INTRODUCTION

Campylobacter jejuni, a Gram-negative, microaerophilic bacterium, is the most frequently isolated foodborne pathogen worldwide. The annual reported incidence in England and Wales is around 50 000, which is thought to be a gross underestimate (CPLS, 2000). C. jejuni is also associated with neuropathies such as Guillain–Barré and Miller–Fisher syndromes (Salloway et al., 1996), where it is postulated that there is molecular mimicry between C. jejuni lipo-oligosaccharide and human gangliosides, leading to an auto-immune response (Yuki, 1997). The primary source of human infection is thought to be the consumption or handling of contaminated chicken and therefore the survival of C. jejuni in poultry house water systems is particularly significant (Pearson et al., 1996; Zimmer et al., 2003).

There is a paradox in that although C. jejuni is extremely widespread, its microaerobic growth requirements mean that the organism does not multiply in the natural aerobic environment. Although not all ‘natural environments’ that C. jejuni inhabits may necessarily be aerobic, it is still difficult to explain the high incidence of infection, particularly as in contrast to several other gastrointestinal pathogens, C. jejuni is rarely transmitted between humans. It has been suggested that C. jejuni maintains itself in the environment by forming a biofilm (Buswell et al., 1998). Biofilms are commonly defined as matrix-enclosed bacterial populations adherent to each other and/or to surfaces or interfaces (Costerton et al., 1995). Aggregates of bacteria not attached to a surface are commonly termed flocs and have many of the characteristics of a biofilm (Hall-Stoodley et al., 2004). Pellicles are aggregates of bacterial cells that form at an air–liquid interface (Friedman & Kolter, 2004).

The biofilm mode of growth is widespread and is of economic importance in diverse ecological niches (Costerton et al., 1995). Monospecies biofilms are formed by many human pathogens such as Pseudomonas aeruginosa (Whiteley et al., 2001), Staphylococcus epidermidis (Mack et al., 2000), Salmonella enteritidis (Solano et al., 2002), Vibrio cholerae (Nesper et al., 2001), Streptococcus gordonii (Loo et al., 2000) and Burkholderia cepacia (Huber et al., 2001). In human infection, biofilms are often less susceptible to antibiotics (Nichols, 1991).

C. jejuni has been observed to autoagglutinate, in Mueller–Hinton broth, minimal essential medium (Golden & Acheson, 2002) and phosphate-buffered saline (Misawa & Blaser, 2000). However, it is unclear if autoagglutination represents a biofilm mode of growth in this species. C. jejuni has been found in preformed biofilms of other bacterial species (Trachoo et al., 2002; Keevil, 2003). Buswell et al. (1998) postulated that C. jejuni in autochthonous biofilms had enhanced survival. C. jejuni adherent to stainless steel

Abbreviations: CLSM, confocal laser scanning microscopy; EPM, extracellular polymeric matrix; SEM, scanning electron microscopy.
coupons were assumed to have formed biofilms by Somers et al. (1994). However, to our knowledge, biofilm formation by C. jejuni has not hitherto been properly demonstrated. We therefore examined C. jejuni, grown in liquid culture, with and without shaking; and on glass or cellulose acetate surfaces by confocal laser scanning microscopy (CLSM) and by scanning electron microscopy (SEM) to determine whether it can use this form of growth. Additionally, selected C. jejuni mutants were tested for their ability to form biofilms to help identify likely surface determinants that may play a role in biofilm formation.

**METHODS**

**Growth.** All C. jejuni cultures were grown in a Variable Atmosphere Incubator (Don Whitley Scientific) under microaerobic conditions (N₂, 85%; O₂, 5%; CO₂, 10%) at 37 °C. For collection of bacterial aggregates, cultures were in Mueller–Hinton broth shaken at 50 r.p.m. on a mini orbital shaker (Stuart Scientific). Ten millilitres of medium was seeded with 100 µl Mueller–Hinton broth containing 10 µl loop of C. jejuni taken from a Columbia blood agar plate containing 6-8% (v/v) horse blood, grown for 2 days, also under microaerobic conditions. Aggregate and planktonic bacteria were grown for 3 days in vented 50 ml tissue flasks (Falcon). For growth of pellicles and attached bacteria, cultures were in 10 ml Brucella broth seeded with C. jejuni from Columbia blood plates to an OD₆₀₀ of 0.1–0.2 in glass test tubes incubated without shaking under microaerobic conditions (see above) at 37 °C for 5 days.

**Bacterial strains and mutants (see Table 1).** C. jejuni strains NCTC 11168 (National Culture Type Collection, London, UK), its stable motile derivative 11168H (Karlyshev et al., 2000) and 81-176 (Black et al., 1988) have been described elsewhere. Clinical isolates were from an Infectious Intestinal Disease case control study (strains 31467, 33106, 32799, 33084 and 31485) and a C. jejuni outbreak in Wales in 2000 (strain 40671). These strains were obtained from the Campylobacter Reference Laboratory (Health Protection Agency, UK). Mutant strains were made in 11168H and included mutations in genes affecting capsular polysaccharide formation (kpsM; encoding a capsule transport protein) (Karlyshev et al., 2000), flagella formation (maf5 is located in the flagellin glycosylation locus, and the maf5 mutant is aflagellate and non-motile) (Karlyshev et al., 2002), N-linked protein glycosylation biosynthesis (pglH, encoding a glycosyltransferase essential for the biosynthesis of the N-linked protein glycosylation pathway) (Linton et al., 2002) and lipo-oligosaccharide biosynthesis (neuB1, encoding a sialic acid synthetase) (Linton et al., 2000). In order to complement the maf5 mutation in 11168H, the maf5 gene was PCR amplified and cloned downstream from the cam promoter from the delivery vector pAV35 (van Vliet et al., 1998) under the control of a cam promoter constitutively expressed in Campylobacter. Integration of the cam maf5 gene fusion cassette into the rrs (16S rRNA)–rrl (23S rRNA) spacer region of one of the three rRNA gene clusters in the chromosome resulted in restoration of motility of the 11168H maf5 mutant (Karlyshev & Wren, 2005). The complemented 11168H maf5 mutant was termed 11168H maf5– (maf5⁺). Mutants flIS, cj0688 and cj0689 were generated via insertion of the kan cassette from the delivery vector pMK30 (van Vliet et al., 1998) into the respective pUC18 derivatives obtained from a random 2 kb library generated in the C. jejuni NCTC 11168 genome sequencing project (Parkhill et al., 2000). All wild-type strains were motile, as were all mutants except maf5 and flIS, which were aflagellate and non-motile mutants. All strains and mutants used in the study had similar growth profiles in Mueller–Hinton liquid culture medium over a 48 h period (data not shown).

**Microscopy.** For CLSM (Zeiss HAL 100), bacteria on glass cover slips were mounted in PBS/glycerol (1:1, v/v) and visualized with

<table>
<thead>
<tr>
<th>Strain</th>
<th>Aggregate*</th>
<th>Attached*</th>
<th>Pellicle*</th>
<th>Hydrophobicity†</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCTC 11168</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>0.004 ± 0.002</td>
</tr>
<tr>
<td>11168H</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>0.023 ± 0.005</td>
</tr>
<tr>
<td>11168H kpsM</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>0.004 ± 0.002</td>
</tr>
<tr>
<td>11168H maf5</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>0.875 ± 0.072</td>
</tr>
<tr>
<td>11168H maf5(maf5⁺)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0.0008 ± 0</td>
</tr>
<tr>
<td>11168H pglH</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0.001 ± 0.001</td>
</tr>
<tr>
<td>11168H neuB1</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>0.001 ± 0</td>
</tr>
<tr>
<td>11168H flIS</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>0.333 ± 0.058</td>
</tr>
<tr>
<td>11168H cj0688</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0.027 ± 0.008</td>
</tr>
<tr>
<td>11168H cj0689</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0.013 ± 0.001</td>
</tr>
<tr>
<td>81-176</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>Not tested</td>
</tr>
<tr>
<td>40671</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0.078 ± 0.027</td>
</tr>
<tr>
<td>31467</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0.031 ± 0</td>
</tr>
<tr>
<td>33106</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>0.046 ± 0.015</td>
</tr>
<tr>
<td>32799</td>
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<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>33084</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>0.038 ± 0.004</td>
</tr>
<tr>
<td>31845</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>0.002 ± 0</td>
</tr>
</tbody>
</table>

* −, absent; +, small (just visible); ++, intermediate; +++, extensive.
†Minimal concentration of ammonium sulphate for cell aggregation of 0–0.05–0.05, 0–0.25–0.05 and 0–25–1.0 M represents strong, medium and weak hydrophobicity, respectively.
Results

In our initial studies we attempted to grow biofilms of the sequenced strain of C. jejuni (NCTC 11168) on glass cover slips or on cellulose acetate filters, either in tissue culture flasks, in 24-well tissue culture plates or in a modified Robbins device (Tyler Engineering) (Domingue et al., 1994). We found that in the modified Robbins device, no bacteria attached to either cellulose acetate filters or glass with a flow rate of 300 ml h⁻¹, as used by Domingue et al. (1994) or with various flow rates down to 10 ml h⁻¹. Nor did C. jejuni NCTC 11168 attach to polystyrene, as assessed by crystal violet staining, in tests to quantify biofilm formation (Nesper et al., 2001) (data not shown). Similarly, in liquid culture with normal shaking (80–100 r.p.m.) there was no growth of bacteria on the plastic of the tissue culture flask, or on the surface of glass cover slips or cellulose acetate filters, and bacteria in the medium were planktonic. However, with shaking at a lower speed (50 r.p.m.) we noted the formation of bacterial aggregates in culture. We also observed the formation of a pellicle at the liquid–gas interface of cultures grown without shaking after 3–6 days, with cells impinging on the walls of the test tubes at the liquid–gas interface strongly attached.

We therefore tested a variety of strains of C. jejuni for their ability to form aggregates and pellicles, and to attach to glass. These included several clinical isolates, NCTC 11168, its stable motile derivative 11168H and several 11168H isogenic mutants. Three strains contained mutations in genes kpsM, neuB1 and pgIH, affecting the biosynthesis of three surface glyco-conjuncts on C. jejuni cells, namely capsular polysaccharide, lipo-oligosaccharide and N-linked glycoproteins respectively. Selection of these mutants was based on the observation that in other bacteria an extracellular matrix in the biofilms may contain polymers of carbohydrate-containing molecules (Danese et al., 2000; Mack et al., 2000; Nesper et al., 2001; Solano et al., 2002; Sherlock et al., 2005). The maf5 and flIS mutants are aflagellate and were chosen because of a possible role of flagella as an adhesin during an initial step of biofilm formation (Kirov et al., 2004; Watnick et al., 2001; Golden & Acheson, 2002). cj0688 and cj0689 encode putative phosphate acetyltransferase and acetate kinase respectively. Since the latter two genes have recently been shown to be involved in the biofilm formation in Escherichia coli (Wolfe et al., 2003) we reasoned that C. jejuni homologues of these genes may also affect biofilm formation.

**Fig. 1.** Aggregates (A), pellicles (B) and attached (C) C. jejuni biofilms. Panels A and B show 11168H, 11168H flIS, 11168H kpsM, 11168H maf5 and 11168H maf5(maf5⁺) from left to right. Panel C shows 11168H standing culture after removal of medium. Aggregate (A) and attached biofilms (B, C) are arrowed. (Tissue culture flasks and test tubes, not to scale.)
Excepting *C. jejuni* wild-type strains 33106, 32799, 33084 and 31485, all strains tested formed aggregates, including the isogenic mutants *pglH*, *kpsM*, *neuB1* and *maf5*. Aggregates in *fliS* and in *cj0688* mutants were reduced. All wild-type strains also formed pellicles and attached to the surface of glass test tubes, including strains 33106, 32799, 33084 and 31485. The *pglH* mutant formed a pellicle and attached to glass in a similar way to wild-type strains; the *kpsM* and *neuB1* mutants formed a noticeably larger pellicle than all other strains. The *maf5* mutant did not form a pellicle or attach to glass but this ability was restored in the complemented strains. Pellicle formation was also reduced in the *fliS* and *cj0688* mutants. Fig. 1 shows examples of the various forms of biofilm. Note that attached bacteria were contiguous with the pellicle, and thus are not easily seen in intact standing cultures (Fig. 1B); Fig. 1C shows attached 11168H bacteria after removal of a portion of medium. The results are summarized in Table 1; all strains were tested

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**Fig. 2.** CLSM of *C. jejuni* 11168H aggregate biofilm on glass coverslip. A, stained with Penner 2 antiserum visualized with Alexa 546; B, stained with SYTO 16; C, Penner 2 and SYTO 16 merged; D, phase-contrast. Bars, 5 μm. E, orthogonal projection from Z-stack of aggregate biofilm.
in triplicate (mutant strains using three clonal isolates). Additionally, to provide information on the potential physical forces responsible for cell–cell interactions, Table 1 includes the relative hydrophobicity values for each strain and mutant strain studied.

We examined aggregates, pellicles and attached forms of bacteria in 11168H and selected mutants. Fig. 2 shows CLSM images of C. jejuni 11168H aggregates on glass cover slips. Under phase-contrast (Fig. 2D) a mass of bacteria can be seen as curved rods which were morphologically identifiable as C. jejuni. These were stained by the nucleic acid specific stain SYTO 16 (Fig. 2B) and Penner 2 antiserum, specific to 11168H, (Fig. 2A, and shown merged in Fig. 2C). The three-dimensional nature of the bacterial mass, characteristic of a biofilm, is shown by orthogonal projection from a Z-stack (Fig. 2E). However, CLSM does not show connection of the bacteria by an extracellular polymeric matrix (EPM), which is a defining characteristic of a biofilm. This is seen by SEM (Figs 3–5).

Fig. 3 shows the three forms of biofilm of C. jejuni 11168H. Fig. 3A shows the bacterial aggregates grown in liquid culture. The extracellular material connecting bacteria appears flattened and extensive. Biofilm bacteria attached to glass (Fig. 3B) resemble the aggregate form, with a flattened and extensive EPM. In the pellicular biofilm (Fig. 3C), however, bacteria are similarly connected via extracellular material but this material is not extensive or flattened.

**Fig. 3.** SEM of C. jejuni 11168H. A, aggregate; B, attached; C, pellicle. Bars, 1 μm.
The aggregated forms of biofilms of isogenic capsular polysaccharide (kpsM) and flagellar (maf5) mutants (Fig. 4) were identical to that of the wild-type, precluding the requirement for capsular polysaccharide or flagella in the aggregate biofilm formation. The kpsM mutant’s attached and pellicular biofilms again resembled those of the wild-type (Fig. 5A, B). However, the maf5 mutant attached minimally to glass (Fig. 5C) and did not form a pellicle (Fig. 1B); cells from the surface of a standing maf5 culture were not connected by an EPM and appeared as planktonic bacteria (Fig. 5D).

To assess the effect of biofilm on resistance to environmental stress, cultures were grown in triplicate for 3 days under microaerobic conditions promoting aggregate formation (see Methods). Aggregates and planktonic bacteria were then separated and transferred to ambient temperature and atmospheric conditions. Viable counts from individual cultures were taken at 3 day intervals. As seen in Fig. 6, while all planktonic bacteria died by 12 days following transfer to ambient conditions, bacterial aggregates survived for up to 24 days. Whereas cell numbers in planktonic cultures fell from $\sim 10^9$ c.f.u. ml$^{-1}$ to zero by day 12, viable cells in aggregates fell from $\sim 10^9$ to $10^2$ c.f.u. ml$^{-1}$ by day 21 and to zero by day 24.

DISCUSSION

Given the public health significance of C. jejuni infection it is important to understand how the organism survives in the environment and enters the food chain. Bacteria in a biofilm are relatively resistant to changes in environmental conditions, to antimicrobial agents and to host immune responses (Hall-Stoodley et al., 2004). It is an attractive hypothesis, therefore, that C. jejuni cells form a biofilm to survive adverse conditions between animal hosts; yet although C. jejuni has been found to colonize biofilms of autochthonous bacteria (Buswell et al., 1998), monospecies biofilm formation by C. jejuni has not previously been demonstrated.

We present data to show that C. jejuni in monoculture can form three distinct biofilms. It can attach to a glass surface (Figs 1C, 3B and 5A), and attachment is perhaps considered a hallmark and necessary first step in the formation of a biofilm. C. jejuni can also form a pellicle at a liquid–gas interface when grown in stationary culture (Figs 1B, 3C and 5B), which has a similar three-dimensional architecture to the attached biofilm, with bacteria connected via an EPM, although the EPM is not flattened. Notably, C. jejuni forms unattached aggregates in liquid culture. Aggregates of this type have sometimes been referred to as flocs, which are said to resemble biofilms (Hall-Stoodley et al., 2004). We have used the term aggregate biofilm to describe the unattached bacteria connected to each other via an EPM and resembling the biofilm attached to glass and the pellicle forming at the liquid–gas interface. We propose that all three forms are true biofilms.

C. jejuni aggregate biofilms have increased resistance to ambient environmental stress, bacteria in the aggregate biofilm decreasing in number but surviving for up to 24 days, in comparison to up to 12 days survival by planktonic bacteria (Fig. 6). Similar experiments comparing pellicular and attached biofilm survival with planktonic bacteria after transfer from microaerobic conditions to ambient temperature and aerobic conditions were also performed. However, pellicles fell from the surface after 10–12 days and were visible as aggregates at the base of the tubes. These aggregates were no longer, by definition, pellicles and experiments were discontinued. It was not possible, therefore, to determine whether the attached and pellicular forms of C. jejuni biofilm conferred the same
resistance to environmental stress. However, cells from the pellicle that were attached to the glass remained viable under atmospheric conditions for several weeks (unpublished data), suggesting that once a biofilm is formed (e.g. at 37 °C) this may provide a survival advantage to cells in the ambient environment. Due to stringent growth requirements C. jejuni cannot be grown at ambient temperature or atmosphere conditions; however we were able to demonstrate aggregate and pellicle formation for strain NCTC 11168 at 30 °C under microaerophilic conditions (unpublished data). The relevance of biofilm formation for C. jejuni in its natural life cycle in the environment can only be speculated upon, as our studies demonstrating biofilm formation were undertaken at 37 °C (or 30 °C). It may be that biofilm formation is possible within the host (e.g. avian, livestock or human), which subsequently confers a survival advantage to the micro-organism in the ambient environment (or a specific microenvironment) before transmission to the next host.

It is noteworthy that C. jejuni is strongly adherent to glass only at the liquid–gas interface, contiguous with the pellicle, in standing culture and does not attach to any surface we have tested with any shear force operating (flow or shaking). There may, however, be conditions in which attachment and subsequent biofilm formation occur that we have not

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**Fig. 5.** SEM of C. jejuni 11168H mutants: A, kpsM attached; B, kpsM pellicle; C, maf5 not attached; D, maf5 pellicle. Bars, 1 μm.
determined. C. jejuni does, however, readily form the aggregate biofilm. It seems likely that aggregation of C. jejuni is initiated by non-specific forces such as hydrophobic interactions, as suggested by the relative hydrophobicity values of the wild-type and mutant strains (Table 1). The relative hydrophobicity was significantly reduced in aflagellate mutants, as previously observed by Misawa & Blaser (2000). Hydrophobic interactions have also been proposed to allow C. jejuni cells to bind to INT 407 cell membranes (Moser & Schroder, 1997). Whether or not subsequent development of the three forms of biofilm utilizes the same mechanisms and produces the same EPM remains to be determined.

Attachment by C. jejuni to a surface appears to be mediated by flagella, as the aflagellate fliS and maf5 mutants do not attach. Non-motile strains also failed to form pellicles, but in this case, attachment to a surface may not be a prerequisite for biofilm formation. A possible explanation may be that within the pellicle an oxygen gradient may form due to limited oxygen consumption; because C. jejuni are aero-tactic (Marchant et al., 2002) this would disadvantage non-motile strains. In addition, other genes may play a part in biofilm formation in this species. We thank Christine Szymanski and Bill Keevil for useful discussion on biofilm formation. We thank Maria McCrossan for assistance with electron microscopy studies. We acknowledge support from the Leverhulme Trust and the Biotechnology and Biological Sciences Research Council.

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REFERENCES


