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Biofilm formation in *Campylobacter jejuni* is increased under aerobic conditions

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ABSTRACT

The microaerophilic human pathogen *Campylobacter jejuni* is the leading cause of food-borne bacterial gastroenteritis in the developed world. During transmission through the food chain and environment, the organism must survive stressful environmental conditions, particularly high oxygen levels. Biofilm formation has been suggested to play a role in environmental survival of this organism. In this work we show that *C. jejuni* NCTC 11168 biofilms developed more rapidly under environmental and food chain-relevant aerobic conditions (20% O₂) when compared to microaerobic conditions (5% O₂, 10% CO₂), although final levels of biofilm were comparable after 3 days. Staining of biofilms with Congo Red gave similar results when compared to the commonly used crystal violet staining. Biofilm formation of non-motile aflagellated strains was lower than those observed with the motile flagellated strain, but nonetheless increased in aerobic conditions, suggesting the presence of flagella-dependent and flagella-independent mechanisms of biofilm formation in *C. jejuni*. Moreover, pre-formed biofilms shed high numbers of viable *C. jejuni* cells into the culture supernatant independent of the oxygen concentration, suggesting continuous passive release of cells into the medium, rather than a condition-specific active mechanism of dispersal. We conclude that under aerobic or stressful conditions, *C. jejuni* adapts to a biofilm lifestyle allowing survival in detrimental conditions, and that such a biofilm can function as a reservoir of viable planktonic cells. The increased formation of biofilm under aerobic conditions is likely to be an adaptation contributing to the zoonotic lifestyle of *C. jejuni.*
INTRODUCTION

Infection with *Campylobacter jejuni* is the leading cause of food-borne bacterial gastroenteritis in the developed world, and is often associated with consumption of undercooked poultry products (18). The UK Health Protection Agency reported over 45,000 laboratory-confirmed cases for England and Wales in 2006 alone, although this is thought to be a five- to tenfold underestimation of the total number of community incidents (20, 43). The symptoms associated with *C. jejuni* infection usually last between 2 to 5 days and include diarrhoea, vomiting, and stomach pains. Sequelae of *C. jejuni* infection include more serious autoimmune diseases like Guillain–Barre syndrome, Miller–Fisher syndrome (19), and Reactive Arthritis (15).

Poultry represents a major natural reservoir for *C. jejuni*, as the organism is usually considered to be a commensal, and can reach densities of up to $1 \times 10^8$ CFU g$^{-1}$ ceecal contents (35). As a result, large numbers of bacteria are shed via faeces into the environment and consequently, *C. jejuni* can rapidly spread through a flock of birds in a broiler house (1). Whilst well adapted to life in the avian host, *C. jejuni* must survive during transit between hosts, and on food products in stressful storage conditions including high and low temperature and atmospheric oxygen levels. The organism must therefore have mechanisms to protect itself from unfavourable conditions.

Biofilm formation is a well characterized bacterial mode of growth and survival, as the surface-attached and matrix-encased bacteria are protected from stressful environmental conditions such as UV radiation, predation, and desiccation (7, 8, 28). Bacteria in biofilms are also known to be >1000-fold more resistant to disinfectants and antimicrobials when compared to their planktonic counterparts (11). Several reports have now shown that *Campylobacter* species are capable of forming a monospecies biofilm (21, 22) and can
colonise a pre-existing biofilm (14). Biofilm formation can be demonstrated under laboratory conditions, and environmental biofilms, from poultry rearing facilities, have been shown to contain *Campylobacter* (5, 32, 44). *Campylobacter* biofilms allow survival of the organism up to twice as long under atmospheric conditions (2, 21) and in water systems (27).

A molecular understanding of biofilm formation in *Campylobacter* is still in its infancy, although there is evidence for the role of flagella and gene regulation in biofilm formation. Indeed, a *flaAB* mutant shows reduced biofilm formation (34), mutants defective in flagellar modification (*cj1337/lmaf5*) and assembly (*fliS*) are defective in adhering to glass surfaces (21), and a proteomic study of biofilm-grown cells shows increased levels of motility-associated proteins including FlaA, FlaB, FliD, FlgG, and FlgG2 (22). Flagella are also implicated in adhesion, biofilm formation and development in other bacterial species including *Aeromonas, Vibrio, Yersinia, and Pseudomonas* (3, 23, 24, 31, 42).

Previous studies on *Campylobacter* biofilms have mostly focused on biofilm formation under standard microaerobic laboratory conditions. In this work we have examined biofilm formation of motile and non-motile *C. jejuni*, in atmospheric conditions that are relevant to the survival of this organism in a commercial context of environmental and food-based transmission.
MATERIALS AND METHODS

C. jejuni strains and growth conditions

Campylobacter jejuni strains were cultured in a MACS-MG-1000 controlled atmosphere cabinet (Don Whitley Scientific) under microaerobic conditions (85% N\textsubscript{2}, 5% O\textsubscript{2}, 10% CO\textsubscript{2}) at 37°C. For growth on plates, strains were either grown on Brucella agar, blood plates (Blood Agar Base 2 (BAB), 1% yeast extract, 5% horse blood (Oxoid)), or BAB with Skirrow supplements (10 µg ml\textsuperscript{-1} vancomycin, 5 µg ml\textsuperscript{-1} trimethoprim, 2.5 IU polymyxin-B). Broth culture was carried out in Brucella broth (Becton, Dickinson & Company). A Jouan EB115 incubator was used for aerobic culture at 37°C and a Sanyo MCO-20 AIC incubator was used for culture in 10% CO\textsubscript{2} in air at 37°C.

Two variants of C. jejuni strain NCTC 11168 were used: a motile strain (11168Mot) and its non-motile (aflagellated) derivative (11168Non-mot). A C. jejuni NCTC 11168 flaAB mutant (11168Mot::flaAB) was created by transformation of the motile strain with chromosomal DNA from C. jejuni strain R2 (81116 flaAB::Km\textsuperscript{R}) (41) using standard protocols (16, 39).

Motility and autoagglutination assays

Motility of C. jejuni was assessed in soft-agar plates, as described previously (21, 22). For soft-agar assays, 5 µl of an overnight culture was spotted on 0.4% agar Brucella plates, left to dry for 30 minutes and incubated under microaerobic conditions for 2 days. Autoagglutination (i.e. cell clumping and sedimentation) was measured as described previously (12, 17), by monitoring the decrease in A\textsubscript{600} following incubation in a cuvette at room temperature in aerobic conditions.
Crystal Violet biofilm assays

Crystal violet staining was used for measuring biofilm formation, as described previously for *C. jejuni* and other bacteria (2, 9, 29). For each assay, a 50 µl single-use glycerol stock, routinely stored at -80°C, was plated on a BAB plate with Skirrow supplements and these cells were used to inoculate fresh Brucella broth. Cultures were grown microaerobically with shaking overnight at 37°C. The overnight culture was diluted to ~ 1 × 10⁹ CFU ml⁻¹ in fresh Brucella broth and 1 ml added to a sterile borosilicate glass test tube. Tubes were incubated without shaking at 37°C under microaerobic, aerobic, or 10% CO₂ in air conditions. Three replicates for each strain in each condition were used for each assay; three independent experiments were conducted. To determine the number of viable cells, prior to crystal violet staining, a sample of the planktonic cells was serially diluted in PBS and dilutions plated on Brucella agar plates. After two days of growth, colonies were counted and CFU ml⁻¹ calculated.

For crystal violet staining, tubes were washed with water then dried at 60°C for 30 minutes. One ml of 1% crystal violet solution was added and the tubes were incubated on a rocker at room temperature for 30 minutes. Unbound crystal violet was washed off with water and the tubes were dried at 37°C. Bound crystal violet was dissolved in 20% acetone in ethanol for 10 minutes, poured into cuvettes and the A₅₉₀ measured.

Microscopy

25 ml of sterile Brucella broth was inoculated with 750 µl overnight culture (~ 1×10⁹ cells). Sterile twin-frost microscope slides (VWR International) were inserted in the tubes and grown without shaking under microaerobic or aerobic conditions. After 1 to 5 days, slides were removed and washed once with distilled water. One side was cleaned and the other side examined using a Nikon Eclipse 50i microscope at ×400 and ×1000 magnification. For crystal
violet staining, slides were stained with 1% crystal violet for 5 minutes then washed with water to remove unbound crystal violet. Microcolony pixel area was measured using ImageJ software (version 1.41; National Institute of Health [http://rsbweb.nih.gov/ij/]).

Congo Red assay

Overnight cultures were prepared as described for the crystal violet biofilm assays. The overnight culture was diluted to ~ $1 \times 10^9$ CFU ml$^{-1}$ in fresh Brucella broth, supplemented with 0.01% (w/v) Congo Red (Hopkin and Williams Ltd, UK), and 1 ml was added to sterile borosilicate glass test tubes (Corning, UK). Tubes were incubated without shaking under either microaerobic, aerobic, or 10% CO$_2$ in air conditions at 37°C for 2 days. Culture supernatant was carefully removed with a pipette and the tubes washed with 500 µl PBS (10 mM phosphate buffer, 137 mM NaCl, pH 7.5) to remove unbound Congo Red. Tubes were dried for 30 minutes at 60°C and developed in 1 ml 50% ethanol in PBS pH 7.5 for 10 minutes before reading the $A_{500}$ of the solution. Three technical replicates were used for each condition, and data were obtained from three independent experiments.

Shedding of viable cells from pre-formed biofilms

Two day-old C. jejuni biofilms were incubated aerobically as described in the biofilm assay section above. Instead of washing and staining with crystal violet, tubes were washed twice with 1 ml sterile PBS and 1 ml fresh sterile Brucella broth added to each tube. Viable cells in the culture supernatant were determined by plating serial dilutions on Brucella agar plates immediately following washing or after 24 hours at 37°C under microaerobic or aerobic conditions. Data were obtained from three independent experiments.
RESULTS

Loss of motility negatively affects biofilm formation by *C. jejuni*

To determine the role of flagellar motility in biofilm formation by *C. jejuni*, we first isolated a non-motile derivative of motile *C. jejuni* NCTC 11168. This approach is based on the relatively high frequency of loss of motility by *C. jejuni* strains (26), and has the advantage of not requiring genetic modification with antibiotic resistance cassettes. We observed that under laboratory growth conditions, the motility of *C. jejuni* NCTC 11168 will diminish following continued culture. Following several rounds of sub-culture an entirely non-motile variant was obtained. This loss of motility was monitored using swarm plates, light microscopy and auto-agglutination assays (data not shown). The non-motile strain did not produce flagella, and had a shorter doubling time compared to the motile strain (100 minutes compared to 120 minutes), perhaps reflecting a diversion of energy away from flagellar biosynthesis, assembly and rotation.

Biofilm formation was compared between the non-motile variant (11168Non-mot) and the motile strain (11168Mot) after static incubation for 2 days at 37°C. Under microaerobic conditions, the motile strain formed > 50% more biofilm when compared to the non-motile strain (Figure 1A). The culture supernatants of both strains contained ~1 x 10⁹ viable cells, suggesting that the difference is not due to a difference in viability between the two strains (Figure 1C). To test if the lack of biofilm phenotype in the non-motile strain was due to the absence of flagella, we constructed a flaAB deletion strain as described in the Materials and Methods section. The 11168Mot::flaAB mutant was confirmed to be non-motile using auto-agglutination, light microscopy and swarm-plates. Biofilm formation of the 11168Mot::flaAB mutant, under microaerobic conditions, was about half that of the motile strain and similar to the non-motile variant (Fig. 1A). Again this difference was not due to differences in viability,
as equivalent numbers of viable cells were recovered from the culture supernatant (Figure 1C).

**Biofilm formation is increased under aerobic conditions**

In the food chain and during transfer between hosts, *C. jejuni* will be exposed to stressful levels of oxygen (>10% O₂). We therefore examined biofilm formation under aerobic conditions where biofilms may be relevant as a survival mechanism. As a control, we also tested 10% CO₂ in air conditions, which is the same CO₂ concentration used during microaerobic culture. Biofilm formation of the motile wild-type was double the level of biofilm observed under aerobic conditions, when compared to microaerobic conditions (Figure 1A). Interestingly, in the presence of 10% CO₂, biofilm formation was reduced to levels similar as observed in microaerobic conditions (Figure 1A). All cultures contained similar numbers of viable cells after the 2 day incubation, suggesting that these observations were not due to differences in viability (Fig. 1C). However, microscopic examination of the culture supernatants from the aerobic and 10% CO₂ in air samples showed many elongated cells suggesting that the cells are stressed (data not shown). After three days incubation, levels of biofilm formation in the aerobic and microaerobic samples were equivalent, suggesting that aerobic conditions result in more rapid biofilm formation when compared to microaerobic conditions (Figure 2).

Incubation in aerobic conditions also stimulated biofilm formation of 11168Non-mot and the 11168Mot::*flaAB* mutant, although overall levels were lower compared to the motile strain (Figure 1A). Interestingly, under 10% CO₂ in air, the level of biofilm formation in all strains is approximately the same. Again, all cultures contained equivalent numbers of viable cells (Fig. 1C). Biofilm formation was not increased under aerobic conditions at 20°C and 4°C as judged by crystal violet staining, suggesting a role for cellular biosynthetic processes...
in biofilm formation (data not shown).

C. jejuni biofilms bind Congo Red

Previous reports have shown that Congo Red binds to the extracellular component of microbial biofilms (10). To establish whether Congo Red can be used as an alternative method for measuring biofilm formation in C. jejuni, we incubated static cultures of C. jejuni in Brucella media supplemented with 0.01% Congo Red under the aforementioned environmental conditions. We observed staining of the C. jejuni biofilms with this dye, and were able to measure the level of staining by dissolving the Congo Red in 50% ethanol. Using this assay, we observed more staining of the motile strain incubated in aerobic conditions compared to microaerobic and 10% CO₂ in air conditions (Figure 1B), supporting the conclusion drawn from the crystal violet assays (Fig. 1A) that aerobic conditions result in increased biofilm formation in C. jejuni.

Motile C. jejuni form a thick biofilm at the air-surface interface

To demonstrate that the data obtained using the crystal violet and Congo Red biofilm assays were a result of C. jejuni cells binding to the borosilicate glass, we observed the formation of biofilms on sterile microscope slides directly using light microscopy. For the motile strain, microcolonies could be observed at the air-surface interface after 1 day incubation under microaerobic conditions. Incubation for more than 1 day resulted in a thick biofilm at the air-surface interface (Figure 3A). After 1 day incubation, microcolonies were approximately tenfold larger in aerobic conditions (median pixel area = 3.7 × 10⁵) compared to microaerobic conditions (median pixel area = 2.5 × 10⁴; p = <0.01, Kruskal-Wallis test). After 2 to 3 days incubation, these microcolonies had developed into a thick biofilm at the air-surface interface (Figure 3B). In contrast to the motile strain, the non-motile strain formed a thin biofilm at the
air-surface interface after 2 days incubation in both microaerobic and aerobic conditions

supporting the crystal violet and Congo Red assays (Figure 3C, 3D).

Campylobacter biofilms passively shed viable cells

Microscopic examination of culture supernatants from 5 day old biofilms grown under microaerobic conditions showed the presence of bacterial flocs in the supernatant, shed from the biofilm. To study the release of such cells from a pre-formed biofilm, we used a 2-day old aerobic biofilms of strain 11168Mot, which were first washed with sterile PBS pH 7.5, and subsequently incubated in fresh Brucella broth for up to 24h. We assayed for viable cells before washing, immediately following the washing and 24 hours incubation in fresh medium.

Before washing, the medium contained ~1 × 10⁹ viable cells (Fig. 4A). Following washing, we observed 1 × 10⁶ (± 1 log) viable cells in the washes (Figure 4A). After 24 hours, static Brucella cultures contained a 3 log increase in viable cells after incubations under both aerobic and microaerobic conditions, reaching viable cell numbers seen in the pre-wash supernatants. When the 11168Mot and the 11168Mot::flaAB mutant strain were compared, there was 1 log fewer viable cells in the wash fractions but no difference in the samples incubated for 24h in aerobic or microaerobic conditions (Figure 4A). The 3 log increase in viable cells seen in aerobic conditions is unlikely to be the result of growth, as it was observed equally in aerobic and microaerobic conditions, and therefore is likely to represent shedding of cells from the pre-formed biofilm. This experiment shows that a C. jejuni biofilm can act as a reservoir of a potentially high number of viable cells.
DISCUSSION

One of the conundrums of zoonotic diseases caused by *C. jejuni* is that the organism is a very successful pathogen which survives transmission in stressful aerobic conditions, but that the organism itself is an obligate microaerophile which survives poorly in controlled aerobic conditions. Compared to other foodborne pathogens like *E. coli* and *Salmonella enterica* serovar Typhimurium, *C. jejuni* has a low infectious dose (500-800 CFU, see reference 4). While this may contribute to infection, it is still unclear what allows the bacterium to survive during transmission in aerobic conditions. Survival in a biofilm would be an explanation, and in our study we have demonstrated that biofilm formation of *C. jejuni* is clearly increased under aerobic conditions, that the presence of flagella-dependent motility results in increased biofilm formation, and that biofilms are a reservoir of viable cells.

It was previously reported that flagellar expression is required for biofilm formation by *C. jejuni* in microaerobic conditions (21, 22, 34), and our results comparing the motile wild-type strain with both a non-motile strain and a *flaAB* mutant are in agreement with these previous studies (21, 34). Likewise, in other bacterial species, loss of flagella and motility defects are often shown to result in a biofilm defect (3, 23, 24, 31, 42). We observed though that the absence of flagella does not completely abolish biofilm formation, as aflagellate *C. jejuni* also display increased biofilm formation under aerobic conditions (Fig. 1A). Hence, in *C. jejuni* biofilms, flagella may improve or facilitate initial attachment or biofilm structuring, but are not essential for this process. Flagellar motility is however likely to be critical for motility towards a pre-existing biofilm. In our experiments, in a growing biofilm, we cannot distinguish between cell division within the biofilm and recruitment of planktonic cells to an existing biofilm, however, an initial attachment stage is necessary for the initiation of biofilm formation. In light of our data, we suggest that there may be both flagella-dependent and
flagella-independent mechanisms of attachment and biofilm formation in *C. jejuni*. In addition to the role of flagella in surface attachment (17), the flagella may also be co-opted as a system for secretion of non-flagellar extracellular proteins, as was shown for FlaC (36), CiaB (25), and FspA (33). These secreted proteins may contribute to the biofilm lifestyle. The correlation between autoagglutination and biofilm formation is in agreement with published experiments (17) that show flagella glycosylation mutants have both an autoagglutination and biofilm defect. Clearly, the non-motile strains used in this study represent the extreme end of this scale given that they are devoid of flagella.

The observation of bacterial flocs in the supernatant of biofilm cultures and the relatively high numbers of cells liberated from a pre-formed biofilm show that viable cells are readily shed from a biofilm (Fig. 4A). In other organisms, biofilm dispersal can be a coordinated response to environmental signals, such as nutrient-induced dispersal in *Pseudomonas aeruginosa* (30) or flow-induced dispersal in *Shewanella oneidensis* (38). *C. jejuni* may lack this coordinated response and instead rely on continual shedding of cells into the environment resulting in new populations of planktonic cells. Under unfavourable conditions, these cells may die or re-attached to an existing biofilm; however, under favourable conditions, the cells will go on to colonize relevant niches, such as the poultry host (visualized in Figure 4B). We observed no difference in shedding between motile and non-motile stains, suggesting this process is independent of flagella and motility. Clearly, in an environmental setting, motility would be crucial for colonization of new niches/host.

The observation that biofilm formation is enhanced under aerobic conditions suggests that *C. jejuni* may be well adapted for survival in the environment in a biofilm. Indeed, under static microaerobic conditions, we can recover viable cells from a biofilm after 50 days of culture (data not shown). The detection of viable cells released by aerobically formed biofilms is consistent with our hypothesis of biofilm-mediated survival of *C. jejuni* during transmission.
in the food chain or environment. Moreover, we can postulate that the biofilm may provide a microaerobic environment suitable for growth or survival, generating viable cells that are eventually shed into the environment. Indeed, our washing assay clearly demonstrates the role of a biofilm as a reservoir of viable cells. A study of Campylobacter in multi-species biofilms showed that the species composition of the biofilm is in flux, with changes of up to 40% every 24 hours demonstrating the role of release of cells from a biofilm (14). Oxygen has been shown to penetrate a P. aeruginosa biofilm to a depth of 90 µm (40) indicating a role of the biofilm in protecting cells from oxygen. In this study, it is not possible to know the growth phase of the planktonic cells in the aerobic culture. However, mutations in genes that affect stationary phase (polyphosphate kinase 1 and the ppGpp biosynthesis protein SpoT) appear to play a role in biofilm formation (6, 29).

A recent study postulated that biofilm-grown cells are poorer colonizers of chicks compared to planktonic cells (13). However, their model of biofilm was agar grown cells, and while this is an adherent lifestyle, it is perhaps not the most appropriate biofilm model. Our data suggests that in the environment, a C. jejuni biofilm will more likely act as a reservoir of motile bacteria that can subsequently colonize chicks.

Many questions remain about the role of biofilm formation as an environmental protection mechanism. We have shown that under a relevant environmental stress, biofilm formation is increased; however, further work is necessary to define the signaling mechanisms underlying this response. A number of regulatory proteins have shown to have a role in biofilm formation in C. jejuni. Deletion of the gene encoding a histidine kinase sensor (cprS) enhances biofilm formation (37) while absence of the global regulator CsrA causes a biofilm defect (9). The data presented herein may shed new light on the role of these regulators with respect to environmental sensing. Indeed, one can speculate that these regulators may be involved in integrating increased oxygen levels into a global transcription response resulting in a change
from a planktonic to a biofilm lifestyle.

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LEGENDS TO FIGURES

Figure 1
Biofilm formation of *C. jejuni* NCTC 11168 motile variant (11168Mot), the non-motile variant (11168Non-mot), and the *flaAB* mutant (11168Mot::*flaAB*) after incubation for 2 days at 37°C in static microaerobic (black bars), aerobic (white bars), and 10% CO₂ in air (grey bars) conditions. Results shown are the average from three independent experiments each performed in triplicate. A) Crystal violet assay for biofilm formation. B) Congo Red assay for biofilm formation. C) Viable planktonic cells from biofilm supernatant from the crystal violet assay. The error bars represent standard deviations calculated from the three independent experiments performed.

Figure 2
Biofilm formation of *C. jejuni* NCTC 11168 motile variant over 3 days under static microaerobic (black bars) and aerobic (white bars) incubation, as determined using crystal violet staining. Results are the average from three independent experiments, each containing triplicate samples. The error bars represent standard deviations calculated from the three independent experiments performed.

Figure 3
Representative light microscopy photographs of *C. jejuni* biofilms after 2 days incubation at 37°C. Slides were stained with 1% crystal violet and photographed at ×400 magnification. A) 11168Mot grown in microaerobic conditions. B) 11168Mot grown in aerobic conditions. C) 11168Non-mot grown in microaerobic conditions. D) 11168Non-mot grown in aerobic conditions.
**Figure 4**

A) Viable cells in the supernatant following 2 days aerobic biofilm formation (Pre-wash – dark grey bars), washing with sterile PBS (Wash – white bars) and following 24 hours static incubation in fresh Brucella broth under either aerobic (black bars) or microaerobic (light grey bars) conditions at 37°C. Values are the means of least three independent experiments, error bars represent standard deviations calculated from the three independent experiments. B) Model of *C. jejuni* biofilm showing the fate of cells following release from a pre-formed biofilm. After release from a biofilm, planktonic cells can proliferate under favourable conditions or may re-attach to an existing biofilm. Cells may also die under conditions that preclude biofilm formation (i.e. in a fast flowing system).
**Figure 1**

A. Crystal violet staining (A$_{590}$)

B. Congo Red staining (A$_{500}$)

C. Viable cells (CFU ml$^{-1}$)
Crystal violet staining (A590)

Day 1  Day 2  Day 3
A. 

B. 

1. Proliferation in favourable conditions 
2. Cell death 
3. Shedding of viable cells into medium or environment 
4. Re-attachment to existing biofilm 

\[ \text{CFU ml}^{-1} \]