Molecular methods to investigate adhesion, transmigration, invasion and intracellular survival of the foodborne pathogen Campylobacter jejuni

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Abstract
Campylobacter jejuni is a spiral-shaped Gram-negative pathogen and major agent of gastrointestinal foodborne illness in humans worldwide. This pathogen encodes numerous described pathogenicity-associated factors involved in important processes including bacterial adhesion to, transmigration across, invasion into and intracellular survival within intestinal epithelial cells. This review article highlights various molecular techniques applied in the studies of each of these individual steps of C. jejuni host cell interactions in vitro including gentamicin protection assay, chemotaxis and motility assays, transwell and intracellular survival assays, G-Lisa, siRNA knockdown, immunohistochemistry, immunofluorescence, electron microscopy and luciferase reporter assays. We discuss the strengths and limitations of the methods as well as the different cell model systems applied. Future work should employ new technologies including modern microscopic, proteomics-based and cell signaling approaches to identify and characterise novel virulence mechanisms, which are crucial to provide fresh insights into the diversity of strategies employed by this important pathogen to cause disease.

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1. Introduction

Zoonoses are a major health problem. An important example is the spiral-shaped flagellated bacterium Campylobacter jejuni which lives as a commensal in the gut of many birds and domestic animals, and thus can enter the food chain of humans in various ways. Infections with C. jejuni by fecal contaminated food products represent the major cause of bacterial gastroenteritis, and may be responsible for as many as 400–500 million cases in the human world population every year (Nachamkin et al., 2008; Dasti et al., 2010; Oyarzabal and Backert, 2011). Our knowledge about the interplay between C. jejuni and its hosts is still limited (Young et al., 2007), but several infection studies using different animal models elucidated the roles of motility and chemotaxis as crucial factors for a successful C. jejuni infection (Hendrixson and DiRita, 2004; Morooka et al., 1985; Nachamkin et al., 1993; Yao et al., 1997). The high motility of C. jejuni (Karim et al., 1998), which is even enhanced in medium with increased viscosity (Ferrero and Lee, 1988; Szymanski et al., 1995), allows this pathogen to efficiently reach its favoured colonisation site, the inner mucus layer of the intestine.

During the infection process C. jejuni interacts with non-phagocytic intestinal epithelial cells and encounters phagocytic immune cells (Babakhani et al., 1993; Black et al., 1988). Electron microscopic examinations of biopsies from patients with Campylobacter coliitis showed C. jejuni closely associated with the colonic epithelial cells as well as inside intestinal cells (van Spreeuwel et al., 1985). Similar observations were made upon C. jejuni infections of infant Macaca mulatta monkeys (Russell et al., 1993) and newborn piglets (Babakhani et al., 1993). Interestingly, in other infection experiments C. jejuni was not seen attached to or inside intestinal epithelial cells of mice (Lee et al., 1986) and chicken (Beery et al., 1988), although C. jejuni was present in high numbers in the mucus within the intestinal crypts. These studies imply that adhesion to and invasion into intestinal epithelial cells are not necessarily essential steps for C. jejuni colonisation of the gut.

An essential objective of ongoing C. jejuni research is to clarify the precise role of bacterial adhesion to and invasion into enterocytes for the pathogenesis of these infections in different hosts. In general it is believed that the diverse clinical manifestations of campylobacteriosis in humans, ranging from a mild, watery to a severe inflammatory and bloody diarrhoea, correlate with the variable virulence potential of different C. jejuni isolates and the immune predisposition of the patients (Allos, 2001; Janssen et al., 2008; van Putten et al., 2009; Dasti et al., 2010). Some studies suggest that C. jejuni strains isolated from patients with diarrhoea and fever adhere to and invade cultured cells in vitro more than C. jejuni isolates from patients with less obvious clinical manifestations or asymptomatic infections (Fauchere et al., 1986). In contrast, other studies have demonstrated that certain C. jejuni isolates from patients with non-inflammatory diarrhoea can exhibit similar invasion capacities in vitro as isolates causing severe colitis (Everest et al., 1992). These studies and other reports demonstrate convincingly that various C. jejuni isolates have significantly different capacities to interact with cultured epithelial cells in vitro.

The establishment of a functional interface with cultured epithelial cells by C. jejuni can be divided into three distinct processes: (i) adherence to the enterocytes, (ii) invasion into the intestinal epithelium cells and (iii) survival inside a defined intracellular compartment (called Campylobacter-containing vacuole; CCV) (Fig. 1). In addition, the translocation of C. jejuni across the intestinal epithelium into the subepithelial space of the lamina propria has been described during infection experiments (Humphrey et al., 1986) and in vitro experiments using polarised cultured epithelial cells were used with the aim to investigate if this process occurs via the transcellular or paracellular route (Fig. 1). During the last decades a main focus in the field of C. jejuni research has been to identify the bacterial factors mediating the efficient interaction with cultured epithelial cells. The methodology that has been used to characterise the interaction of C. jejuni with host cells in vitro will be presented and discussed in this review.

2. General aspects of experimental design

The efficiency by which C. jejuni interacts with cultured host cells depends on the specific properties of C. jejuni strains and the cultured epithelial cell lines. Consequently, the outcomes of in vitro assays being used to characterise the adherence, invasion, translocation or survival capacities of C. jejuni vary considerably in different studies. In addition, variable experimental settings as listed below make direct comparisons of published studies often difficult.

2.1. Selection of C. jejuni strains

Studies using C. jejuni isolates from various host origins for in vitro infection experiments with cultured epithelial cell lines suggest that no obvious host tropism occurs: C. jejuni isolates from humans, chicken or pigs are capable to adhere to and invade human, avian and porcine cell lines (Biswas et al., 2000; Gripp et al., 2011). However, C. jejuni isolates adhere to and invade cultured cell lines of certain host or tissue origins with different efficiencies (Larson et al., 2008; Poly et al., 2007; Wine et al., 2008), and the adherence and/or invasion capabilities between strains vary significantly (Biswas et al., 2000; Fauchere et al., 1986; Fearney et al., 2008; Newell et al., 1985b; Zheng et al., 2008). While a positive correlation between adherence and invasion properties of C. jejuni isolates seems to exist (Biswas et al., 2000), it was shown that efficient adhesion is not sufficient for an effective invasion process (Song et al., 2004; Konkel et al., 1992a; Kim et al., 2008; Javed et al., 2012; Christensen et al., 2009). To perform in vitro infection experiments with C. jejuni, the strain of choice should exhibit efficient adherence and invasion characteristics but also robustly infect animal models (Ahmed et al., 2002; Hiett et al., 2008; Seal et al., 2007). Such strains allow the critical evaluation of in vitro characterised pathogen–host interactions in more complex infection models (Hermans et al., 2011; Bereswill et al.,...
2.2. Cell line choice and cell confluence

The permissiveness by which \textit{C. jejuni} interacts with a wide range of different eukaryotic cell types is striking. While \textit{C. jejuni} adheres to different cell lines to similar extend (Konkel et al., 1992a), its internalisation efficiency varies dependent on the cell lines. It was suggested that \textit{C. jejuni} invades epithelial cell lines of human origin more efficiently than cell lines of non-human origin (Konkel et al., 1992a). Thus \textit{C. jejuni} infection experiments have been most commonly studied with the human intestinal cell lines Caco-2, T84 and INT-407 (Tables 1–4). In vitro invasion of porcine IPEC-1 and IPEC-J2 small intestinal epithelial cell by \textit{C. jejuni} has been described as well (Gripp et al., 2011; Naikare et al., 2006). Furthermore, \textit{C. jejuni} is able to interact in vitro with cell lines of non-intestinal origin from various organisms like human HeLa cells, liver-derived HepG2 cells, LMH chicken hepatocellular carcinoma epithelial cells, and the African green monkey kidney derived COS-1 and Vero cell lines (Biswas et al., 2006; Flanagan et al., 2009; Konkel et al., 2007; Larson et al., 2008; Novik et al., 2010; Watson and Galan, 2008; Coote et al., 2007). Only few studies have worked with primary intestinal epithelial cell preparations from chicken (Byrne et al., 2007; Van Deun et al., 2008) or human biopsy material (Byrne et al., 2007).

The choice of cell line depends on the specific experimental question. Many studies have examined the adherence, invasion and survival capacity of \textit{C. jejuni} during infection of non-differentiated INT-407, T84 or Caco-2 cells. The used cell confluence also influences the outcome of infection experiments. The term confluence refers to the coverage of cells on the given petri dish surface. For example, 100% confluence means the petri dish is completely covered by a monolayer of cells (confluent), whereas 50% confluence means that half of the dish is covered by cells (semi-confluent). Interestingly, \textit{C. jejuni} invades cells of a semi-confluent cell monolayer more efficiently than cells of a confluent cell monolayer (Hu et al., 2008). Furthermore, \textit{C. jejuni} infects Caco-2 less and T84 cells more efficiently than INT-407 cells (Poly et al., 2008; Wine et al., 2008). The use of INT-407 cells is preferred when investigating the detailed intracellular localisation and trafficking of \textit{C. jejuni}: Co-localisation studies with transfected GFP-tagged phagolysosomal marker proteins have been used to investigate this...
process (Watson and Galan, 2008) and cultured INT-407 or COS-1 cell lines can be transfected more easily than T84 or Caco-2 cells.

Studies of the translocation properties of C. jejuni isolates across an intestinal epithelium layer in vitro require polarizing cell lines like T84 (Chen et al., 2006; Monteville and Konkel, 2002; Wine et al., 1992; Zheng et al., 2008), Caco-2 (Everest et al., 1992; Grant et al., 1993; Harvey et al., 1999; Hu et al., 2008; Konkel et al., 1992a), MDCK-I (Wine et al., 2008) or MKN-28 cells (Boehm et al., 2012), which express tight and adherens junctions thus properly connecting neighboring cells (Fig. 1). For this purpose, these cell lines are commonly grown as tight and adherens junctions thus properly connecting neighboring cells (Fig. 1). For this purpose, these cell lines are commonly grown as tight and adherens junctions thus properly connecting neighboring cells (Fig. 1).

Mucus colonisation has been seen as a critical step in the infection process of C. jejuni (Beery et al., 1988; Lee et al., 1986). Until recently appropriate in vitro models have been missing to investigate the interaction of Campylobacter with mucus as most cultured cell lines generally do not synthesise and secrete mucin (Alemka et al., 2012). Lately it has been shown that the HT29-derivative E12 cell line produces a mucus layer when grown and differentiated on transwell filters increasing the binding efficiency of C. jejuni to E12 cells and subsequently its internalisation (Alemka et al., 2012; Behrens et al., 2001).

For immunological studies the significant variation in the dynamics of Campylobacter infection in vitro requires the use of MOIs of 10 to 500 for most infection studies, especially to compare the invasion capabilities of different C. jejuni strains or to investigate the intracellular fate of C. jejuni by microscopy. Using these MOIs up to 11 bacteria have been found inside infected cultured cells using fluorescence microscopy. COS-1 cells grown in 15 cm cell culture dishes were infected with C. jejuni in a ration 1:1000 to reach the critical number of intracellular bacteria for the intracellular proteome analysis of C. jejuni (Liu et al., 2012).

2.4. Motility and growth phase of bacteria

Many studies have undoubtedly proven that flagellum-driven motility of C. jejuni is a crucial requirement for the infection process in vivo and to make contact with host cells (Young et al., 2007; Guerry, 2007). Interestingly, recent studies have shown that motility is necessary but not sufficient for the efficient invasion process in vitro as the clinical isolates C. jejuni BH-01-0142, CG8421 and CG8486 are fully motile when compared to strain C. jejuni 81-176 but show significantly reduced invasion capability (Poly et al., 2007, 2008). Nevertheless, up to now many identified C. jejuni mutants severely (3 magnitudes or more) impaired in their capability to invade cultured epithelial cells, are negatively affected in their motility. Consequently, comparing the capability of different C. jejuni isolates to interact with host cells (Biswas et al., 2000; Ø Crónin and Backert, 2012), or investigating specific mutants for invasion defects

Table 1

<table>
<thead>
<tr>
<th>Applied method</th>
<th>Cell model used</th>
<th>Bacterial factors involved</th>
<th>Host factors involved</th>
<th>C. jejuni strains used</th>
<th>References</th>
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<tbody>
<tr>
<td>ALA</td>
<td>INT-407, Caco-2</td>
<td>ND</td>
<td>ND</td>
<td>NC, 81-176</td>
<td>McSweeney and Walker (1986); Russell and Blake (1994)</td>
</tr>
<tr>
<td>LM</td>
<td>HeLa</td>
<td>ND</td>
<td>ND</td>
<td>Human isolates</td>
<td>Fauchere et al. (1986)</td>
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<tr>
<td>MLA</td>
<td>Caco-2</td>
<td>ND</td>
<td>ND</td>
<td>Human isolates</td>
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</tr>
<tr>
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<td>INT-407</td>
<td>FlaA</td>
<td>ND</td>
<td>81116</td>
<td>Grant et al. (1993)</td>
</tr>
<tr>
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<td>FlaA, CheY</td>
<td>ND</td>
<td>81-176</td>
<td>Yao et al. (1994, 1997)</td>
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<tr>
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<td>GaE</td>
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<td>ND</td>
<td>81-176</td>
<td>Bacon et al. (2001)</td>
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<tr>
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<td>INT-407</td>
<td>CadF</td>
<td>Fibronectin</td>
<td>F38011, 81-176</td>
<td>Monteville and Konkel (2002); Monteville et al. (2003); Konkel et al. (2005)</td>
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<tr>
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<td>PseA</td>
<td>ND</td>
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<td>Guerry et al. (2006)</td>
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<td>Bachtiar et al. (2007)</td>
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<tr>
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<td>IntPIC, chPic</td>
<td>ND</td>
<td>ND</td>
<td>81-176, chicken and human isolates</td>
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<tr>
<td>CBA</td>
<td>Caco-2, LMH</td>
<td>CapA</td>
<td>ND</td>
<td>11168, F38011</td>
<td>Ashgar et al. (2007); Flanagan et al. (2009)</td>
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<td>CBA</td>
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<td>Cj1461</td>
<td>ND</td>
<td>81-176</td>
<td>Kim et al. (2008)</td>
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<td>LMH, INT-407</td>
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<td>Fibronectin</td>
<td>F38011</td>
<td>Flanagan et al. (2009); Konkel et al. (2010)</td>
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<td>SodB</td>
<td>ND</td>
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<td>Brandsted et al. (2005); Baek et al. (2011); Hoy et al. (2012)</td>
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<td>Caco-2</td>
<td>Cst-II</td>
<td>ND</td>
<td>81-176, GB11</td>
<td>Louwen et al. (2012)</td>
</tr>
</tbody>
</table>

Abbreviations: ALA (3H-acetic acid labelling assay); CBA (CFU-based adherence assay); IFM (immunofluorescence microscopy); IOS (inside/outside staining); LM (light microscopy); MLA (15S-methionine labelling assay); hPic (human primary intestinal cells), chPic (chicken primary intestinal cells).

Due to space limitations only a few key references are provided.

Identified as an adhesin in C. jejuni strain TG9011, but no effect observed with JlpA mutants in other isolates such as 11168 or 81-176 (van Alphen et al., 2008; Novik et al., 2010). Another report showed that JlpA is not required for adhesion of strain F38011 to chicken LMH cells (Flanagan et al., 2009).
require the thorough pre-testing of their motility properties. Only if the tested strains exhibit a similar high motility both on semi-solid motility agar plates and in liquid cultures, observed differences in adherence or invasion properties are potentially mediated by motility-independent virulence factors. Motility pre-tests with semi-solid motility agar plates are not always sufficient as C. jejuni mutants can lose motility upon cultivation in liquid medium and behave like the wild-type strain on semi-solid motility agar plates (Goon et al., 2006; Novik et al., 2010).

Motility is an important phase variable property of C. jejuni (Hendrixson, 2006, 2008; Karlyshev et al., 2002). C. jejuni motility is preferentially lost during in vitro cultivation (Gaynor et al., 2004), but can be regained throughout infection experiments (Jones et al., 2004; Caldwell et al., 1985). Especially during the process of generating isogenic C. jejuni mutants the resulting strains have to be screened for altered motility in comparison to the wild-type strain. To compensate the negative effect of slight motility alterations on the adherence efficiency of C. jejuni strains, many in vitro infection studies take advantage of an additional centrifugation step. For this purpose, C. jejuni can be enforced to interact with the epithelial cell monolayer using a centrifugation step for 5 min at 1000 × g. This represents a suitable method to synchronise the infection process in order to overcome variations in the interaction of C. jejuni strains with cultured epithelial cells that are due to variable motility properties rather than altered adherence or invasion characteristics.

The motility of C. jejuni is not only phase variable as discussed above, but also growth-phase dependent. It peaks during the late logarithmic growth phase and diminishes during the stationary phase (Wösten et al., 2004). These findings imply that the growth phase of C. jejuni plays an additional role for the in vitro infection process (Konkel et al., 1992a) and it was shown that C. jejuni from mid-logarithmic growth cultures showed higher invasion efficiency than bacteria from early stationary phase (Hu and Kopecko, 1999). An important disadvantage of using agar plate grown C. jejuni for infection experiments is that the bacteria vary significantly in shape, flagellation, motility and viability (Ng et al., 1985a). Thus, before infection, the subcultivation of C. jejuni in liquid culture is highly recommended (Ng et al., 1985a). It has been shown that C. jejuni growth in liquid broth medium led to a higher percentage of highly motile C. jejuni cells with similar shape.

3. Host cell adhesion by C. jejuni

Though the precise molecular mechanisms involved in the attachment of C. jejuni to eukaryotic cells are still unknown, several studies have provided evidence in recent years that the adhesion process of C. jejuni is a multifactorial event (Young et al., 2007; Ó Cróinín and Backert, 2012). This is reflected by the circumstances that most of the described adherence-defective mutants exhibit only reduced rather than severely abolished interactions with the eukaryotic cells wherein the mutants reach adherence level of about 20% to 50% of the wild-type strain. These observations imply that redundancy may exist in the adhesion process and suggest that the co-operative action of several factors of C. jejuni is required to mediate optimal adherence to host cells.

3.1. Recovery of cell-associated bacteria

Traditionally, the adherence of C. jejuni to eukaryotic cells has been investigated by cell association experiments (Everest et al., 1992; Fauchere et al., 1986; Russell and Blake, 1994). These approaches enumerate all bacteria that adhere to and invade eukaryotic cells but fail to distinguish between these two populations and therefore provide no direct information about the number of bacteria solely attached to the surface of the host cell at a given time point of infection. Determination of colony forming units (CFU) has generally been used to characterise...
the attachment or association of C. jejuni to cultured eukaryotic cells and to identify bacterial factors involved in the adherence process (Table 1). The outcome of these assays depends on the plating efficiency of C. jejuni, which varies for different culture media (Ng et al., 1985b). Therefore, culture variations have also to be considered when comparing different studies.

For the standard CFU-based adherence (CBA) assay C. jejuni strains are added with a defined MOI to cultured epithelial cells in 24 well tissue plates. Non-adherent C. jejuni cells are removed by a repeated washing procedure before epithelial cells are lysed with 0.1% Triton X-100, 0.1% sodium deoxycholate (DOC) solutions or physical rupture. With this approach all cell-associated bacteria, either attached to the membranes or released from the intracellular compartments of infected cells, are spread in serial dilutions on appropriate agar plates to determine the bacterial counts. The cell association efficiency of C. jejuni is then calculated by dividing the number of bacteria determined by plate counting (CFU) after lysing the cultured epithelial cells by the total number of bacteria in the infection inoculum. This assay does not allow the direct quantification of extracellular attached bacteria as all cell-associated bacteria including intracellular bacteria are recovered after cell lysis. The accurate number of adherent bacteria can only be calculated when cell association experiments are performed in combination with a gentamicin protection assay (see below) that allows the determination of intracellular bacteria (Section 6.1).

The CBA assay does not require expensive laboratory equipment, is easy to handle and enables the fast and economical test of C. jejuni mutants for their adherence properties. As such the assay has been successfully used to screen C. jejuni transposon mutant libraries for mutants with reduced capability to interact with cultured epithelial cells (Golden and Acheson, 2002; Javed et al., 2010; Novik et al., 2010). Since the experimental procedure usually involves the use of 0.1% DOC or 0.1% Triton X-100 to lyse the epithelial cell monolayer, potential false positive hits (Novik et al., 2010) are recommended as adherence factor by screening a transposon mutant library of C. jejuni T90H01 to HEP-2 cells (Jin et al., 2001), it is not defective for invasion (Flanagan et al., 1998) lost the metabolic fitness required for the ef- cient interaction as consequence for its inability to catabolise growth-supporting glucogenic amino acids. A similar phenotype has been described for the liquid scintillation counter. Similar to the CBA assay described above, this approach does not allow the direct quantification of extracellular attached bacteria, which have to be determined in combination with a gentamicin protection assay.

To date, most C. jejuni mutants defective for efficient interaction (adhesion/invasion) with cultured epithelial cells have been characterised by the CBA/invasion assay (Table 1). This approach identified motility and chemotaxis (Yao et al., 1994, 1997; Tareen et al., 2010, 2011) as critical factors mediating the interaction of C. jejuni with host cells, but revealed also the important role of glycan surface structures for the adherence/invasion process of C. jejuni. In particular, inactivation of the capsule biosynthesis genes pksM (Bacso et al., 2001) and pksP (Bachtar et al., 2007), the LOS synthesis gene gale (Fry et al., 2000) and the genes pgfB and pgfE (Szymanski et al., 2002) involved in the N-glycosylation of C. jejuni proteins resulted in a adherence defective phenotype. The C. jejuni 81-176 pseA mutant, unable to modify flagellin with the acetamidino form of pseudaminic acid, shows a decreased adherence to INT-407 cells (Guerry et al., 2006). Furthermore the in- volvement of the fibronectin-binding protein CadF (Monteville et al., 2003), the fibronectin-like protein A FlpA (Flanagan et al., 2009), the autotransporter protein CapA (Ashgar et al., 2007) and the HtrA protease (Brøndsted et al., 2005; Bak et al., 2011; Hoy et al., 2012) in the adherence process have been identified by CBA assays. The surface-exposed lipoprotein JlpA has been described to facilitate the binding of C. jejuni TCH0911 to HEP-2 cells (Jin et al., 2001) but is surprisingly dispensable for the adherence of C. jejuni F38011 to chicken LMH hepatocellular car- cinoma epithelial cells (Flanagan et al., 2009). Interestingly, although the JlpA mutant shows a reduced adherence phenotype by 20% of wild-type level (Jin et al., 2001), it is not defective for invasion (Flanagan et al., 2009; Novik et al., 2010; van Alphen et al., 2008). Another putative lipoprotein, the hypothetical protein C0497 of the hyper-invasive strain C. jejuni 01/51, has been identified as adherence factor by screening a transposon mutagenesis library for invasion defective mutants (Javed et al., 2010).

Though the antigenic protein Peb1A was originally identified as potential pathogenicity factor of C. jejuni 81-176 mediating adhesion to HeLa cells and invasion into INT-407 cells (Pei et al., 1998), it has been recently characterised as a periplasmic aspartate/glutamate-binding protein (Leon-Kemps Mdel et al., 2006; Muller et al., 2007) required for the utilisation of certain amino acids as growth substrates in vitro (Hofreuter et al., 2008; Leon-Kemps Mdel et al., 2006). These findings question the primary function of Peb1A as adhesin/invasin, especially since peb1A mutants exhibited no significant invasion defects in recent infection studies (Flanagan et al., 2009; Novik et al., 2010; van Alphen et al., 2008). It is feasible that the overnight-culture of the C. jejuni 81-176 peb1A mutant used for the in vitro infection experiment by Pei et al. (1998) lost the metabolic fitness required for the efficient invasion process as consequence for its inability to catabolise growth-supporting glucogenic amino acids. A similar phenotype has been described for the
aspA and aspB mutants, defective in the catabolism of aspartate and glutamate, respectively (Guccione et al., 2008). Both mutants are recovered with reduced CFU in invasion assays, which can be compensated by fumarate supplementation in the culture medium (Novik et al., 2010). Though Peb1A plays probably no role in the direct interaction with host cells in vitro, it has a crucial role for the proliferation and persistence of C. jejuni in vivo as demonstrated in various animal infection studies (Flanagan et al., 2009; Hofreuter et al., 2012; Pei et al., 1998).

3.2. Immunofluorescence microscopy (IFM)

The above described CBA assay alone is not suitable to characterise the interaction of C. jejuni with individual cultured host cells. In order to gain more detailed information about the adherence process of C. jejuni, IFM studies have gained increased importance in recent years. The availability of C. jejuni strains expressing fluorescence proteins like GFP, its derivatives or mCherry (Miller et al., 2000; Mixter et al., 2003; Krause-Gruszczynska et al., 2007a; van Alphen et al., 2008; Guerry et al., 2006) has facilitated studies imaging the interaction of C. jejuni with cultured host cells. Alternatively, immunostaining experiments with antibodies raised against C. jejuni (Apel et al., 2012; Byrne et al., 2007; Hu and Kopecko, 1999; Konkel et al., 2007; Louwen et al., 2012; Monteville et al., 2003; Novik et al., 2009, 2010; Watson and Galan, 2008) or direct labelling of C. jejuni cells by using a fluorescent dye like FITC (Song et al., 2004), Texas-Red (Buelow et al., 2011) or TAMRA (Alema et al., 2010; Byrne et al., 2007) have been successfully employed to investigate the infection process in vitro. Especially the inside outside staining approach overcomes the restriction of the above-described CFU-based assay and allows a precise quantification of the C. jejuni subpopulations which are extracellularly attached to the cultured cells or residing intracellularly (Apel et al., 2012; Byrne et al., 2007; Pryjma et al., 2012; Watson and Galan, 2008). IFM experiments have been also used to complement the results gained from CBA assay (Table 1). Recently, IFM-based infection experiments have demonstrated that efficient adherence of C. jejuni wild-type strains to Caco-2 cells occurs within 15 minutes and that ganglioside-like LOS enhances this interaction (Louwen et al., 2012). Consequently mutants with an inactivated sialyltransferase cstII gene, which are unable to produce a ganglioside-like LOS, exhibit a significant adhesion defect (Louwen et al., 2012).

4. Cellular invasion of C. jejuni

4.1. Gentamicin protection assay (GPA) and microscopic methods

Early reports investigated intestinal biopsies from human patients using immunohistochemistry and transmission electron microscopy (TEM) and have shown that C. jejuni can be found in compartments within gut tissue cells in vivo (van Spreeuwel et al., 1985). Later, a multitude of studies have demonstrated that C. jejuni can invade and survive within a number of cultured cell lines in vitro, notably studies using non-polarised INT-407, Hep-2 or HeLa and polarised T84, MKN-28 or Caco-2 cells (Table 2). Mutagenesis of numerous C. jejuni genes involved in adhesion, glycosylation, capsular formation, bacterial motility and host adaptation or survival have been implicated to play a role during invasion (Grant et al., 1993; Szymanski et al., 2002; Yao et al., 1994; Pei et al., 1998; Bacon et al., 2001; Konkel et al., 2001; Hendrixson and DiRita, 2004; Kakuda and DiRita, 2006; Krause-Gruszczynska et al., 2007b; Watson and Galan, 2008; Novik et al., 2010). A widely-used but indirect laboratory method to monitor adherence and invasion is the gentamicin protection assay (GPA). This method uses the antibiotic substance gentamicin to kill all extracellular bacteria after infection, but is unable to penetrate host cells. Thus, intracellular bacteria stay alive and can be quantified as CFU on agar plates. However, this method is indirect and also bears some discussed limitations (Ó Cróinin and Backert, 2012). Direct visualisation of entering or intracellular C. jejuni in vitro infected cells was performed by IFM, live cell imaging (LCI) and higher resolution approaches such as TEM or scanning electron microscopy (SEM), which were applied in some but not all studies (Table 2). For example, IFM was used to investigate the association of internalised C. jejuni with microtubules and the molecular motor protein dynein (Hu and Kopecko, 1999), filamentous actin (Konkel et al., 1992a; Rivera-Amill et al., 2001; Krause-Gruszczynska et al., 2007a), the Golgi protein GM130 (Watson and Galan, 2008) and the small Rho GTPases Rac1 and Cdc42 (Krause-Gruszczynska et al., 2007a; Eucker and Konkel, 2012).

In addition, Louwen et al. (2008, 2012) investigated whether sialylation of C. jejuni LOS structures, generating human nerve ganglioside mimetics, is important for intestinal epithelial invasion. They showed that C. jejuni isolates expressing ganglioside-like LOS bound to and invaded Caco-2 cells in larger numbers than ΔcstII mutants or natural isolates lacking such structures as determined by GPA and

**Table 4** Methods applied to study C. jejuni intracellular survival in cultured epithelial cellsa.

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<tr>
<td>ISA</td>
<td>Hep-2</td>
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<tr>
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<td>Lamp-1, CD63</td>
<td>ISA-176, 108</td>
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a Abbreviations: IFM (immunofluorescence microscopy); ISA (intracellular survival assay); LDS (live/dead staining), LRA (luciferase reporter assay).
b Co-localisation of the indicated marker proteins occurs in a certain temporal fashion during the CCV maturation and represents merely an indirect indication for the host factors being involved in CCV development.
c Due to space limitations only a few key references are provided.
parallel experiments using an isogenic downstream signalling to ERK1/2 and GTPases (Boehm et al., 2011). Another tyrosine residue, Y-925, which has a proposed role in FAK bacterial uptake. Moreover, FAK was shown to be phosphorylated at time-dependent fashion, and correlating with the time points of 3-kinase (PI3-kinase) are involved in signalling cascades permitting protein (MAP) kinases, protein kinase C (PKC) and phosphatidylinositol combined with certain functional assays. For example, GPA combined (or two) of the above quantification results of various groups provide a rather confusing picture. In numerous reports, the small interfering RNA (siRNA) approach can be employed to investigate if a given host factor plays a role in the bacterial entry process of C. jejuni (Krause-Gruszczynska et al., 2007a, 2011; Boehm et al., 2011). In agreement with some of the inhibitor studies described above, C. jejuni infection, which was not seen during infection with mutants of CadF (Monteville et al., 2003). These data support the view that the CadF → fibronectin interaction is upstream of various tyrosine kinases and other signalling molecules involved in C. jejuni-triggered host cell invasion.

4.4. Application of GTPase activation assays

The GTPases Rac1, Cdc42 and RhoA are the best-characterised members of the small Rho family of GTP-binding proteins. They act as guanine nucleotide regulated switches, transmitting extracellular signals to regulate normal intracellular functions as well as invasion of various microbial pathogens (Boquet and Lemichez, 2003; Tegtmeyer et al., 2011). With the help of specific inhibitors (e.g. compactin) and GTPase-regulating toxins (e.g. CNFs, Toxin B) it was further shown by GPA, GPA and other methods that Rac1 and Cdc42 (but not RhoA) play a role in the cellular entry process of C. jejuni (Krause-Gruszczynska et al., 2007a). The uptake of C. jejuni into INT-407 cells is accompanied by a time-dependent accumulation of both increased Rac1-GTP and Cdc42-GTP levels as determined by classical CRIB-domain pulldown and G-LISA approaches (Krause-Gruszczynska et al., 2007a, 2011; Eucker and Konkel, 2012; Boehm et al., 2011). The induction of maximal GTPase activities by C. jejuni appears to require the fibronectin-binding proteins CadF and FlpA, the flagellum and the Campylobacter invasion antigen CiaC as well as the activities of FAK, Src, EGF receptor, PDGF receptor and PI3-kinase, as confirmed by inhibitor experiments (Krause-Gruszczynska et al., 2007a, 2011; Eucker and Konkel, 2012). GPA studies with wild-type and mutant C. jejuni also indicated that CiaC is required for re-localising Rac1 to the site of bacterial attachment (Eucker and Konkel, 2012).

4.5. Reporter assay to monitor infection of Cia proteins into host cells

One pressing question in the C. jejuni field is whether Cia and probably other pathogenicity-associated proteins can be delivered from the bacterium into the host cell cytoplasm in order to function as invasion-triggering molecules. To address this question, Neal-McKinney and Konkel (2012) utilised a reporter assay approach based on the adenylate cyclase domain of the Bordetella pertussis CyaA toxin expressed as a fusion construct with Cia proteins. The adenylate cyclase enzyme is inactive in the bacterium and activated in the presence of the eukaryotic factor calmodulin. Delivered adenylate cyclase then catalyses the production of cAMP from isotope-labelled ATP given to infected cells, which can be easily monitored in the assays. Expression of a CiaC-adenylate cyclase fusion protein exhibited significantly increased cAMP levels during infection with wild-type C. jejuni as compared to mutants of FlgK, FlgK and FlgL flagellar hook proteins (Neal-McKinney and Konkel, 2012). Similar results were obtained for another Cia protein, CiaI, suggesting that the flagellum is necessary for the delivery process (Neal-McKinney and Konkel, 2012). However, more functional studies are necessary to investigate this fascinating hypothesis in greater detail.

4.6. siRNA knockdown and useful mouse knockout cell lines

To investigate if a given host factor plays a role in C. jejuni invasion, the small interfering RNA (siRNA) approach can be employed to downregulate the expression of any gene of interest. This method was exemplarily used to confirm the importance of Cdc42 (Krause-Gruszczynska et al., 2011) and Rac1 (Boehm et al., 2011) for C. jejuni host cell entry. In addition, the siRNA method was applied to identify guanine exchange factors (GEFs), signalling compounds that can interact with GTPases and promote the active, quantitative IFM. Various TEM and SEM studies were then applied to visualise tightly bound C. jejuni to various host cell types or intracellular bacteria (Oelschlaeger et al., 1993; Hu et al., 2008; Watson and Galan, 2008; Eucker and Konkel, 2012). Investigation of infected INT-407 and other cells indicated that C. jejuni can induce membrane ruffling in a contact-dependent fashion (Krause-Gruszczynska et al., 2007a; Eucker and Konkel, 2012) and is associated with a specific host cell penetration mechanism, first entering with its flagellar tip followed by the opposite flagellar end (Krause-Gruszczynska et al., 2007a, 2011; Boehm et al., 2011). The reason for these discrepancies is unknown. In addition, the siRNA method was applied to investigate this fascinating hypothesis in greater detail.
GTP-bound state by facilitating the exchange of GDP for GTP. In this way, shown to host cell GEFs (Tiam-1 and DOCK180) were downregulated and shown to mediate Rac1 activation downstream of FAK (Boehm et al., 2011; Eucker and Konkel, 2012). In addition, another GEF, Vav2, appears to act downstream of FAK, EGF receptor, PDGF receptor and PI3-kinase and stimulates the generation of elevated Cdc42-GTP levels (Krause-Gruszczynska et al., 2011). These studies were complemented with experiments using available fibroblast cell lines from knockout mice. Such knockout cell lines have the great advantage over siRNA studies that the respective gene of interest is completely deleted in the genome, and thus not even small traces of protein can be expressed. In fact, SEM and GPA studies showed that C. jejuni is severely impaired in its capability to enter cell lines derived from fibronectin−/−, integrin−β1/−/−, focal adhesion kinase (FAK)−/−/−, Vav1/2−/−/− and Src−/−/−/Yes−/−/−/Fyn−/−/− (SYF) triple knockout mice, but can efficiently invade the corresponding wild-type control cells (Krause-Gruszczynska et al., 2011; Boehm et al., 2011). Finally, infection of integrin−β1/−/− knockout cells stably expressing the integrin−β1 point mutant TT788/89AA exhibiting proper FAK signaling but defects in extracellular fibronectin fibril formation, and integrin−β1/−/−Y783/795F cells, which have a pronounced defect in FAK autophosphorylation at Y-397, revealed large defects in C. jejuni invasion (Krause-Gruszczynska et al., 2011). Interestingly, the activation of Rac1 and Cdc42 by C. jejuni associated with the induction of membrane ruffling and filopodia formation was also significantly abrogated in infected fibronectin−/−/−, integrin−β1/−/−, FAK−/− and SYF−/−/−/− knockout cells, although the bacteria expressed intact flagella and adhered to cells (Krause-Gruszczynska et al., 2011; Boehm et al., 2011). Collectively, these results strongly support the view that C. jejuni infection induces fibronectin−/integrin-dependent signal transduction to stimulate their own uptake by host target cells using signalling to activate the Rho GTPase members Rac1 and Cdc42. 4.7. Application of dominant-negative and constitutive active expression constructs

A common method in cell biology is the expression of dominant-negative (DN) and constitutive-active (CA) constructs in order to investigate the importance of a given factor of interest. A dominant-negative mutation adversely affects the normal, wild-type gene product within the same cell. This commonly occurs if the protein can still interact with the same elements as the wild-type protein, but inhibits its function. In contrast, a constitutively active protein is a mutated gene product whose activity is constant and active. Canonical point mutations that affect the GTPase cycle are well-described and lead to the generation of CA or DN Rho GTPase constructs (Heasman and Ridley, 2008). To confirm that Rac1 and Cdc42 activity was required for C. jejuni invasion, INT-407 cells were transfected with the DN alleles of Rac1 (Rac1-T17N) or Cdc42 (Cdc42-T21N). Transfection of INT-407 cells with both DN-Rac1 and DN-Cdc42 resulted in a significantly reduced bacterial internalisation as determined by GPA and IFM; while expression of either DN-Rac1 or DN-Cdc42 alone was less effective (Krause-Gruszczynska et al., 2007a). When CA GTPase constructs were expressed the most pronounced effect was observed with CA-Rac1 (Rac1-ΔT61L) whose expression stimulated C. jejuni invasion −4.7-fold as compared to the empty vector control, while moderate effects (−1.7-fold increase) were seen with CA-Cdc42 (Cdc42-ΔQ61L) (Krause-Gruszczynska et al., 2007a). Thus, activation of Rac1 and Cdc42 are involved in C. jejuni invasion. In addition, overexpression of DN constructs of caveolin-1 but not GTPase dynamin-II substantially inhibited C. jejuni uptake, suggesting that caveolin-1 in the classical caveolae structures of cell surfaces may also play a role in the uptake process (Watson and Galan, 2008). Finally, expression of DN forms PDGF and EGF receptors significantly reduced the amount of recovered intracellular C. jejuni by GPA, confirming the involvement of both receptor tyrosine kinases in the uptake of C. jejuni (Krause-Gruszczynska et al., 2011). 4.8. Application of other transient expression constructs

To further investigate the potential involvement of lipid rafts or caveolae in C. jejuni invasion, Watson and Galan (2008) examined the acquisition of caveolin-1 and flotillin-1, two markers associated with these membrane domains, to invading bacteria. Expression of the GFP (green fluorescence protein)-tagged proteins and LCI have shown that C. jejuni acquired caveolin-1–GFP and flotillin-1–GFP immediately after uptake (Watson and Galan, 2008). Quantification of this association determined that at early time points during infection, 60% of the C. jejuni containing vacuoles (CCVs) co-localised with both caveolin-1–GFP and flotillin-1–GFP. The association, however, was transient since at later points after infection, only ~10% of the CCVs were seen in association with these markers. In addition, the signalling of FAK and Vav2 was further investigated using transient transfection of point mutant constructs exhibiting specific signalling defects. First, FAK−/−/− knockout cells re-expressing wild-type FAK restored the capability of C. jejuni to invade these cells as determined by GPA, while expression of FAK-mutants that were either not capable of autophosphorylation (FAKY397F), impaired kinase activity (FAK K454R), mutated in a binding site for the adapter protein Grb2 (FAKY925F), or mutated in several proline residues of two PxxP-motifs (FAKAPR1/2) necessary for association with SH3-domain containing factors such as Graf and p130CAS, significantly reduced the invasion rates of C. jejuni by about 35–50% (Boehm et al., 2011). This indicates an important role of FAK signalling downstream of fibronectin and integrins in facilitating efficient uptake of C. jejuni. Second, INT-407 cells were transiently transfected with wild-type Vav2 and different Vav2 mutants that were either impaired in EGF-receptor-dependent phosphorylation of Vav2 (Vav2 Y172/159F), lacking the primary phosphatidylinositol-3,4,5-triphosphate binding site (Vav2 R425C) or were unable in binding to activated EGF receptor (Vav2 W673R and Vav2 G693R) (Tamás et al., 2003). GPA revealed that overexpression of either Vav2 mutant construct significantly reduced the number of intracellular C. jejuni, further confirming the importance of Vav2 in bacterial uptake (Krause-Gruszczynska et al., 2011). These findings also support the view that Vav2, by binding to and signaling through C. jejuni-induced EGF/PDGF receptors and PI3-kinase activation pathways, may contribute Cdc42 activation during infection (Krause-Gruszczynska et al., 2011). 5. C. jejuni transmigration across polarised epithelial cells

Cell polarity of the epithelium is an important functional and protective feature in the gut system in vivo. While most of the above studies were done with non-polarised cells, polarised intestinal epithelial cells exhibit apical and basolateral surfaces that are separated by tight and adherens junctions (Fig. 1). They form microvilli structures with a well-defined brush border and express several defined marker proteins. Various important gut pathogens such as Salmonella, Shigella and Listeria, however, developed strategies to cross this epithelial barrier, gain access to submucosal tissues, trigger tissue damage and cause disease in humans. Generally, there are two different mechanisms, described as the paracellular and the transcellular transmigration routes (Kazmierczak et al., 2001; Tegtmeier et al., 2011). Pathogens utilising the paracellular mechanism cross the epithelial barrier by passage between neighbouring epithelial cells and overcome the tight and adherens junction complexes. In contrast, pathogens specialised on the transcellular mechanism invade host cells at the apical membrane followed by intracellular trafficking and exit these cells at the basolateral membrane (Balkovetz and Katz, 2003). 5.1. Transwell assays and electron microscopy

Polarised cell models are particularly valuable for the study of microbic effects on cell barrier permeability, transepithelial electrical resistance (TER), mode of transmigration and cell invasion. To investigate whether
C. jejuni can cross the epithelial cell barrier reflective of a virulence mechanism, the transwell filter system has been applied (Table 3). For this purpose, various cell lines were seeded and differentiated for a few weeks in transwells. TER values were followed over time to ensure proper cell monolayers and junction formation, which have been confirmed by SEM illustrating the presence of normal microvilli and well-defined brush borders (Konkel et al., 1992c; Hu et al., 2008) as well as IFM staining for E-cadherin (adherens junction marker) and JAM (junction adhesion molecule, tight junction marker) as described (Boehm et al., 2012). SEM and TEM studies were used to compare the interaction of C. jejuni with non-polarised INT-407 cells with polarised Caco-2 cells. The efficiency of C. jejuni invasion of Caco-2 cells was two- to three fold less as compared to INT-407 cells (Hu et al., 2008). C. jejuni bound to most INT407 cells and entered about two-thirds of the host cells over 2 h (two bacteria/cell). In contrast, only 11–17% of differentiated Caco-2 cells were observed to bind and internalise either C. jejuni strains 81-176 or NCTC1168, and a small percentage of infected Caco-2 cells contained 5–20 internalised bacteria per cell after 2 h (Hu et al., 2008). Further SEM demonstrated that C. jejuni were present intercellularly between two neighboring cells as well as intracellularly in CCV compartments (Konkel et al., 1992c; Hu et al., 2008). It was therefore suggested that C. jejuni can translocate across polarised Caco-2 cell monolayers both by passing through and between cells as summarised in Fig. 1.

5.2. Determination of CFU and functional studies

Migration of C. jejuni through polarised cells was confirmed by determination of CFU in the lower chamber of transwells, GPA and other functional assays. Chloramphenicol, an inhibitor of bacterial protein synthesis, reduced the translocation rates of C. jejuni (Konkel et al., 1992c). C. jejuni adherence, internalisation, and translocation were also inhibited at lower temperatures as examined at 20 °C and 4 °C compared to 37 °C (Konkel et al., 1992c). These data indicate that adherence, penetration, and translocation of C. jejuni require active bacterial and host cell processes. However, similar to the TEM studies described above some researchers supported the paracellular route as major transmigration pathway (Grant et al., 1993; Monteville and Konkel, 2002; Boehm et al., 2012), while other groups favoured the transcellular mode (Brás and Ketley, 1999; Kalischuk et al., 2009; Louwen et al., 2012) or proposed a mix of both (Konkel et al., 1992c; Hu et al., 2008). Because bacterial penetration through tight and adherents junctions might be expected to cause a loss in TER, one major argument for the transcellular mode is that TER values often did not change upon C. jejuni infection. In addition, the application of lipid raft inhibitors to the apical surface reduced the number of transmigrated CFU (Brás and Ketley, 1999; Kalischuk et al., 2009). Interestingly, C. jejuni induced not only its own internalisation and translocation but also that of commensal Escherichia coli, without affecting TER (Kalischuk et al., 2009).

Major arguments for the paracellular route came from competition experiments with soluble fibronectin and the observation that invasion-defective ΔactA or ΔactD mutants transmigrated as effectively as wild-type C. jejuni (Monteville and Konkel, 2002). In addition, ΔactA/B and motility mutants were unable to pass polarised Caco-2 or MKN-28 cells suggesting that certain flagellum-mediated activities play a role (Grant et al., 1993; Boehm et al., 2012).

Interestingly, two other types of mutants, LOS-deficient ΔcstII and ΔhtrA serine protease which are highly motile, exhibited strong deficiencies in C. jejuni transmigration across polarised cells (Boehm et al., 2012; Louwen et al., 2012). This suggests that bacterial motility per se is not required for transmigration, but cstII- and htrA-mediated cell binding maybe involved. Another important observation is that HtrA was found to be secreted into the C. jejuni cell culture supernatant (Hoy et al., 2012; Boehm et al., 2012). Infection studies and in vitro cleavage assays showed that HtrA cleaves-off the extracellular TGF domain of E-cadherin on epithelial cells. Deletion of the htrA gene or expression of a protease-deficient S197A point mutant in C. jejuni led to severe defects in E-cadherin cleavage and bacterial transmigration across polarised MKN-28 cell monolayers (Boehm et al., 2012). Thus, cleavage of host junctional proteins like E-cadherin (and maybe others) by secreted HtrA may explain how C. jejuni can transmigrate paracellularly between neighboring cells. Interestingly, C. jejuni HtrA was unable to cleave the fibronectin receptor although this has been seen for its HtrA counterpart in H. pylori (Hoy et al., 2010; Boehm et al., 2012). This observation is in agreement with earlier findings that fibronectin is a major host cell factor necessary for C. jejuni binding and invasion of non-polarised cells. But can C. jejuni invade polarised cells from basolateral surfaces? The observation that ΔcadF mutants can transmigrate through but not invade into polarised cells would support this idea (Monteville and Konkel, 2002). Basolateral engulfment of C. jejuni in non-polarised Chang cells has been also shown by TEM and called subversion, but studies on this phenotype indicated that the CadF, JlpA or PEB1 adhesins were not required (van Alphen et al., 2008). Thus, the processes of C. jejuni transmigration and basolateral invasion at the molecular level are still not clear. In future, more direct microscopic and genetic approaches are necessary to investigate in detail the involved bacterial and host factors.

6. Intracellular survival of C. jejuni

While the entry process into eukaryotic cells by C. jejuni has been the focus of many studies in the past (Ö Cróinin and Backert, 2012), only a few recent reports have examined the survival of C. jejuni within eukaryotic cells. Consequently, by comparison to other invasive enteropathogenic bacteria, the intracellular fate of C. jejuni within eukaryotic cells is still poorly understood. It has been generally suggested that C. jejuni is able to persist within cultured epithelial cells in vitro for 1–3 days in a membrane-surrounded compartment termed the CCV (Buelow et al., 2011; Day et al., 2000; Gaynor et al., 2005; Naikare et al., 2006; Watson and Galan, 2008; Bouwman et al., 2013). Methods that have been applied to characterise the intracellular fate of C. jejuni are summarised in Table 4 and described below.

6.1. Intracellular survival assay (ISA)

Most studies investigating the intracellular survival capability of C. jejuni have used a CFU-based intracellular survival assay (ISA) demonstrating that C. jejuni is able to survive within various types of non-phagocytic cells for 24 h (Gaynor et al., 2005; Watson and Galan, 2008) or even up to 72 h (Konkel et al., 1992b; Naikare et al., 2006). The experimental settings of the ISA approach are similar to the GPA assay described in Section 4.1. After an initial infection time, cultured cells are treated with a high dose of gentamicin to kill extracellular bacteria. The follow-up cell lysis performed for invasion assays to release the intracellular bacteria is skipped; instead the cultured epithelial cells are further cultivated in cell culture medium for various time periods to investigate the survival capacity of C. jejuni. During this extended incubation time the experimental procedure varies significantly between studies as gentamicin is either omitted (Konkel et al., 1992b; Naikare et al., 2006) or added in concentrations of 10 μg/ml (Condon et al., 2007; Louwen et al., 2012; Watson and Galan, 2008), 20 μg/ml (Novik et al., 2010) or 50 μg/ml (Konkel et al., 1992b; Van Deun et al., 2008; Bouwman et al., 2013) to the cell culture medium. After this incubation and subsequent washing steps, C. jejuni is finally released from the cells by treatment with detergents or by physical sheering, and intracellular bacteria CFUs are quantified by plating of serial dilutions. The survival is often presented as absolute number of intracellular bacteria recovered over time (Buelow et al., 2011; Condon et al., 2007; Gaynor et al., 2005; Watson and Galan, 2008). Alternatively, the survival rate is calculated either by dividing the numbers of recovered bacteria in relation to the inoculum or by dividing the number of bacteria recovered at the end of an infection experiment in relation to the number of invaded bacteria recovered about four hours after infection.
Until now, only few C. jejuni mutants with intracellular persistence defects have been identified using the ISA test (Table 4). Inactivation of the stringent response regulator SoxP results, among other defects, in a reduced intracellular survival capability (Gaynor et al., 2005). Polyphosphate kinase 1 and 2 (Ppk1 & Ppk2) mediating polyphosphate metabolism also support the viability of C. jejuni in the CCVs (Candon et al., 2007; Gangaih et al., 2010). Furthermore, Cial, a newly identified member of the Campylobacter invasion antigen family, is suggested to be involved in efficient intracellular survival (Buelow et al., 2011). An active superoxide dismutase SodB is not only required for efficient adherence and invasion (Novik et al., 2010), but also for intracellular persistence (Novik et al., 2010; Pesci et al., 1994). It is surprising in this context that the catalase KatA, involved in the detoxification of reactive oxygen species, is apparently not required by C. jejuni to survive within Hep-2 cells (Day et al., 2000). In addition, aspA (aspartate ammonia-lyase) and frdA (fumarate reductase A) mutants, potentially defective in fumarate respiration, also showed a reduced survival capability (Liu et al., 2012). Only one mutation has so far been identified that leads to slightly increased intracellular survival: Inactivation of CprS, the sensor kinase of a two-component regulatory system involved in Campylobacter planktonic growth regulation, improves intracellular fitness (Svensson et al., 2009).

The ISA technique requires attentiveness to avoid misleading results because extracellular growing bacteria that have survived the high dose gentamicin treatment can be mistaken for intracellular bacteria recovered from the CCVs. It was also suggested that intracellular C. jejuni, which are protected from gentamicin treatment, are able to escape their host cells and re-infect other cells (Van Deun et al., 2008). In addition, a so-called subversion mechanism, the basolateral invasion of epithelial cells accompanied by preceding migration into a subcellular, gentamicin-protected space, of certain C. jejuni has been suggested (van Alphen et al., 2008; Bouvman et al., 2013). Few extracellular bacteria that survive gentamicin treatment can skew survival experiments as in vitro studies have demonstrated that various cell culture media with or without serum supplementation can promote the growth of certain C. jejuni isolates. In particular, the high glutamine concentration (4 mM) present in certain cell culture media provides a suitable growth substrate for C. jejuni isolates expressing a secreted, active γ-glutamyltranspeptidase (Hofreuter et al., 2008). So the continuous presence of low gentamicin concentrations up to 20 μg/ml for the extended incubation period during survival experiments is highly recommended to prevent the ongoing proliferation of extracellular C. jejuni that have potentially survived the initial gentamicin treatment. Higher gentamicin concentrations over a long time period are problematic and can lead to the killing of intracellular bacteria in the CCVs since gentamicin is not completely excluded from eukaryotic cells (Elingshorst, 1994).

Numerous survival studies using ISA have clearly demonstrated that the number of intracellular, viable bacteria declines substantially during the first 24 h. Recent reports showed that the significant decrease in the number of intracellular surviving bacteria after 24 h reflects a less efficient recovery with the standard cultivation methods used rather than a reduced viability of intracellular C. jejuni. This phenomenon is due to pronounced physiological adaptation of C. jejuni to the specific environment present in the CCVs (Liu et al., 2012; Watson and Galan, 2008). The metabolic alterations of C. jejuni are most prominently reflected by a general metabolic down shift and changes in the respiratory activity (Liu et al., 2012) that allows better adaptation to oxygen-independent respiration. It was further shown that the physiological adaptations of C. jejuni to the CCV negatively affect its efficient recovery from this intracellular compartment unless specific cultivation methods are used. Consequently, C. jejuni isolated from eukaryotic cells 24 h after infection can be recovered with higher CFUs when cultivated under very low oxygen/anerobic conditions than under microaerobic or 10% CO2 cultivation conditions (Liu et al., 2012; Watson and Galan, 2008). After 48 h, the transfer of the recovered C. jejuni colonies to a microaerobic or 10% CO2 atmosphere is required for maximal growth. This is in agreement with previous observations demonstrating that the in vitro growth of C. jejuni is restricted in anerobic atmosphere (Sellars et al., 2002). Moreover, it was recently suggested that C. jejuni undergoes an intracellular metabolic reprogramming that reduces its recovery on standard Mueller–Hinton cultivation plates after survival or invasion experiments (Pryjma et al., 2012). Addition of 20 mM sodium sulfite, a suitable respiratory electron donor substrate for C. jejuni (Myers and Kelly, 2005), enhanced the recovery of C. jejuni wild-type and especially of its formate dehydrogenase mutants from infected host cells (Pryjma et al., 2012).

Although the intracellular survival of C. jejuni in epithelial cells is well documented, there are conflicting data about the survival capability of C. jejuni in phagocytic cells. Some studies have described that C. jejuni can persist for several days in isolated BALB/c macrophages, in the murine J774A.1 macrophage cell line (Kiehlbauch et al., 1985), in human monocytes and in the human monocyte cell line 28SC (Hickey et al., 2005) as well as in murine peritoneal macrophages (Day et al., 2000). In contrast, other reports have shown that C. jejuni is efficiently killed by chicken peripheral macrophages (Myszewski and Stern, 1991), macrophages derived from human peripheral monocytes (Wassenaar et al., 1997), and murine bone marrow-derived macrophages (Watson and Galan, 2008). Experimental variances like the use of different phagocytic immune cells, different C. jejuni isolates, variable MOIs and infection times and the usage or non-usage of gentamicin to prevent the extracellular growth of C. jejuni as discussed previously (Wassenaar et al., 1997) could be reasons for the conflicting observations. Furthermore, the altered induction of immune cell differentiation and activation could influence C. jejuni survival in these experiments. Further studies are required to clarify the capability of C. jejuni to survive within macrophages of certain hosts and to identify bacterial factors that are involved in this potential process.

As the ISA is based on the recovery of intracellular bacteria by standard cultivation methods, potential survival defects of mutants have to be characterised in more detail. Follow up experiments like the live/dead staining procedure described below can provide additional information to exclude the possibility that the observed phenotype is a defect in culturability of investigated C. jejuni strains upon release from the CCV rather than a loss of their intracellular viability.

6.2. Live/Dead Staining (LDS)

Several reports have investigated the in vitro viability of C. jejuni upon various environmental stresses using DNA-specific dyes that allow the discrimination of live from dead bacteria according to their membrane integrity (Cameron et al., 2012; Ica et al., 2012; Asakura et al., 2007). This live/dead staining (LDS) technique has been successfully applied as an alternative to the ISA to investigate the survival of C. jejuni within the human mononcytic 28 SC cells (Hickey et al., 2005), the intestinal T84 epithelial cells (Novik et al., 2010; Watson and Galan, 2008) and the waterborne protozoa Tetrahymena pyriformis and Acanthamoeba castellanii (Snelling et al., 2005). The LDS method does not depend on bacterial growth and overcomes therewith the problem of enumerating viable but non-culturable C. jejuni that can be missed by ISA experiments. Instead viable intracellular C. jejuni cells are identified by staining with a cell viability kit and can be counted visually by IFM or enumerated by flow cytometry.

LDS-based survival experiments are set up as described in Section 6.1 with the following modifications: At the end of the experiment host cells are lysed and the released C. jejuni cells are not tested for viability by direct plating and enumerating but separated from the cell debris by a low speed spin. Subsequently, the bacteria in the supernatant are stained with a mixture of two solutions: one dye is membrane permeable and stains all cells (e.g. thiazole orange) whereas the second dye is membrane impermeable (e.g. propidium iodide) and
only enters dead bacterial cells with disrupted membranes. Using IFM C. jejuni cells that remain viable inside the CCV can be seen as green, whereas dead C. jejuni cells are stained red (Watson and Galan, 2008). Direct counting by microscopic examination is possible but time consuming. In addition, remaining cell debris particles of lysed host cells could interfere with the precise counting by fluorescence microscopy.

The use of flow cytometry can overcome these problems and makes it possible to quantify the stained viable and dead C. jejuni cells of a mixed population (Novik et al., 2010; Watson and Galan, 2008). The FACS (fluorescence activated cell sorting) analysis method has to be carefully adapted and optimised for each C. jejuni isolate, as this pathogen has rather small dimensions (Walker et al., 1986) in comparison to other bacteria and different strains vary in their tendencies to self-aggregate (Golden and Acheson, 2002; Misawa and Blaser, 2000).

6.3. Microscopic methods

TEM studies have revealed that C. jejuni remains tightly surrounded by a host-derived membrane after invasion into epithelial cells in vitro and in vivo (Babakhani et al., 1993; De Melo et al., 1989; Hu et al., 2008; Konkel et al., 1992b; van Spreeuwel et al., 1985; Watson and Galan, 2008). Recent infection studies with COS-1 cells suggest that C. jejuni 81–176, once internalised, actively subverts the classical endocytic pathway to establish its intracellular compartment: Shortly after invasion, C. jejuni resides in an early-endosomal-like compartment as demonstrated by IFM experiments in which C. jejuni co-localises with the antibody-stained endocytic marker EEA-1, with the transfected GFP-fusion proteins of the GTPases Rab4 and Rab5 as well as with a PX-domain-containing probe (Ellson et al., 2002) for phosphoinositide 3-phosphate presence (Watson and Galan, 2008). Subsequently, the CCV co-localises transiently with a transfected GFP-tagged late endosomal marker protein Rab7. One hour after invasion the CCV can be efficiently stained by IFM using an antibody against the late endosomal/lysosomal marker protein Lamp1. At this time point only very limited co-localisation with the previously detected EEA-1, Rab4, Rab5 and Rab7 occurs (Watson and Galan, 2008). A similar maturation of the CCV during the first 2 h after invasion was recently described upon C. jejuni infection of Caco-2 cells (Lounwe et al., 2012). Though marked with Lamp1 the CCV does not acquire lysosomal properties supported by the observation that cathepsin B is not detected by IFM staining in significant amounts on the vesicle where C. jejuni resides (Watson and Galan, 2008). It was shown recently that C. jejuni Cia1 may contribute to the exclusion of cathepsin D from the CCV (Buelow et al., 2011). About 5 h after invasion C. jejuni resides in a survival- permissive vacuolar compartment that is localised in the perinuclear region of the host cells as demonstrated by TEM (Hu and Kopecko, 1999; Watson and Galan, 2008) and can be immuno-stained with the Golgi marker protein GM130 (Watson and Galan, 2008). The translocation of the CCV from the cell periphery to the perinuclear space could be mediated by the vesicle transport machinery involving microtubules and dynein motor protein (Hu and Kopecko, 1999; Watson and Galan, 2008). Infection experiments with polarised Caco-2 islands revealed that C. jejuni resides in CCVs that can be immuno-stained with antibodies against Lamp1 and CD63 but not with GM130 (Bouwman et al., 2013). Further studies have to clarify if the perinuclear localisation of the CCV varies with the usage of non-polarised or polarised cultured epithelial cells.

C. jejuni resides in the non-spacious CCV during its entire intracellular persistence. This vacuole usually contains a single C. jejuni bacterium (Hu et al., 2008; Watson and Galan, 2008) suggesting that no obvious intracellular replication of C. jejuni occurs in vitro. Only occasionally multiple bacteria have been observed in one CCV but it is unclear if this event is the consequence of intracellular C. jejuni replication or the fusion of several phagosomes containing individual C. jejuni cells. The latter seems more feasible: While inside/outside IFM showed up to 11 bacteria inside infected cultured epithelial cells (Novik et al., 2009), that C. jejuni does not replicate intracellularly is supported by experiments based on the previous described survival assays (see Sections 6.1 and 6.2) where after 24 h no more bacteria can be recovered from infected cells than after 4 h.

7. Conclusions and perspectives

C. jejuni is a predominant foodborne pathogen causing enterocolitis in humans worldwide. This zoonotic bacterium represents part of the commensal flora in many wild and domestic animals. Its importance for humans attracted many researchers worldwide to investigate the molecular mechanisms of associated infections. However, despite the high prevalence of C. jejuni induced disease and research progress made in recent years, our knowledge is still relatively limited as compared to other invasive pathogens such as Salmonella, Listeria, Shigella or Legionella. Efforts with rodent and chicken model systems have been made to study C. jejuni infection in vivo. Currently, disease manifestations such as gastroenteritis can be achieved in gnotobiotic, immunodeficient and CH mice with a defined gut flora, but each model system has limitations (Beresswill et al., 2011; Haag et al., 2012). No model alone seems ideal to investigate pathogenicity and virulence mechanisms of C. jejuni. In vivo and in vitro research studies performed in the last two decades revealed that C. jejuni exhibits remarkable features during infection. Several key processes such as glycosylation, molecular mimicry, tissue entry and intracellular survival have been described. Many genes have been discussed to be involved in the above processes including the capsule, flagellum, LOS, Cadf, HtrA, respiratory factors and others. Future studies should combine in vivo and in vitro studies and should take advantage of powerful modern technologies including high-resolution IFM and LCI as well as proteomics-based and cellular signal transduction approaches. It will be particularly important to investigate in future, the detailed mechanisms by which C. jejuni enters polarised epithelial cells from apical and/or basolateral surfaces as well as how it survives and spreads intracellularly.

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