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Lipooligosaccharide Core Truncations Affect the Ability of *Campylobacter jejuni* to Attach to Glass and Form Biofilms under Aerobic Conditions

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Abstract

The aim of this study was to investigate the effects of lipooligosaccharide (LOS) core truncations on the ability of *Campylobacter jejuni* to attach to glass and form biofilms under aerobic conditions. We compared the wild-type and previously constructed glycosyltransferase mutants of two *C. jejuni* strains, NCTC 11168 and 81-176, in terms of the numbers of attached cells, biofilm formation, autoagglutination (AAG) activity, and extracellular DNA (eDNA) release. All LOS mutants of NCTC 11168, except *cj1138* mutant, and 81-176 *waaC* mutant with most severe LOS truncation, exhibited increased attachment and biofilm formation. Conversely, *waaC* and *lgtF* mutant of both NCTC 11168 and 81-176 showed significantly reduced AAG activity. There was no significant difference between all LOS mutants and the wild-type with respect to eDNA production. The biofilm formation levels correlated significantly with the attachment numbers ($p < 0.05$, $R^2 = 0.95$) but not with the AAG activity and eDNA levels ($p \geq 0.48$, $R^2 \leq 0.72$). These results suggest that various LOS core truncations have different impacts on *C. jejuni* attachment, biofilm formation under aerobic conditions, and the AAG activity levels, whereas they may not affect eDNA production. Bacterial attachment, but not the AAG activity and eDNA release levels, may cause changes in *C. jejuni* biofilm formation because of LOS core truncations.

Discipline: Food
Additional key words: Autoagglutination activity, extracellular DNA release

Introduction

Campylobacter jejuni is an important foodborne bacterial pathogen, which is frequently associated with gastrointestinal diseases in humans worldwide (Kirkpatrick & Tribble 2011). Attachment to abiotic surfaces plays an important role in the survival of *C. jejuni* in food systems and the environment, thereby leading to infections (Nguyen et al. 2012). Because of the importance of bacterial attachment, we previously investigated factors that potentially influence the ability of *C. jejuni* to attach to abiotic surfaces, thereby elucidating the mechanisms of attachment. We found that cell surface hydrophobicity affected the ability of many *C. jejuni* strains to attach to abiotic surfaces such as stainless steel and glass (Nguyen et al. 2011). Although it has been suggested that capsular polysaccharides (CPS) and lipooligosaccharides (LOS) contribute to the degree of hydrophobicity of various bacteria (Guerry & Szymanski 2008), CPS removal and LOS core truncation caused by *kpsE* and *waaF* mutation, respectively, did not affect cell surface hydrophobicity and attachment in *C. jejuni* (Nguyen et al. 2013). Various LOS core truncations caused by the mutation of various genes encoding heptose biosynthesis enzymes and glycosyltransferases were found to increase the cell surface hydrophobicity of both *C. jejuni* NCTC 11168 and 81-176 strains (Iwata et al. 2013). However, it is currently unknown whether LOS core truncations in these sugars affect the attachment ability of *C. jejuni*.

In addition to surface attachment, biofilm formation by *C. jejuni* has a role in enhanced survival (Joshua et al. 2006,

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Reeser et al. 2007). Flagella and motility play an important role in the biofilm formation of *C. jejuni*, although the mechanisms remain unclear (Guerry 2007). Autoagglutination (AAG) activity may also be a factor associated with biofilm formation, because biofilm formation is not only involved in cell-surface interactions but also cell-cell interactions (Guerry 2007). Many bacterial species can make biofilm comprising a unique mixture of hydrated extracellular polymeric substances such as polysaccharides, proteins, lipids, and extracellular DNA (eDNA), but only eDNA has been observed in the biofilm matrix of *C. jejuni* (Svensson et al. 2009). Given the microaerophilic growth requirements of *C. jejuni*, most previous studies performed biofilm assays under standard microaerophilic laboratory conditions (Joshua et al. 2006, Reeser et al. 2007). However, these conditions do not accurately represent the conditions that the organism may encounter in the atmospheric environment. Reuter et al. (2010) reported that *C. jejuni* can form biofilms under aerobic conditions and that biofilms may play important roles in the zoonotic lifecycle of *C. jejuni*. The effects of LOS core truncations on biofilm formation by *C. jejuni* have not been investigated under aerobic conditions. However, the LOS core truncations caused by *waaF* or *lgtF* mutations reportedly enhanced biofilm formation by *C. jejuni* strain 81-176 under microaerobic conditions (Naito et al. 2010). In contrast, the LOS core truncations caused by *galT* or *cstII* (*cjj1157*) mutations did not affect biofilm formation by this strain under the same growth conditions (Naito et al. 2010). These results suggest that different LOS core truncations have distinct impacts on biofilm formation by *C. jejuni* under microaerobic conditions. However, it is unclear whether similar results can be achieved under aerobic conditions.

Accordingly, in the present study, we aimed to determine how various LOS core truncations affect the ability of *C. jejuni* to attach to an abiotic surface such as glass, and form biofilms under aerobic conditions. We compared the attachment and biofilm formation abilities of the wild-type and various glycosyltransferase mutants, which we constructed previously from *C. jejuni* NCTC 11168 and 81-176 strains (Table 1) (Iwata et al. 2013). These glycosyltransferase mutants had similar motility levels compared with the wild type in both the NCTC 11168 and 81-176 strains (Iwata et al. 2013). We also determined the factors associated with bacterial attachment and biofilm formation, including the AAG activity and eDNA levels, for all the glycosyltransferase mutants and wild-type strains. We determined (1) whether various LOS core truncations affect the AAG activity and eDNA production levels in *C. jejuni* and (2) if the AAG activity and eDNA release are involved in determining the changes in *C. jejuni* biofilm formation due to LOS core truncations.

Materials and methods

1. Bacterial strains and culture conditions

The wild-type bacterial strains and glycosyltransferase mutants used in this study and descriptions of their LOS core structures are presented in Table 1. All strains were stored at −80°C in Mueller-Hinton broth (Becton, Dickinson and Company) supplemented with 30% glycerol. Prior to each experiment, working cultures were prepared by inoculating 50 µl of glycerol stock into 3 ml of Brucella broth (Becton, Dickinson and Company), followed by incubation at 42°C for 48 h under microaerobic conditions, which were generated using an AnaeroPack®-MicroAero sachet (Mitsubishi Gas Chemical Co., Inc., Japan). Depending on the assay, 100 µl of the working culture was either inoculated into Brucella broth or plated onto Brucella agar, followed by microaerobic incubation at 42°C.

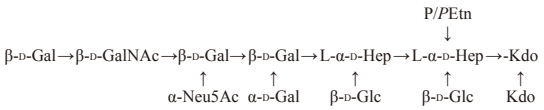
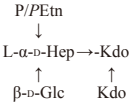
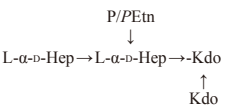
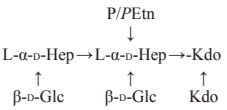
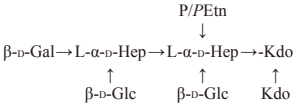
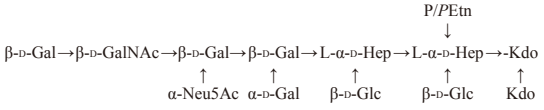
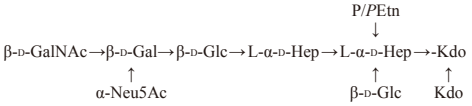
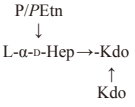
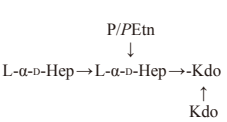
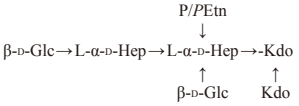
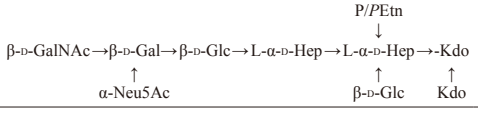
2. Attachment assays

Sterilized precleaned single end frosted microscope slides (76 × 26 × 1 mm thick, Matsunami Glass Industries Ltd, Osaka, Japan) were used for the attachment assays, as described previously (Nguyen et al. 2010). Briefly, *C. jejuni* strains were grown overnight under microaerobic conditions at 42°C in Brucella broth without shaking, then diluted to an OD_{600} of 0.05 (ca 2×10^7 CFU/ml) using fresh Brucella broth. Aliquots (40 ml) of *C. jejuni* culture were then transferred to 50-ml centrifugation tubes, into which sterile glass slides were vertically immersed. Cell attachment was allowed to occur for 30 min at 25°C under atmospheric conditions. After attachment, the slides were removed, rinsed twice with fresh Brucella broth, stained with 0.01% (w/v) acridine orange for 20 min, and observed under an epifluorescence microscope to quantify the attached cells. In total, 50 fields per slide were scanned and the fluorescent cells were enumerated. The counts were calculated as log cells/cm^2.

3. Biofilm and microscopic assays

Biofilm formation on glass by *C. jejuni* under aerobic conditions was analyzed using the Crystal Violet (CV) staining assay, as described by Reuter et al. (2010), but with some modifications. Briefly, overnight *C. jejuni* cultures were prepared as described for the attachment assay and then diluted to an OD_{600} of 0.05 (ca 2×10^7 CFU/ml) using fresh Brucella broth, 1 ml of which was added to new sterile glass tubes and incubated for five days at 25°C under atmospheric conditions. Following incubation, the medium was removed and the tubes were stained with 1 ml of 1% (w/v) CV for 30 min at room temperature. The tubes were washed twice with sterile distilled water to remove unbound CV and then dried for 1 h at 42°C. Bound CV was dissolved by adding 1 ml of 20% acetone in ethanol for 15 min. Biofilm

Table 1. Bacterial strains used in this study*

Bacterial strains	Description	LOS core structure	Sources
11168WT	*C. jejuni* NCTC 11168 wildtype; human isolate		(Parkhill *et al*. 2000)
11168Δ*waaC*	NCTC 11168 derivative, *waaC* mutant (*waaC* encodes heptosyltransferase I that adds the first Hep to Kdo)		(Iwata *et al*. 2013)
11168Δ*waaF*	NCTC 11168 derivative, *waaF* mutant (*waaF* encodes heptosyltransferase II that adds the second Hep to the first)		(Iwata *et al*. 2013)
11168Δ*lgtF*	NCTC 11168 derivative, *lgtF* mutant (*lgtF* encodes the glycosyltransferase that adds the Glc)		(Iwata *et al*. 2013)
11168Δ*cj1136*	NCTC 11168 derivative, *cj1136* mutant (*cj1136* encodes the glycosyltransferase that adds the first Gal to the second Hep)		(Iwata *et al*. 2013)
11168Δ*cj1138*	NCTC 11168 derivative, *cj1138* mutant (*cj1138* encodes the glycosyltransferase that adds the second Gal to the first)		(Iwata *et al*. 2013)
11168Δ*waaC*c	11168Δ*waaC*/complemented in trans from pUOA18Km which is *Escherichia coli-C. jejuni* shuttle vector		(Iwata *et al*. 2013)
81-176WT	*C. jejuni* 81-176 wildtype; human isolate		(Black *et al*. 1988)
81-176Δ*waaC*	81-176 derivative, *waaC* mutant (*waaC* encodes heptosyltransferase I that adds the first Hep to Kdo)		(Iwata *et al*. 2013)
81-176Δ*waaF*	81-176 derivative, *waaF* mutant (*waaF* encodes heptosyltransferase II that adds the second Hep to the first)		(Iwata *et al*. 2013)
81-176Δ*lgtF*	81-176 derivative, *lgtF* mutant (*lgtF* encodes the glycosyltransferase that adds Glc)		(Iwata *et al*. 2013)
81-176Δ*galT*	81-176 derivative, *galT* mutant (*galT* encodes the glycosyltransferase that adds Gal to Glc)		(Iwata *et al*. 2013)
81-176Δ*waaC*c	81-176Δ*waaC*/complemented in trans from pUOA18Km		(Iwata *et al*. 2013)

* WT, wild-type; NCTC, National Collection of Type Cultures; LOS, lipooligosaccharide; Kdo, 2-keto-3-deoxy-d-*manno*-octulosonic acid; P/PEtn, phosphate or phosphoethanolamine; Hep, Heptose; Glc, Glucose; Gal, Galactose; GalNAc, N-acetylgalactosamine; Neu5Ac, N-acetylneuraminic acid.

formation was quantified by measuring the absorbance at 570 nm using a spectrophotometer.

For the microscopic assays, 25 ml of the remaining bacterial cultures was transferred to 50-ml centrifugation tubes, into which sterile glass slides were inserted and incubated for five days at 25°C under atmospheric conditions. After incubation, the slides were removed, washed once with sterile distilled water, and stained with 1 ml of 1% (w/v) CV for 5 min before examining for biofilms using a microscope at 1,000 × magnification.

4. AAG activity assay

The AAG activity assay was performed as previously described by Misawa and Blaser (2000). Briefly, *C. jejuni* strains grown microaerobically on Brucella agar for 24 h were resuspended in 150 mM phosphate-buffered saline (PBS, 8.5 g/l NaCl, 0.2 g/l KCl, 1.44 g/l Na_2HPO_4, and 0.24 g/l KH_2PO_4, pH 7.4) to reach an OD_{600} of 1.0. A volume of 2 ml of cell suspensions was then transferred to 15-ml falcon tubes and incubated at room temperature under atmospheric conditions. After 24 h, 1 ml of the upper phase was carefully removed and OD_{600} was measured.

5. eDNA quantification

eDNA was quantified as described previously (Nakao et al. 2012). After five days of incubation at 25°C under aerobic conditions, as described for the biofilm assay, the bacterial culture was centrifuged (17,400 × g, 5 min) at 4°C. The supernatant was collected and diluted 100-fold with Tris (10 mM)-EDTA (5 mM) (pH 8.0) buffer. The amount of double-stranded DNA (dsDNA) in the supernatant was then measured using Quant-iT PicoGreen dsDNA reagent and kit (Invitrogen).

6. Statistical analysis

All experiments were performed in triplicate. Unless otherwise stated, the quantitative data are presented as means and the error is represented by standard deviation. Significant differences between datasets were determined using a one-way ANOVA (Tukey's method) with Minitab (Minitab 15, Minitab Inc., Minneapolis, MN, USA). The significance levels of the correlations between datasets were determined using fitted-line regression plots (Minitab 15).

Results and discussion

1. Effects of LOS core truncations on *C. jejuni* attachment to glass and biofilm formation under aerobic conditions

To determine the effects of various LOS core truncations on *C. jejuni* attachment to glass and biofilm formation, the numbers of bacterial cells attached to the glass surface and biofilm formation in an atmospheric environment were compared for *C. jejuni* NCTC 11168 and 81-176, and a series of glycosyltransferase mutants. The glycosyltransferase mutants exhibited increased or similar attachment and biofilm formation abilities compared with the wild-type, suggesting that LOS core truncations may increase or have no effects on *C. jejuni* attachment and biofilm formation (Table 2). In particular, both heptosyltransferase I mutants, 11168Δ*waaC* and 81-176Δ*waaC*, attached to glass in significantly ($p < 0.05$) higher numbers and formed larger biofilms than their parent strain, suggesting that LOS truncation of the core of Hep I may increase *C. jejuni* attachment and biofilm formation under aerobic conditions. Truncation of the LOS core of Hep I shows that the *C. jejuni* LOS core lacked the entire oligosaccharide chain, except Kdos (Iwata et al. 2013), which indicates that loss of the LOS core oligosaccharide chain may be required to reduce attachment by *C. jejuni* and biofilm formation.

The LOS core truncations caused by *waaF* or *lgtF* mutations have been shown to enhance biofilm formation by *C. jejuni* 81-176 under microaerobic conditions (Naito et al. 2010). However, under aerobic conditions, these results were only achieved for the NCTC 11168 strain and not the 81-176 strain, which suggests that the incubation conditions may affect the role of the LOS core in biofilm formation by *C. jejuni*. In addition, 11168Δ*cj1136* and 11168Δ*cj1138*, but not 81-176Δ*galT*, exhibited enhanced biofilm formation compared with the wild-type, which shows that the truncations of LOS core caused by these mutations increased biofilm formation by the NCTC 11168 strain but not the 81-176 strain. Based on these results, it appears that LOS core truncations may affect *C. jejuni* biofilm formation in a strain-dependent manner. Similarly, LOS core truncations may also affect *C. jejuni* attachment in a strain-dependent manner because an increase in the attachment numbers with some mutants such as Δ*waaF* was only observed in the NCTC 11168 strain, but not in the 81-176 strain, and also in other wild-type strains in our previous study (Nguyen et al. 2013).

To verify the biofilm results obtained from the CV assays, we used light microscopy to examine those formed by all glycosyltransferase mutants and the wild-type on glass slides in Brucella broth in an atmospheric environment after five days. Multiple photographic images were acquired for each sample, but only one image each for 11168WT, 11168Δ*waaC*, 81-176WT, and 81-176Δ*waaC* biofilms is presented in Fig. 1 In agreement with the results of the CV assays, all glycosyltransferase mutants of the NCTC 11168 strain were found to form larger biofilms than the wild type. For the 81-176 strain, only the 81-176Δ*waaC* mutant formed larger biofilms than the wild type, whereas the other mutants formed biofilms that resembled those formed by the wild type. Complementation of *waaC* gene restored attachment to glass and biofilm formation in NCTC 11168

and 81-176 (Table 2).

2. Effects of LOS core truncations on *C. jejuni* AAG activity and eDNA release

The effects of various LOS core truncations on the *C. jejuni* AAG activity levels are shown in Table 2. In general, glycosyltransferase mutants had weaker or similar AAG activity levels compared with the wild type, thereby suggesting that LOS core truncations may reduce or have no effects on the AAG activity of *C. jejuni*. In particular, the Δ*waaC* and Δ*lgtF* mutants exhibited significantly ($p < 0.05$) lower AAG activity levels compared with the wild type in both the NCTC 11168 and 81-176 strains. However, there were no significant differences ($p > 0.05$) in the AAG activity levels of the *waaF*, *cj1136*, *cj1138*, or *galT* mutants and their parental strains.

In terms of eDNA release, there were no significant ($p > 0.05$) differences in the amounts of eDNA (ca 15 ng/ml) produced by the wild type and all glycosyltransferase mutants after five days of incubation under aerobic conditions (Table 2). These findings suggest that LOS core truncations do not affect eDNA release by *C. jejuni*.

3. Correlations between attachment numbers, AAG activity, eDNA release, and biofilm formation levels

The AAG activity and eDNA release levels have both been suggested as having roles in biofilm formation by *C. jejuni* (Guerry 2007, Svensson et al. 2009). However, correlation analysis showed that the AAG activity ($p = 0.5$, $R^2 = 0.72$) and eDNA levels ($p = 0.48$, $R^2 = 0.03$) had no significant correlations with the biofilm formation levels in all the strains tested, thereby suggesting that the AAG activity level and eDNA release may not have key roles

Table 2. Effects of LOS core truncations from different sugars on the attachment, biofilm formation, AAG activity, and eDNA release levels of *C. jejuni*

Bacterial strains	Attachment numbers (log cell/cm^2) *	5-d biofilms (OD_{570}) †	AAG activity (OD_{600}) ‡	eDNA level (ng/ml) §
11168WT	4.38 ± 0.06 ^{b}¶	0.303 ± 0.071 ^{b}	0.417 ± 0.050 ^{c,d}	14.34 ± 0.44 ^{a}
11168Δ*waaC*	4.89 ± 0.06 ^{e}	0.759 ± 0.112 ^{d}	0.593 ± 0.042 ^{e}	14.28 ± 0.42 ^{a}
11168Δ*waaF*	4.74 ± 0.04 ^{d}	0.526 ± 0.036 ^{c}	0.313 ± 0.089 ^{c}	14.92 ± 0.44 ^{a}
11168Δ*lgtF*	4.64 ± 0.04 ^{d}	0.520 ± 0.085 ^{c}	0.603 ± 0.059 ^{e}	14.58 ± 0.71 ^{a}
11168Δ*cj1136*	4.61 ± 0.10 ^{c,d}	0.495 ± 0.027 ^{c}	0.507 ± 0.004 ^{d}	14.00 ± 0.38 ^{a}
11168Δ*cj1138*	4.44 ± 0.08 ^{b,c}	0.473 ± 0.069 ^{c}	0.352 ± 0.096 ^{c,d}	15.05 ± 1.05 ^{a}
11168Δ*waaCc*	4.44 ± 0.04 ^{b}	0.371 ± 0.107 ^{b,c}	0.418 ± 0.039 ^{c,d}	14.52 ± 0.38 ^{a}
81-176WT	3.89 ± 0.13 ^{a}	0.134 ± 0.042 ^{a}	0.139 ± 0.022 ^{a,b}	14.59 ± 0.61 ^{a}
81-176Δ*waaC*	4.35 ± 0.08 ^{b}	0.325 ± 0.065 ^{b}	0.290 ± 0.094 ^{c}	13.76 ± 0.52 ^{a}
81-176Δ*waaF*	4.13 ± 0.16 ^{a,b}	0.216 ± 0.059 ^{a,b}	0.095 ± 0.023 ^{a}	14.04 ± 0.55 ^{a}
81-176Δ*lgtF*	4.06 ± 0.06 ^{a}	0.202 ± 0.084 ^{a,b}	0.236 ± 0.020 ^{c}	15.09 ± 0.54 ^{a}
81-176Δ*galT*	3.92 ± 0.06 ^{a}	0.140 ± 0.020 ^{a}	0.151 ± 0.027 ^{b}	15.17 ± 0.35 ^{a}
81-176Δ*waaCc*	3.75 ± 0.08 ^{a}	0.129 ± 0.017 ^{a}	0.142 ± 0.040 ^{a,b}	14.09 ± 0.71 ^{a}

* Bacterial attachment was determined based on the number of cells attached to the surface. Glass microscope slides were exposed to cell suspension in Brucella broth at 25°C under an atmospheric environment. After 30 min of exposure, the slides were stained with acridine orange and the number of attached cells was quantified by epifluorescence microscopy.

† Biofilms were measured using the crystal violet assay after five days of incubation in Brucella broth in glass tubes at 25°C under an atmospheric environment.

‡ The AAG activity was determined based on a decrease in the OD_{600} of the upper phase of the cell suspension after 24-h incubation. Cells grown on Brucella agar for 24 h were resuspended in phosphate-buffered saline (pH 7.4) to an OD_{600} of 1.0. The cell suspensions were incubated without shaking at room temperature for 24 h and the OD_{600} of the upper phase was measured. A lower OD_{600} value reflected a higher AAG activity.

§ The eDNA levels in the supernatants of Brucella cultures following the 5-day incubation under aerobic conditions were measured using a Quant-iT PicoGreen dsDNA reagent and kit (Invitrogen).

¶ Representative results are shown as means ± standard deviations, which were calculated based on triplicate experiments. Values in the same column labeled with the same letter do not differ significantly ($p > 0.05$).

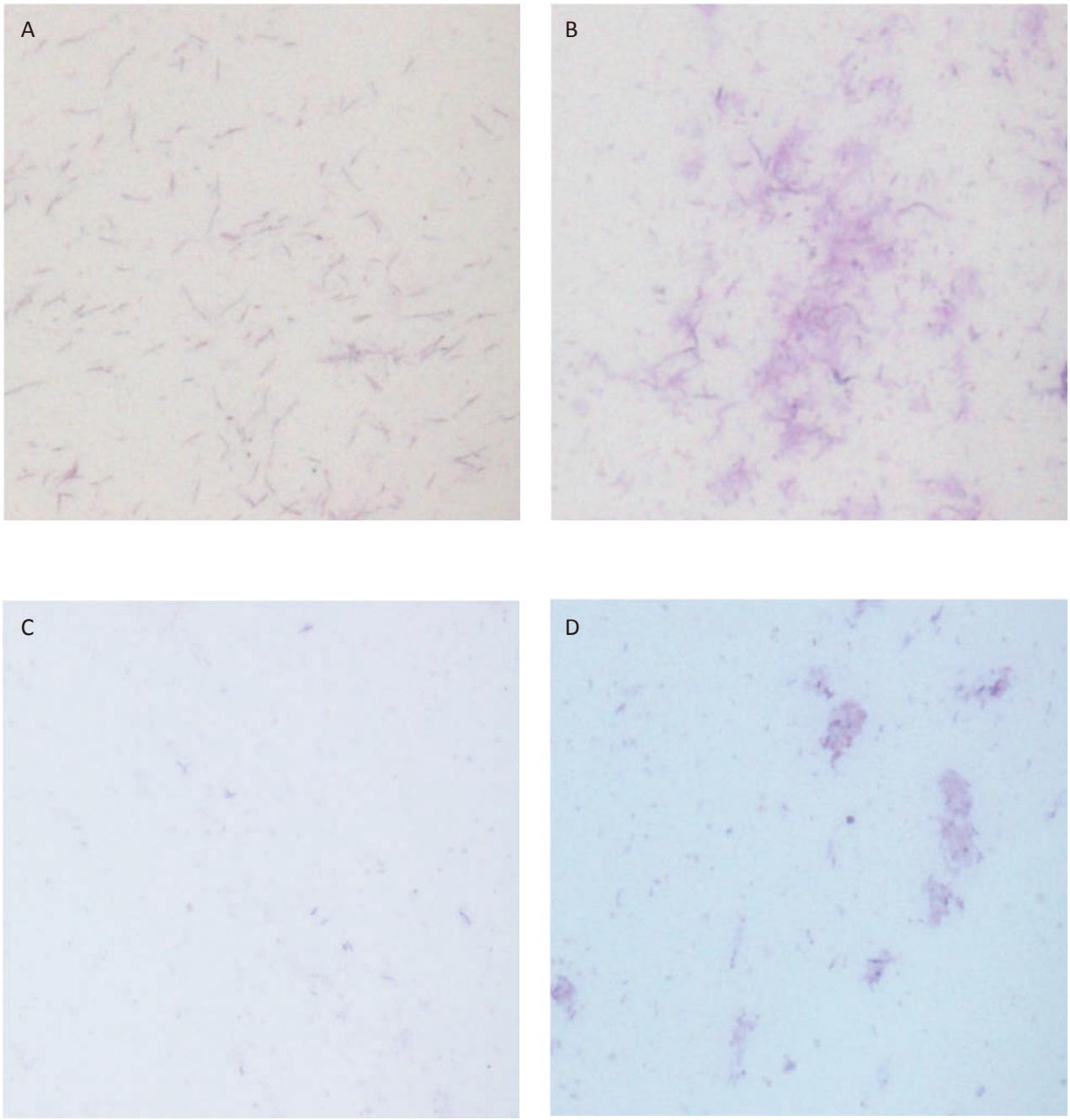


Fig. 1. Representative light microscopy images of *C. jejuni* biofilms on glass slides

Prior to microscopic examination, the slides were stained with 1% crystal violet and the images were acquired at 1,000× magnification. The images show biofilms formed by 11168 wild-type (A), 11168Δ*waaC* (B), 81-176 wild-type (C), and 81-176Δ*waaC* (D) after five days of incubation in Brucella broth under aerobic conditions.

in determining *C. jejuni* biofilm formation under aerobic conditions. However, there was a significant and positive correlation ($p < 0.05$, $R^2 = 0.95$) between the attachment numbers and biofilm formation, thereby indicating that changes in attachment due to LOS core truncations may affect biofilm formation by *C. jejuni*.

Conclusion

In summary, the results of the present study demonstrate that LOS core truncations may either increase or have no effects on the *C. jejuni* attachment and biofilm formation under aerobic conditions, depending on the core oligosaccharide structure. These results highlight the important roles

of the chemical structure or sugar composition of the LOS core oligosaccharide in reducing bacterial attachment and biofilm formation by *C. jejuni* or maintaining the planktonic mode of growth. The changes in *C. jejuni* biofilm formation after LOS core truncations can be predicted based on the attachment ability of glycosyltransferase mutants, but not by their AAG activity or eDNA release levels.

Acknowledgments

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