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Inverse Correlation between Extracellular DNase Activity and Biofilm Formation among Chicken-Derived *Campylobacter* Strains

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Campylobacter jejuni and *Campylobacter coli* are important foodborne pathogenic bacteria, particularly in poultry meat. In this study, the presence of extracellular DNase activity was investigated for biofilm-deficient *Campylobacter* strains versus biofilm-forming *Campylobacter* strains isolated from chickens, to understand the relationship between extracellular DNase activity and biofilm formation. A biofilm-forming reference strain, *C. jejuni* NCTC11168, was co-incubated with biofilm non-forming strains isolated from raw chickens or their supernatants. The biofilm non-forming strains or supernatants significantly prohibited the biofilm formation of *C. jejuni* NCTC11168. In addition, the strains degraded pre-formed biofilms of *C. jejuni* NCTC11168. Degradation of *C. jejuni* NCTC11168 biofilm was confirmed after treatment with the supernatant of the biofilm non-forming strain 2-1 by confocal laser scanning microscopy. Quantitative analysis of the biofilm matrix revealed reduction of extracellular DNA (16%) and proteins (8.7%) after treatment. Whereas the biofilm-forming strains *C. jejuni* Y23-5 and *C. coli* 34-3 isolated from raw chickens and the *C. jejuni* NCTC11168 reference strain showed no extracellular DNase activity against their own genomic DNA, most biofilm non-forming strains tested, including *C. jejuni* 2-1, *C. coli* 34-1, and *C. jejuni* 63-1, exhibited obvious extracellular DNase activities against their own or 11168 genomic DNA, except for one biofilm non-former, *C. jejuni* 22-1. Our results suggest that extracellular DNase activity is a common feature suppressing biofilm formation among biofilm non-forming *C. jejuni* or *C. coli* strains of chicken origin.

Keywords: *Campylobacter*, biofilm, extracellular DNase, inhibition, extracellular DNA, chicken

Introduction

Campylobacter strains are among the most common foodborne pathogenic bacteria causing gastrointestinal disease and are highly associated with poultry [1]. *Campylobacter* species are microaerophilic, but can survive under normal atmospheric conditions. The annual reported incidence of *Campylobacter* infections in the UK is confirmed to be around 80,000 cases; however, the outbreak of cases is underestimated, and actual figures may be up to 9-fold higher than the measured numbers [2]. In addition, *Campylobacter* species are associated with neurological diseases such as Guillain-Barré and Miller-Fisher syndromes [3].

A biofilm is the protective polymeric matrix of biosecreted substances and consists of extracellular DNA, protein molecules, and a few polysaccharides. Biofilms can trap substantial nutrients to increase the survival rate of bacterial cells under harsh conditions. Bacterial biofilm formation is an important survival strategy against antimicrobials, physiological conditions, or chemical agents. *Campylobacter* species are also known to form biofilms on abiotic surfaces, including food contact surfaces such as stainless steel, glass, and plastics [4–7]. *Campylobacter* biofilms also have

increased resistance against external stresses, such as oxygen, compared with single cells [5]. Although the presence of monospecies *Campylobacter* biofilm in food-processing environments remains controversial [8], it is necessary to understand the mechanism of biofilm formation in *Campylobacter* species because their biofilm appear to be associated with survival in different environments. For example, the biofilm formation of *Campylobacter* increases under stressful conditions such as aerobic environments [9]. In addition, food-associated environments such as chicken juice can enhance the biofilm formation of *Campylobacter jejuni* [10]. Biofilms formed on food contact surfaces can increase the risk of cross-contamination in food processing.

Although protein molecules and polysaccharides are known to be key factors in the biofilm matrix, extracellular DNA also plays an important role in biofilm formation [11, 12]. Extracellular DNA is a major component of *Campylobacter* biofilms, and extracellular DNase activity is known to affect biofilm formation [13–15]. In addition, a previous study demonstrated that transposon-mediated insertional mutation of *flgA* in *C. jejuni* NCTC11168 resulted in the absence of flagella and a significant decrease in biofilm formation, strongly supporting that *flgA* is essential for flagellar biosynthesis and flagellar-mediated motility or that the presence of flagella is important in the biofilm formation of *C. jejuni* NCTC11168 [16]. Although it has been widely examined, the mechanism of biofilm formation in *Campylobacter* remains poorly understood. In particular, the presence of extracellular DNase activity among *Campylobacter* strains isolated from chickens, a major source of human infection, remains largely unknown. In addition, the role of extracellular DNase activity is poorly understood in the biofilm formation of the *Campylobacter* natural isolates. Therefore, this study was conducted to investigate the presence of extracellular DNase activity in biofilm-forming versus biofilm non-forming *Campylobacter* strains isolated from raw chicken meats, to understand the effects of this activity on biofilm formation by *Campylobacter*.

Materials and Methods

Strains and Growth Conditions of *Campylobacter*

The *Campylobacter* strains used in this study were *C. jejuni* NCTC11168, an *flgA* insertional mutant derived from *C. jejuni* NCTC11168 [16], biofilm non-forming strains *C. jejuni* 2-1, *C. jejuni* 22-1, *C. coli* 34-1, and *C. jejuni* 63-1, and biofilm-forming strains *C. jejuni* Y23-5 and *C. coli* 34-3 isolated from commercially purchased raw chickens [7]. The strains were cultured on tryptic soy agar supplemented with 5% (v/v) sheep blood from −80°C stocks at 37°C under microaerobic conditions (6–12% O_2, 5–8% CO_2) using AnaeroPack-MicroAero (Mitsubishi Gas Chemical Co., Japan).

Co-Culture Biofilm Assay

A co-culture biofilm assay was conducted as previously described with some modifications [16]. *Campylobacter* strains were grown on BAP at 37°C for 48 h under microaerobic conditions. Next, the cells were suspended in Mueller-Hinton broth (MHB) at OD_{600} 0.1–0.2 and spread on the blood agar plate (BAP) at 100 µl. After incubation for 14–15 h at 37°C under microaerobic conditions, the cells grown on the plates were resuspended in MHB at OD_{600} 0.005–0.014 using cell scrapers. An equal volume of the two different cell suspensions was pooled, and 100 µl was inoculated into sterile 96-well polystyrene plates (Spl, Korea). After inoculation, the plates were incubated at 37°C under microaerobic conditions for 72 h. After incubation, planktonic cells were removed by pipetting up and down 4 times. The wells were washed twice to remove loosely attached cells using 150 µl of deionized water by pipetting up and down 4 times and then the cells were dried at 37°C. Next, 100 µl of 1% crystal violet was added followed by incubation at room temperature for 30 min to stain the attached biofilms in the wells. The crystal violet was removed after the reaction, and the wells were thoroughly washed with flowing cold tap water followed by rinsing with deionized water and tapping on a paper towel to remove the remaining water. After the moisture was completely removed at 37°C, the remaining crystal violet was dissolved in 100 µl of 30% methanol and 10% acetic acid by pipetting up and down. The absorbance intensity of crystal violet was measured at 590 nm using the Nanoquant Infinite M200 Pro microplate reader (Tecan, Switzerland).

Filtered Supernatant-Added Monoculture Biofilm Assay

The monoculture biofilm assay was conducted in the presence of filtered supernatants from other bacterial cultures. The cell suspensions prepared as above were filtered through sterile 0.22-µm polyvinylidene fluoride membrane filters (Merck Millipore, USA). The filtered supernatant was mixed with the cell suspension of monoculture in equal volumes, and 100 µl was inoculated into 96-well plates. The biofilm assay was conducted as described above.

Degradation of Pre-Existing Biofilms with Biofilm Non-Forming Strains

The *C. jejuni* NCTC11168 strain was incubated in 96-well plates under the same conditions as the co-culture biofilm assay and washed with sterile deionized water after biofilm formation. The cultures of biofilm non-forming strains were inoculated into each well and incubated at 37°C for 14–18 h. After incubation, the biofilm was quantified as described above.

Confocal Laser Scanning Microscopy (CLSM)

The *C. jejuni* biofilm was grown under the conditions described above. For static biofilms, a 6-well polystyrene plate was used.

After 72 h incubation, the supernatant was removed gently. *Campylobacter jejuni* 2-1 strain suspensions prepared as described above were filtered through sterile 0.22-μm polyvinylidene fluoride membrane filters (Merck Millipore). The filtered supernatant was added to the preestablished *C. jejuni* NCTC11168 biofilm and incubated at room temperature for 30 min. Next, the biofilm was stained in sterile 0.85% NaCl solution containing 10 μM Syto60 (Thermo Scientific, USA), a red-fluorescent, membrane-permeable dye that stains live bacteria, and 0.5 μM TOTO-1 (Thermo Scientific), a green-fluorescent dye that stains extracellular DNA or DNA of bacteria with compromised membranes, as previously described with some modifications [17]. Imaging was performed on a Leica TCS SP8 STED Confocal Laser Scanning Microscope (Leica, Germany). The excitation wavelengths were 633 and 488 nm and emission pathlengths were 650–750 and 490–560 nm for Syto60 and TOTO-1, respectively. All images were captured with a 63× objective and analyzed using Leica Application Suite X software (Leica).

Quantitative Analysis of Extracellular DNA, Proteins, and Carbohydrates in Biofilm Matrix

Extracellular polymeric substances were extracted from the biofilm as described by Wu and Xi [18]. The 11168 strain was incubated for 72 h in a 6-well polystyrene plate with 3 ml per well under microaerobic conditions to form the biofilm. Next, the supernatant was replaced with that of strain 2-1 filtered at OD_{600} 0.01. After the reaction for 30 min at room temperature, the samples were washed with 3 ml of phosphate-buffered saline (PBS) and suspended in 1 ml of Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and 0.85% NaCl at a 1:1 dilution mixture by scraping off the well bottom and surface. The biofilm samples from three different wells were pooled and filtered through a 0.22-μm syringe-driven filter (Merck Millipore) and used to quantify extracellular DNA, carbohydrates, and proteins. Extracellular DNA was extracted using the MasterPure Gram Positive DNA Purification Kit according to the manufacturer's instructions (Epicentre, USA). Briefly, 300 μl of lysed biofilm samples was mixed with 175 μl of MPC protein precipitation reagent. The debris was pelleted by centrifugation at 4°C for 10 min at 10,000 ×*g*. The supernatant was transferred to a clean microcentrifuge tube. RNase A (5 μg/μl) was added at 1 μl to each sample and mixed thoroughly. After incubation at 37°C for 30 min, 500 μl of isopropanol was added and the tubes were inverted 30–40 times. DNA was pelleted by centrifugation at 4°C for 10 min at 10,000 ×*g*. The isopropanol was removed and the pellet was rinsed with 70% ethanol. After removing the ethanol, the DNA was resuspended in 35 μl of TE buffer. Protein was extracted according to the BCA Protein Assay Kit instructions (Thermo Scientific). Biofilm samples (25 μl) were mixed with 200 μl working reagent mixture (50:1 ratio of reagent A:reagent B). The mixture of samples and working reagent was incubated at 37°C for 30 min. After the reaction, the absorbance of the samples was measured at 562 nm, and the protein concentration in the samples was estimated based on the standard curve of bovine serum albumin at different concentrations. To quantify the carbohydrate contents, the Total Carbohydrate Assay Kit (Sigma, USA) was used, with glucose used as a standard. H_2SO_4 (150 μl) was added to 30 μl of samples and then incubated with shaking at 400 rpm for 15 min at 90°C by covering the plate to protect the samples from light. After the reaction, 30 μl of developer was added and the plate was shaken at 300 rpm for 5 min at room temperature. Absorbance was measured at 490 nm.

Genomic DNA Extraction

To extract genomic DNA from the *Campylobacter* strains, *Campylobacter* cells grown on BAP were suspended at OD_{600} 0.3–0.5 in PBS, and 1 ml of the cells was pelleted by centrifugation at 9,400 ×*g* at 4°C for 10 min. After removing the supernatant, the pellet was resuspended in 200 μl of PBS and the manufacturer's protocol was followed to extract genomic DNA using the Easy-DNA kit (Invitrogen, USA). Genomic DNA was extracted using the MasterPure Gram Positive DNA Purification Kit (Epicentre).

Extracellular DNase Activity Assay

The extracellular DNase activity of *Campylobacter* cells was measured as previously described [14]. The cells were grown as described above and suspended in PBS at OD_{600} 1.0 after 14–15 h incubation. The cell suspensions (1 ml) were centrifuged at 9,400 ×*g*, at 4°C for 10 min. The cell pellets were washed twice with PBS and then resuspended in 1 ml of PBS. A small portion (50 μl) (~10^8 CFU) of the cell suspensions and 10 μl of the extracted genomic DNA (2–3 μg) were mixed and incubated for 0, 1, 2, and 3 h at 37°C with shaking at 300 rpm using an Eppendorf ThermoMixer C (Eppendorf, Germany). After the reaction, the samples were centrifuged at 21,000 ×*g*, 4°C for 5 min. The supernatants were transferred to other microtubes and stored at −20°C until analysis. The samples (20 μl) were loaded and run on a 1% agarose gel to evaluate the degradation of genomic DNA.

Band Intensity

Band intensities after genomic DNA degradation were investigated using Image Lab Software ver. 3.0 with a Gel Doc EZ imager (Bio-rad, USA). Significant differences in the degradation of genomic DNA among the reaction times were determined from the decrease in band intensity.

Statistical Analysis

All data were analyzed by one-way analysis of variance using SPSS 18 software (SPSS, Inc., USA). Significant differences in data were determined by Duncan's test at $p < 0.05$. The experimental results were expressed as the mean ± standard deviation.

Results

Inhibition of Biofilm Formation in *C. jejuni* NCTC11168 Following Co-Culture with Biofilm Non-Forming Strains

Biofilm-forming *C. jejuni* strain 11168 and each biofilm

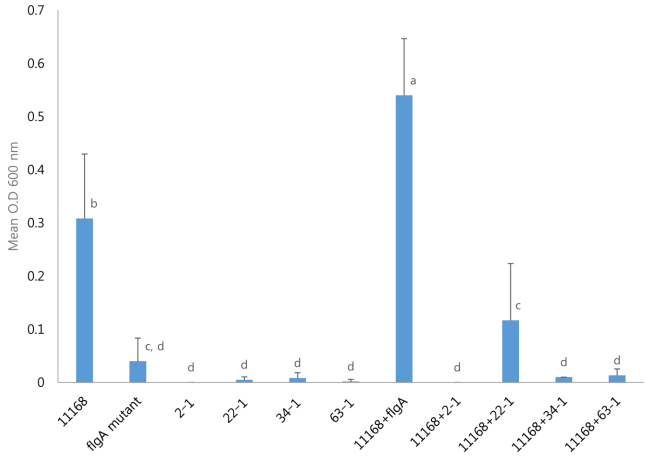

Fig. 1. Inhibition of biofilm formation when biofilm-forming *C. jejuni* strain NCTC11168 was co-cultured with biofilm non-forming strains at 37°C for 72 h.

Different letters (a–d) next to bars indicate significant differences ($p < 0.05$). The measurements were normalized after the deduction of Mueller-Hinton broth only (negative control) for nonspecific binding. The data are based on three independent experiments conducted in triplicate.

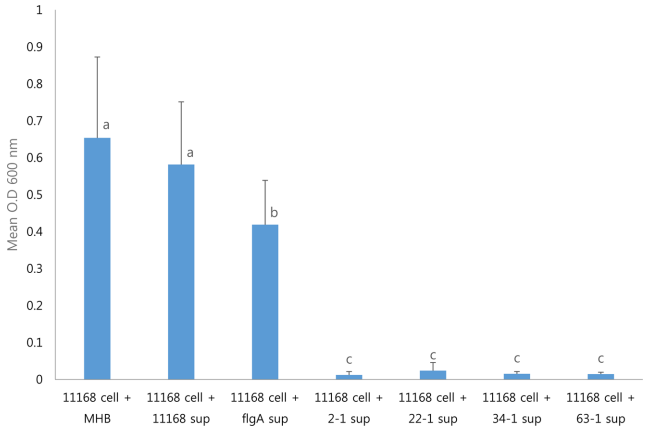

Fig. 2. Inhibition of biofilm formation when biofilm-forming *C. jejuni* strain NCTC11168 was incubated with the supernatants (sup) of biofilm-non-forming strains at 37°C for 72 h.

Different letters (a–c) next to bars indicate significant differences ($p < 0.05$). The measurements were normalized after the deduction of Mueller-Hinton broth only (negative control) for nonspecific binding. The data are based on three independent experiments conducted in triplicate.

non-forming strain, *C. jejuni* 2-1, *C. jejuni* 22-1, *C. coli* 34-1, or *C. jejuni* 63-1, were mixed and incubated at 37°C for 72 h to investigate biofilm formation ability in different mixed cultures (Fig. 1). Biofilm-forming strain 11168 effectively formed a biofilm on the polystyrene surface, whereas all single strains of biofilm non-formers formed very little or no biofilms. In the co-culture biofilm assay with two mixed strains, strain 11168 and the biofilm non-forming strain, the *flgA* mutant, an isogenic and aflagellated mutant derived from strain 11168, showed the most biofilm formation. This suggests that the presence of a biofilm non-forming strain in a co-culture biofilm assay does not reduce biofilm formation. In contrast, no or very little biofilm formed when strain 11168 was co-incubated with strain 2-1, 34-1, or 63-1 (Fig. 1). In contrast to other co-culture assays of biofilm non-forming strains, significant but partial biofilm inhibition was detected when strain 22-1 was used.

Inhibition of Biofilm Formation in Strain 11168 Incubated with Supernatant of Biofilm Non-Forming Strains

We tested the biofilm formation of strain 11168 in the presence of supernatants of biofilm non-forming strains to investigate whether the supernatants inhibit the biofilm formation of strain 11168 (Fig. 2). Slightly significant inhibition of biofilm formation of strain 11168 was observed in the presence of the supernatant from the *flgA* mutant, compared with the 11168 supernatant or MHB (Fig. 2). No or very little biofilm formation was observed in strain 11168 in the presence of strain 2-1, 22-1, 34-1, or 63-1 supernatants. The strain 22-1 supernatant also significantly inhibited the biofilm formation of strain 11168. Based on these results, the supernatants of biofilm non-forming strains can inhibit the biofilm formation of strain 11168.

Degradation of Pre-Existing Biofilms with Biofilm Non-Forming Strains

The degradation of pre-existing biofilms was studied with biofilm non-forming strains to investigate whether they can not only inhibit biofilm formation, but also degrade pre-existing biofilms. Biofilm non-forming strains were added to pre-existing biofilms and incubated at 37°C for 14–18 h, and the amount of biofilm formed was measured. The degradation of pre-existing biofilms by biofilm non-forming strains yielded similar results as the co-culture assay (Fig. 3). Strains 2-1, 34-1, and 63-1 completely degraded the pre-existing biofilms of strain 11168. Similar to the co-culture assay (Fig. 1), the pre-existing biofilm of strain 11168 was partially but significantly degraded by strain 22-1 (Fig. 3). As expected, the biofilm was not degraded by the *flgA* mutant strain. These data clearly show that biofilm non-forming strains isolated from raw chickens can

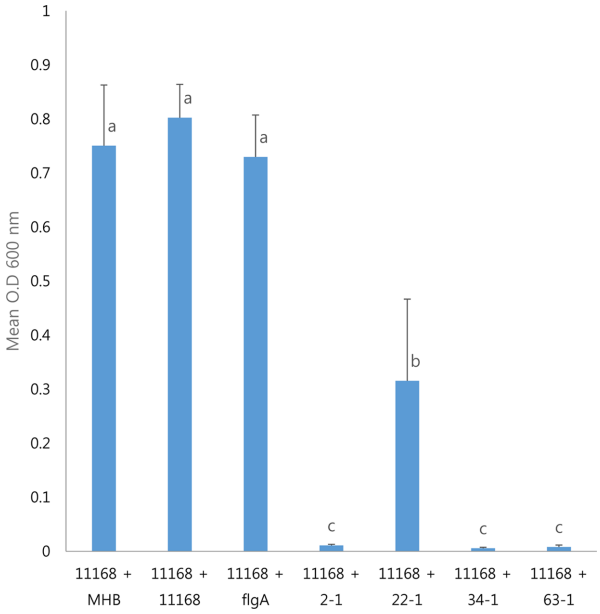

Fig. 3. Degradation of pre-existing *C. jejuni* NCTC11168 biofilm by adding biofilm non-forming strains.

Biofilm non-forming strains were added after 72 h incubation of biofilm-forming 11168. Different letters (a–c) next to bars indicate significant differences ($p < 0.05$). The measurements were normalized after the deduction of Mueller-Hinton broth only (negative control) for nonspecific binding. The data are based on three independent experiments conducted in triplicate.

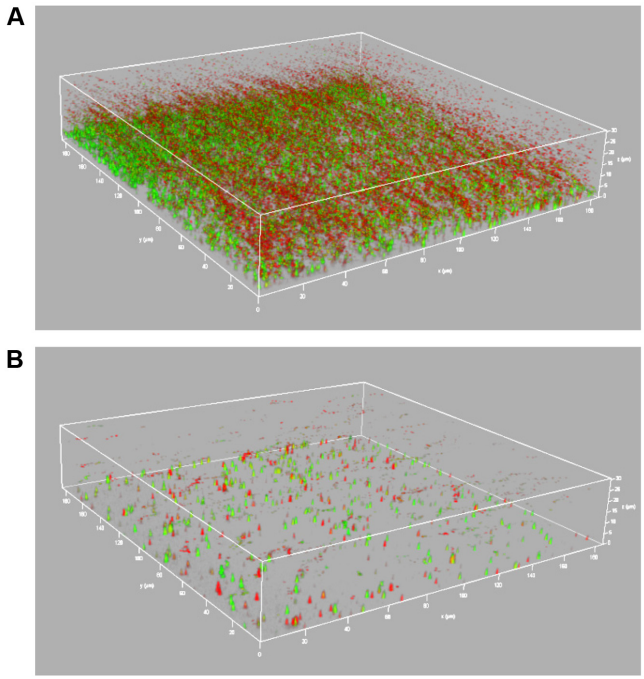

Fig. 4. Three-dimensional confocal laser scanning microscopy Z-stack images of biofilms grown for 72 h under static conditions in 6-well microtiter plates.

Live bacteria were stained by SYTO-60 (red), and extracellular DNA or dead bacteria by TOTO-1 (green). (**A**) *C. jejuni* NCTC11168 biofilms and (**B**) *C. jejuni* NCTC11168 biofilms after treatment with *C. jejuni* 2-1 strain supernatant for 30 min at room temperature.

degrade pre-existing biofilms of strain 11168.

To confirm the degradation of pre-existing biofilms by biofilm non-forming strains, strain 11168 biofilm was treated with the supernatant of strain 2-1 for 30 min at room temperature and observed by CLSM (Fig. 4). The biofilm mass was greatly reduced after treatment with the supernatant, confirming the biofilm-degrading effect.

Quantitative Analysis of Extracellular DNA, Carbohydrate, and Protein Contents in Biofilm Matrix after Treatment with Supernatant of Strain 2-1

Extracellular DNA, carbohydrate, and protein contents were measured in the biofilm of strain 11168 before and after treatment with the supernatant of strain 2-1, a biofilm non-forming strain, for 30 min at room temperature (Fig. 5). Before treatment, the amount of extracellular DNA, carbohydrates, and proteins for the surface area of 1 cm^2 were 319 ng, 1.39 mg, and 172 μg, respectively. After treatment, the amounts were 269 ng, 1.41 mg, and 157 μg, respectively. Overall, the amounts of extracellular DNA and proteins were decreased by 16% and 8.7%, respectively, whereas carbohydrates were not reduced (Fig. 5).

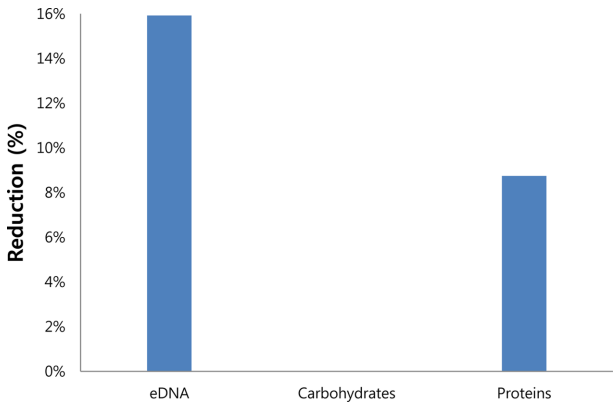

Fig. 5. Reductions (%) in extracellular DNA (eDNA), carbohydrates, and proteins in the *C. jejuni* NCTC11168 biofilm matrix after treatment with supernatant of strain 2-1, a biofilm non-forming strain, for 30 min at room temperature.

Extracellular DNase Activity of Biofilm Non-Forming Strains

Extracellular DNA plays an important role in the biofilm

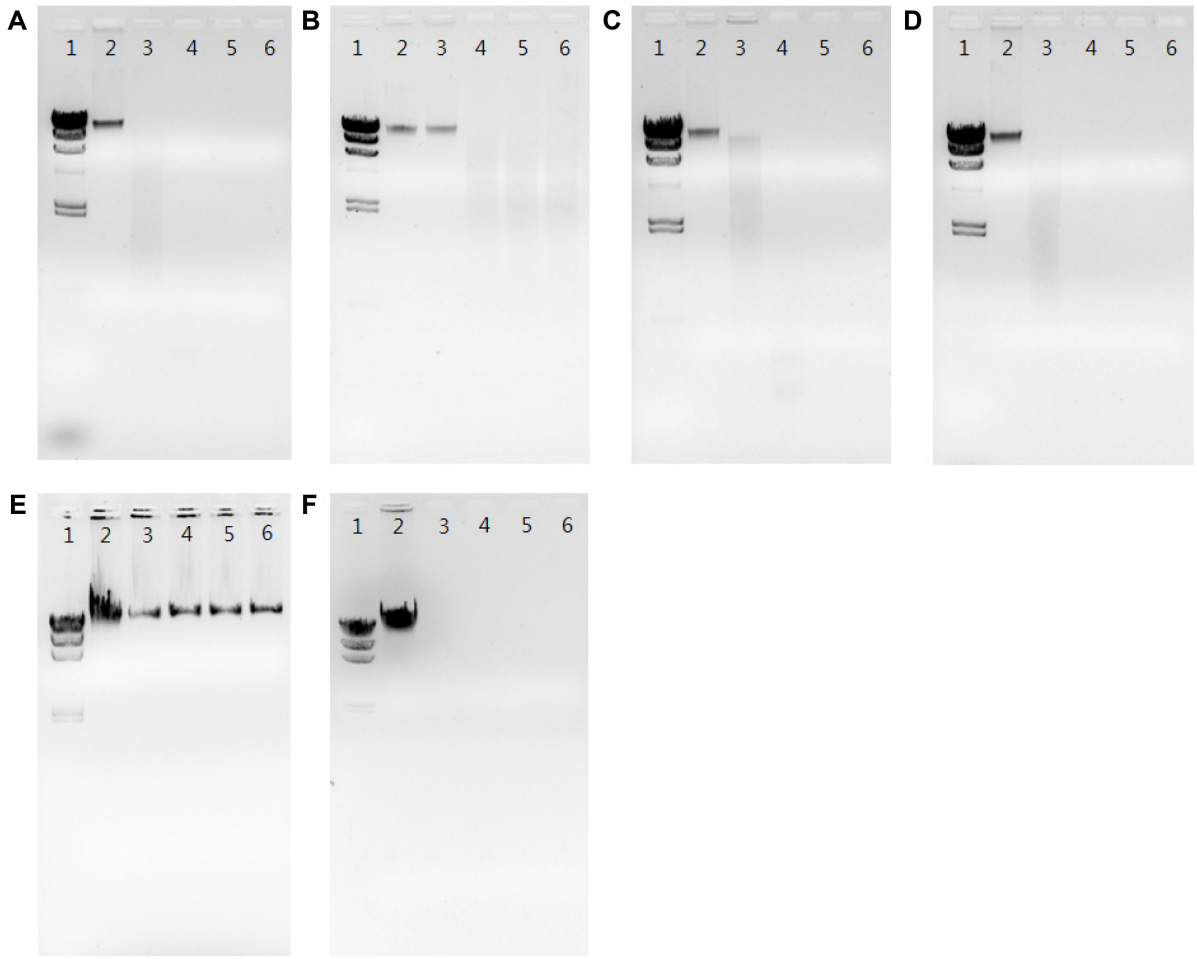

Fig. 6. Extracellular DNase activities of biofilm non-forming *Campylobacter* strains on *C. jejuni* NCTC11168 genomic DNA.
(**A**) *C. jejuni* 2-1, (**B**) *C. jejuni* 22-1, (**C**) *C. coli* 34-1, (**D**) *C. jejuni* 63-1, (**E**) *flgA* mutant derived from *C. jejuni* NCTC11168, (**F**) DNase I treatment (+ control). Bacterial cells were incubated with *C. jejuni* NCTC11168 genomic DNA at 37°C for 3 h. Strains 2-1, 22-1, 34-1, 63-1, and DNase I treatments degraded 11168 genomic DNA after the reaction. Lane 1: Trackit λ DNA/HindIII fragment; lane 2: genomic DNA only; lanes 3–6: genomic DNA incubated with the cells or DNase I for 0, 1, 2, and 3 h, respectively.

structures of many bacteria including *C. jejuni* [12, 13, 15, 19, 20]. Extracellular DNase activity plays an important role in biofilm modulation by acting on extracellular DNA [21, 22]. Thus, in this study, the extracellular DNase activity of biofilm non-forming strains was assessed by measuring the extent of genomic DNA degradation during incubation with the cells. First, genomic DNA of the standard strain 11168 was mixed with the cells of biofilm non-forming strains, and then the degradation of genomic DNA was assessed (Fig. 6). All investigated biofilm non-forming strains showed a strong ability to degrade the genomic DNA of standard strain 11168, demonstrating that these strains have extracellular DNase activities. All biofilm non-formers, except for strain 22-1, immediately degraded the genomic DNA of strain 11168 (Fig. 6). For strain 22-1, unlike other strains, immediate degradation of genomic DNA was not detected, but the degradation was clearly observed after 1 h incubation (Fig. 6B). In contrast, immediate degradation (0 h) of the standard strain's genomic DNA was detected in strains 2-1, 34-1, and 63-1.

To understand the inability of biofilm non-forming strains to form biofilms, biofilm non-forming strains were mixed with their own genomic DNA and degradation was assessed (Fig. 7). Similar to the reaction with standard strain 11168 genomic DNA, strains 34-1 and 63-1 showed immediate degradation of self-DNA in the reaction. Strain

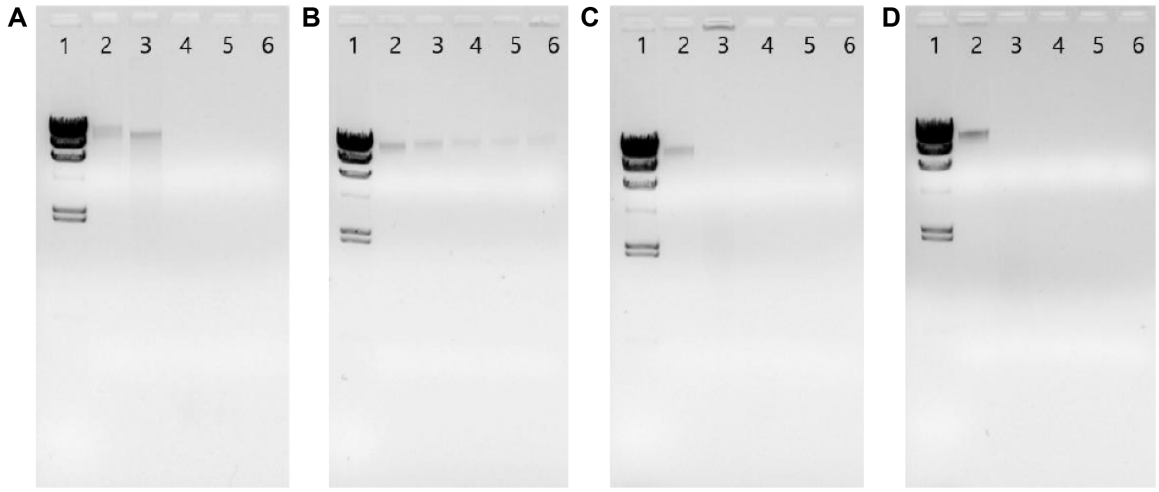

Fig. 7. Extracellular DNase activities of biofilm non-forming *Campylobacter* strains on their own genomic DNA.
(**A**) *C. jejuni* 2-1, (**B**) *C. jejuni* 22-1, (**C**) *C. coli* 34-1, and (**D**) *C. jejuni* 63-1. Bacterial cells were incubated with their own genomic DNA at 37°C for 3 h. Except for strain 22-1, strains 2-1, 34-1, and 63-1 degraded their own genomic DNA after the reaction. Lane 1: Trackit λ DNA/Hind III fragment; lane 2: genomic DNA only; lanes 3–6: genomic DNA incubated with the cells for 0, 1, 2, and 3 h, respectively.

2-1, however, exhibited lower extracellular DNase activity against self-DNA (Fig. 7A) compared with the reaction with standard strain 11168 genomic DNA (Fig. 6A), in which immediate degradation of self-genomic DNA was not detected. In contrast to other biofilm non-forming strains, the band intensities of strain 22-1 were only slightly decreased based on the analysis of gel band intensity. This result indicates that strain 22-1 has very weak extracellular DNase activity against self-genomic DNA (Fig. 7B).

Absence of Extracellular DNase Activity of Biofilm-Forming Strains

Biofilm-forming strains *C. jejuni* 11168, *C. jejuni* Y23-5, and *C. coli* 34-3 were tested to investigate the presence of extracellular DNase activity against self-DNA (Fig. 8). The intensities of bands for strains 11168 and Y23-5 were nearly unchanged after 3 h incubation, while the intensities of bands for strain 34-3 showed slight decreases during incubation. These data suggest that the biofilm-forming

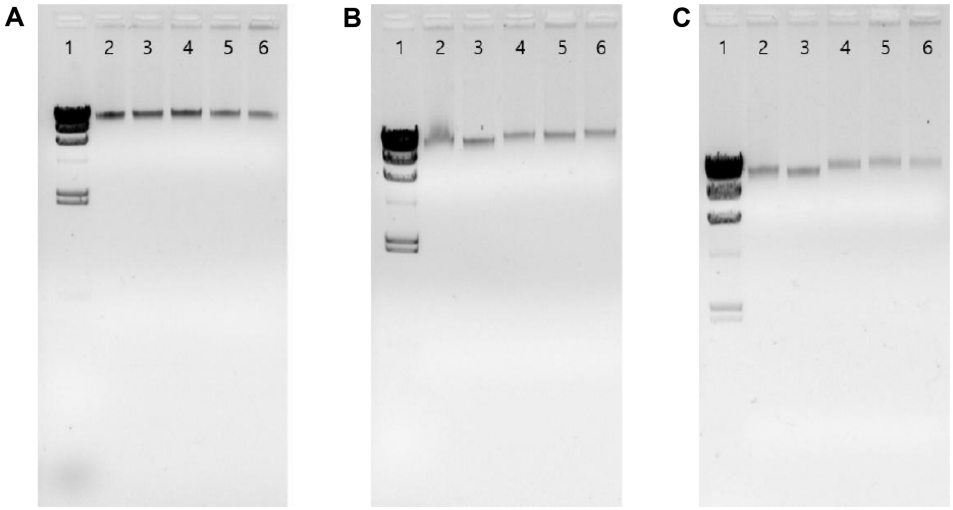

Fig. 8. Absence of extracellular DNase activity of biofilm-forming *Campylobacter* strains on their own genomic DNA.
(**A**) *C. jejuni* NCTC11168, (**B**) *C. jejuni* Y23-5, and (**C**) *C. coli* 34-3. Bacterial cells were incubated with their own genomic DNA at 37°C for 3 h. All three strains were unable to degrade their own genomic DNA. Lane 1: Trackit λ DNA/Hind III fragment; lane 2: genomic DNA only; lanes 3–6: genomic DNA incubated with the cells for 0, 1, 2, and 3 h, respectively.

strains did not degrade their own genomic DNA or only slightly degraded genomic DNA during 3 h incubation, revealing the absence of, or very weak, extracellular DNase activity (Fig. 8).

Discussion

In the life cycle of bacteria, biofilms play an important role and cause serious problems in a wide range of food industries such as brewing [23], seafood processing [24], dairy processing [25], meat processing [26], and poultry processing [27]. Some bacteria can form biofilms in close association with surfaces and interfaces [28]. It has also been shown that biofilms can increase the survival rate of bacterial cells [29].

A biofilm has an extracellular matrix that allows bacterial cells to remain hydrated and trap nutrients to maintain the metabolic activity of cells in the biofilm [13]. Previous studies on biofilm formation indicated that extracellular DNA is essential and plays an important role in biofilm formation of many bacterial species, including *C. jejuni* and *C. coli* [7, 12, 13, 15, 19, 20, 30, 31]. Although the percentage of extracellular DNA in biofilms differs depending on the biofilm-forming species, extracellular DNA is among the major components of most biofilms in addition to proteins and polysaccharides [19].

Previous studies have shown that exogeneous addition of DNase can reduce bacterial biofilms and be used for biofilm control [7, 21, 32–34]. For example, the biomass of biofilms in *E. coli* and *Staphylococcus aureus* was decreased when the added DNase I concentration was increased [34]. DNase I treatment also degraded the biofilms of *Haemophilus influenzae* VT 450-2006, *Klebsiella pneumoniae* VT 1367, *Pseudomonas aeruginosa* ATCC 27853, *Streptococcus pyogenes* VT 59, and *Acinetobacter baumannii* VT 126 [35] as well as the biofilms of *C. jejuni* and *C. coli* [7].

Similarly, the expression of extracellular nucleases from bacterial cells can modulate bacterial biofilm formation [22, 36–38]. For example, Mann *et al*. [22] demonstrated that staphylococcal thermonuclease is involved in the degradation of extracellular DNA during biofilm development of *S. aureus*. In another study, Tran *et al*. [39] found that a *Ralstonia solanacearum* mutant lacking extracellular DNases formed thicker biofilm than wild-type biofilm. In another study, Seper *et al*. [38] showed that two extracellular nucleases, Dns and Xds, can modulate and control extracellular DNA in the biofilm matrix of *Vibrio cholerae*.

Recently, Brown *et al*. [14] found that *C. jejuni* RM1221, a biofilm non-forming strain, has extracellular DNase activity, whereas *C. jejuni* NCTC11168 and 81116, biofilm-forming strains, do not. Although *C. jejuni* RM1221 was originally isolated from chicken, the prevalence of extracellular DNase activity among biofilm non-forming *Campylobacter* strains of chicken origin is largely unknown. Consistent with the previous study, our study demonstrated that biofilm non-forming strains isolated from chicken samples had extracellular DNase activities, whereas the biofilm-forming strains from chicken samples did not have these activities (Figs. 6–8). Although there is no direct evidence that such extracellular DNase activities affected the biofilm formation of *Campylobacter* strains in our study, such a highly inverse correlation between extracellular DNase activities and biofilm-forming abilities strongly suggests that extracellular DNase activities can greatly affect biofilm formation among food-associated *Campylobacter* strains. The hypothesis is further supported by the observation that biofilm formation was inhibited or the pre-formed biofilm was degraded by the biofilm non-forming strains (Figs. 1–4). Additionally, the reduction of extracellular DNA in the biofilm matrix was clearly observed after treatment with the supernatant of the biofilm non-forming strain 2-1 (Fig. 5). However, we still cannot exclude other possibilities, such as protease activity, considering that the reduction of proteins in the biofilm matrix was also substantial (Fig. 5) [40, 41]. For example, self-produced extracellular proteases inhibited the biofilm formation of *Staphylococcus aureus* or group A *Streptococcus* [42–44]. Furthermore, the inhibitory effect of extracellular protease can occur in a cross-species manner. For example, the supernatant of *Staphylococcus epidermidis* inhibited biofilm formation or degraded pre-formed biofilms of *S. aureus*, for which serine protease was found to be responsible [45]. This protease can degrade several biofilm formation-associated proteins such as Atl, FnBPA, and Spa [46]. In addition, the supernatant of *Bdellovibrio bacteriovorus*, containing proteases, inhibited or degraded biofilm formation of *S. aureus* [47]. Serine protease is highly conserved across species and exists in *C. jejuni*, suggesting that this enzyme has a similar function in biofilm inhibition or degradation in *Campylobacter* [48]. Based on our study, the presence of such extracellular DNase activity may be a common feature among food-associated *Campylobacter* strains unable to form biofilms.

Motility is a very well-known feature in bacterial biofilm formation, including for *Campylobacter* [6, 16, 49]. Such weak extracellular DNase activity of biofilm non-forming strain 22-1 supports that extracellular DNase activity is not the only factor involved in *Campylobacter* biofilm formation. In fact, the strain was non-motile (data not shown),

suggesting that the lack of motility was the main reason for the poor biofilm formation rather than extracellular DNase activity.

Overall, our study suggests that extracellular DNase activity is a common feature among biofilm non-forming *Campylobacter* strains in food-associated environments. Additionally, the absence of extracellular DNase activity may be one of the major determinants in biofilm formation among natural *Campylobacter* strains in addition to well-established factors such as motility or the presence of flagella [6, 15, 16]. Therefore, extracellular DNase may be useful for controlling *Campylobacter* biofilms.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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