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Review

Campylobacter–Acanthamoeba interactions

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Campylobacter jejuni is a foodborne pathogen recognized as the major cause of human bacterial enteritis. Undercooked poultry products and contaminated water are considered as the most important sources of infection. Some studies suggest transmission and survival of this bacterial pathogen may be assisted by the free-living protozoa *Acanthamoeba*. The latter is known to play the role of a host for various pathogenic bacteria, protecting them from harsh environmental conditions. Importantly, there is a similarity between the mechanisms of bacterial survival within amoebae and macrophages, making the former a convenient tool for the investigation of the survival of pathogenic bacteria in the environment. However, the molecular mechanisms involved in the interaction between *Campylobacter* and *Acanthamoeba* are not well understood. Whilst some studies suggest the ability of *C. jejuni* to survive within the protozoa, the other reports support an extracellular mode of survival only. In this review, we focus on the studies investigating the interaction between *Campylobacter* and *Acanthamoeba*, address some reasons for the contradictory results, and discuss possible implications of these results for epidemiology. Additionally, as the molecular mechanisms involved remain unknown, we also suggest possible factors that may be involved in this process. Deciphering the molecular mechanisms of pathogen–protozoa interaction will assist in a better understanding of *Campylobacter* lifestyle and in the development of novel antibacterial drugs.

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Introduction

Free-living protozoa, such as amoebae, are widespread in nature. They have been isolated from a wide variety of public water supplies, swimming pools, bottled water, ventilation ducts, soil, surgical instruments and contact lenses (Sandström *et al.*, 2011). Amoebae are the dominant bacterial consumers, contributing to recycling of nutrients and maintaining the structure of the microbial community (Greub & Raoult, 2004). These micro-organisms have been the subject of intensive investigation due to their ability to capture prey by phagocytosis, act as vectors and hosts for pathogenic organisms, and their ability to produce serious human infections (Siddiqui & Khan, 2012a).

Campylobacter bacteria, known to be the most important enteric pathogen, are sensitive to environmental stress outside their warm-blooded hosts (Park, 2002). It was reported that amoebae may act as a protective environmental host for this pathogen; thus, creating a problem for human and animal health. However, there are varying accounts in the literature on the types of interaction between *Campylobacter* and *Acanthamoeba*.

In this review, we perform critical analysis of these data and discuss possible reasons for the conflicting results. We also indicate *Campylobacter* factors that may be involved in the interaction of this pathogen with amoeba. Elucidation of the molecular mechanisms of *Campylobacter–Acanthamoeba* interaction is important for public health, since foodborne pathogens and amoebae co-occur in the same environments (Vaerewijck *et al.*, 2014).

Abbreviations: CDT, cytolethal distending toxin; CPS, capsular polysaccharide; LOS, lipooligosacharides.

Acanthamoeba

Acanthamoeba is a genus of amoebae that includes free-living protozoan pathogens commonly found in soil and water and characterized by spine-like structures on their surface, known as acanthopodia. They contain one or more contractile vacuoles, whose function is to expel water for osmotic regulation, digestive vacuoles, lysosomes and glycogen-containing vacuoles (Siddiqui & Khan, 2012c). *Acanthamoebae* have two stages in their life cycle: an active trophozoite stage, in which they exhibit vegetative growth when the cells divide mitotically and when they feed on organic particles and microbes; and a dormant double-walled cyst stage with minimal metabolic activity, which amoebae adopt when under harsh environmental conditions (Siddiqui & Khan, 2012c). Although the trophozoites are the infective forms, both amoebal forms can enter the human host through a variety of routes, including the eyes, causing severe blinding keratitis, and also through nasal passages or skin lesions, from where they may further

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invade the nervous system, causing granulomatous encephalitis (Khan, 2006). *Acanthamoeba* keratitis is a rare disease that can affect anyone, but it is most common in individuals who wear contact lenses (Khan, 2006).

Historically, these micro-organisms feed on bacteria to fulfil their nutritional requirements; thus, regulating bacterial populations in the environment and contributing to the balance in ecosystems (Khan, 2006; Rønn *et al.*, 2002). An increasing number of micro-organisms, including pathogenic bacteria and viruses, have been described as benefiting from their relationships with amoebae (Thomas *et al.*, 2010). These associations are of great concern to human, animal and ecosystem health. By serving as their hosts and being highly resistant to physical and chemical stresses, they enable pathogenic bacteria to survive under harsh environmental conditions, which would normally kill them. In addition, the amoebae aid in their transmission to susceptible hosts, leading to infection (Siddiqui & Khan, 2012a; Thomas *et al.*, 2010).

The first report describing the ability of coliform bacteria to survive ingestion by protozoa was published in 1988 (King *et al.*, 1988). Although the interaction between *Legionella pneumophila* and *Acanthamoeba* was most widely studied and established (Hoffmann *et al.*, 2014), many other micro-organisms were found to benefit from their relationship with this protozoan micro-organism (Thomas *et al.*, 2010). Among the bacteria known to be hosted by *Acanthamoeba* are: *Acinetobacter* spp., *Aeromonas* spp., *Bacillus* spp., *Burkholderia* spp., *Campylobacter* spp., *Enterobacter* spp., *Escherichia coli*, *Helicobacter pylori*, *Klebsiella pneumoniae*, *Mycobacterium* spp., *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Serratia* spp., *Shigella* spp., *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Vibrio cholerae*, *Yersinia* spp. and others (Thomas *et al.*, 2010). Different types of interaction between bacteria and protozoa have been described, with possible outcomes including intracellular survival and multiplication of bacteria leading to amoebal lysis, or intracellular lysis of bacteria, followed by its digestion by amoebae. In addition, the presence of amoebae may stimulate extracellular survival and multiplication of bacteria.

Micro-organisms residing inside amoeba are linked to disease outbreaks caused by contaminated water, as these become more resistant to disinfectants (Winiecka-Krusnell & Linder, 2001). For example, internalization of *L. pneumophila* by amoebae results in an enhanced ability of these bacteria to invade macrophages, increased virulence and resistance to antibiotics (Barker & Brown, 1995; Winiecka-Krusnell & Linder, 2001).

Campylobacter

Campylobacter is a genus of spiral-shaped, Gram-negative, non-spore-forming microaerophilic bacteria. These organisms can be found in water and other environmental sources, as commensal organisms in some animals and in some food products (Dasti *et al.*, 2010). Despite being fastidious under cultivation in laboratory conditions, they can survive in the environment for long periods of time (Joshua *et al.*, 2006). Among various pathogenic *Campylobacter* species (e.g. *C. lari*, *C. hyointestinalis*, *C. fetus*, *C. coli*), *C. jejuni* is the most frequent cause of human infections (Altekruse *et al.*, 1999; Gebhart *et al.*, 1985; Moore *et al.*, 2005; Dasti *et al.*, 2010).

Most commonly, disease arises after the consumption of contaminated poultry products, raw milk or water, where *Campylobacter* can associate with protozoa and form biofilms. In humans, *C. jejuni* can invade the gut epithelial layer, resulting in an acute, self-limited gastrointestinal illness characterized by fever, diarrhoea and abdominal cramps (Young *et al.*, 2007). The most important complication preceded by *C. jejuni* infection is the Guillain–Barré syndrome, which is a demyelinating disease of the peripheral nervous system leading to paralysis, which can last for several weeks and requires intensive medical care (Vucic *et al.*, 2009). Unfortunately, there is no licensed vaccine against this enteric pathogen (Albert, 2014).

Interaction between *Acanthamoeba* and *Campylobacter*

There have been a number of reports on the interaction between *Campylobacter* and *Acanthamoeba* with somewhat contradictory results. Whilst the majority of studies suggest intracellular survival of *C. jejuni* in *Acanthamoeba* (Axelsson-Olsson *et al.*, 2005, 2007, 2010a, b; Snelling *et al.*, 2005, 2008; Baré *et al.*, 2010; Olofsson *et al.*, 2013), the others indicate that bacteria can only survive extracellularly (Bui *et al.*, 2012a, b; Dirks & Quinlan, 2014). These conflicting results may be explained by a variation in the *Campylobacter* and *Acanthamoeba* species studied, or by the different methodologies used. The variability in structures of lipooligosaccharides (LOS) and capsular polysaccharides (CPS) in different *C. jejuni* strains can influence the outcome of infection in the host, since these structures participate in cell adhesion and invasion (Backert & Hofreuter, 2013). Invasiveness of *C. jejuni* is both bacterial-strain dependent (Backert & Hofreuter, 2013) and host cell line dependent (Poly *et al.*, 2007). For instance, *C. jejuni* strain CG8486 was shown to be far less invasive compared with *C. jejuni* 81-176 towards INT-407 intestinal cells, but both showed the same phenotype upon infection of Caco-2 intestinal cells (Poly *et al.*, 2007). The outcomes of these experiments may also result from variation in experimental conditions. For example, although the m.o.i. in most studies was 100 bacteria per amoeba, in other experiments it was as low as 1 (Snelling *et al.*, 2008) and as high as 1000 bacteria per amoeba (Dirks & Quinlan, 2014) (Table 1). It is important to take into account the m.o.i. factor as it may have an effect on the efficiency of infection (Backert & Hofreuter, 2013).

It was observed, that maximal internalization level of *C. jejuni* strain 81-176 is achieved at 2 and 4 h at m.o.i. values of 200 and 20, respectively (Hu & Kopecko, 1999). Importantly, as no gentamicin was used in some of these

Table 1. Interactions described for different *Campylobacter* and *Acanthamoeba* species

Co-culturing experiments were performed aerobically in PYG medium (peptone yeast extract glucose) or in PAS (amoeba saline buffer solution).

Campylobacter	*Acanthamoeba*	Co-culture conditions	Survival	Multiplication	Comment	Reference
*C. jejuni**	*A. polyphaga* Linc Ap-1	m.o.i. 1 : 100; 4, 10, 25, 30 and 37 °C; 1 h	IC	IC	IC multiplication of *C. jejuni*, followed by amoebal lysis, occurs at 37 °C	Axelsson-Olsson *et al.* (2005)
C. jejuni NCTC 11351; *C. coli* NCTC 11366	*A. castellanii* CCAP 1501/10	m.o.i. 1 : 1; 25 °C; 1, 3, 6 and 24 h and 3 days	IC	nd	Increased resistance of *Campylobacter* to industrial disinfection; *Campylobacter* and protozoa were shown to co-exist	Snelling *et al.* (2005)
C. jejuni NCTC 11351, F4382, F6555, 1153, 6137 and 02A364; *C. coli* NCTC 11366; *C. lari* NCTC 11352; *C. hyointestinalis* CCUG 20822	*A. polyphaga* Linc Ap-1	37 °C; 24–96 h	IC†	IC†	Development of a novel enrichment method (ACC) for *Campylobacter* spp. described	Axelsson-Olsson *et al.* (2007)
C. jejuni NCTC 11168	*A. castellanii* CCAP 1501/10	m.o.i. 1 : 10; 25 °C; 3 h	IC	ND	Colonization of broilers by *C. jejuni* internalized by amoeba	Snelling *et al.* (2008)
C. jejuni NCTC 11351; *C. coli* NCTC 11366; *C. lari* NCTC 11352; *C. hyointestinalis* CCUG 20822	*A. polyphaga* Linc Ap-1; *A. castellanii**; *A. rhysodes**	m.o.i. 1 : 10; 10 and 37 °C; several days and 96 h, respectively	IC	IC	Whilst campylobacters are able to survive in other eukaryotic species, *Acanthamoeba* spp. also promote bacterial replication	Axelsson-Olsson *et al.* (2010a)
C. jejuni NCTC 11351	*A. polyphaga* Linc Ap-1; *A. castellanii**; *A. rhysodes**	m.o.i. 1 : 100; acidified PBS added after invasion; 32 °C; 0, 5 and 20 h	IC	ND	Internalization of *C. jejuni* by amoeba was triggered by moderately acidic conditions	Axelsson-Olsson *et al.* (2010b)
C. jejuni NCTC 11351, C40, 1-2, H397 and 442	*A. castellanii* ATCC 30234	m.o.i. 1 : 100; 25 and 37 °C; 2 weeks (also micro-aerobically)	IC	Not able to multiply IC	Environmental conditions play a crucial role in this interaction; IC survival observed at least for 24 h	Baré *et al.* (2010)
C. jejuni NCTC 11168	*A. castellanii* ATCC 30234	m.o.i. 1 : 100; 25 and 37 °C; 3 h	EC (37 °C only)	EC	*C. jejuni* is rapidly degraded by amoeba; depletion of dissolved oxygen by amoeba is a major contributor of this interaction	Bui *et al.* (2012a)
C. jejuni NCTC 11168	*A. castellanii* ATCC 30234	m.o.i. 1 : 100; 25 °C; 3 h	EC	ND	Pre-exposure to outside environmental stresses did not prime bacteria for resistance to IC killing by amoeba	Bui *et al.* (2012b)
C. jejuni 81-176	*A. polyphaga* Linc Ap-1	m.o.i. 1 : 20; RT; 1, 24, 48, 72 and 96 h	IC	ND	Viable bacteria were inside amoebal vacuoles; this interaction is dependent on bacterial viability	Olofsson *et al.* (2013)
C. jejuni NCTC 11168	*A. castellanii* ATCC 30010	m.o.i. 1 : 1000; RT; 2 h	EC	ND	Lack of consistency in results	Dirks & Quinlan (2014)

EC, Extracellular survival or multiplication in co-culture medium observed; IC, intracellular survival or multiplication within amoeba demonstrated; m.o.i., amoeba per bacteria ratio; ND, not determined; RT, room temperature.

*Denotes a lack of a strain name.

†Intracellular survival claimed, but not supported experimentally.

studies, the figures referring to intracellular bacterial numbers (calculated as c.f.u.) could be misleading. Nevertheless, some of these data were complemented by microscopy, allowing visualization and semiquantitative analysis of internalized bacteria (Axelsson-Olsson *et al.*, 2007, 2010a, b; Snelling *et al.*, 2008). Table 1 comprises a summary of the methodology used and main conclusions made in these studies, the details of which are discussed below.

Evidence for the intracellular mode of survival of *Campylobacter* in *Acanthamoeba*

In 2005, it was reported that *C. jejuni* can infect *Acanthamoeba polyphaga in vitro* and multiply inside this host (Axelsson-Olsson *et al.*, 2005). In that study, the authors investigated intracellular survival of a clinical isolate of *C. jejuni* at several temperatures (4, 10, 25 and 30 °C) and multiplication at 37 °C during co-culture with *A. polyphaga* (Table 1). The intracellular bacteria were visualized using phase-contrast microscopy techniques. First, it was observed that at all temperatures tested, soon after inoculation into amoebic cultures, *C. jejuni* cells were aggregated in certain locations of the *A. polyphaga* cell wall, confirming the bacterial ability to adhere to this host. In addition, 1 h after inoculation, live and motile bacteria were seen inside amoebic vacuoles at all temperatures tested.

The authors reported that *C. jejuni* cells survived for longer periods when present inside amoebic vacuoles than in culture medium alone. Surprisingly, it was found that *C. jejuni* could survive inside *A. polyphaga* for more than 60 days at 10 °C. Aerobic incubation at 37 °C resulted in intracellular multiplication of *C. jejuni* and lysis of amoebae, as detected by phase-contrast microscopy. Overall, the results demonstrated that at low temperatures, typical to natural water sources, *Campylobacter* could enter and remain viable within the amoebae, and it is also able to replicate at increased temperatures. This study suggests that these bacteria may employ a mechanism of survival within *A. polyphaga* as a means of escaping adverse environmental conditions (Axelsson-Olsson *et al.*, 2005).

In the same year, the results from another study examining the intracellular survival of *Campylobacter* in waterborne protozoa were published (Snelling *et al.*, 2005). Snelling and co-workers suspected that failure to reduce the *Campylobacter* contamination of reared poultry could be partially due to *Campylobacter* resistance to disinfection in broiler drinking water after their internalization by waterborne protozoa. In this study, broilers from different farms were tested for the presence of *Campylobacter*, followed by confirmation of the presence of bacterial and eukaryotic micro-organisms in the drinking water. Since in this study *Campylobacter* and protozoa were shown to co-exist in the poultry farm water systems, potential interaction between these micro-organisms was investigated. To assess intracellular survival, co-cultures of *Acanthamoeba castellanii* infected with *C. jejuni* and *C. coli* were incubated at 25 °C (Table 1). Although after 24 h of co-incubation *Campylobacter* cells inside food vacuoles of *A. castellanii* were viable, the bacteria did not survive after additional 2 days of incubation.

The microscopy methods used to examine co-cultures clearly showed that *Campylobacter* was present within amoebic vacuoles, supporting intracellular survival of this enteric pathogen within *Acanthamoeba*. Another important finding was a delayed decline in the viability of the tested *Campylobacter* spp. strains in co-cultures. For instance, *Campylobacter* was able to survive for an extra 36 h in the presence of amoebae, compared to their survival in medium without amoebae. Additionally, it was shown that *Campylobacter* internalized by protozoa were significantly more resistant to Virudine (a disinfectant widely used in the poultry industry) than the planktonic *Campylobacter*. Collectively, these findings suggest that the presence of protozoa and their interaction with *Campylobacter* in water supplies of reared poultry may lead to an increase risk of the broilers being colonized by this pathogen (Snelling *et al.*, 2005).

A novel method for the isolation and enrichment of *Campylobacter* species named *Acanthamoeba–Campylobacter* co-culture (ACC) was described by Axelsson-Olsson *et al.* (2007). The detection of *Campylobacter* from non-clinical samples often involves an enrichment step in a *Campylobacter*-specific broth before plating on blood agar, which enhances the yield of bacteria (Bolton & Robertson, 1982; Corry *et al.*, 1995). In contrast to conventional culture methods used for isolation of *Campylobacter* spp., the ACC method does not require the use of blood or a microaerobic gas environment. The aim of that study was to investigate the growth kinetics of different *Campylobacter* species during co-culture with *A. polyphaga* over the course of 96 h (Table 1). The authors claimed that all *Campylobacter* strains tested entered and proliferated within amoebae within 24 h, which resulted in successful enrichment of bacteria. However, no evidence (e.g. using gentamicin protection assay or microscopy) supporting the intracellular survival of bacteria was provided. Although the authors considered that the ACC method is a quick, efficient, simple and sensitive technique for *Campylobacter* enrichment, and that it could also be set up for other fastidious bacteria, it remains unclear whether the enrichment occurred due to intracellular or extracellular multiplication (Axelsson-Olsson *et al.*, 2007).

In a subsequent study, it was suggested that intra-amoebal *C. jejuni* could colonize broilers (Snelling *et al.*, 2008). In experiments on the investigation of the ability of *C. jejuni* strain NCTC 11168 residing inside *A. castellanii* to colonize broilers, these poultry were orally challenged by: (i) Page's amoeba saline solution (PAS); (ii) *C. jejuni* in its standard growth medium; and (iii) *A. castellanii* with internalized *C. jejuni*, incubated for 3 h at 25 °C. After different numbers of days post-challenge, seven broilers from each group were euthanized for c.f.u. enumeration (Table 1). As expected, no *C. jejuni* were found in broilers from the negative group (i). The broilers were colonized by *C. jejuni* residing inside amoeba (group iii) as efficiently as with a suspension of *C. jejuni* cells (group ii).

As mentioned above, *Campylobacter* bacteria are considered fragile organisms with fastidious growth requirements and reduced ability to tolerate environmental stress when compared to other foodborne pathogens (Park, 2002). It was found that *Campylobacter* spp. can potentially use a wide variety of unicellular eukaryotic organisms as hosts for survival in the environment (Axelsson-Olsson *et al*., 2010a). To reach this conclusion, different species of protozoa, such as amoebae, flagellate protozoa, algae and ciliate protozoa, were challenged with different *Campylobacter* species. Two different assays were performed: the survival assay, in which the co-cultures were incubated at 10 °C for several days; and the replication assay, in which the co-cultures were incubated at 37 °C for 96 h (Table 1).

The authors observed variation in survival rates among different *Campylobacter* species depending on which eukaryotic species was used. Compared with other bacterial species, *C. jejuni* was found to have the longest ability to survive when co-cultured with all protozoa tested. Among *Campylobacter* spp., *C. coli* and *C. hyointestinalis* were less able to survive in protozoa than *C. jejuni* and *C. lari*. In general, the authors observed that bacteria survived longer when co-cultured with all the eukaryotic organisms tested, especially inside algae and *Acanthamoeba* species, compared to when cultured in medium alone. Growth curves obtained by c.f.u. counts showed that only *Acanthamoeba* species promoted replication of *Campylobacter* spp., with *C. jejuni* showing the best replication rate. In contrast, *C. hyointestinalis* was shown to have the lowest replication rate in all three *Acanthamoeba* species. The authors concluded that they had confirmed the intracellular multiplication of *Campylobacter* inside *A. polyphaga* (Axelsson-Olsson *et al*., 2010a). However, this was not supported by microscopy, as was done in other studies (Axelsson-Olsson *et al*., 2005; Griekspoor *et al*., 2013).

Two articles published in 2010 reported the effect of environmental stresses on the interaction between *C. jejuni* and *Acanthamoeba* spp. (Axelsson-Olsson *et al*., 2010b; Baré *et al*., 2010). Axelsson-Olsson and co-authors investigated whether internalization of *C. jejuni* by *Acanthamoeba* affects bacterial tolerance to low pH. As amoebae are naturally resistant to many environmental conditions that are lethal to *Campylobacter*, they may be suitable hosts increasing survival of these bacteria. In this study, *A. polyphaga* was challenged with *C. jejuni* and the co-culture medium was replaced by acidified PBS at different pH levels (1 to 5) (Table 1). Fluorescence microscopy was used to visualize attached and internalized *C. jejuni* cells in *A. polyphaga*. It was observed that at all pH tested, *C. jejuni* incubated in the presence of *A. polyphaga* (attached or internalized) survived longer than when in culture medium alone. Similar survival rates at low pH were observed when *C. jejuni* was co-cultured with other *Acanthamoeba* species. These findings confirm that protozoa may act as protective hosts for *Campylobacter* against harsh environmental conditions, such as low pH (Axelsson-Olsson *et al*., 2010b).

Baré and co-workers investigated the influence of temperature and oxygen on the interaction between various strains of *C. jejuni* with *A. castellanii* (Baré *et al*., 2010). In order to test the ability of several *C. jejuni* strains to survive and replicate within this host, the co-cultures were incubated under aerobic and microaerobic conditions at 25 and 37 °C, over 2 weeks (Table 1). The results showed that, contrary to what was described previously (Axelsson-Olsson *et al*., 2007), co-culturing did not increase the *C. jejuni* population, but only resulted in a delayed decline in viability. An increase in long-term survival of *Campylobacter*, up to 6 days longer, especially under microaerobic conditions, was also observed.

CLSM experiments using co-cultures grown at 25 °C under aerobic conditions revealed *C. jejuni* cells attached to amoebic cells after 30 min of incubation. As previously reported by Axelsson-Olsson *et al*. (2005), bacteria showed a strong tendency to gather at certain positions on the amoebae cell walls. It was observed that large numbers of bacteria were inside organelles 3 h after infection. However, after 24 h, the number of intracellular bacteria was significantly decreased. Despite the fact that the authors did not apply gentamicin during their *in vitro* experiments, these data were supported by microscopy. The study suggested intracellular survival of *C. jejuni* within *A. castellanii* for a certain period, without multiplication (Baré *et al*., 2010).

Experiments with human epithelial cells and *C. jejuni*, demonstrating that bacterial viability is important for bacterial entry (Konkel & Cieplak, 1992), prompted Olofsson and others to investigate whether bacterial viability is also required for the internalization of *C. jejuni* by the amoebae (Olofsson *et al*., 2013). Bacterial suspensions with live and dead (heat inactivated) *C. jejuni* strain 81-176 were added to *A. polyphaga* and incubated at room temperature for periods of between 1 and 96 h (Table 1). For quantification of adhered/internalized viable and heat-killed *C. jejuni* in *A. polyphaga*, a bacterial viability kit was used. In order to determine the intracellular localization of viable and heat-killed bacteria in *A. polyphaga*, bacteria and amoebic lysosomes were stained with CTC (5-cyano-2,3-ditolyl tetrazolium chloride) solution and with dextran, respectively.

First, it was shown that, during co-incubation with *A. polyphaga*, both viable and heat-killed bacteria were internalized, but that the kinetics of internalization and the total number of internalized bacteria were dependent on bacterial viability. Specifically, it was shown that the internalization was significantly higher with viable compared to heat-killed bacteria. It was also observed that, after 1 h of co-incubation, 90 % of viable bacteria were attached to the amoebic surface, whilst 10 % were found inside amoebic vacuoles. At later stages, from 24 to 96 h, more than 80 % of viable bacteria were seen inside vacuoles. In contrast, heat-killed bacteria were hardly seen attached to the surface of amoebae at any point, but were found inside the vacuoles.

In addition, it was observed that viable bacteria were equally present in both non-digestive and digestive vacuoles, but over

time viable bacteria were found in higher numbers in the non-digestive vacuoles, in contrast with heat-killed bacteria. Interestingly, it was also shown that viable and heat-killed *C. jejuni* were taken up into different types of *A. polyphaga* vacuoles. Viable bacteria were predominantly localized to small vacuoles distributed throughout the amoebae, where the bacteria were densely packed (Fig. 1). In contrast, heat-killed bacteria were mostly gathered in one giant spacious vacuole within the amoebae, often located near the amoebic membrane. Collectively, the authors concluded that both viable and heat-killed bacteria were processed for degradation in acidic vacuoles, but that viable bacteria could escape this degradative pathway, suggesting that *C. jejuni* is able to survive within amoeba (Olofsson *et al.*, 2013).

Acanthamoeba assists survival of *Campylobacter* without phagocytosis

Recently, three studies supporting the role of amoebae in enhancing extracellular survival of *C. jejuni* have been published (Bui *et al.*, 2012a, b; Dirks & Quinlan, 2014). In 2012, Bui and colleagues investigated the effect of amoeba-mediated depletion of dissolved oxygen on the survival of *C. jejuni* when in co-culture with *A. castellanii* (Bui *et al.*, 2012a). The aims of the study were to investigate the intracellular survival of *C. jejuni* within *A. castellanii* at both 25 and 37 °C under aerobic conditions, whether *C. jejuni* can replicate inside amoeba at 37 °C in aerobic conditions and to find out whether *C. jejuni* could benefit from the presence of amoebae to grow extracellularly in aerobic conditions. Gentamicin was added to the culture wells to kill extracellular bacteria, followed by 0, 5 and 24 h incubation before lysing the cells (Table 1). CLSM was used to visualize the intracellular environment where, at 25 °C, it was observed that immediately after gentamicin treatment (0 h), a significant percentage of motile bacteria was internalized and confined to tight vacuoles within the amoebae. However, at 5 and 24 h after gentamicin treatment, the number of internalized bacteria decreased significantly, confirming a previous finding (Baré *et al.*, 2010).

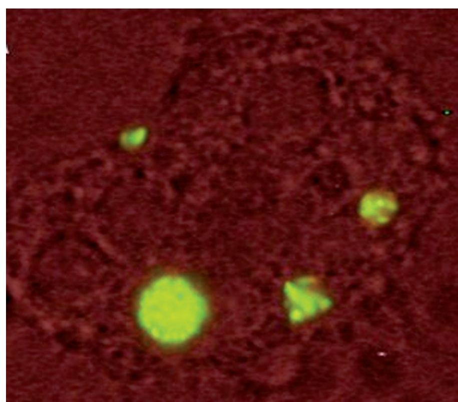


Fig. 1. Detection of viable *C. jejuni* 81-176 cells (green fluorescence) in *A. polyphaga* vacuoles using fluorescence microscopy after 1 h of co-incubation at room temperature (Olofsson *et al.*, 2013). Reproduced with the permission of the editor (under the terms of the Creative Commons Attribution license – http://creativecommons.org/licenses/by/3.0).

According to these data, *C. jejuni* rapidly loses viability inside amoebae at 25 °C. Although bacteria remain viable intracellularly for 5 h, they are eventually destroyed within the vacuoles. The same experiment was performed at 37 °C. However, microscopy revealed no intracellular *C. jejuni* inside *A. castellanii* after 24 h of gentamicin treatment, demonstrating that at this temperature *A. castellanii* may only support an extracellular mode of bacterial survival and growth. Under aerobic conditions at 37 °C the number of recovered bacteria in the co-culture medium increased significantly over time, demonstrating that *C. jejuni* can survive and replicate in co-culture, but not inside this protozoa.

Further, to understand how *C. jejuni* can survive and multiply under aerobic conditions in co-cultures, the authors hypothesized that amoebae could modify the oxygen level in the co-culture medium in a way that is beneficial for the survival and multiplication of *C. jejuni*. The researchers observed a decrease in the dissolved oxygen levels in cultures of *A. castellanii* with or without *C. jejuni*. These data support the previous hypothesis, in which *A. castellanii* cells may reduce the dissolved oxygen level, leading to the promotion of survival and replication of *C. jejuni* (Bui *et al.*, 2012a).

In a follow-up report by Bui and others, the effects of different environmental stresses on the uptake and survival of *C. jejuni* in *A. castellanii* were investigated (Bui *et al.*, 2012b). It was hypothesized that exposure of *C. jejuni* to stress conditions increases bacterial resistance to phagocytosis and intracellular killing by amoeba.

To test this hypothesis, the effects of heat, low nutrient, oxidative and osmotic stresses on the uptake and intracellular survival of *C. jejuni* in amoebae were tested. 'Non-stressed' controls were also included in all assays. *A. castellanii* was challenged with the bacterial cells pre-exposed to different stress conditions. The co-cultures were then incubated for 3 h at 25 °C under aerobic conditions and intracellular survival was monitored by lysing amoeba at 0, 5 and 24 h after gentamicin treatment (Table 1). It was observed that pre-exposure of *C. jejuni* to heat, starvation and osmotic but not oxidative stress increased bacterial susceptibility to intracellular killing by amoeba. Immediately after gentamicin treatment, both viable *C. jejuni* non-stressed and pre-exposed to stress were confined to tight amoebic vacuoles (Fig. 2a). However, at 5 h post-treatment, fewer internalized bacteria could be seen inside the amoebae vacuoles (Fig. 2b).

These data confirm the observations from the previous study (Bui *et al.*, 2012a), and suggest not only that pre-exposure of *C. jejuni* to different stresses strongly compromises the ability

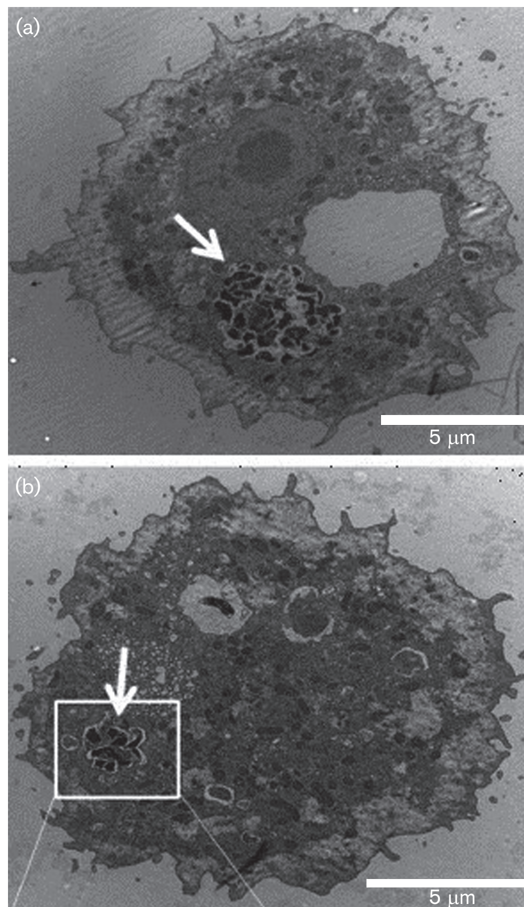


Fig. 2. Detection of *C. jejuni* NTCT 11168 cells (indicated by white arrows) within vacuoles of *A. castellanii* trophozoites at 0 (a) and 5 h (b) after gentamicin treatment using transmission electron microscopy (Bui *et al.*, 2012b). Reproduced with permission of the editor (under the terms of the Creative Commons Attribution license – http://creativecommons.org/licenses/by/2.0).

of *C. jejuni* to survive within amoebae, but also that *C. jejuni* is unable to survive for long periods within this host (Bui *et al.*, 2012b). Despite that both studies suggest that *C. jejuni* is unable to survive inside amoeba for more than 5 h (Bui *et al.*, 2012a, b), even the short period that *C. jejuni* may remain viable inside this host may still increase the risk of infection in poultry.

The extracellular survival of *C. jejuni* in the presence of *A. castellanii* was also supported by a study by Dirks & Quinlan (2014). The authors modified the currently used gentamicin protection assay (Friis *et al.*, 2005) in order to increase the accuracy of quantification of bacterial internalization rate, and used a modified protocol for the investigation of interaction between *C. jejuni* and amoebae. *A. castellanii* cells were mixed with *C. jejuni* NCTC 11168 cells in centrifuge tubes. Co-cultures were then incubated for 2 h at room temperature to allow *C. jejuni* to enter the amoebae and treated with gentamicin (Table 1). The integrity of the amoebae was accessed and confirmed by using a trypan blue exclusion procedure. The results were found to be inconsistent between the experiments. In some tests, higher bacterial numbers were recovered after co-incubation with *A. castellanii*, suggesting bacterial internalization and a protective effect from amoeba, whilst in other tests no protective effect of amoebae on bacteria was detected, with a higher recovery of *C. jejuni* in the absence of amoeba.

The authors concluded that *C. jejuni* cells do not survive upon internalization by *A. castellanii*, but they do survive extracellularly for extended periods. However, since in a large number of experiments the authors actually demonstrated the recovery of *C. jejuni* after gentamicin treatment, to our opinion this study does support the possibility of the intracellular survival of *C. jejuni* in *Acanthamoeba*, rather than merely extracellular survival (Dirks & Quinlan, 2014).

C. jejuni interaction with tissue culture host cells

There is a remarkable similarity between macrophages and amoebae, particularly in cellular structure, molecular motility, biochemical physiology and ability to capture prey by phagocytosis. Consequently, the molecular mechanisms involved in bacterial interaction with these host cells may also have some similarities (Siddiqui & Khan, 2012b).

Although epithelial and phagocytic cells are different, the mechanisms of bacterial interaction with these targets may have some similarity. The ability of *C. jejuni* to invade human intestinal cells *in vitro* was found to be strain dependent, with *C. jejuni* strain 81-176 possessing superior invasive properties (Hu & Kopecko, 1999). Strain to strain variation may be attributed to differences in the structures of molecules involved in host–pathogen interaction, including LOS and CPS (Parkhill *et al.*, 2000; Gilbert *et al.*, 2002; Karlyshev *et al.*, 2005; Guerry *et al.*, 2006). Several *in vitro* studies involving intestinal epithelial (INT407) and human colon (Caco-2) cell lines led to the establishment of the hypothetical model for *C. jejuni* interaction with host cells. It was suggested that, following attachment to enterocytes *in vivo*, *C. jejuni* invades intestinal epithelium cells, which allows the bacteria to survive inside a defined intracellular compartment, the *Campylobacter*-containing vacuole (Backert & Hofreuter, 2013). Subsequent studies allowed the identification of bacterial factors involved in adhesion and invasion, including fibronectin-binding protein CadF, cell-binding factor PEB1, capsule-biosynthesis protein CapA, serine protease HtrA, fibronectin-binding-protein FlpA, flagellar subunits FlaA and FlaC, flagellar biosynthetic FlhB, LOS, CPS, cytolethal distending toxin (CDT) and Cia antigens (Konkel *et al.*, 1997, 2004, 2010; Monteville *et al.*, 2003; Ashgar *et al.*, 2007; Baek *et al.*, 2011; Wassenaar *et al.*, 1991; Song *et al.*, 2004; Backert & Hofreuter, 2013).

The interaction between *Campylobacter* and epithelial cells may also be assisted by protein secretion systems

responsible for the delivery of effector molecules (Filloux *et al.*, 2008; Young *et al.*, 2007). For instance, the flagella export apparatus of *C. jejuni*, which functions as a type III secretion system (T3SS), was reported to be involved in host cell invasion (Samuelson *et al.*, 2013). Additionally, a type VI protein secretion system is used to target cells by injection of antibacterial toxins, and is also required for *C. jejuni* adherence to, and invasion of, host cells and for colonization (Bleumink-Pluym *et al.*, 2013; Lertpiriyapong *et al.*, 2012). Different protein secretion systems were shown to be important for survival of other bacterial pathogens within amoeba. Examples are the T3SS and type IV secretion system, the former being important for both *E. coli* and *P. aeruginosa* survival within amoebae, and the latter for intracellular multiplication of *L. pneumophila* inside this protozoa (Siddiqui *et al.*, 2011; Abd *et al.*, 2008; Bandyopadhyay *et al.*, 2004).

Among the other factors known to be required for intracellular survival of *Campylobacter* within host cells, are: superoxide dismutase SodB; aspartate ammonia lyase AspA; aspartate aminotransferase AspB; the *Campylobacter* invasion antigen CiaI; guanosine-3-pyrophosphohydrolase SpoT; polyphosphate kinase Ppk1; heptosyltransferase WaaF (required for LOS formation); the alkaline phosphatase PhoX (a substrate of the TAT transport system); fumarate reductase flavoprotein FrdA; the sensor kinase CprS; and a hypothetical virulence protein VirK (Novik *et al.*, 2009, 2010; Buelow *et al.*, 2011; Gaynor *et al.*, 2005; Candon *et al.*, 2007; Naito *et al.*, 2010; Drozd *et al.*, 2011; Liu *et al.*, 2012; Svensson *et al.*, 2009). While the adherence, entry and intracellular survival processes of *C. jejuni* for epithelial host cells have been widely studied, its intracellular survival within macrophages is still poorly understood (Backert & Hofreuter, 2013); however, it was previously reported that *C. jejuni* could survive inside murine macrophages (Kiehlbauch *et al.*, 1985; Day *et al.*, 2000; Hickey *et al.*, 2005).

Potential factors involved in the interaction between *C. jejuni* and amoeba

Elucidation of possible *C. jejuni* factors that may be involved in the interaction with amoeba is assisted by consideration of such factors identified in other bacterial pathogens. Orthologous *Campylobacter* proteins can be identified by using the BLAST similarity search. In addition, one can consider *C. jejuni* factors required for invasion of other host cells, particularly macrophages sharing some similarity with protozoa. A summary of our findings using the NCTC 11168 genome as a reference strain is presented in Table 2. Similar genes/gene products were also identified in the genome of a highly invasive stain 81-176 (data not shown).

As previously mentioned, flagella contribute to adhesion and invasion (Ramos *et al.*, 2004). The *Burkholderia pseudomallei* flagellin structural protein FliC and the *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*S.* Typhimurium) secretion system apparatus protein SsaU were found to be necessary for the bacterial adherence/entry and survival inside *Acanthamoeba*, respectively (Inglis *et al.*, 2003; Bleasdale *et al.*, 2009). Despite the absence of FliC in *C. jejuni*, the protein shares some amino acid sequence and functional similarity with flagella proteins FlaA and FlaB (Table 2). In addition, the *S.* Typhimurium SsaU shares high functional and sequence similarity with *Campylobacter* protein FlhB (Table 2). Also, the *L. pneumophila* FlaA, important for its interaction with amoeba (Dietrich *et al.*, 2001), shares significant sequence similarity with *C. jejuni* FlaA and FlaB (Table 2).

Various types of outer-membrane protein may be involved in bacterial interaction with amoeba. It was reported that the outer-membrane protein OmpA was crucial for invasion and intracellular survival of *E. coli* within *Acanthamoeba* (Alsam *et al.*, 2006). Although *C. jejuni* strain 11168 has a putative OmpA protein (Cj0599), CadF shares the highest similarity with OmpA from *E. coli*. In contrast, it was shown that in *V. cholerae* the lack of outer-membrane protein OmpA suppresses *Acanthamoeba* viability, and its survival inside this host is enhanced in the absence of this protein (Valeru *et al.*, 2014). Instead, the transcriptional regulator ToxR, which modulates expression of the outer-membrane proteins OmpU and OmpT in *V. cholerae*, was found to be necessary for the survival of this pathogen within amoebae (Valeru *et al.*, 2012), but no potential orthologues of these proteins could be found in *C. jejuni*. However, *K. pneumoniae* outer-membrane protein OmpA, important for the resistance to phagocytosis by the amoeba *Dictyostelium discoideum* (March *et al.*, 2013), shares the highest similarity with *C. jejuni* CadF. In addition to OmpA, the outer-membrane protein OmpK36 and LPS of *K. pneumoniae* were also found to be important for the resistance to phagocytosis. Although no OmpK36 orthologues could be found in *C. jejuni*, the latter does produce a porin protein (PorA, also known as the major outer-membrane protein, MOMP), which was shown to be important for bacterial attachment to epithelial cells (Moser *et al.*, 1997; Mahdavi *et al.*, 2014). Moreover, the *L. pneumophila* outer-membrane efflux protein TolC, which plays a role in multidrug resistance, and is important for the organism's virulence, invasion and intracellular multiplication inside amoebae (Ferhat *et al.*, 2009), shares sequence and functional similarity with the *C. jejuni* multidrug efflux pump protein CmeC. So far, there are no studies suggesting involvement of this factor in *C. jejuni* invasion of host cells.

E. coli sialic acid synthases, NeuB and NeuD, were found to be essential for bacterial invasion and survival within *Acanthamoeba* (Jung *et al.*, 2007). *E. coli* NeuD shares sequence similarity with the *C. jejuni* glycotransferase PglD involved in protein glycosylation. Also, other glycosylation proteins, PglB and PglE, were reported to be involved in *Campylobacter* host cell adhesion and invasion (Szymanski *et al.*, 2002). Sialic synthases are responsible for the synthesis of NeuNAc (N-acetyl neuraminic acid), the acetylated derivative of sialic acid, constituting the

Table 2. Hypothetical factors of *Campylobacter* that may play a role in interaction with amoeba

Amino acid sequence similarities to such factors found in other bacteria are shown. The selection of hits was based on the threshold levels of ⩾40 and ⩾20 % for query cover and amino acid sequence identities, respectively. Only proteins sharing functional similarities are listed. –, No name assigned.**Legionella* strain Paris, except for FliA and RpoS data.

Bacterial species	Bacterial factor	Note	Reference	*C. jejuni* NCTC 11168	
				Gene product	Identity (%) [query cover (%)]
B. pseudomallei strain 1106b	FliC	Flagellin structural protein	Inglis *et al.* (2003)	FlaA/Cj1339c and FlaB/Cj1338c	33 [96]
E. coli strain APEC O1	OmpA	Outer-membrane protein A	Alsam *et al.* (2006)	CadF /Cj1478c and OmpA/Cj0599	29 [48] and 30 [40]
	PhoB	Phosphate regulon/transcriptional regulatory protein	Chekabab *et al.* (2012)	–/Cj0355c	28 [97]
K. pneumoniae strain 342	OmpA	Outer-membrane protein A	March *et al.* (2013)	CadF/Cj1478c and OmpA/Cj0599	27 [74] and 30 [47]
*L. pneumophila**	FlaA	Flagellin	Dietrich *et al.* (2001)	FlaA/Cj1339c and FlaB/Cj1338c	34 [86]
	AroB	3-Dehydroquinate synthase	Polesky *et al.* (2001)	AroB/Cj1008c	41 [95]
	FeoB	Ferrous iron transport protein B	Robey & Cianciotto (2002)	FeoB/Cj1398	30 [94]
	FliA	Flagellar biosynthesis sigma factor	Heuner & Steinert (2003)	FliA/Cj0061c	30 [97]
	CsrA	Carbon storage regulator	Forsbach-Birk *et al.* (2004)	CsrA/Cj1103	35 [84]
	RpoS	RNA polymerase sigma factor	Abu-Zant *et al.* (2006)	RpoD /Cj1001	40 [95]
	AnkH and AnkJ	Ankyrin proteins	Habyarimana *et al.* (2008)	–/Cj0834c	43 [43] and 40 [71]
	TolC	Outer-membrane efflux protein	Ferhat *et al.* (2009)	CmeC/Cj0365c	23 [70]
	PrmB	Two-component regulator system signal sensor kinase	Al-Khodor *et al.* (2009)	DccS/Cj1222c	26 [74]
	AnkB	Ankyrin protein B	Price *et al.* (2010)	–/Cj0834c	27 [55]
	ClpP	ATP-dependent Clp protease proteolytic subunit	Li *et al.* (2010)	ClpP/Cj0192c	70 [88]
P. aeruginosa strain PAO1	Lon	Intracellular protease	Breidenstein *et al.* (2012)	Lon/Cj1073c	38 [95]
	TypA	Ribosome-binding GTPase	Neidig *et al.* (2013)	TypA/Cj0039c	56 [99]
S. Typhimurium strain 798	PhoP	DNA-binding transcriptional regulatory protein	Bleasdale *et al.* (2009)	–/Cj0890c	28 [97]
	SsaU	Secretion system apparatus protein	Bleasdale *et al.* (2009)	FlhB/Cj0335	32 [96]

polysaccharide capsule (Daines *et al.*, 2000). Similar to *E. coli*, it is worthwhile to investigate a possible role of the capsule in the interaction of *C. jejuni* with amoeba. Some previous studies demonstrated that *Campylobacter* capsule is required for adherence and invasion to epithelial cells (Bacon *et al.*, 2001; Karlyshev & Wren, 2001; Bachtiar *et al.*, 2007). In another pathogen, the Gram-positive *Streptococcus suis*, it was also found that the capsule is important for its virulence towards amoeba *D. discoideum* (Bonifait *et al.*, 2011).

Although NeuB of *E. coli* is involved in capsule formation, the *C. jejuni* NeuB is required for LOS biosynthesis (Linton *et al.*, 2000). It was reported that sialylated LOS contributes to epithelial invasion by *C. jejuni* (Louwen *et al.*, 2008). In addition, Cst-II sialyltransferase of *C. jejuni*, which is involved in the biosynthesis of a ganglioside-like LOS structure, was found to be important for *C. jejuni* invasion and intracellular survival inside host cells (Louwen *et al.*, 2012).

A phosphate regulon protein, PhoB, known to be required for bacterial adaptation to low level inorganic phosphate and virulence (Crépin *et al.*, 2011), contributes to *E. coli* survival inside *Acanthamoeba* (Chekabab *et al.*, 2012). An orthologue of PhoB protein can be found in *C. jejuni* (Table 2). Remarkably, despite low amino acid sequence similarity, both *Campylobacter* and *E. coli* proteins are DNA-binding response regulators involved in phosphorelay signal transduction systems. Additionally, the *S.* Typhimurium DNA-binding protein PhoP, which was also shown to be involved in bacterial survival inside *Acanthamoeba* (Bleasdale *et al.*, 2009), shares sequence and functional similarity with *C. jejuni* sensory transduction transcriptional regulator Cj0890c (Table 2). The *L. pneumophila* global stress response regulator RpoS, also

involved in DNA-binding, shares significant amino acid and functional similarity with *C. jejuni* RpoD (*C. jejuni* lacks an RpoS-encoding gene) (Table 2). However, the *L. pneumophila* two-component regulator system signal sensor kinase PrmB, important for replication within amoeba (Al-Khodor *et al.*, 2009), shares low similarity but similar function with the *C. jejuni* two-component sensor histidine kinase DccS (Table 2). The latter was found to be important for *C. jejuni* colonization *in vivo*, but is dispensable for growth *in vitro* (MacKichan *et al.*, 2004).

The intracellular protease Lon and the ribosome-binding GTPase TypA are important for *P. aeruginosa* survival after invading amoeba *D. discoideum*, and are involved in ATP- and GTP-binding, respectively (Breidenstein *et al.*, 2012; Neidig *et al.*, 2013). A similar role could be predicted for *Campylobacter* Lon protein (Table 2), which was found to be necessary for invasion of epithelial cells (Cohn *et al.*, 2007). Orthologues of *L. pneumophila* ATP-dependent Clp protease proteolytic subunit ClpP and carbon storage regulator CsrA were also found in *C. jejuni*. The ClpP and CsrA proteins in these bacteria share similarity both in amino acid sequences and functions (Table 2). Remarkably, both ClpP and CsrA proteins of *C. jejuni* are important for *C. jejuni* host cell invasion (Fields & Thompson, 2008; Cohn *et al.*, 2007).

Two proteins involved in iron transport, IroT and FeoB (ferrous iron transport protein B), were also identified as important for *L. pneumophila* replication inside amoebae (Portier *et al.*, 2014; Robey & Cianciotto, 2002). *C. jejuni* FeoB orthologues share significant sequence identity with *L. pneumophila* FeoB (Table 2). Additionally, it was reported that *C. jejuni* FeoB has a major role in its intracellular survival into host epithelial cells (Naikare *et al.*, 2006). Although no orthologues of *L. pneumophila* IroT could be found in *C. jejuni*, other proteins that are involved in iron transport may also contribute to the interaction with amoeba.

Bacterial protein secretion systems are crucial for virulence and survival within hosts (Filloux *et al.*, 2008). The T3SS was found to be required for *E. coli* and *P. aeruginosa* for interaction with amoebae (Siddiqui *et al.*, 2011; Abd *et al.*, 2008). In particular, *P. aeruginosa* cytotoxin ExoU secreted by this system was reported to be necessary for intracellular survival within amoebae (Pukatzki *et al.*, 2002). Despite the presence of T3SS in *C. jejuni*, there are no proteins sharing sequence similarity with this cytotoxin. Still, the *Campylobacter* CiaB and CiaC, secreted via the T3SS, were shown to be required for maximal invasion of epithelial cells by *C. jejuni* (Christensen *et al.*, 2009; Konkel *et al.*, 1999). In 2012, it was reported that the virulence factors exported by the ESX-1 secretion system of *Mycobacterium marinum* were necessary for intracellular survival and multiplication within *Acanthamoeba* (Kennedy *et al.*, 2012). Nevertheless, this secretion system is mainly present in mycobacteria and other Gram-positive bacteria, and it is related to the type VII secretion system, which is not found in *C. jejuni*.

L. pneumophila operon *bdhA–patD* was found to be important for virulence and replication of the *Legionella* inside amoebae (Aurass *et al.*, 2009). The *C. jejuni* 3-ketoacyl-ACP reductase FabG shares significant identity with *Legionella* 3-hydroxybutyrate protein BdhA, both have oxidoredutase molecular function. However, the *Legionella bdhA* gene is co-transcribed with gene *patD*, encoding a patatin-like protein PatD (Aurass *et al.*, 2009), and no similarity was found between the latter and *C. jejuni* proteins.

The ankyrin proteins AnkB, AnkH and AnkJ required for replication of *Legionella* inside amoeba (Habyarimana *et al.*, 2008; Price *et al.*, 2010), share sequence similarity with C. *jejuni* 11168 ankyrin Cj0834c. In addition, another *C. jejuni* ankyrin protein Cj1386 was shown to be important for colonization and pathogenesis, although a role of this protein in host cell invasion has not been investigated (Flint *et al.*, 2012). Also, it would be interesting to investigate the *C. jejuni* bacterial factors already known to be important for intracellular survival of the bacteria in macrophages, such as catalase KatA and the iron-binding Dps protein (Day *et al.*, 2000; Theoret *et al.*, 2011).

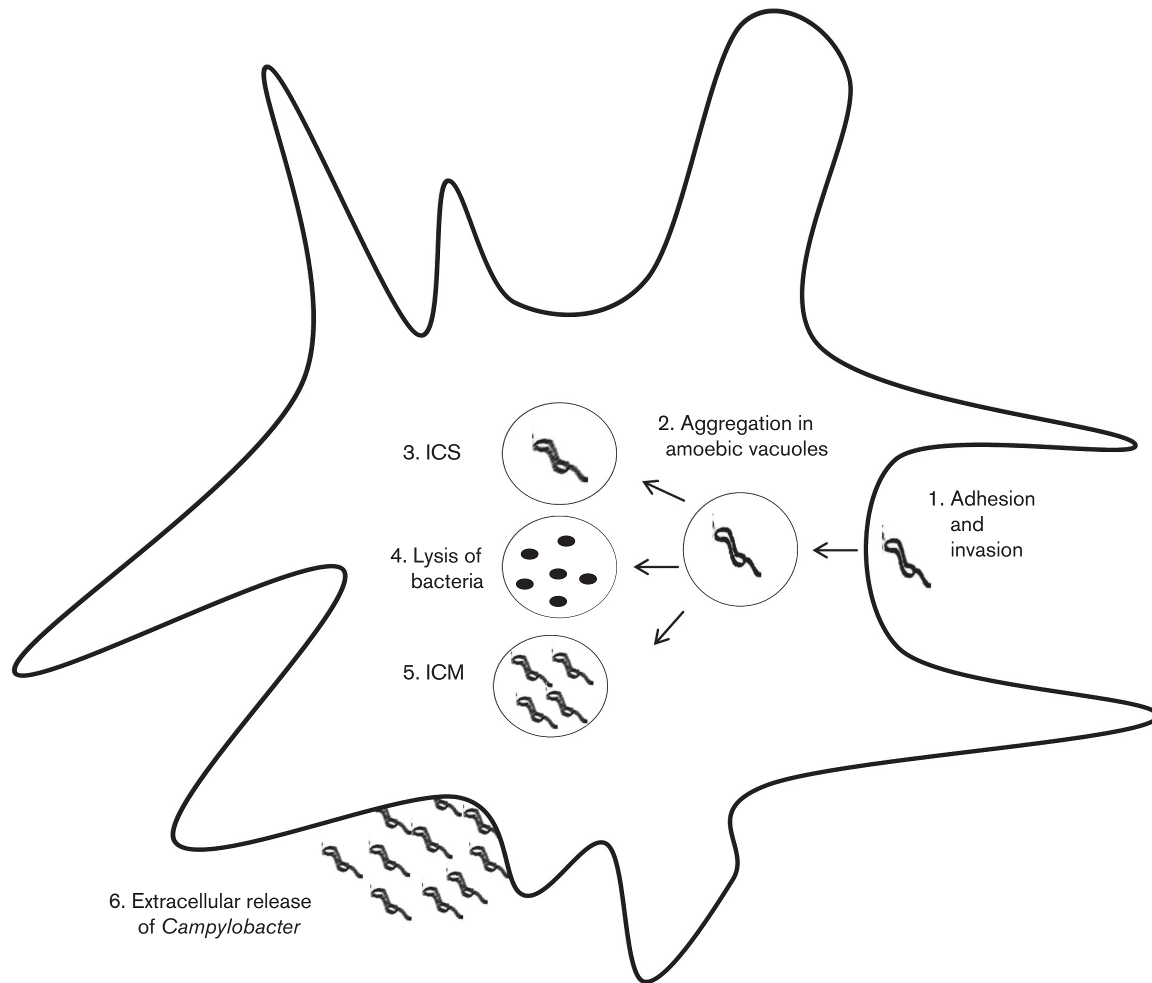
As mentioned above, *C. jejuni* strain 81-176 is more invasive than strain NCTC 11168 (Backert & Hofreuter, 2013). However, no factors additional to those listed in Table 2 for the reference strain 11168 could be found in the former. Moreover, the similarity and coverage figures for these factors are almost identical among these two strains. One possible explanation for the difference in invasion activities could be the different levels of expression of these genes in these strains. In addition, a structural variation of such cell-surface molecules as LOS and CPS may also contribute to a strain to strain variation in the ability of the bacteria to invade amoeba. Indeed, it was suggested that the difference in invasion efficiency of *C. jejuni* strains towards tissue culture cells could be associated with a difference in their CPS structures (Young *et al.*, 2007).

Based on the information available, we suggest a model describing a mechanism of interaction between amoebae and *C. jejuni*. Fig. 3 summarizes possible outcomes of *C. jejuni* interaction with this protozoan host. Selection of the genes and their products is based on either functional or amino acid sequence similarities with those found in other bacteria, or is based on factors shown to be important for *C. jejuni* infection of other host cells.

Conclusion

This review provides an up to date summary of studies investigating *Campylobacter–Acanthamoeba* interactions. In general, all researchers agree that *Campylobacter* survival is increased in the presence of *Acanthamoeba*, but there is a disagreement on whether the interaction occurs intracellularly or extracellularly. Although intracellular survival seems to have been proven by the majority of the studies described here, some researchers remain sceptical about this.

Even if *Campylobacter* is able to reside within amoeba for a



Potential gene products involved in the interaction between *Campylobacter* and amoeba		
Adhesion and invasion	ICS	ICM
CapA; CiaB/C; Cst-II; FlaC; FlpA; HtrA; PEB1/3; PglB/E; PorA; SodB; AroB; CmeC; CadF; ClpP; CsrA; KpsE; KpsM; KpsT; FlaA; FlaB; FlhB; Lon	AspA/B; CiaI; CprS; Cst-II; Dps; FrdA; KatA; PhoX; Ppk1; SodB; SpoT; VirK; WaaF; Ank; AroB; ClpP; CsrA; DccS; FlhB; FliA; Lon; OmpA; RpoD; TypA; CadF; FeoB	Ank; AroB; ClpP; CsrA; DccS; FeoB; FliA; RpoD

Fig. 3. Hypothetical model of the interaction of *C. jejuni* with amoeba, highlighting potential stages, outcomes and gene products involved. (1) Adhesion and invasion via phagocytosis; (2) aggregation of bacteria in amoebic vacuoles; (3) intracellular survival without multiplication (ICS); (4) lysis of bacteria; (5) intracellular multiplication (ICM); (6) lysis of amoebal cells and bacterial release. The gene products shown are hypothetical and based on either functional or amino acid sequence similarities with those found in other bacteria (blue), or gene products shown to be important for *C. jejuni* adherence, invasion of and intracellular survival in epithelial cells (green), or both (red). Although only some gene products related to the production of specific structures are shown, e.g. KpsE, KpsM and KpsT for CPS, or WaaF and Cst-II for LOS, these structures *per se* are predicted to be involved in the interaction with amoeba.

short time period, this may be of epidemiological importance as this could still represent a sufficient period of time for this eukaryotic organism to be a source of transmission of this pathogen. In addition, in the environment, the protozoa may be subjected to repeated cycles of infection by the pathogen. The presence of amoebae in water sources in poultry farms may increase the risk of infection with *Campylobacter* as this has been previously reported. Further investigation is, therefore, required to resolve these conflicting findings.

We suggest possible factors that may be involved in the interaction between *Campylobacter* and *Acanthamoeba*. Exploration of this topic is important for a better understanding of the mechanisms and genetic features of *Campylobacter* contributing to its interaction and survival within amoebae; thus, enhancing our understanding of the risk of infection. A promising approach in this direction would be a gene expression study (transcriptomics analysis) aimed at the identification of the bacterial genes differentially regulated during invasion.

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