotting analysis with the antibody was conducted. Also, the continuously expressed proteins on the gels were analyzed by MALDI-TOF MS. The result in immunoblotting was that the commercial whole-cell antibody of *C. jejuni* reacted with the spots on the gels (Fig. 3). The spot proteins, which were confirmed as flagellin proteins when they were analyzed by MALDI-TOF MS, were expressed at the same time and were epitope proteins in both microaerobic and aerobic exposures (Table 1). Lazaro *et al.* reported that expressions of flagellin did not decrease. When microorganisms were exposed to external stress, they adapted to the stress after many physiological properties were modified [15]. Many foodborne pathogens are similarly adaptable and one of their modified is a viable but nonculturable (VBNC) cell [17]. When the VBNC cells were compared with injured cells, the injured cells had the property of regrowth. The VBNC cells also had property of resuscitation, which would possibly increase camplyobacteriosis [8, 13]. In this study, the morphology of *Campylobacter* was changed into the coccoid form (VBNC cell) in aerobic conditions, whereas the cells were not usually detected traditionally incubating in food. When there is contamination like *Campylobacter* in food, detection techniques have advanced beyond such methods as RT-PCR, ELISA, florescence microscope, and flow cytometry. However, these methods could be applied to the products after food processing. Thus, the best way to control foodborne pathogens requires detection on time during processing. An immunosensing system would be such a method using an antibody-antigen reaction, and has an immune response advantage. Therefore, these results might indicate that flagellins are expressed even during aerobic exposure, and their antibodies could be utilized as an immunosensor for the detection of any kind of cell of *C. jejuni* influenced by the different environmental oxidative stresses in food.

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Table 1. Identification of proteins in microaerobic and aerobic exposures of *C. jejuni* detected by whole-cell antibody.

Spot no.	Accession no.	Protein name	MW/pI	No. of peptides matched	Coverage (%)
1	S15286	Flagellin	59,599/5.56	15	24
2	AAK73701	Flagellin A	57,161/5.29	5	12
3	AAK73701	Flagellin A	57,161/5.29	7	11
4	S15286	Flagellin	59,599/5.56	8	11

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