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Amoebae and algae can prolong the survival of *Campylobacter* species in co-culture

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ABSTRACT

Several species of free-living amoebae can cause disease in humans. However, in addition to the direct pathogenicity of e.g. *Acanthamoebae* and *Naegleria* species, they are recognized as environmental hosts, indirectly involved in the epidemiology of many pathogenic bacteria. Although several studies have demonstrated intracellular survival of many different bacteria in these species, the extent of such interactions as well as the implications for the epidemiology of the bacterial species involved, are largely unknown and probably underestimated. In this study, we evaluated eight different unicellular eukaryotic organisms, for their potential to serve as environmental hosts for *Campylobacter* species. These organisms include four amoebozoas (*Acanthamoeba polyphaga, Acanthamoeba castellanii, Acanthamoeba rhysodes* and *Hartmanella vermiformis*), one alveolate (*Tetrahymena pyriformis*), one stramenopile (*Dinobryon sertularia*), one eugoenozoa (*Euglena gracilis*) and one heterolobosea (*Naegleria americana*). *Campylobacter* spp. including *Campylobacter jejuni, Campylobacter coli* and *Campylobacter lari* are the most common cause of gastroenteritis in the western world. Survival and replication of these three species as well as *Campylobacter hyointestinalis* were assessed in co-cultures with the eukaryotic organisms. *Campylobacter* spp. generally survived longer in co-cultures, compared to when incubated in the corresponding growth media. The eukaryotic species that best promoted bacterial survival was the golden algae *D. sertularia.* Three species of amoebozoas, of the genus *Acanthamoeba* promoted both prolonged survival and replication of *Campylobacter* spp. The high abundance in lakes, ponds and water distribution networks of these organisms indicate that they might have a role in the epidemiology of campylobacteriosis, possibly contributing to survival and dissemination of these intestinal pathogens to humans and other animals. The results suggest that not only *C. jejuni*, but a variety of *Campylobacter* spp. can interact with different eukaryotic unicellular organisms.

1. Introduction

Studies on the interactions between unicellular eukaryotic organisms and bacteria have brought the importance of these phenomena in nature to our attention. This research has increased since the early 1980's when Rowbotham described that the human pathogen *Legionella pneumophila* could survive and replicate in free-living amoebae of the genera *Acanthamoeba*, *Naegleria* and *Hartmanella* (Rowbotham, 1980, 1986). Since then the interaction of *Legionella pneumophila* with amoebae have been extensively studied and the importance of the amoeba host as a vector for dissemination and survival of the bacterium is well established (reviewed in Greub and Raoult, 2004). Free-living amoebae such as *Acanthamoeba* spp. have been particularly well studied and have been shown to host a wide variety of bacterial species including many human pathogens such as *Vibrio cholerae*, *Listeria monocytogenes*, *Mycobacterium* spp. and *Helicobacter pylori*, to mention a few (reviewed in Thomas et al., 2009). Other unicellular species shown to host intracellular bacteria include *Tetrahymena pyriformis* and *Dictyostelium discoideum*. The latter has been used as a model organism for testing virulence mechanisms of *Legionella* spp., *Mycobacterium* spp. and *Vibrio* spp. and in this way giving an insight to the pathogenesis of the bacteria (reviewed in Hilbi et al. (2007)).

Our group and others have described the survival and replication of *Campylobacter* spp. in different species of the genus *Acanthamoeba* (Axelsson-Olsson et al., 2005, 2007; King et al., 1988; Snelling et al., 2005). This suggests that in addition to warm blooded hosts, *Campylobacter* spp. might use water bound organisms as hosts for survival and potentially for replication in the environment.

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Interactions between *Campylobacter* spp. and free-living amoebae such as *Acanthamoeba* spp. is not a very surprising phenomenon. *Achantamoeba* spp. is very common and can be found in most aquatic settings (reviewed in Khan, 2003). Free-living amoebae are often abundant in proximity to humans (reviewed in Rodriguez-Zaragoza, 1994 and Bare et al., 2009) and many species are part of the biofilm in water supply systems, where they graze the bacterial flora (Parry, 2004). *Campylobacter jejuni* is the leading cause of bacterial enteritis in the western world, and an increasing number of epidemiological studies have demonstrated that water is an important factor for the spread of these bacteria (Carrique-Mas et al., 2005; Hanninen et al., 2003; Jones, 2001; Nygard et al., 2004; Schonberg-Norio et al., 2004; Studahl and Andersson, 2000). Many sporadic cases and outbreaks have been shown to originate from environmental sources including lakes, springs and even water distribution networks (reviewed in Humphrey et al., 2007). Most *Campylobacter* spp. are microaerophilic, requiring a reduced oxygen gas environment for optimal growth (Bolton and Coates, 1983) and have a narrow range of growth temperatures at 37–43 °C (Skirrow, 1994). They are considered as fragile organisms with particular growth requirements and less able to tolerate environmental stress than other food borne pathogens (Park, 2002), and hence they would be predicted to benefit from using unicellular aquatic organisms as intermediate and temporary vectors between warm blooded hosts.

The aim of this study was to determine if a number of unicellular eukaryotic organisms, some known to host bacterial spp. including human pathogens, could also serve as hosts for *Campylobacter* spp. We studied eight different eukaryotic species to assess the ability of four *Campylobacter* spp. to survive and replicate in co-cultures with these organisms. Among those are three species of *Acanthamoebae*, that are freshwater amoebae belonging to the amoebozoa group of unicellular organisms, abundant in moist habitats (Khan, 2003). *Naegleria americana* and *Hartmanella vermiformis* are fresh water amoebae and interactions of the latter with *Legionella pneumophila* have been the focus of many studies (Atlas, 1999; Rowbotham, 1983). *T. pyriformis*, a freshwater ciliate, is abundant in ponds and shallow water. It feeds on bacteria, and has been shown to support intracellular survival of *Salmonella enterica, Escherichia coli* and *Legionella pneumophila* (Brandl et al., 2005; Harb et al., 2000; Steinberg and Levin, 2007). The mixotrophic algae *Dinobryon sertularia* and *Euglena gracilis* are mainly found in freshwater lakes and ponds (Bird and Kalff, 1986; Graham et al., 2004), but can also be found in coastal marine or brackish waters where they feed on bacteria in addition to their photosynthetic capacity.

We found that the *Campylobacter* spp. in this study generally survived longer in the co-cultures, compared to when cultured in growth media alone. Species of the genus *Acanthamoeba* supported both intracellular survival and replication of all *Campylobacter* spp. in the study.

2. Material and methods

2.1. Bacteria

Bacterial strains of *C. jejuni* (CCUG 11284), *Campylobacter coli* (LMG 6440), and *Campylobacter lari* (LMG 8846) were cultured on conventional blood agar plates (Columbia agar II, containing 8% (vol/vol) whole horse blood) at 42 °C in a microaerobic gas environment, using the CampyGen gas generating system (CN0025A, Oxoid, Ltd., Basingstoke, UK) and BBL GasPak™ system (BD, Franklin Lakes, NJ, USA). A strain of *Campylobacter hyointestinalis* (CCUG 20822) was cultured using similar media and gas environment but at 37 °C instead of 42 °C. Bacterial cells were harvested after 24 h, suspended in Peptone–Yeast extract–Glucose (PYG) medium and diluted to a final concentration of approximately 10^3 cfu/ml for the replication study and approximately 10^6 cfu/ml for the survival study.

2.2. Eukaryotic organisms

Eight species of unicellular eukaryotic organisms were used in the study: *Acanthamoeba polyphaga* (Linc Ap-1), *Acanthamoeba castellanii and Acanthamoeba rhysodes*, the latter two were originally isolated from patients with keratitis and provided by J. Winiecka–Krusnell, Swedish Institute for Infectious Disease Control (SMI), Sweden., *H. vermiformis* (CCAP 1534/7A), *N. americana* (CCAP 1518/1G), *E. gracilis* (CCAP 1224/5Z) and *D. sertularia* (CCAP 917/2), were purchased from Culture collection of algae and protozoa (CCAP), Argyll Scottland UK. The ciliate *T. pyriformis* was provided by A. Andersson, Umeå University, Sweden. Trophozoites of *A. polyphaga*, *A. castellanii* and *A. rhysodes* were maintained aerobically at 27 °C in PYG medium, at the bottom of 25 cm^2 culture flasks, as previously described (Axelsson-Olsson et al., 2007). *H. vermiformis* were grown on non-nutrient agar according to the manual from CCAP in the presence of bacteria in a north facing window. The *H. vermiformes*-cultures were let to grow for several weeks to allow almost confluent layer of the amoeba and to minimize the bacteria present. *N. americana* were grown in Modified Chang's Serum–Casein–Glucose–Yeast Extract medium (MC) at 27 °C in the dark according to the manual from CCAP. *T. pyriformis* where grown in Peptone–Yeast medium (PPY) at RT in the dark. *E. gracilis* where grown in *Euglena gracilis* Medium 1:1 with Jaworski's Medium (EG:JM) in a north facing window and *D. sertularia* where grown in Modified Woods Hole Medium (MWC) in a north facing window, both according to the manuals from CCAP. Axenic cultures of *H. vermiformis* and *D. sertularia* were not obtained, but these species were grown in the presence of *Campylobacter*-selective antibiotics (modified Bolton broth selective supplement, SR0208; Oxoid, Ltd., Basingstoke, UK) to minimize the growth of xenic bacteria. All eukaryotic species were grown in 12-well culture plates to a density of 10^6 cells/ml to allow comparison between the different species for both the survival and replication study (see below).

2.3. Detection methods

The survival of *Campylobacter* cells was assessed by conventional plating on blood agar plates. To increase the sensitivity (i.e. the chance to detect very low concentrations of viable bacteria) we also used the *Acanthamoeba Campylobacter* co-culture (ACC) method, previously described by our group (Axelsson-Olsson et al., 2007). The ACC method is a very sensitive enrichment method for *Campylobacter* spp. Briefly, *A. polyphaga* are cultured at the bottom of 12 well polystyrene plates in PYG medium at 27 °C until confluent. Samples (100 μl) of *Campylobacter* spp. from the co-cultures are inoculated into the wells of the *A. polyphaga* plate. The plate is then incubated aerobically at 37 ° for 48 h. At these conditions the four *Campylobacter* spp. in the study replicate with rapid kinetics as shown in a previous study (Axelsson-Olsson et al., 2007). Positive samples are confirmed both by microscopic observation and by plating on blood agar.

2.4. Survival

The ability of the different *Campylobacter* spp. to survive within the eukaryotes was determined by setting up co-cultures at 10 °C. *C. jejuni, C. lari, C. coli* and *C. hyointestinalis* were added in triplicates to cultures of the eight different eukaryotic species, at a ratio of 10 bacteria per eukaryotic cell in a total volume of 2 ml. Control plates