Listeria monocytogenes does not survive ingestion by *Acanthamoeba polyphaga*

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Listeria monocytogenes is a ubiquitous bacterium capable of infecting humans, particularly pregnant women and immunocompromised individuals. Although the intracellular invasion and pathogenesis of listeriosis in mammalian tissues has been well studied, little is known about the ecology of *L. monocytogenes*, and in particular the environmental reservoir for this bacterium has not been identified. This study used short-term co-culture at 15, 22 and 37 °C to examine the interaction of *L. monocytogenes* strains with *Acanthamoeba polyphaga* ACO12. Survival of *L. monocytogenes* cells phagocytosed by monolayers of trophozoites was assessed by culture techniques and microscopy. *A. polyphaga* trophozoites eliminated bacterial cells within a few hours post-phagocytosis, irrespective of the incubation temperature used. Wild-type *L. monocytogenes* and a phenotypic listeriolysin O mutant were unable to either multiply or survive within trophozoites. By contrast, *Salmonella enterica* serovar Typhimurium C5 cells used as controls were able to survive and multiply within *A. polyphaga* trophozoites. The data presented indicate that *A. polyphaga* ACO12 is unlikely to harbour *L. monocytogenes*, or act as an environmental reservoir for this bacterium.

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INTRODUCTION

Listeria monocytogenes is a well-known Gram-positive, opportunistic, intracellular bacterial pathogen capable of infecting humans and animals (Allerberger, 2003; Vázquez-Boland *et al.*, 2001). *L. monocytogenes* is ubiquitous in the environment and can survive and grow in a wide range of environmental conditions (Roberts & Wiedmann, 2003; Vázquez-Boland *et al.*, 2001). This versatility may explain the ability of *L. monocytogenes* to contaminate a variety of foods and food-processing environments (Roberts & Wiedmann, 2003). Foods contaminated with this bacterium represent a serious health concern in developed countries, as food-borne outbreaks of listeriosis are characterized by a high case mortality rate (20–30 %) (Herd & Kocks, 2001; Roberts & Wiedmann, 2003; Vázquez-Boland *et al.*, 2001; Wing & Gregory, 2002).

The mechanism of intracellular invasion, dissemination and survival of *L. monocytogenes* in mammalian cells has been well characterized *in vitro* (Cossart, 2002; Cossart & Sansonetti, 2004). However, comparatively little is known about the ecology of this intracellular pathogen in natural habitat, and unlike other intracellular pathogenic bacteria, no host reservoir has been identified (Zhou *et al.*, 2007). If

indeed *L. monocytogenes* lacks a host reservoir, it is intriguing that this pathogen has maintained an ability to invade and parasitize mammalian cells in the absence of selection for the genes necessary for internalization by host cells, escape from the host phagolysosome to the cytoplasmic compartment, and infection of neighbouring host cells.

Studies of interactions between amoebae and various intracellular bacterial pathogens have suggested that free-living amoebae are able to harbour bacterial pathogens (Harb *et al.*, 2000; Molmeret *et al.*, 2005). For example, *Salmonella enterica*, *Legionella pneumophila*, and species of *Shigella*, *Burkholderia*, *Mycobacterium* and *Chlamydia*, are able to survive and multiply within *Acanthamoeba* spp. (Bozue & Johnson, 1996; Gaze *et al.*, 2003; Greub & Raoult, 2004; Matz & Kjelleberg, 2005; Steinert *et al.*, 1998; Winiacka-Krusnell & Linder, 1999). As amoebae feed on bacteria by phagocytosis, it is believed that bacterial pathogens may have evolved survival strategies to counter predation pressures by these cells. Interestingly, these strategies share similarities with those required for parasitism of mammalian cells. For example, gene products required for survival of *L. pneumophila* within amoebae are also required to parasitize mammalian macrophages (Gao *et al.*, 1997; Segal & Shuman, 1999). Furthermore, passage of *L. pneumophila* and *Mycobacterium avium* selects for strains with increased invasiveness and virulence (Cirillo *et al.*, 1994, 1997). Indeed, the ability of some bacterial

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Abbreviations: TEM, transmission electron microscopy.

pathogens to parasitize both amoebae and mammalian hosts has led to the view that obligate and facultative bacterial pathogens may have evolved as a result of adaptation to intracellular growth and survival within amoebae and other protozoa (Cirillo, 1999; Greub & Raoult, 2002, 2004; Molmeret *et al.*, 2005).

Studies of the interaction between *Listeria monocytogenes* cells and *Acanthamoeba* spp. have reported markedly different results. Ly & Müller (1990) reported that *L. monocytogenes* cells are able to survive and multiply within a strain of *Acanthamoeba* following co-culture, although ideal controls were not used. Zhou *et al.* (2007) concluded that *L. monocytogenes* cells could remain viable in the presence of *Acanthamoeba castellanii* cells and that the bacterial cells survived within amoeba trophozoites, but they found no evidence that the bacteria could replicate within the amoeba during co-culture at 37 °C. Huws *et al.* (2008) also reported that *L. monocytogenes* was unable to survive or multiply within *Acanthamoeba polyphaga* cells during co-culture at 37 °C. Although this temperature is required for expression of factors involved in parasitization of mammalian cells by *L. monocytogenes*, it is not representative of environmental conditions under which *A. polyphaga* and other amoebae are likely to encounter *L. monocytogenes*.

To better clarify the potential for *A. polyphaga* trophozoites to harbour *L. monocytogenes* cells, we co-cultured *L. monocytogenes* with a strain of *A. polyphaga* at 15, 22 and 37 °C. These temperatures were chosen to mimic those of food-processing environments, natural environments and the temperature encountered by the bacterial pathogen in mammalian hosts, respectively. *A. polyphaga* was selected as a host for co-cultures because several studies have reported this amoeba was able to harbour bacterial pathogens (Axelsson-Olsson *et al.*, 2005; Gao *et al.*, 1997; Gaze *et al.*, 2003; Greub & Raoult, 2002; Kahane *et al.*, 2001; Lamothe *et al.*, 2004; Landers *et al.*, 2000; Newsome *et al.*, 1998; Steinert *et al.*, 1998; Thom *et al.*, 1992).

METHODS

Amoebae and culture conditions. *A. polyphaga* AC012 was provided by Brett Robinson (South Australian Water Quality Centre, Bolivar, South Australia). Axenic cultures of this amoeba isolate were prepared by subculture on non-nutrient agar (NNA) plates spread with heat-killed *E. coli* cells, or by culture in Proteose peptone-Yeast extract-Glucose (PYG) medium (Rowbotham, 1983 with later modifications of Greub & Raoult (2002). All cultures were routinely examined to rule out contamination by bacteria.

Monolayers of axenic cultures of *A. polyphaga* were routinely prepared by culture in PYG supplemented with antibiotics (10 µg gentamicin ml⁻¹; 200 µg ml⁻¹ each of streptomycin and penicillin), in 25 cm² flasks (Falcon 3018; Becton Dickinson) at 22 °C. Confluent monolayers of amoebae attached to the bottom of the flasks were washed three times with modified Neff's Amoeba Saline (AS) (Smirnov & Brown, 2004). Antibiotic-free PYG medium was added and the flasks were incubated overnight at 22 °C. Finally the monolayers were washed three times in AS buffer and the cells were

harvested in AS buffer by tapping the flasks. Amoeba cell counts were determined by Trypan blue staining and counting in a haemocytometer.

Bacterial strains and culture conditions. *Listeria monocytogenes* strain DRDC8 is a dairy isolate previously shown to be pathogenic for mice (Francis & Thomas, 1996). Strain LLO17 is an avirulent listeriolysin O (*hly*) mutant of DRDC8 (Francis & Thomas, 1996). *L. monocytogenes* strain 2T was isolated from a turkey-processing plant and strain KE504 was a clinical isolate from the King Edward Memorial Hospital for Women, Perth, Australia. All *L. monocytogenes* strains were routinely cultivated in Brain Heart Infusion (BHI) broth or on BHI agar plates, incubated at 37 °C overnight with aeration. *Escherichia coli* DH5α and *Salmonella enterica* serovar Typhimurium strain C5 (laboratory collection, Adelaide University) were cultivated in Luria broth and Nutrient broth (Oxoid), respectively, with incubation at 37 °C.

When required, strain DRDC8 was cultured in Amoeba-Conditioned Medium (ACM) prepared as previously described (Marolda *et al.*, 1999) although with some modifications. Amoebae cultured in PYG medium in 25 cm² flasks as described above until near-confluent were washed three times with 10 ml sterile AS buffer. Sterile AS buffer (5 ml) was added to the flask and the monolayer incubated overnight at 22 °C. The culture supernatant was recovered and filter-sterilized by passage through a 0.22 µm pore size membrane filter.

E. coli DH5α cells used to feed the amoebae were cultured as described above, harvested by centrifugation and washed in AS buffer to remove residual nutrients, then resuspended in AS and stored at 4 °C. Where required, washed cell suspensions were killed by heating for 20 min at 80 °C.

Co-cultivation of amoebae and bacteria. The growth of amoebae on lawns of bacteria was assessed as follows. Washed bacterial cell suspensions (~10⁸ cells) were spread on the surface of NNA and ~10³ amoeba cells in AS were spotted on the centre of the plates, followed by incubation for 30 min to allow the amoeba cells to settle. The plates were incubated at 22 °C and 37 °C to allow the amoebae to feed on the bacteria and grow to the margin of the plates. The mean diameter of the trophozoite growth zone and the morphology of the amoebae were assessed daily. All growth experiments were done in triplicate.

Co-culture of *L. monocytogenes* with monolayers of amoeba cells was performed in 24-well tissue culture trays. A suspension of AC012 (100 µl containing 10⁵ cells ml⁻¹) was mixed with 800 µl AS in each well and incubated at 22 °C for 1 h to allow the trophozoites to settle and form a monolayer. Washed bacterial cells were added to achieve an m.o.i. of ~100 bacterial cells per amoeba. The trays were then centrifuged (180 g, 5 min) to sediment the bacterial cells onto the surface of the trophozoites, followed by incubation at 22 °C for 2 h. Amoeba cells present as monolayers were then washed with 1 ml AS, followed by the addition 1 ml AS containing 50 µg gentamicin ml⁻¹ and incubated at room temperature for 1 h to kill extra-amoebic bacterial cells. The majority of extra-amoebic bacterial cells and antibiotics were removed by three washes with AS, followed by addition of 1 ml AS to each well. Co-cultures were incubated at 15, 22 or 37 °C. As co-cultures at 15 °C were incubated for extended periods, 10 µl of an *E. coli* DH5α suspension (10⁷ heat-killed cells) was added daily to prevent starvation and early encystment of trophozoites. Control experiments were set up under the same experimental conditions in AS or AS supplemented with ACM and/or heat-killed *E. coli* DH5α.

Total viable counts of bacteria (intra-amoebic plus remaining extra-amoebic bacteria) present in each well were determined as follows. AS (1 ml) containing Triton X-100 (0.3%, v/v), was added to the

contents of each well to lyse trophozoites. Samples of appropriately diluted lysate (100 ml) from each well were plated in triplicate on BHI agar with incubation at 37 °C; colony counts were determined after 24 h and expressed as c.f.u. ml⁻¹. To count viable intra-amoebic bacteria, infected amoebae monolayers were first washed twice with 1 ml volumes of AS to remove the majority of extra-amoebic bacteria. Amoeba cells were then lysed by addition of 1 ml AS containing Triton X-100 (0.3 %, v/v). Samples (100 ml) of appropriately diluted lysate were then plated in triplicate on BHI agar and incubated at 37 °C; colony counts were determined after 24 h. Counts of amoebae in each well were estimated using a haemocytometer. Three dilutions of each well were counted and the mean counts determined. All experiments were done in triplicate.

Immunofluorescence microscopy. The intra-amoebic location of *L. monocytogenes* cells was assessed using an indirect immunofluorescence staining technique. Amoebae were co-cultured with bacteria in 24-well tissue culture trays at 22 °C as described above (m.o.i. 100), except that the amoebae were placed on the surface of sterile 13 mm diameter round glass coverslips. Following incubation, the coverslips were gently rinsed in AS buffer, fixed in formalin solution (10 %, v/v) for 30 min then washed three times in PBS. The amoebae were then lysed by addition of Triton X-100 (0.3 %, v/v, in AS buffer) for 1 min, washed in PBS and incubated with (diluted 1/50 in PBS) polyclonal rabbit anti-*Listeria* primary antibody (Difco *Listeria* poly O antisera type 1, 4) for 30 min. Unbound primary antibody was removed by washing three times in PBS, and the preparations were then treated with an FITC-labelled secondary antibody (Anti-Rabbit Immunoglobulin F(ab)₂ Fraction Affinity Isolated Fluorescein Conjugated; Silenus). Each preparation was then washed, dried, mounted on glass microscope slides in Mowiol 4-88 containing an anti-bleaching agent (*p*-phenylene diamine) and examined with a fluorescence microscope. *Salmonella* Typhimurium C5 cells were labelled similarly, except that a polyclonal rabbit anti-*Salmonella* Typhimurium antiserum was used as the source of primary antibody.

Transmission electron microscopy (TEM). Monolayers of amoebae were prepared and washed, as described above, in 25 cm² flasks. Suspensions of *L. monocytogenes* cells were added (m.o.i. 50) and the co-culture incubated for 2 h at 22 °C. Samples of uninfected control amoebae were also prepared in an identical manner. The amoeba cells were pelleted by centrifugation (180 g, 7 min), then processed for TEM as described by Gao *et al.* (1997) and Smirnov & Brown (2004). Ultra-thin sections of resin-embedded amoeba cells were stained with uranyl acetate and Reynolds' lead citrate and examined in Philips EM300 transmission electron microscope.

Statistical analysis. Student's *t*-test was used to compare the groups and controls. *P*-values of <0.05 were considered statistically significant.

RESULTS

Growth of amoebae on lawns of bacteria

The ability of *A. polyphaga* AC012 to survive and grow on clinical and environmental isolates of *L. monocytogenes*, and *E. coli* DH5 α , was determined by culture on the surface of NNA medium spread with suspensions of these bacteria. During incubation at 22 °C the growth zone diameter and number of amoebae increased steadily, irrespective of the type of bacteria used, and by 3 days incubation the amoebae completely covered the NNA plate surface (Fig. 1). Although about 50 % of the amoebal population

Fig. 1. Growth of *A. polyphaga* on *L. monocytogenes* and *E. coli* cells. Growth zone diameters of *A. polyphaga* AC012 on the surface of NNA medium spread with *L. monocytogenes* strains DRDC8, 2T, KE504 and the listeriolysin mutant LLO17 were determined. *E. coli* DH5 α was used as a control food source. The percentage of amoeba cells present as cysts was determined by direct observation of 10 randomly selected fields. Data are the means of three independent NNA plates. (*P*>0.05).

was present as cysts after 3 days incubation, there was no evidence to indicate killing of amoebae by bacteria on co-culture plates, or growth of bacteria. When co-cultured at 37 °C for 3 days, most amoeba cells were present as cysts. This observation indicated that the amoebae were unable to grow at 37 °C.

The diameters of the growth zones obtained for *A. polyphaga* AC012 cells cultured on lawns of *E. coli* and different strains of *L. monocytogenes* were not significantly different (*P*>0.05). No differences in the morphology of *A. polyphaga* trophozoites were observed at any stage of incubation on bacterial lawns. The cytoplasmic vacuoles of trophozoites grown on *L. monocytogenes* were not enlarged compared to those of cells grown on lawns of heat-killed *E. coli*. No amoeba cell debris was found on the co-culture plates when the *A. polyphaga* cells were co-cultured with different types of bacteria.

Co-culture of *L. monocytogenes* DRDC8 with monolayers of *A. polyphaga* AC012

Quantitative data describing the interaction of *L. monocytogenes* and *A. polyphaga* were obtained by infection of

monolayers of amoeba cells. Counts of viable intra-amoebic bacteria decreased from $\sim 10^3$ to ~ 10 c.f.u. ml^{-1} within the first 2–3 h of co-culture at 22 and 37 °C ($P < 0.05$) (Figs 2a and 3a). Total counts of bacteria (extra-plus intra-amoebic bacteria) also decreased significantly, although at a slower rate than that observed for counts of intra-amoebic bacteria ($P < 0.05$). Clearly some extra-amoebic bacteria became prey for amoebae so that over of the entire period of co-cultivation, the total number of bacteria decreased. Although counts of amoeba cells did not change significantly, about 20 % of trophozoites exhibited morphological changes typical of encystment (Figs 2b and 3b).

Co-culture at 15 °C required extended incubation for 96 h to quantitatively determine the fate of phagocytosed bacteria (Fig. 4). As expected, the rate of decline in the number of both total and intra-amoebic bacteria was slower than that observed for co-cultures incubated at higher temperatures (Fig. 4a). Nevertheless, counts of intra-amoebic *L. monocytogenes* cells decreased from $\sim 10^5$ to $\sim 10^3$ c.f.u. ml^{-1} within 48 h ($P < 0.05$), whereas counts of extra- plus intra-amoebic bacteria decreased only slightly by the end of the co-culture experiment (96 h)

Fig. 2. Counts of *L. monocytogenes* and *A. polyphaga* during co-culture at 22 °C. (a) Counts of intra-amoebic bacteria (□) and the total viable count of bacteria (■) (intra-amoebic plus extra-amoebic bacteria). (b) Counts of amoebae (●) and the percentage of amoeba cells present as cysts (○). Data are means $\pm$ SD of three replicate experiments (error bars not shown where smaller than symbols).

Fig. 3. Counts of *L. monocytogenes* and *A. polyphaga* during co-culture at 37 °C. (a) Counts of intra-amoebic bacteria (□) and the total viable count of bacteria (■) (intra-amoebic plus extra-amoebic bacteria). (b) Counts of amoebae (●) and the percentage of amoeba cells present as cysts (○). Data are means $\pm$ SD of three replicate experiments (error bars not shown where smaller than symbols).

(Fig. 4a). This indicated that growth of extra-amoebic bacteria had probably occurred. Counts of amoeba cells in co-culture decreased slightly from $\sim 10^5$ to $\sim 10^4$ ml^{-1} over the first 24 h of co-culture, but counts increased over the next 24 h and remained essentially unchanged ($\sim 5 \times 10^5$ ml^{-1}) for the remainder of the experiment. By the end of the experiment, up to 25 % of all amoeba cells had undergone morphological changes typical of encystment (Fig. 4a).

As controls, the viability of *L. monocytogenes* was assessed by incubation at 22 °C in both AS buffer and ACM containing heat-killed *E. coli* (Fig. 4b). After incubation for 96 h in AS buffer, the number of viable bacteria declined from $\sim 10^5$ c.f.u. ml^{-1} to $\sim 10^4$ c.f.u. ml^{-1} after 96 h incubation ($P < 0.05$). However, when *L. monocytogenes* was suspended in ACM containing killed *E. coli* cells, the counts of viable bacteria increased about fivefold relative to counts at the beginning of the experiment. This trend was similar to that obtained for the total viable counts of bacteria in co-cultures. Rapid loss of viability when cells were suspended in AS buffer versus maintenance of viability when incubated in amoeba conditioned medium clearly showed that the reduction in the number of intra-amoebic

Fig. 4. Counts of *L. monocytogenes* and *A. polyphaga* during co-culture at 15 °C. (a) Total viable counts of bacteria (■), counts of intra-amoebic bacteria (▲), counts of amoebae cells (●) and percentage of amoeba cells present as cysts (○) during co-culture. (b) Viability of *L. monocytogenes* cells during incubation in AS buffer (▲) and ACM plus heat-killed *E. coli* (■) at 15 °C. Data are means $\pm$ SD of three replicate experiments (error bars not shown where smaller than symbols).

bacteria during co-culture with amoebae was not caused by loss of viability of the bacterial cells, but rather by active predation and killing by amoeba trophozoites. Furthermore these control experiments showed that factors released by amoebae into AS buffer may have stimulated growth of *L. monocytogenes*. Identical trends were obtained when the haemolysin mutant LLO17 variant of DRDC8 and other isolates of *L. monocytogenes* were used in co-culture assays (data not shown).

To ensure that the results obtained reflected killing of *L. monocytogenes* by *A. polyphaga* and were not an artefact of the co-culture method used, we carried out co-cultures with *A. polyphaga* and *Salmonella* Typhimurium strain C5 as control experiments. Previous research has indicated that this bacterium is capable of survival and multiplication within *A. polyphaga* (Gaze *et al.* 2003). During co-culture for 6 h at 22 °C, counts of intra-amoebic C5 increased slightly, from $\sim 2 \times 10^6$ c.f.u. ml⁻¹ to $\sim 3 \times 10^6$ c.f.u. ml⁻¹ (data not shown). Thus unlike *L. monocytogenes*, *Salmonella* Typhimurium C5 was capable of growth and multiplication within amoebae at 22 °C.

Fluorescence microscopy

Micrographs of immunolabelled *L. monocytogenes* DRDC8 cells internalized by trophozoites 2 h post-infection at 22 °C are shown in Fig. 5. Intra-amoebic bacteria were distributed as clusters of cells, presumably within vacuoles. Counts of intra-amoebic DRDC8 cells per trophozoite cell varied from one or two bacteria to as many as fifty, although only a small percentage of trophozoites contained maximum numbers of labelled DRDC8 cells. However, over a period of 5 h post-infection, counts of intra-amoebic bacteria gradually decreased. After 5 h of co-culture, labelled intra-amoebic bacterial cells were not found (Fig. 5d). No bacterial cells were observed within cyst forms of amoeba cells (data not shown). These results directly correlated with the bacteriological data described above. By contrast, the number of immunolabelled *Salmonella* Typhimurium C5 cells located within trophozoites increased during the course of co-culture at 22 °C (Fig. 6). After 4 h of co-culture, most amoeba cells contained large clusters of internalized C5 cells.

Transmission electron microscopy

More detailed observation of *L. monocytogenes* cells internalized within *A. polyphaga* AC012 was performed by TEM. Sections of amoeba cells infected with bacteria 2 h post-infection showed the bacteria to be confined to tight vacuoles within the host amoeba cytoplasm (Fig. 7). Each vacuole in infected cells apparently contained a single bacterial cell in close juxtaposition with the vacuole membrane. Typically, each vacuole was surrounded by mitochondria and other vesicles presumed to be lysosomes. The cell walls of internalized *L. monocytogenes* cells were still intact, with the thick peptidoglycan layer typical of Gram-positive bacterial cells clearly visible. No section of internalized bacterial cells examined showed evidence of cell division.

By contrast, sections of bacteria within vacuoles of amoebae 4–5 h post-infection showed features characteristic of a loss of cellular integrity. These cells did not have the thick cell wall typical of sections of intact bacteria, and significant loss of electron-dense material from the bacterial cytoplasm was evident. Lysosome-like vesicles surrounding vacuoles containing bacterial cells were observed and some of these were partially fused with the vacuoles that contained bacterial cells. Taken together, these results indicated that phagocytosed *L. monocytogenes* are rapidly destroyed within host vacuoles. No evidence was obtained to indicate intra-amoebic survival or replication of *L. monocytogenes* in trophozoites or cystic forms of *A. polyphaga*.

DISCUSSION

Bacteria are a primary food source for heterotrophic protists such as amoebae. However, not all bacteria are

Fig. 5. Localization of *L. monocytogenes* within *A. polyphaga* during co-culture at 22 °C. Immunofluorescence micrographs show *L. monocytogenes* within vacuoles of *A. polyphaga* cells. (a, b) 2 h post-infection, (c) 3 h post-infection, (d) 4 h post-infection. Note the reduction in numbers of intra-amoebic bacteria over the 4 h of co-culture and the loss of the distinct margins of the bacterial cells. Bars, 20 μm.

digested following ingestion. For example there are now a number of reports that facultative intracellular bacterial pathogens are able to evade digestion and under appropriate conditions are able multiply within the protist host cell (Gao *et al.*, 1997; Gaze *et al.*, 2003; Greub & Raoult, 2002; Kahane *et al.*, 2001; Lamothe *et al.*, 2004; Landers *et al.*, 2000; Newsome *et al.*, 1998; Steinert *et al.*, 1998). In view of the fact that *L. monocytogenes* is a ubiquitous, facultative intracellular pathogen responsible for serious food-borne infections, it was of interest to determine whether this bacterium is capable of survival within protozoa such as amoebae. Survival within these hosts might indicate a potential reservoir of these bacterial pathogens.

The growth of *A. polyphaga* AC012 on lawns of different strains of *L. monocytogenes* at 22 and 37 °C provided good

evidence that *Acanthamoeba* trophozoites use these bacteria as a food source. Furthermore, the absence of cellular debris typical of dead trophozoite cells or any change in the size or number of trophozoite cytoplasmic vacuoles indicated that *L. monocytogenes* cells do not interfere with either the growth or encystment of trophozoites. Although amoeba trophozoites encysted early without growth during co-culture at 37 °C, this likely to be related to the fact that this incubation temperature is close to the maximum physiological growth limit (38–40 °C) for this organism (Daggett *et al.*, 1982).

These observations are different from those reported for co-culture of *A. polyphaga* with other bacterial pathogens. Marolda *et al.* (1999) reported that during co-culture, *A. polyphaga* trophozoites developed large cytoplasmic vacuoles that contained live, motile *Burkholderia cepacia*

Fig. 6. Localization of *Salmonella* Typhimurium C5 within *A. polyphaga* AC012 during co-culture at 22 °C. Immunofluorescence micrographs show *Salmonella* Typhimurium in vacuoles of *A. polyphaga* cells following co-culture at (a) 2 h and (b) 4 h post-infection. Note the clumps of *Salmonella* cells within the vacuoles of amoeba cells. Bars, 20 µm.

Fig. 7. TEM of *L. monocytogenes* cells within vacuoles of *A. polyphaga* trophozoites. Electron micrographs show sections of (a, b) an infected amoeba cell with intact bacterial cells (B) within vacuoles of infected amoebae cells 2 h post washing, and (c) a partially degraded bacterial cell within a vacuole of an infected amoeba cell 4 h post-washing. Note the vacuolar membrane (M) enclosing bacterial cells and the loss of the cell wall (BW) of bacteria between 2 and 4 h post washing. Small lysosome-like vacuolar structures (L) and mitochondria (Mit.) are arrowed.

cells. Furthermore, these trophozoites exhibited delayed encystment in the presence of *B. cepacia* cells. Similarly, *Salmonella* Typhimurium has been reported to grow and survive within *A. polyphaga* during plate co-culture (Gaze *et al.*, 2003). These discrepancies may reflect the different abilities among bacteria to avoid killing within amoeba trophozoites.

Quantitative assays that tracked the fate of viable *L. monocytogenes* following co-culture with monolayers of *A. polyphaga* indicated that trophozoites were able to eliminate intra-amoebic *L. monocytogenes* cells within 2–5 h of co-cultivation. This conclusion is consistent with data for longer-term co-culture studies (Akya & Thomas, 2009; Huws *et al.*, 2008; Zhou *et al.*, 2007) and is supported by direct microscopic observation of *L. monocytogenes* cells internalized by *A. polyphaga*. Typically, within 4–5 h of co-culture at 15, 22 and 37 °C, intact *L. monocytogenes* cells were not found within either trophozoites or cysts. This outcome is significant for several reasons. The co-culture temperatures used in this study are representative of those across a food supply chain continuum. Many foods are processed at cooler temperatures of about 15 °C, prior to storage at refrigeration temperatures required to limit growth of pathogenic bacteria. It was therefore of interest to determine whether heterotrophic protists could harbour *L. monocytogenes* in the food-processing environment and provide a protective environment that would allow the bacteria to escape the rigorous sanitation systems normally deployed in these environments. The 22 °C temperature used is a common environmental temperature at which both *L. monocytogenes* and protozoa such as *Acanthamoeba* spp. can grow. Therefore at both 15 and 22 °C there is a potential opportunity for interaction between naturally occurring *L. monocytogenes* and *Acanthamoeba* spp. Co-cultures at 37 °C provided an opportunity to examine interactions under conditions known to favour expression of virulence factors required for escape from the phagolysosome to the cytoplasm of mammalian cells. However, unlike mammalian cells, *A. polyphaga* AC012 trophozoites were able to kill *L. monocytogenes* cells 2–5 h after phagocytosis. Indeed *A. polyphaga* AC012 was apparently able to kill an avirulent listeriolysin O-deficient variant of strain DRDC8 just as well as the virulent wild-type parental strain that has been shown to be virulent for mice (Francis & Thomas, 1996). Together, these data indicate that *A. polyphaga* may inactivate *L. monocytogenes* cells before they can express proteins critical for access to the cytoplasmic compartments of amoeba cells. Huws *et al.* (2008) also found no evidence of intracellular survival or replication of *L. monocytogenes* within *A. polyphaga*. Furthermore, Zhou *et al.* (2007) also concluded that listeriolysin O expression by the *L. monocytogenes* strains tested did not significantly change the outcome of bacterial interaction with *A. castellanii*.

The fact that the total number of amoeba cells during co-culture remained almost unchanged over the duration of short-term co-culture experiments (5 h) indicated that

amoeba trophozoites that predate/phagocytose *L. monocytogenes* are not killed, but instead can use these bacteria as a food source. Consequently, *L. monocytogenes* cells are unable to either survive long-term within *A. polyphaga* cells, or grow and multiply within these cells to an extent that the host amoeba cells undergo lysis. This observation was confirmed by microscopic examination of amoebae co-cultured with *L. monocytogenes*. In addition our data support work reported by Zhou *et al.* (2007) showing that *L. monocytogenes* did not significantly reduce the number of amoeba cells during co-culture.

TEM provided the most definitive evidence that *L. monocytogenes* cells within amoeba trophozoites are not able to escape the phagosomal vacuole, enter the cytosol and replicate, as is the case for infected mammalian cells. Loss of the bacterial cell wall integrity indicated that bacteria are degraded within vacuoles in a short period of time (2–5 h). The concentration of mitochondria and lysosome-like vesicles at the periphery of vacuoles containing *L. monocytogenes* cells suggested that these structures play an active role in degradation of internalized bacteria.

The survival and multiplication of *Salmonella* Typhimurium C5 within *A. polyphaga* is consistent with previous research by Brandl *et al.* (2005) showing that *Salmonella enterica* strains resisted killing by a *Tetrahymena* sp., whereas *L. monocytogenes* cells were killed by this ciliate. Clearly *Salmonella* spp. express molecular mechanisms that allow the bacteria to avoid killing by amoeba trophozoites during both short- and long-term co-cultivation. These observations highlight the fact that different bacterial pathogens have evolved different mechanisms to avoid killing by phagocytic cells. This variation extends to different strains of the same pathogen. For example, although different strains of *B. cepacia* interact with *A. polyphaga*, not all strains of this bacterial pathogen are able to survive equally well within amoeba cells (Marolda *et al.*, 1999; Taylor *et al.*, 2003). However, this is clearly not the case for *L. monocytogenes* strains. Clinical and environmental strains of *L. monocytogenes* were killed equally well by *A. polyphaga* AC012 trophozoites.

The maintenance of viability of *L. monocytogenes* cells in ACM together with the slow loss of viability in non-nutrient medium indicated that the killing of bacteria by amoebae in co-cultures is not due to toxic compounds released by amoeba cells. This observation is consistent with previous reports showing that extracellular bacteria can maintain high populations in the presence of *Acanthamoeba* spp. (Ly & Müller, 1990; Zhou *et al.*, 2007). The observed reduction of viable intra-amoebic bacterial cells during co-culture with *A. polyphaga* trophozoites therefore represents an active intracellular killing process.

In conclusion, phagocytosed *L. monocytogenes* cells are rapidly degraded by *A. polyphaga* during co-culture at a variety of temperatures. It is therefore unlikely that *A. polyphaga* AC012, at least, can act as an environmental

reservoir for *L. monocytogenes* under the conditions used. However, more research needs to be done under conditions that more closely mimic natural niches to uncover complex interactions between *L. monocytogenes* and amoebae. For example, it would be interesting to determine whether survival of *L. monocytogenes* strains within the phagosomes of *A. polyphaga* AC012 is dependent on the level of expression of the cytolytic protein listeriolysin O, or presence of other types of bacteria that are adapted for survival within this niche. It will also be of interest to determine what host factors are important in determining the different outcome of infection of heterotrophic protists compared with that observed for mammalian phagocytic cells.

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