Biofilm Formation by *Campylobacter jejuni* Is Increased under Aerobic Conditions

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Received 5 August 2009/Accepted 31 January 2010

Infection with *Campylobacter jejuni* is the leading cause of food-borne bacterial gastroenteritis in the developed world and is often associated with the consumption of undercooked poultry products (19). The United Kingdom Health Protection Agency reported more than 45,000 laboratory-confirmed cases for England and Wales in 2006 alone, although this is thought to be a 5- to 10-fold underestimation of the total number of community incidents (20, 43). The symptoms associated with *C. jejuni* infection usually last between 2 and 5 days and include diarrhea, vomiting, and stomach pains. Sequelae of *C. jejuni* infection include more-serious autoimmune diseases, such as Guillain-Barré syndrome, Miller-Fisher syndrome (18), and reactive arthritis (15).

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

Biofilm formation is a well-characterized bacterial mode of growth and survival, where 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 more resistant to disinfectants and antimicrobials than their planktonic counterparts (11). Several reports have now shown that *Campylobacter* species are capable of forming a monospecies biofilm (21, 22) and can colonize a preexisting 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 the organism to survive up to twice as long under atmospheric conditions (2, 21) and in water systems (27).

Molecular understanding of biofilm formation by *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*) 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 and in biofilm formation and development in other bacterial species, including *Aeromonas*, *Vibrio*, *Yersinia*, and *Pseudomonas* species (3, 23, 24, 31, 42).

Previous studies of *Campylobacter* biofilms have focused mostly on biofilm formation under standard microaerobic laboratory conditions. In this work we have examined the formation of biofilms by motile and nonmotile *C. jejuni* strains under 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% N₂, 5% O₂, 10% CO₂) at 37°C. For growth on plates, strains were either grown on brucella agar, on blood plates (Blood Agar Base no. 2 [BAB], 1% yeast extract, 5% horse blood [Oxoid]), or on BAB with Skirrow supplements (10 μg ml⁻¹ vancomycin, 5 μg ml⁻¹ trimethoprim, 2.5 IU polymixin B). Broth culture was carried out in brucella broth (Becton, Dickinson and Company). A Jouan EB115 incubator was used for aerobic culture at 37°C, and a Sanyo MCO-20AIC incubator was used for culture under 10% CO₂ in air at 37°C.

Two variants of C. jejuni strain NCTC 11168 were used: a motile strain (11168Mot) and its nonmotile (flagellate) 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:Km³) (41) using standard protocols (16, 39).

Motility and autoagglutination assays. The motility of C. jejuni was assessed on soft agar plates as described previously (2). For soft-agar assays, 5 μl of an overnight culture was spotted onto brucella medium supplemented with 0.4% agar, left to dry for 30 min, 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 A₅₅₀ following incubation in a cuvette at room temperature under aerobic conditions.

Crystal violet biofilm assays. Crystal violet staining was used for measuring biofilm formation, as described previously for C. jejuni (12). For crystal violet staining, slides were examined biofilm formation under aerobic conditions, where C. jejuni biofilm assays were reduced to levels similar to those obtained. This loss of motility was monitored using swarm plates, light microscopy, and autoagglutination assays (data not shown). The nonmotile strain did not produce flagella and had a shorter doubling time than the motile strain (100 min compared to 120 min), perhaps reflecting a diversion of energy away from flagellar biosynthesis, assembly, and rotation.

Biofilm formation by the nonmotile variant (11168Non-mot) and the motile strain (11168Mot) was compared after static incubation for 2 days at 37°C. Under microaerobic conditions, the motile strain formed >50% more biofilm than the nonmotile strain (Fig. 1A). The culture supernatants of both strains contained ~1 × 10⁷ viable cells, suggesting that differences in viability were significant. To test if the lack of a biofilm phenotype for the nonmotile strain was due to the absence of flagella, we constructed a flaAB deletion strain as described in Materials and Methods. The 11168Mot::flaAB mutant was confirmed to be nonmotile by using autoagglutination, light microscopy, and swarm plates. The level of biofilm formation by the 11168Mot::flaAB mutant, under microaerobic conditions, was about half that of the motile strain and similar to that of the nonmotile variant (Fig. 1A). Again, this difference was not due to differences in viability, but due to the absence of flagella. We used the 11168Mot::flaAB mutant, under microaerobic conditions, as a control for this experiment.

Biofilm formation is increased under aerobic conditions. In the food chain and during transfer between hosts, C. jejuni is 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, which is the same CO₂ concentration used during microaerobic culture. The level of biofilm formation by the motile wild-type strain under aerobic conditions was double that observed under microaerobic conditions (Fig. 1A). Interestingly, in the presence of 10% CO₂, biofilm formation was reduced to levels similar to those observed under microaerobic conditions (Fig. 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 supernatants from cultures grown under aerobic conditions and those grown under 10% CO₂ in air showed many elongated cells, suggesting that the cells were stressed...
After 3 days of incubation, levels of biofilm formation in the aerobic and microaerobic samples were equivalent, suggesting that aerobic conditions result in more-rapid biofilm formation than microaerobic conditions (Fig. 2).

Incubation under aerobic conditions also stimulated biofilm formation by 11168Non-mot and the 11168Mot::flaAB mutant, although overall levels were lower than those for the motile strain (Fig. 1A). Interestingly, under 10% CO2 in air, the levels of biofilm formation by all strains were 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 medium supplemented with 0.01% Congo red under the environmental conditions mentioned above. We observed staining of the C. jejuni biofilms with this dye, and we 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 when it was incubated under aerobic conditions than when it was incubated under microaerobic conditions or under 10% CO2 in air (Fig. 1B), supporting the conclusion drawn from the crystal violet assays (Fig. 1A) that aerobic conditions result in increased biofilm formation by C. jejuni.

The motile C. jejuni strain forms a thick biofilm at the air-surface interface. To demonstrate that the data obtained using the crystal violet and Congo red biofilm assays were results of the binding of C. jejuni cells to the borosilicate glass, we observed the formation of biofilms on sterile microscope slides directly by using light microscopy. For the motile strain, microcolonies could be observed at the air-surface interface after 1 day of incubation under microaerobic conditions. Incubation for more than 1 day resulted in a thick biofilm at the air-surface interface (Fig. 3A). After 1 day of incubation, microcolonies were approximately 10-fold larger under aerobic conditions than under microaerobic conditions (median pixel area, 3.7 × 105) than under microaerobic conditions (median pixel area, 2.5 × 104) (P < 0.01 by the Kruskal-Wallis test). After 2 to 3 days of incubation, these microcolonies had developed into a thick biofilm at the air-surface interface (Fig. 3B). In contrast to the motile strain, the nonmotile strain formed a thin biofilm at the air-surface interface after 2 days of incubation under both microaerobic and aerobic conditions, supporting the findings of the crystal violet and Congo red assays (Fig. 3C and D).
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, shed from the biofilm, in the supernatant. To study the release of such cells from a preformed biofilm, we used 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 as long as 24 h. We assayed for viable cells before washing, immediately after washing, and after 24 h of incubation in fresh medium. Before washing, the medium contained $1 \times 10^9$ viable cells (Fig. 4A). After washing, we observed $1 \times 10^6$ (±1 log) viable cells in the washes (Fig. 4A). After 24 h of incubation under either aerobic or microaerobic conditions, static brucella broth cultures showed a 3-log-unit increase in the number of viable cells, equivalent to the prewash supernatants. When the 11168Mot strain and the 11168Mot::flaAB mutant strain were compared, there were 1 log fewer viable cells in the wash fractions, but no difference was observed in the samples incubated for 24 h under aerobic or microaerobic conditions (Fig. 4A). The 3-log-unit increase in the number of viable cells seen under aerobic conditions is unlikely to be the result of growth, since it was observed equally under aerobic and microaerobic conditions; therefore, it is likely to represent the shedding of cells from the preformed 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 during transmission under stressful aerobic conditions, yet it is an obligate microaerophile which survives poorly under controlled aerobic conditions. Compared to other foodborne pathogens, such as Escherichia coli and Salmonella enterica serovar Typhimurium, C. jejuni has a low infectious dose (500 to 800 CFU [see reference 4]). While this may contribute to infection, it remains unclear what allows the bacterium to survive during transmission under aerobic conditions. Survival in a biofilm would be an explanation, and in our study we have demonstrated that the level of biofilm formation by C. jejuni is clearly increased under aerobic conditions, that the presence of flagellum-dependent motility results in increased biofilm formation, and that biofilms are a reservoir of viable cells.

It has been reported previously that flagellar expression is required for biofilm formation by C. jejuni under microaerobic conditions (21, 22, 34), and our results comparing the motile wild-type strain with both a nonmotile strain and a flaAB mutant are in agreement with the findings of these previous studies (21, 34). Likewise, in other bacterial species, loss of flagella and motility defects have often been 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, since aflagellate C. jejuni strains also display increased...
biofilm formation under aerobic conditions (Fig. 1A). Hence, in \textit{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 toward a preexisting 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 flagellum-dependent and flagellum-independent mechanisms of attachment and biofilm formation in \textit{C. jejuni}. In addition to the role of flagella in surface attachment (17), the flagella may also be coopted as a system for the secretion of nonflagellar extracellular proteins, as has been 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) showing that flagellar glycosylation mutants have both an autoagglutination and a biofilm defect. Clearly, the nonmotile 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 supernatants of biofilm cultures and the relatively high numbers of cells liberated from a preformed 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 \textit{Pseudomonas aeruginosa} (30) or flow-induced dispersal in \textit{Shewanella oneidensis} (38). \textit{C. jejuni} may lack this coordinated response and may instead rely on continual shedding of cells into the environment, resulting in new populations of planktonic cells. Under unfavorable conditions, these cells may die or reattach to an existing biofilm; however, under favorable conditions, the cells will go on to colonize relevant niches, such as the poultry host (visualized in Fig. 4B). We observed no difference in shedding between motile and nonmotile stains, suggesting that this process is independent of flagella and motility. Clearly, in an environmental setting, motility would be crucial for the colonization of new niches/hosts.

The observation that biofilm formation is enhanced under aerobic conditions suggests that \textit{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 \textit{C. jejuni} during transmission in the food chain or the 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 \textit{Campylobacter} in multispecies biofilms showed that the species composition of the biofilm is in flux, with changes of as much as 40% every 24 h, demonstrating the role of release of cells from a biofilm (14). Oxygen has been shown to penetrate a \textit{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 the 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 than planktonic cells (13). However, those investigators’ model of the biofilm was agar-grown cells, and while this is an adherent lifestyle, it is perhaps not the
most appropriate biofilm model. Our data suggest 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, the level of biofilm formation is increased; however, further work is necessary to define the signaling mechanisms underlying this response. A number of regulatory proteins have been shown to have a role in biofilm formation by C. jejuni. Deletion of the gene encoding a histidine kinase sensor (cprS) enhances biofilm formation (37), while the absence of the global regulator CsrA causes a biofilm defect (9). The data presented here 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.

ACKNOWLEDGMENTS

This work is supported by the Institute Strategic Programme Grant from the Biotechnology and Biological Sciences Research Council (BBSRC) of the United Kingdom to the IFR. We acknowledge the members of the Campylobacter research group at the IFR for helpful comments and suggestions. We also acknowledge Carmen Pin for statistical advice.

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