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.

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).

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
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 ecosystem health. By serving as their hosts and being highly resistant to physical and chemical stresses, they enable

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 microorganisms 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 amoebae aid in their transmission to susceptible hosts, leading to infection (Siddiqui & Khan, 2012a; Thomas et al., 2010).

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 (Axelson-Olsson et al., 2005, 2007, 2010a, b; Snelling et al., 2005, 2008; Baré et al., 2010; Olffson 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
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<td>IC</td>
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<td><em>C. jejuni</em> NCTC 11351; C40, 1-2, H397 and 442</td>
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<td>m.o.i. 1 : 100; 25 and 37 °C; 2 weeks (also micro-aerobically)</td>
<td>IC</td>
<td>Not able to multiply IC</td>
<td>Pre-exposure to outside environmental stresses did not prime bacteria for resistance to IC killing by amoeba.</td>
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<tr>
<td><em>C. jejuni</em> NCTC 11168</td>
<td><em>A. castellanii</em> ATCC 30234</td>
<td>m.o.i. 1 : 100; 25 and 37 °C; 3 h (37 °C only)</td>
<td>EC</td>
<td>EC</td>
<td>Viable bacteria were inside amoebal vacuoles; this interaction is dependent on bacterial viability</td>
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<td><em>C. jejuni</em> NCTC 11168</td>
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<td>m.o.i. 1 : 100; 25 °C; 3 h</td>
<td>EC</td>
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<td><em>C. jejuni</em> 81-176</td>
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<td>IC</td>
<td>ND</td>
<td>Viable bacteria were inside amoebal vacuoles; this interaction is dependent on bacterial viability</td>
<td>Olofsson et al. (2013)</td>
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<td><em>C. jejuni</em> NCTC 11168</td>
<td><em>A. castellanii</em> ATCC 30010</td>
<td>m.o.i. 1 : 1000; RT; 2 h</td>
<td>EC</td>
<td>ND</td>
<td>Lack of consistency in results</td>
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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.
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-amoebl 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 amoeba, where the bacteria were densely packed (Fig. 1). In contrast, heat-killed bacteria were mostly gathered in one giant spacious vacuole within the amoeba, 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 amoeba 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).

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...
amoebae and treated with gentamicin (Table 1). The cells in centrifuge tubes. Co-cultures were then incubated cells were mixed with A. castellanii, suggesting bacterial internalization and a protective effect from amoeba, whilst in other tests no protective effect of amoeba 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, cytotoxins 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

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**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).
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 amoeba (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

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 strain 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

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 Cial; guanosine-3-pyrophosphohydrolase SpoT; polyphosphate kinase Ppk1; heatosyltransferase 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).
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 phospho-relay 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

### 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.

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>Bacterial factor</th>
<th>Note</th>
<th>Reference</th>
<th>Gene product</th>
<th>Identity (%)</th>
<th>C. jejuni NCTC 11168</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. pseudomallei strain 1106b</td>
<td>FliC</td>
<td>Flagellin structural protein</td>
<td>Inglis et al. (2003)</td>
<td>Flia/Cj1339c and Flab/Cj1338c</td>
<td>33 [96]</td>
<td></td>
</tr>
<tr>
<td>E. coli strain APEC O1</td>
<td>OmpA</td>
<td>Outer-membrane protein A</td>
<td>Alsm et al. (2006)</td>
<td>CadF/Cj1478c and OmpA/Cj0599</td>
<td>29 [48] and 30 [40]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PhoB</td>
<td>Phosphate regulon/transcriptional regulatory protein</td>
<td>Chekabab et al. (2012)</td>
<td>–/Cj0355c</td>
<td>28 [97]</td>
<td></td>
</tr>
<tr>
<td>K. pneumoniae strain 342</td>
<td>OmpA</td>
<td>Outer-membrane protein A</td>
<td>March et al. (2013)</td>
<td>CadF/Cj1478c and OmpA/Cj0599</td>
<td>27 [74] and 30 [47]</td>
<td></td>
</tr>
<tr>
<td>L. pneumophila*</td>
<td>FlaA</td>
<td>Flagellin</td>
<td>Dietrich et al. (2001)</td>
<td>FlaA/Cj1339c and Flab/Cj1338c</td>
<td>34 [86]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AroB</td>
<td>3-Dehydroquinate synthase</td>
<td>Polesky et al. (2001)</td>
<td>AroB/Cj1008c</td>
<td>41 [95]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FeoB</td>
<td>Ferrous iron transport protein B</td>
<td>Robey &amp; Cianciotto (2002)</td>
<td>FeoB/Cj1398</td>
<td>30 [94]</td>
<td></td>
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<tr>
<td></td>
<td>Flia</td>
<td>Flagellar biosynthesis sigma factor</td>
<td>Heuner &amp; Steiertz (2003)</td>
<td>Flia/Cj0061c</td>
<td>30 [97]</td>
<td></td>
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<tr>
<td></td>
<td>Rpos</td>
<td>RNA polymerase sigma factor</td>
<td>Abu-Zant et al. (2006)</td>
<td>RpoD/Cj1001</td>
<td>40 [95]</td>
<td></td>
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<tr>
<td></td>
<td>AnkH and AnkJ</td>
<td>Ankyrin proteins</td>
<td>Habayarimana et al. (2008)</td>
<td>–/Cj0834c</td>
<td>43 [43] and 40 [71]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tolc</td>
<td>Outer-membrane efflux protein</td>
<td>Ferhat et al. (2009)</td>
<td>Cme/Cj0365c</td>
<td>23 [70]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Prmb</td>
<td>Two-component regulator system signal sensor kinase</td>
<td>Al-Khodor et al. (2009)</td>
<td>Dcss/Cj1222c</td>
<td>26 [74]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Clpp</td>
<td>ATP-dependent Clp protease proteolytic subunit</td>
<td>Li et al. (2010)</td>
<td>ClpP/Cj0192c</td>
<td>70 [88]</td>
<td></td>
</tr>
<tr>
<td>P. aeruginosa strain PAO1</td>
<td>Lon</td>
<td>Intracellular protease</td>
<td>Breidenstein et al. (2012)</td>
<td>Lon/Cj1073c</td>
<td>38 [95]</td>
<td></td>
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<tr>
<td></td>
<td>Typa</td>
<td>Ribosome-binding GTPase</td>
<td>Neidig et al. (2013)</td>
<td>TypA/Cj0039c</td>
<td>56 [99]</td>
<td></td>
</tr>
<tr>
<td>S. Typhimurium strain 798</td>
<td>Phop</td>
<td>DNA-binding transcriptional regulatory protein</td>
<td>Bleasdale et al. (2009)</td>
<td>–/Cj0890c</td>
<td>28 [97]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SsaU</td>
<td>Secretion system apparatus protein</td>
<td>Bleasdale et al. (2009)</td>
<td>FliB/Cj0335</td>
<td>32 [96]</td>
<td></td>
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</tbody>
</table>

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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 oxidoreductase 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
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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