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Biofilm formation in *Campylobacter jejuni*

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The major gastrointestinal pathogen *Campylobacter jejuni* is shown to exist as three forms of monospecies biofilm in liquid culture. It attaches to a glass surface; forms an unattached aggregate (floc); and forms a pellicle at the liquid–gas interface. The three forms of biofilm resemble each other when examined by scanning electron microscopy. The biofilm mode of growth confers protection against environmental stress, the microaerobic bacteria in flocs surviving up to 24 days at ambient temperature and atmosphere compared to 12 days survival by planktonic bacteria. The wild-type strains *C. jejuni* 33106, 32799, 33084 and 31485 did not form flocs, and floc formation was reduced in strains mutant in a putative flagellar protein (FliS) and in a phosphate acetyltransferase (Cj0688). All other strains tested, including strains with mutations affecting capsular polysaccharide (*kpsM*), flagella (*maf5*), protein glycosylation (*pglH*) and lipo-oligosaccharide (*neuB1*) formed flocs. Similarly, all strains tested formed a pellicle and attached to glass except the aflagellate mutant *maf5*; pellicle formation was reduced in *fliS* and *cj0688* mutants. Different mechanisms, therefore, may control formation of different forms of biofilm. It is proposed that these poorly characterized forms of growth are important for the persistence of *C. jejuni* in the environment and may in part explain the high incidence of Campylobacter-associated food borne disease.

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 (N_2, 85 %; O_2, 5 %; CO_2, 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 a 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 OD_{600} 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^r gene from the delivery vector pAV35 (van Vliet *et al.*, 1998) under the control of a cam^r promoter constitutively expressed in *Campylobacter*. Integration of the cam^r*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^r cassette from the delivery vector pJMK30 (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 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 1. Strains of *C. jejuni* used and their ability to form aggregates, pellicles and attached biofilms and their relative hydrophobicity

Strain	Aggregate*	Attached*	Pellicle*	Hydrophobicity†
NCTC 11168	+++	++	++	0·004±0·002
11168H	++	++	++	0·023±0·005
11168H *kpsM*	+++	+++	+++	0·004±0·002
11168H *maf5*	++	−	−	0·875±0·072
11168H *maf5*(*maf5*$^+$)	++	++	++	0·0008±0
11168H *pglH*	++	++	++	0·001±0·001
11168H *neuB1*	++	+++	+++	0·001±0
11168H *fliS*	+	−	+	0·333±0·058
11168H *cj0688*	+	+	+	0·027±0·008
11168H *cj0689*	++	++	++	0·013±0·001
81-176	++	++	++	Not tested
40671	++	++	++	0·078±0·027
31467	++	++	++	0·031±0
33106	−	++	++	0·046±0·015
32799	−	++	++	0·038±0·004
33084	−	++	++	0·038±0·004
31845	−	++	++	0·002±0

*−, absent; +, small (just visible); ++, intermediate; +++, extensive.

†Minimal concentration of ammonium sulphate for cell aggregation of 0·005–0·05, 0·05–0·25 and 0·25–1·0 M represents strong, medium and weak hydrophobicity, respectively.

SYTO 16 (Molecular Probes). For indirect immunofluorescence tests (IFATs), Penner 2 antiserum was used at 1 : 100 and visualized with Alexa 546 (Molecular Probes). For SEM, aggregates were transferred to cover slips with a wide-bore pipette; pellicles were transferred by dipping cover slips through the surface of standing cultures. Attached bacteria were obtained by breaking the glass test tube and processing bacteria *in situ*. Bacteria were fixed for 2 h in 3 % glutaraldehyde made up in 0·2 M sodium cacodylate buffer pH 7·4. The samples were then washed in the same buffer for 75 min prior to post-fixing in 1 % osmium tetroxide in the 0·2 M sodium cacodylate buffer for 105 min. Subsequent washing in 0·2 M buffer for a further 25 min was followed by the storage at 4 °C in 0·2 M sucrose in 0·2 M sodium cacodylate overnight. The samples were then washed in two changes of MilliQ water over 145 min and dehydrated through a graduated series of ethanol (15–30 min per step; 30 %, 50 %, 70 %, 90 %, 2 × 100 %). They were subsequently air-dried in a ventilated covered container and sputter-coated in an Edwards S150 sputter coater. The samples were examined on a JEOL JSM25III scanning electron microscope and photographed using Kodak 120 TP black and white film. Kodak D19 developer was used for developing the rolls of film and the images were printed on Agfa multi-grade paper using an Agfa Print Processor and Agfa chemistry.

Hydrophobicity assay. The relative hydrophobicity of cells was determined using the 'salting out' method of Misawa & Blaser (2000). Hydrophobicity was assessed on the minimum concentration of ammonium sulphate permitting aggregation of cells using a serial doubling dilution of a 4 M solution.

Survival assay. For comparison of survival of aggregate and planktonic bacteria, 10 ml cultures in Mueller–Hinton liquid medium were grown at 50 r.p.m. for 3 days at 37 °C and transferred to test tubes. Aggregates were allowed to settle under gravity for 10 min, washed three times in broth and resuspended in 10 ml fresh Mueller–Hinton liquid medium. Similarly, planktonic bacteria in the supernatant were centrifuged at 4000 r.p.m., washed three times in broth and resuspended in 10 ml fresh Mueller–Hinton liquid medium. Cultures were then stored at ambient temperature and atmospheric conditions for the duration of the assay. At 3 day intervals viable bacterial counts were determined on Columbia blood-agar plates. Experiments were performed in triplicate.

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 *pglH*, affecting the biosynthesis of three surface glyco-conjugants 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.

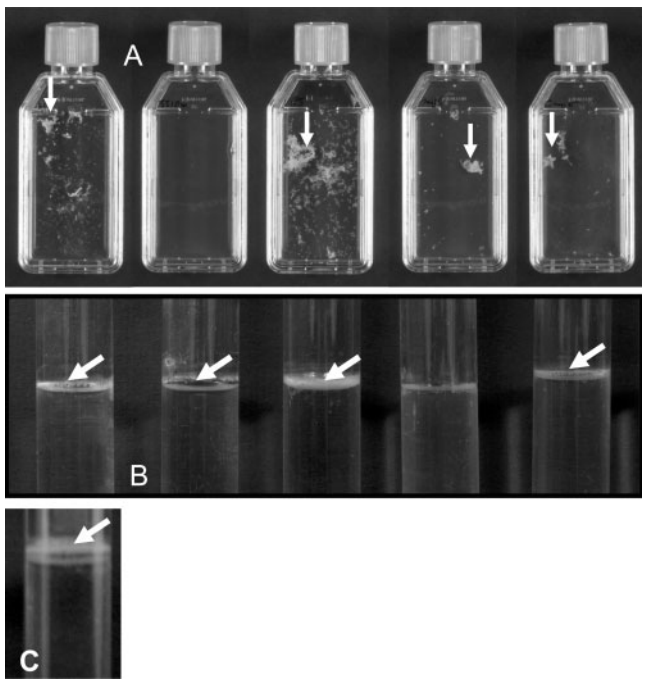

Fig. 1. Aggregates (A), pellicles (B) and attached (C) *C. jejuni* biofilms. Panels A and B show 11168H, 11168H *fliS*, 11168H *kpsM*, 11168H *maf5* and 11168H *maf5*(*maf5*$^{+}$) from left to right. Panel C shows 11168H standing culture after removal of medium. Aggregate (A) and attached biofilms (B, 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

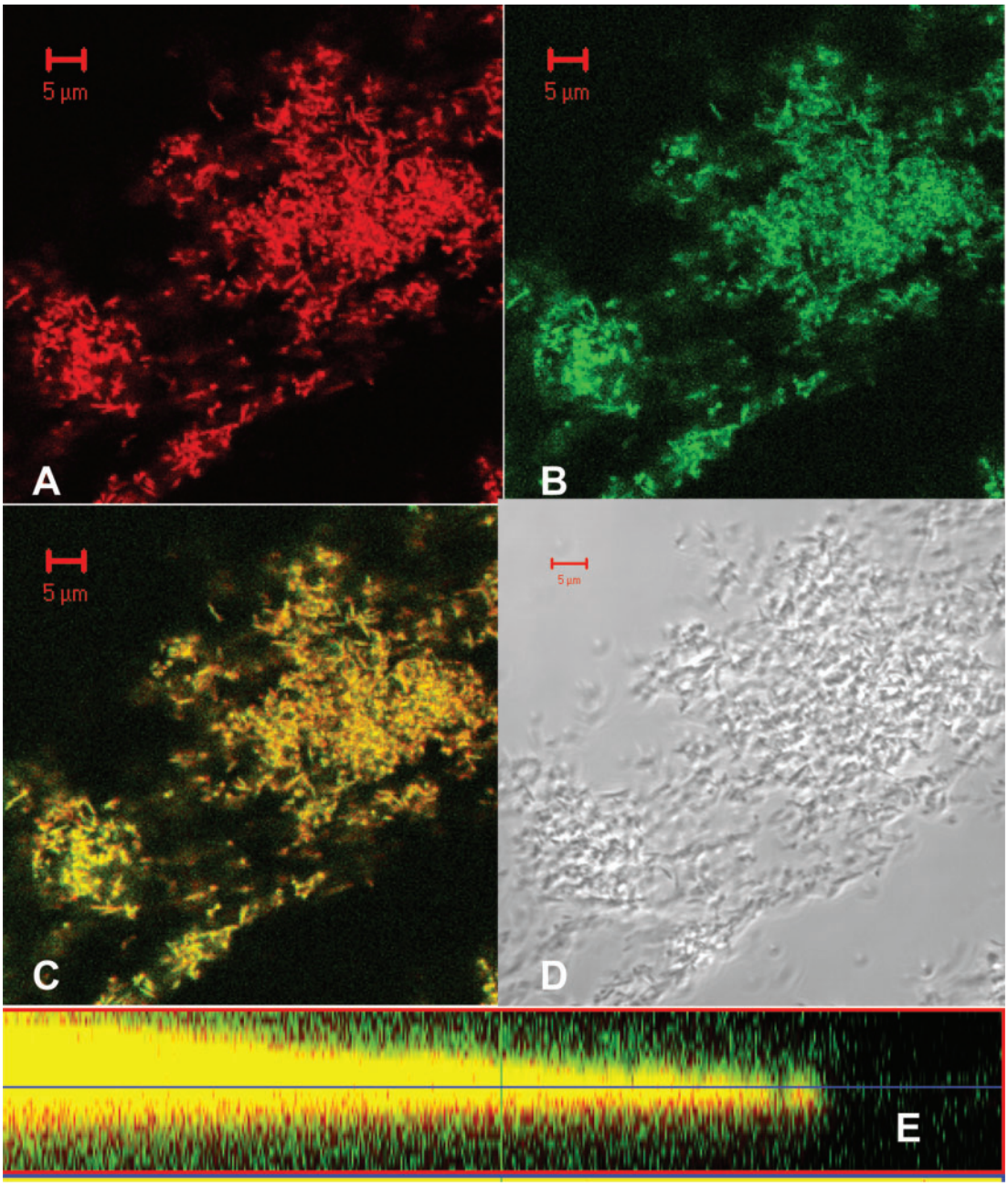

Fig. 2. CLSM of *C. jejuni* 11168H aggregate biofilm on glass coverslip. A, stained with Penner 2 antiserum visualized with Alexa 546; B, stained with SYTO 16; C, Penner 2 and SYTO 16 merged; D, phase-contrast. Bars, 5 µm. E, orthogonal projection from Z-stack of aggregate biofilm.

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.

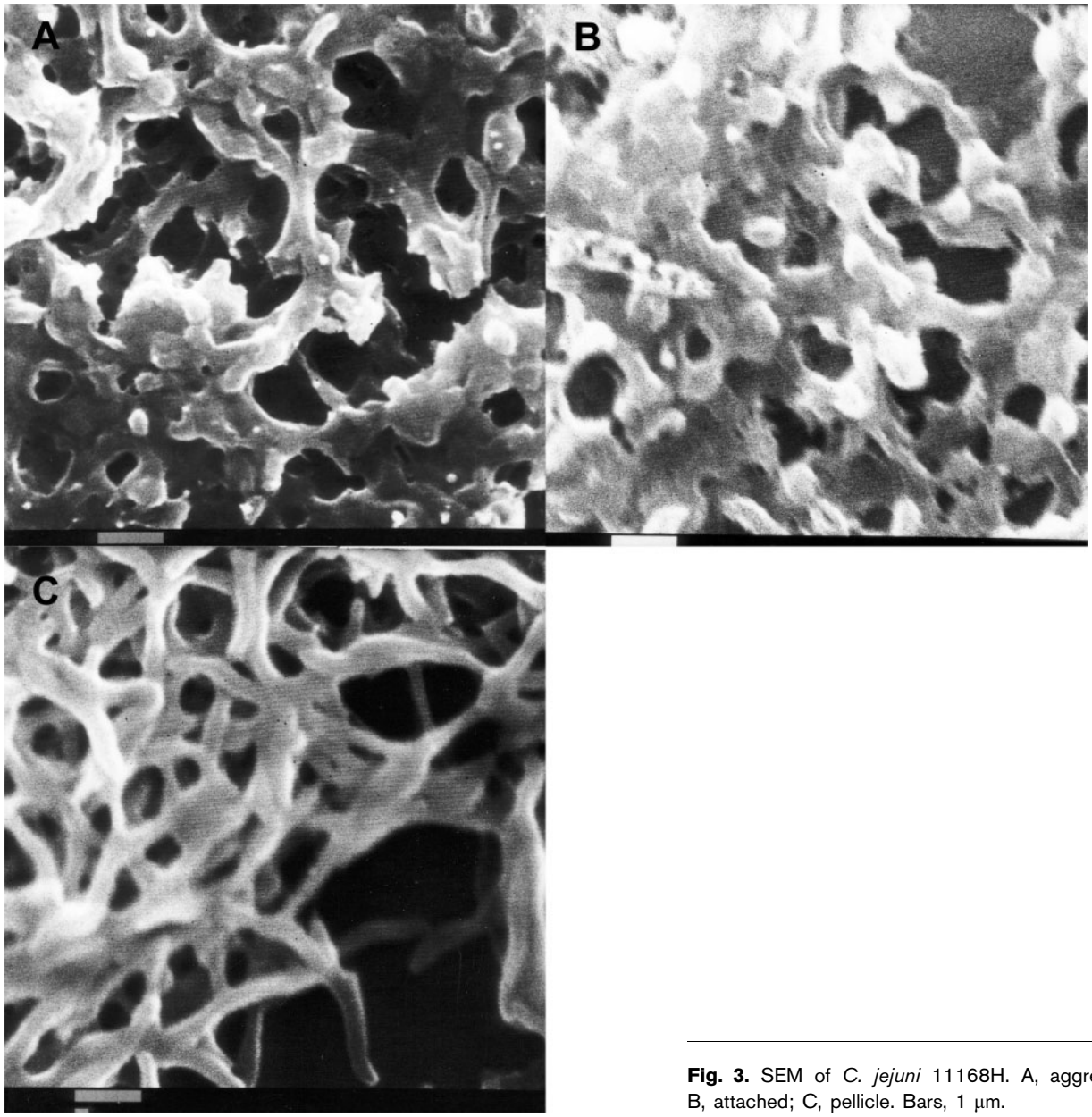

Fig. 3. SEM of *C. jejuni* 11168H. A, aggregate; B, attached; C, pellicle. Bars, 1 μm.

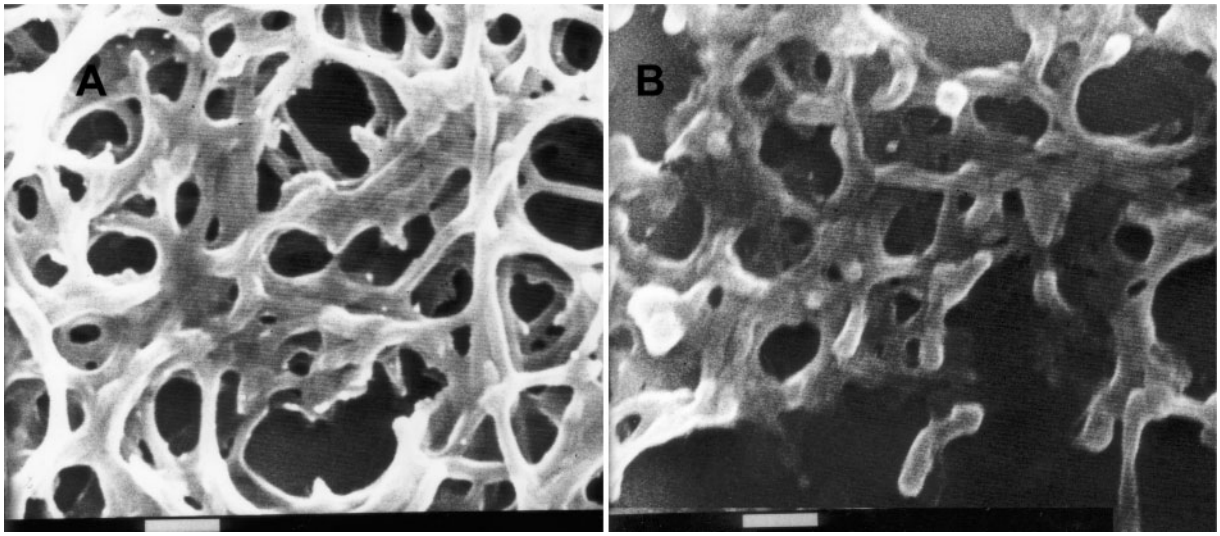

Fig. 4. SEM of aggregates formed by *C. jejuni* 11168H mutants: A, *kpsM*; B, *maf5*. Bars, 1 μm.

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

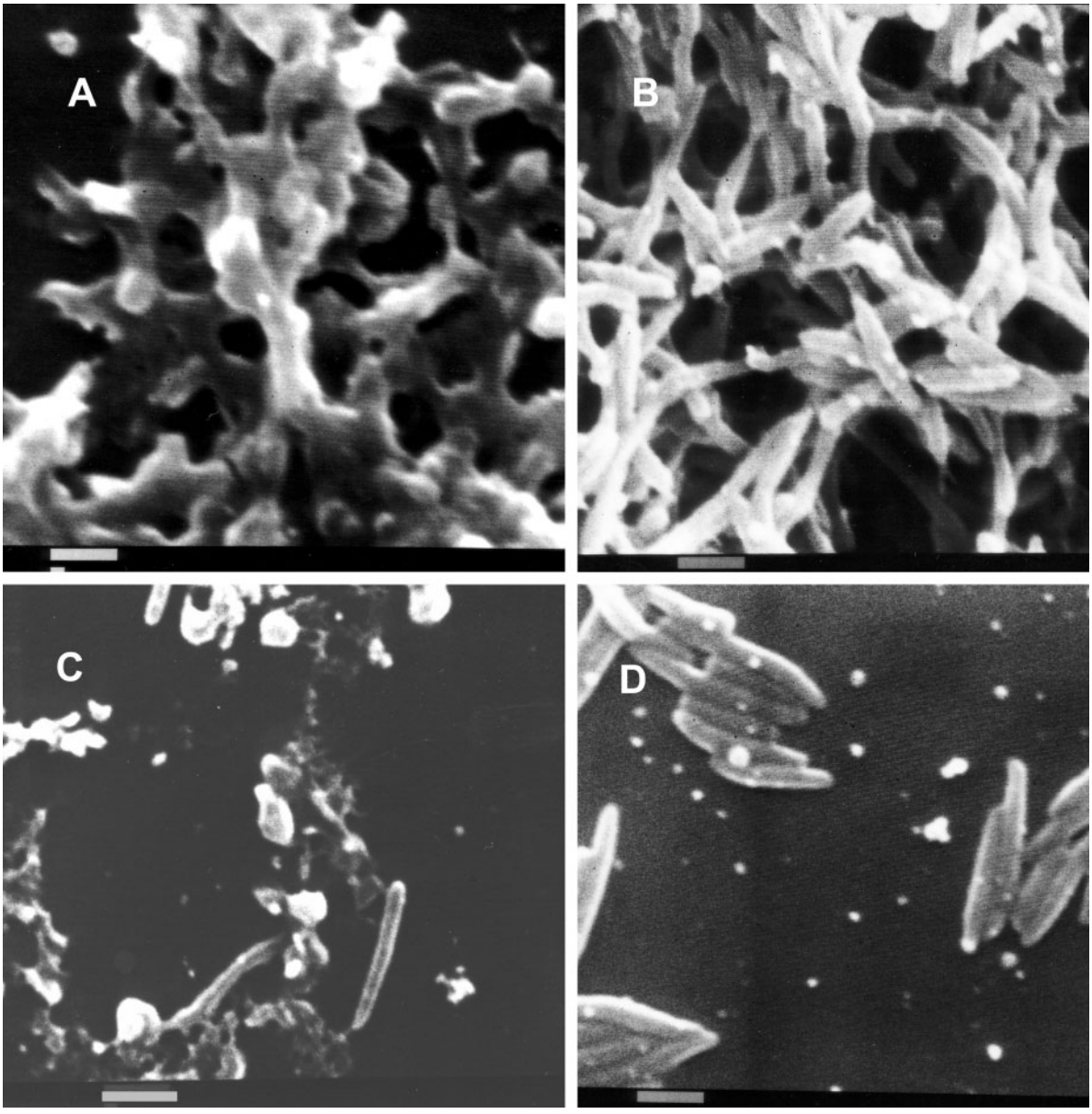

Fig. 5. SEM of *C. jejuni* 11168H mutants: A, *kpsM* attached; B, *kpsM* pellicle; C, *maf5* not attached; D, *maf5* pellicle. Bars, 1 μm.

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

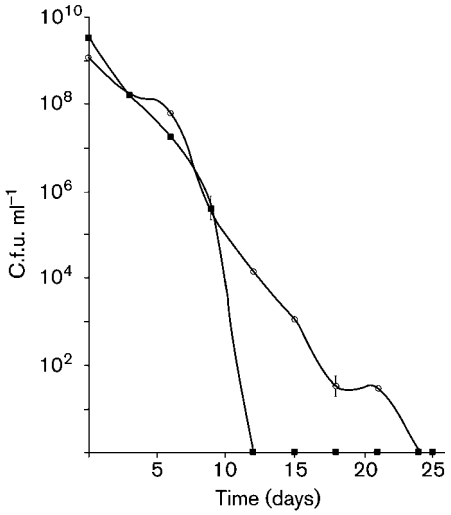

Fig. 6. Survival times of *C. jejuni* 11168H as aggregates (○) and as planktonic bacteria (■). The error bars (some of which are smaller than the symbols) represent SD (n = 3).

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 aerotactic (Marchant *et al.*, 2002) this would disadvantage non-motile strains. In addition, other genes may play a part in biofilm formation. Attachment is also reduced in the *cj0688* mutant and it is noteworthy that the *E. coli* counterpart mutation (*acp*) also affected biofilm formation (Wolfe *et al.*, 2003). In *E. coli* this *acp* gene encodes a phosphate transferase involved in the maintenance of the acetyl phosphate pool and also plays a crucial role in the cells' response to different environmental conditions such as nutrient concentration. This latter characteristic may be important in biofilm formation. By contrast, although *tibA* genes, which are responsible for glycosylation in enterotoxigenic *E. coli*, are important for biofilm formation (Sherlock *et al.*, 2005), the *pgl* genes that encode a general glycosylation pathway in *C. jejuni* (Szymanski & Wren, 2005) appear to have no role in biofilm formation in this species.

Laboratory-passaged strains 11168H and 81-176, and a number of clinical isolates, form the aggregate biofilm, as well as strains with mutations in genes for capsular polysaccharide (*kpsM*), flagella (*maf5*), glycoproteins (*pglH*), lipo-oligosaccharide (*neuB1*) and acetate kinase (*cj0689*). However, a number of wild-type strains, which possess flagella and are motile, do not form the aggregate biofilm; and strains mutant in another flagellar protein (FliS) and in a phosphate acetyltransferase (Cj0688) form a severely reduced aggregate biofilm. In addition, *maf5* and *fliS* do not form a pellicle or attach to glass. Different molecular mechanisms, therefore, may control formation of the different forms of biofilm.

Given that aggregate biofilms were readily formed by the capsule-deficient mutant *kpsM*, the question remains what constitutes the EPM in *C. jejuni* biofilms. An alternative polysaccharide structure has been recently suggested that allows the Calcofluor white staining of *C. jejuni* cells (McLennan *et al.*, 2005). Calcofluor white binds β-(1–4) and β-(1–3) sugar linkages and appears independent of the capsule. However, although the EPM of other bacterial biofilms is characteristically composed of polysaccharide, the genome sequence of *C. jejuni* does not show any obvious candidates for a biofilm polysaccharide other than the *kps* capsular polysaccharide gene cluster. It is possible, therefore, that the constitution of the biofilm may be modified capsular polysaccharides or poly-amino acid (Ornek *et al.*, 2002), or it may be composed of extracellular DNA (Harris & Mitchell, 1973; Whitchurch *et al.*, 2002).

In conclusion, *C. jejuni* in monoculture can attach to surfaces and form a biofilm, and can form a pellicle at both 37 °C and 30 °C. It also forms a biofilm growing unattached and this aggregate biofilm has increased resistance to environmental stress. This may be relevant to the survival of the organism in the environment and in the epidemiology of *C. jejuni* infection.

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