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Biofilm Formation by *Campylobacter jejuni* Is Increased under Aerobic Conditions[▽]

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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 the environment, the organism must survive stressful environmental conditions, particularly high oxygen levels. Biofilm formation has been suggested to play a role in the 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% O_2) than under microaerobic conditions (5% O_2, 10% CO_2), although final levels of biofilms were comparable after 3 days. Staining of biofilms with Congo red gave results similar to those obtained with the commonly used crystal violet staining. The level of biofilm formation by nonmotile aflagellate strains was lower than that observed for the motile flagellated strain but nonetheless increased under aerobic conditions, suggesting the presence of flagellum-dependent and flagellum-independent mechanisms of biofilm formation in *C. jejuni*. Moreover, preformed biofilms shed high numbers of viable *C. jejuni* cells into the culture supernatant independently of the oxygen concentration, suggesting a 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 under detrimental conditions, and that such a biofilm can function as a reservoir of viable planktonic cells. The increased level of biofilm formation under aerobic conditions is likely to be an adaptation contributing to the zoonotic lifestyle of *C. jejuni*.

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 1×10^8 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 >1,000-fold 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

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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_2, 5% O_2, 10% CO_2) 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 polymyxin 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% CO_2 in air at 37°C.

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

Motility and autoagglutination assays. The motility of *C. jejuni* was assessed on soft-agar plates, as described previously (22). 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 A_{600} 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* and other bacteria (2, 9, 29). For each assay, a 50-μl single-use glycerol stock, routinely stored at −80°C, was plated onto 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 $\sim 1 \times 10^9$ CFU ml^{-1} in fresh brucella broth, and 1 ml was added to a sterile borosilicate glass test tube. Tubes were incubated without shaking at 37°C under microaerobic or aerobic conditions, or under 10% CO_2 in air. Three replicates for each strain under 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 phosphate-buffered saline (PBS), and dilutions were plated onto brucella agar plates. After 2 days of growth, colonies were counted, and CFU counts ml^{-1} were calculated.

For crystal violet staining, tubes were washed with water and then dried at 60°C for 30 min. One milliliter of a 1% crystal violet solution was added, and the tubes were incubated on a rocker at room temperature for 30 min. 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 min and was then poured into cuvettes, and the A_{590} was measured.

Microscopy. A 25-ml volume of sterile brucella broth was inoculated with 750 μl of an overnight culture ($\sim 1 \times 10^9$ cells). Sterile twin-frosted microscope slides (VWR International) were inserted into the tubes, and cultures were 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 was examined using a Nikon Eclipse 50i microscope at magnifications of ×400 and ×1,000. For crystal violet staining, slides were stained with 1% crystal violet for 5 min and were then washed with water to remove unbound crystal violet. The microcolony pixel area was measured using ImageJ software (version 1.41; National Institutes 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 $\sim 1 \times 10^9$ CFU ml^{-1} in fresh brucella broth supplemented with 0.01% (wt/vol) Congo red (Hopkin and Williams Ltd., United Kingdom), and 1 ml of this solution was added to sterile borosilicate glass test tubes (Corning, United Kingdom). Tubes were incubated without shaking under microaerobic or aerobic conditions, or under 10% CO_2 in air, at 37°C for 2 days. The culture supernatant was carefully removed with a pipette, and the tubes were 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 min at 60°C and were developed in 1 ml of 50% ethanol in PBS (pH 7.5) for 10 min before the A_{500} of the solution was read. Three technical replicates were used for each condition, and data were obtained from three independent experiments.

Shedding of viable cells from preformed biofilms. Two-day-old *C. jejuni* biofilms were incubated aerobically as described under "Crystal violet biofilm assays" above. Instead of being washed and stained with crystal violet, tubes were washed twice with 1 ml sterile PBS, and 1 ml fresh sterile brucella broth was added to each tube. Viable cells in the culture supernatant were determined by plating serial dilutions on brucella agar plates immediately after washing or after 24 h 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 nonmotile 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. After several rounds of subculture, an entirely nonmotile variant was 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 $\sim 1 \times 10^9$ viable cells, suggesting that the difference was not due to a difference in viability between the two strains (Fig. 1C). 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; equivalent numbers of viable cells were recovered from the culture supernatants (Fig. 1C).

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% O_2). We therefore examined biofilm formation under aerobic conditions, where biofilms may be relevant as a survival mechanism. As a control, we also tested 10% CO_2 in air, which is the same CO_2 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% CO_2, 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% CO_2 in air showed many elongated cells, suggesting that the cells were stressed

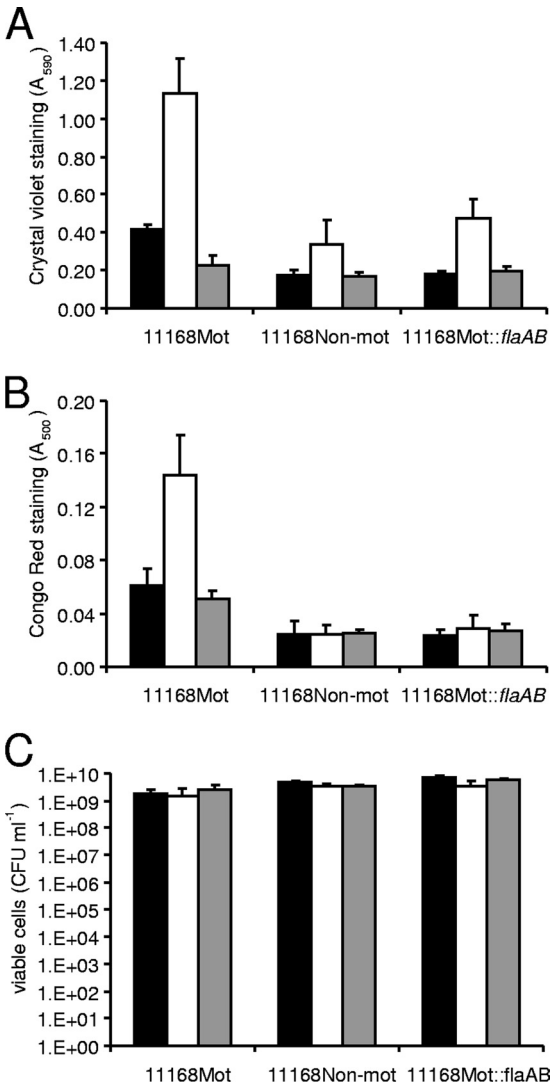

FIG. 1. Biofilm formation by the *C. jejuni* NCTC 11168 motile variant (11168Mot), the nonmotile variant (11168Non-mot), and the *flaAB* mutant (11168Mot::*flaAB*) after incubation for 2 days at 37°C under static microaerobic (filled bars) or aerobic (open bars) conditions or under 10% CO_2 in air (shaded bars). Results shown are averages 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 supernatants from the crystal violet assay. The error bars represent standard deviations calculated from the three independent experiments performed.

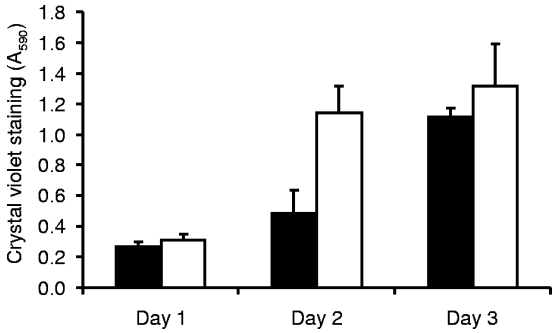

FIG. 2. Biofilm formation by the *C. jejuni* NCTC 11168 motile variant over 3 days under static microaerobic (filled bars) and aerobic (open bars) incubation conditions, as determined using crystal violet staining. Results are averages from three independent experiments, each containing triplicate samples.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

(data not shown). 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% CO_2 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% CO_2 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 (median pixel area, 3.7×10^5) than under microaerobic conditions (median pixel area, 2.5×10^4) (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).

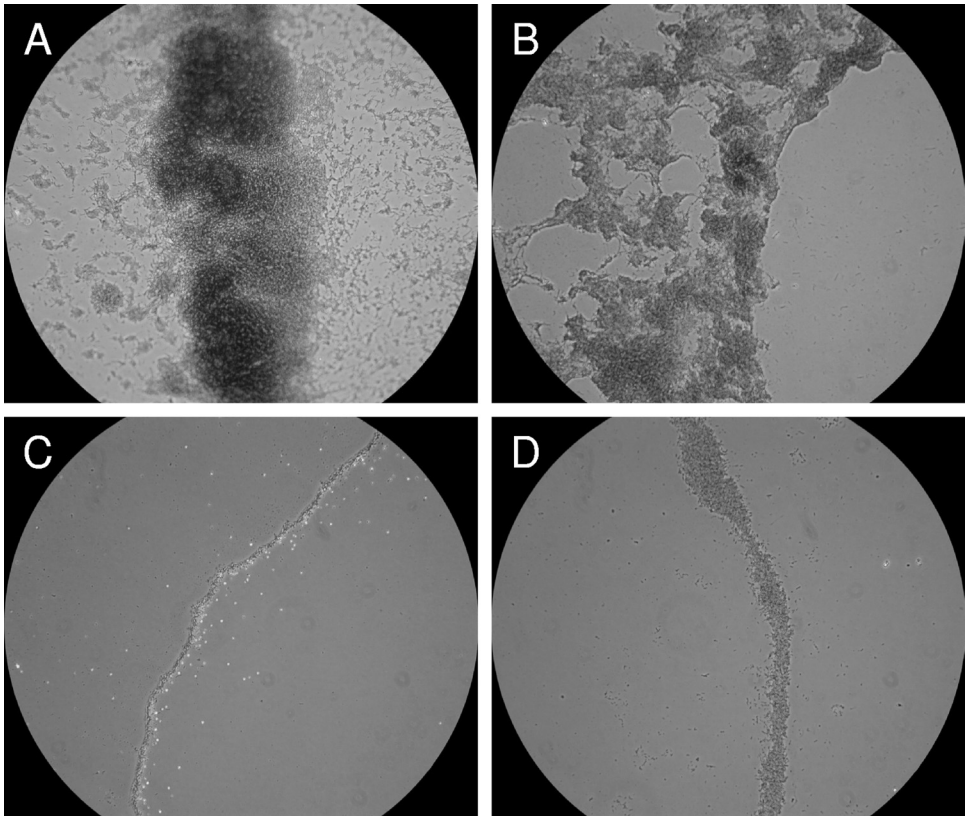

FIG. 3. Representative light microscopy photographs of *C. jejuni* biofilms after 2 days of incubation at 37°C. Slides were stained with 1% crystal violet and were photographed at ×400 magnification. Images show 11168Mot (A and B) and 11168Non-mot (C and D) grown under microaerobic (A and C) and aerobic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

***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 $\sim 1 \times 10^9$ viable cells (Fig. 4A). After washing, we observed 1×10^6 (± 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

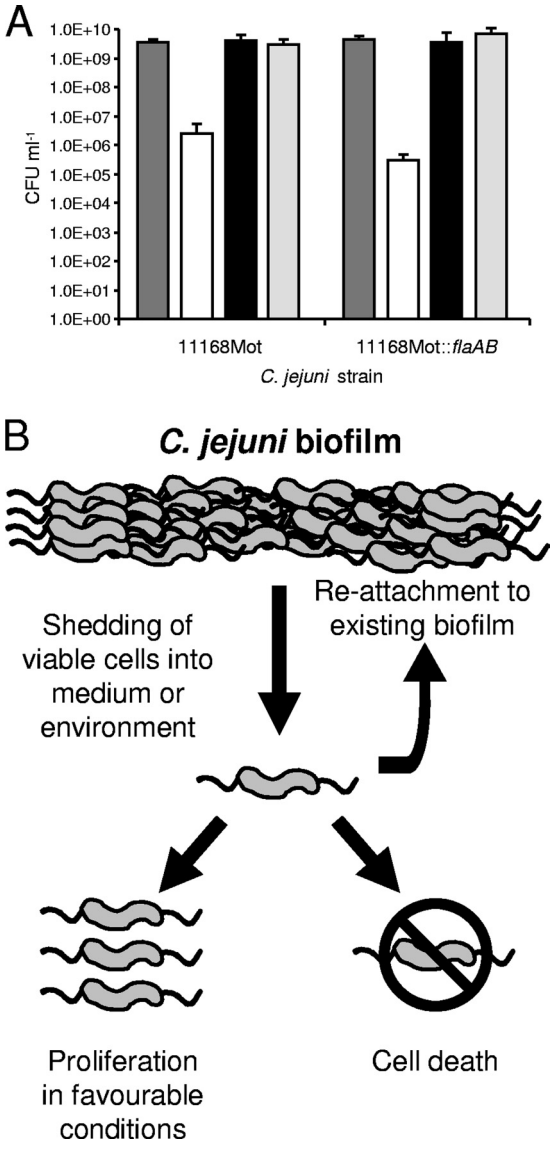

FIG. 4. (A) Viable cells in the supernatant after 2 days of aerobic biofilm formation (prewash) (dark shaded bars), after a wash with sterile PBS (open bars), and after 24 h of static incubation in fresh brucella broth under either aerobic (filled bars) or microaerobic (light shaded bars) conditions at 37°C. Values are means from least three independent experiments, and error bars represent standard deviations calculated from the three independent experiments. (B) Model of a *C. jejuni* biofilm showing the fate of cells following release from a preformed biofilm. After release from a biofilm, planktonic cells can proliferate under favorable conditions or may reattach to an existing biofilm. Cells may also die under conditions that preclude biofilm formation (i.e., in a fast flowing system).

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 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 *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 *Pseudomonas aeruginosa* (30) or flow-induced dispersal in *Shewanella oneidensis* (38). *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 *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 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 *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 *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.

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