Microbiology (1994), 140, 1253–1259 Printed in Great Britain

REVIEW ARTICLE

Trojan Horses of the microbial world: protozoa and the survival of bacterial pathogens in the environment

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Keywords: biofilms, intra-amoebal growth, legionella, listeria, protozoa

The resistance of bacteria to protozoal predation

Protozoa, of which there are thousands of species, are ubiquitous in diverse natural habitats such as fresh and salt water, moist soils and even dry sands. The role of free-living protozoa in terrestrial and aquatic environments as predators of bacteria is widely acknowledged. Predation by protozoa has a significant effect in controlling bacterial populations in soil, and the degradation of bacteria undoubtedly contributes to the maintenance of soil fertility (Foster & Dormaar, 1991; Weekers *et al.*, 1993). Likewise, protozoa play an integral part in the cycling of nutrients in aquatic food chains (Porter, 1984; Wright & Coffin, 1984).

However, it is clear from other studies concerning the interaction of bacteria and protozoa that not all bacteria are suitable food sources for amoebae (Singh, 1942, 1946; Danso & Alexander, 1975; Weekers *et al.*, 1993). Alexander (1981) reported that some Gram-negative bacteria were able to survive grazing by protozoa, which could be due to the inability of the amoebae to take-up or to kill and digest internalized bacteria. Alternatively, bacteria may be able to resist engulfment by defence mechanisms such as toxins, toxic pigments or outer-membrane structures (Weekers *et al.*, 1993). There has been a number of reports (Preer *et al.*, 1974; Hall & Voelz, 1985; Fritsche *et al.*, 1993) of bacteria surviving as endosymbionts of free-living protozoa, thus demonstrating adaptation to the intracellular environment. The discovery that *Legionella pneumophila* infects and multiplies within some species of free-living amoebae (Rowbotham, 1980) has confirmed the ability of bacteria to exploit a normally hostile intracellular environment to ensure survival. Indeed, survival and intracellular growth of bacterial species in protozoa may well prime pathogenic bacteria for virulence. However, the potential role of protozoa as reservoirs for human pathogens does not appear to have received adequate attention. This article examines the importance of protozoa in the maintenance, survival and protection of pathogenic bacteria in natural and man-made ecosystems.

Protozoa and interactions with bacterial pathogens

Legionellaceae

Although at least 34 *Legionella* species have been identified, *Leg. pneumophila* is the primary cause of Legionnaires' disease, a serious form of atypical pneumonia. *Leg. pneumophila* can infect and multiply within *Hartmannella*, *Acanthamoeba* and *Naegleria* species, which are ubiquitous in moist soil and aquatic environments (see Table 1). Legionellae can also survive and multiply within ciliated protozoa of the genus *Tetrahymena*, a freshwater bacteriovore (Fields *et al.*, 1984). Following phagocytosis by acanthamoebae, legionellae multiply within the cytoplasm, evading the host lysosomal attack so that after 36–48 h a single vesicle of motile legionellae fills most of the amoebal cell. The final effect of infection is lysis of the cell and liberation of many motile bacteria into the environment. Rowbotham (1986) has studied electron micrographs of *Leg. pneumophila* growing within *Acanthamoeba polyphaga* in the later stages of infection. It has been estimated that an amoebal vesicle of 10 μm diameter, with 90% of the space occupied by bacteria measuring 0·32 × 0·60 μm, could contain approximately 10^4 bacterial cells. *Leg. pneumophila* is now known to infect five genera of amoebae (Fields, 1993), whereas other species of Legionella have a more specialized host range (Fields *et al.*, 1990).

Recently, a group of legionella-like amoebal pathogens (LLAPs) have been reported (Rowbotham, 1993), which are bacilli that infect and multiply in the cytoplasm of amoebae but so far have not been found to grow on laboratory media. LLAPs may be of considerable importance because they are capable of causing pneumonia and induce a serological response in infected patients

Table 1. Bacterial pathogens surviving within protozoa

Bacterial species	Protozoal host	Outcome of protozoal uptake	Selected references
Legionella pneumophila	*Acanthamoeba* *Naegleria*	Multiplication, cell lysis	Rowbotham (1980)
Legionella pneumophila	*Tetrahymena*	Multiplication, cell lysis	Fields *et al.* (1984)
Legionella-like amoebal pathogens (LLAPs)	*Acanthamoeba* *Hartmannella*	Multiplication, cell lysis	Rowbotham (1993)
Listeria monocytogenes	*Acanthamoeba*	Multiplication, cell lysis	Ly & Müller (1990a)
	Tetrahymena	Multiplication, cell lysis	Ly & Müller (1990b)
Vibrio cholerae	*Acanthamoeba* *Naegleria*	Multiplication	Thom *et al.* (1992)
Edwardsiella tarda *Aeromonas salmonicida* (Fish pathogens)	*Tetrahymena*	Multiplication	King & Shotts (1988)
Mycobacterium leprae	*Acanthamoeba*	Survival	Jadin (1975)
Opportunist mycobacteria	*Acanthamoeba*	Survival	Krishna-Prasad & Gupta (1978)
Pseudomonas, *Alcaligenes* and *Bacillus* species	*Hartmannella*	Survival	Tyndall *et al.* (1991)
Coliform organisms (including *Salmonella typhimurium*)	*Tetrahymena* *Acanthamoeba*	Survival	King *et al.* (1988)

(Rowbotham, 1993). PCR studies of amplified DNA coding for the 16S rRNA of LLAP (type 3) have shown that this organism is a member of the genus *Legionella* (Fry *et al.*, 1991). Based on rRNA analysis it has been suggested that LLAPs may be a new group of Legionella (Rowbotham, 1993) and that they are closely related to *Sarcobium lyticum*, an obligate intracellular parasite of soil amoebae, first described in Poland by Drozanski (1991).

Although the interactions between legionellae and amoebae were observed *in vitro* with axenically grown amoebae (Rowbotham, 1980), it has been shown that amoebae isolated directly from river-water sediment contained *Leg. pneumophila* after they were washed and lysed (Harf *et al.*, 1987). More recently, soil amoebae of the genus *Vahlkampfia* and *Hartmannella* were found to be infected by *Leg. longbeachae* (Steele, 1993). Dry potting soil compost containing such amoebae has been associated with a number of cases of pneumonia in Southern Australian states caused by *Leg. longbeachae*. It is now apparent that intra-protozoal growth of legionellae is a primary mechanism for the survival and multiplication of the bacterium in natural habitats (Wadowsky *et al.*, 1988; Fields *et al.*, 1989) and that Legionella are not simply and only free-living bacteria *per se*, but have a highly evolved host/parasite relationship, described as protozootic (Rowbotham, 1993), for their survival in natural ecosystems.

Other bacterial species surviving in amoebae

Legionella are not the only human pathogens capable of survival in protozoal hosts (see Table 1). Reports suggest that the fate of internalized bacteria falls into three main groups; those which multiply and cause lysis of amoebal cells such as Legionella and Listeria; those which multiply without causing cell lysis (*Vibrio cholerae*); and those which survive without multiplication (certain coliform organisms and mycobacteria).

Listeria are a diverse group of environmental bacteria often considered to be soil micro-organisms (Seeliger, 1988), but some species infect humans and other mammals and invade host cells, including macrophages. Ly & Müller (1990a) reported that *Listeria monocytogenes* infection of *Acanthamoeba castellanii* causes cell lysis and death. *L. monocytogenes* can also grow in coculture with *Tetrahymena pyriformis* and causes cell lysis after 8–15 d incubation (Ly & Müller, 1990b). Panikov *et al.* (1993) have confirmed that *L. monocytogenes* grows within *T. pyriformis* and calculated a generation time of 14·4 h, compared to a generation time of 7 h for legionellae. *L. monocytogenes* infects nutrient-depleted axenic cultures of *A. polyphaga*, resulting in a 1–2 log increase in numbers after 3 d incubation (J. Barker, unpublished data). The infection appears to induce formation of thin-walled precysts containing many motile listeria.

Other environmental bacteria capable of growth in *T. pyriformis* include *Edwardsiella tarda* and *Aeromonas salmonicida*, two common bacterial fish pathogens (King & Shotts, 1988). In addition, environmental species of opportunist mycobacteria (including *Mycobacterium avium*) survive within *A. castellanii* and when amoeba-cell division occurs, ingested bacteria are passed to the progeny (Krishna-Prasad & Gupta, 1978). Jadin (1975) has found that *Mycobacterium leprae* is capable of growth in *Acanthamoeba culbertsoni* and suggested that free-living

amoebae may be environmental vectors for mycobacteria including *M. leprae* (Jadin, 1987).

Further evidence of the role of amoebae in the maintenance of pathogens in the environment has been reported by Thom *et al.* (1992), who found that *V. cholerae* multiplied after ingestion by *Naegleria* and *Acanthamoeba* species. *V. cholerae* also survived within cysts of *Naegleria* and was recovered from the excysting amoeba. Free-living amoebae may help to maintain *V. cholerae* in natural waters in parts of the world where there is no evident association with cases of clinical cholera (Colwell *et al.*, 1977; Bashford *et al.*, 1979), thus acting as reservoirs for possible cholera infections.

Protozoa: their role in the protection of environmental bacteria

Bacteria internalized by protozoa may be given unique protection when the protozoa form cysts. Under adverse conditions, Rowbotham (1986) observed that *Acanthamoeba* containing legionellae encyst, leading to the formation of a precyst or a mature thick-walled cyst which traps motile or non-motile legionellae. *Legionella* species have been detected in sewage works and concentrations were not appreciably reduced by either primary or secondary treatment processes (Palmer *et al.*, 1993). This finding could be related to the protection provided by protozoa which are ubiquitous inhabitants of sewage-treatment plants. The resistance of amoebal cysts to extremes of temperature (Chang, 1978; Biddick *et al.*, 1984) and to the effects of biocides (De Jonckheere & Van de Voorde, 1976) may contribute to the difficulties in eradicating legionellae from contaminated water systems using conventional disinfectant procedures. *A. polyphaga* cysts infected with *Leg. pneumophila* protected the bacterium from the action of chlorine (Kilvington & Price, 1990) and isothiazolone biocides (Kilvington, 1990); bacteria were recovered from excysting amoebae. Not only does the amoebal cyst offer a mechanism for bacteria to evade hostile environmental conditions, but it also provides a means by which the bacteria can spread and colonize new habitats by it being blown through the air (Kingston & Warhurst, 1969).

Coliform bacteria internalized by protozoa in natural ecosystems may give protection against external antagonists (King *et al.*, 1988). *Salmonella typhimurium* and *Shigella sonnei* survived ingestion by laboratory strains of *A. castellanii* and *T. pyriformis* and were shielded from the activity of free chlorine. Bacteria were cultured from chlorine-treated protozoans well after the time required for 99% inactivation of extracellular cells. Thus, organisms trapped within amoebae could be responsible for the persistence of coliform bacteria in chlorine-treated water supplies (Goshko *et al.*, 1983; Hudson *et al.*, 1983). The role of free-living amoebae in harbouring environmental bacteria has been confirmed by the presence of heterotrophs, including *Pseudomonas* and *Alcaligenes* species, isolated from *Hartmannella* trophozoites and cysts, found in well-water samples (Tyndall *et al.*, 1991).

The control and eradication of *L. monocytogenes* in food production is a continuing problem, as Listeria are often isolated from moist surfaces in food-processing plants (Frank *et al.*, 1990; Nelson, 1990). The isolation of these organisms highlights problems in cleaning and disinfection, but a contributory factor to the survival of the organism's in such environments is likely to be through association with adherent biofilms (Ren & Frank, 1993). Although it is widely acknowledged that biofilm bacteria are generally more resistant to treatment with biocides (Costerton *et al.*, 1987; Gilbert *et al.*, 1990), Listeriae ingested or encysted by amoebae would have an increased chance of surviving disinfection procedures. Yet the possible role of free-living amoebae in promoting the survival of Listeria in the environment has not been explored.

Recently eye-wash stations in hospitals were found to contain *Leg. pneumophila*, *Pseudomonas* species and *Acanthamoeba* (Paszko-Kolva *et al.*, 1991). The presence of the *Acanthamoeba* probably had a significant role in allowing the bacterial species to survive, as amoebal lysis resulting from infection with legionellae would release nutrients, encouraging the growth of other bacteria.

As well as providing physical protection from adverse conditions, growth in amoebae may alter the physiology of bacteria. *Leg. pneumophila* cells grown within *Acanthamoeba* and then freed from the amoebal host were significantly resistant to treatment with biocides compared to bacteria grown *in vitro* (Barker *et al.*, 1992). For example, only a 10-fold kill was achieved with an isothiazolone derivative against intra-amoebal-grown legionellae, whereas a 1000-fold kill was observed for cells grown *in vitro*. Intra-amoebal growth may also affect bacterial survival after the death of the host. *Leg. pneumophila* has been recovered after growth in *A. polyphaga* and storage at 4 °C for 6 months with only small reductions in the viable count (J. Barker, unpublished data). These results are compatible with the finding that the physiology of legionellae has altered as a result of their growth within the amoebae and produced a radically altered phenotype (Barker *et al.*, 1993).

Bacterial survival within host protozoa

Although some bacterial species survive ingestion by protozoa, under certain environmental conditions the same organisms are eradicated. At low temperatures acanthamoebae may phagocytose and digest *Leg. pneumophila* as food (Anand *et al.*, 1983), or evict the phagosomes containing legionellae as faecal vesicles (T. Rowbotham, personal communication). Alternatively, at higher temperatures after infection with the same strain of *Legionella*, the amoebae can be heavily parasitized so that after 24 h they become packed with motile legionellae. Growth temperature may be an important factor in determining the virulence of *Leg. pneumophila*. Mauchline *et al.* (1993) have shown that in continuous culture, under defined conditions, *Leg. pneumophila* maintains its virulence in animal pathogenicity tests. When the growth temperature

was decreased from 37 °C to 24 °C, virulence was attenuated because none of the animals died.

As with other micro-organisms the virulence of *Legionella* species is almost certainly governed by the products of many genes (Miller *et al.*, 1989). The molecules of potential importance to the intracellular survival of *Leg. pneumophila* have been extensively reviewed (Dowling *et al.*, 1992; Horwitz, 1993; Hacker *et al.*, 1993). However, the uptake of bacteria by protozoal hosts is an important stage in the infective process. Previous studies (King *et al.*, 1991) have indicated that *Leg. pneumophila* infects *Hartmannella vermiformis* by a microfilament-independent mechanism (it is not inhibited by cytochalasin D). Conversely, an inhibitor of adsorptive pinocytosis (methylamine) blocked infection of *H. vermiformis*, which suggests that receptor-mediated endocytosis is necessary for infection of the amoebae. Further work by Fields *et al.* (1993) has indicated that entry, not attachment, of virulent *Leg. pneumophila* is the limiting step in infection of axenically grown *H. vermiformis*. Hodinka & Wyrick (1986) have described receptor-mediated endocytosis as a means of avoiding phagosome/lysosome fusion for chlamydiae in mouse fibroblast (L-929) cells. This method of uptake is suspected to direct the organism into vesicles that do not fuse with the lysosome.

Ultrastructural examination of infected *Hartmannella* has revealed that, immediately after ingestion, single *Leg. pneumophila* cells are found in endosomes. It has been suggested by Fields (1993) that in *H. vermiformis*, the endosome containing the legionellae fuses with the endoplasmic reticulum of the host cell and that this becomes the site for bacterial multiplication. An examination of the uptake of the intracellular parasite Brucella by Vero-cells has revealed that it multiplies within the endoplasmic reticulum and so avoids attack by lysozyme enzymes (Detilleux *et al.*, 1990).

Protozoa, biofilms and bacterial evolution

The importance of biofilms in the maintenance and survival of micro-organisms in the general environment is widely acknowledged (Costerton *et al.*, 1987; Characklis & Marshall, 1990). Biofilms not only serve to allow for the growth of micro-organisms in water systems but also protect from antimicrobial substances (Keevil *et al.*, 1990; Brown & Gilbert, 1993). Biofilms are a major source of *Legionella* species in both man-made (Rowbotham, 1993) and natural aquatic systems (Marrao *et al.*, 1993). The concentration of bacteria within the biofilms provides excellent opportunities for attack by predators, such as protozoa, and parasites, such as bacteriophages and *Bdellovibrio* species (Characklis *et al.*, 1990). The biofilm/water interface also attracts ciliates, flagellates and amoebae, which graze the surface, seeking food. Although Legionella is an effective parasite of certain species of protozoa, it in turn is susceptible to predation by *Bdellovibrio* (Tomov *et al.*, 1982; Richardson, 1990), a bacterium associated with the biofilm environment (Starr & Seidler, 1971). Perhaps it should be no surprise that environmental bacteria such as *Legionella*, *Listeria* and *Vibrio* species have evolved so that they are capable of surviving and multiplying within biofilm predators such as amoebae, as it offers protection in adverse conditions. Indeed it has been suggested (King *et al.*, 1988) that resistance to digestion by predatory protozoa was an evolutionary prerequisite of bacterial pathogenicity and a survival mechanism for bacteria in aquatic environments. In support of this hypothesis, Cianciotto & Fields (1992) have reported that the *Leg. pneumophila mip* gene potentiates intracellular infection of protozoa and human macrophages. The *mip* gene (Cianciotto *et al.*, 1989), which is responsible for the production of a 24 kDa surface protein, appears to be required for optimal intracellular infection and may be involved in resistance to intracellular killing. Thus the ability of *Leg. pneumophila* to parasitize macrophages and hence to cause human disease may be a consequence of its adaptation to intracellular growth within protozoa. Mip analogues have been found in other intracellular parasites, including *Coxiella*, *Chlamydia* and *Neisseria* (Bangsborg *et al.*, 1991; Dumais-Pope *et al.*, 1993). It would be tempting to speculate that Mip-related proteins are critical for the virulence of intracellular pathogens. However, virulence of intracellular parasites is almost certainly multifactorial and not dependent on the expression of individual phenotypic traits (Horwitz, 1993). The bacterial pathogens capable of survival and/or multiplication in protozoa include *Listeria*, *Legionella*, *Mycobacterium* and *Vibrio*. With the exception of *V. cholerae* their pathogenesis in the human host involves intracellular invasion and replication in phagocytic cells. However, it has been noted that in a study of the phylogenic relationship between *Chlamydia*, an obligate intracellular parasite, and other bacteria, that ribosomal RNA from *Chlamydia* hybridized preferentially with DNA from *V. cholerae* (Palme & Falkow, 1986).

Concluding remarks

Although the evolutionary role of protozoa in the development of bacterial pathogenesis is debatable, there is no doubt of the importance of bacteria/protozoa interactions in terms of human disease. They allow survival, replication and distribution of some species of pathogenic bacteria in the natural environment. The intracellular niche affords protection against adverse environmental conditions and treatment with biocides, such as chlorine. It has been suggested that amoebae may act as vectors for the direct transmission of *Leg. pneumophila* to the human host, through inhalation of amoebal vesicles containing bacilli (Rowbotham, 1992; O'Brien & Bhopal, 1993).

Intra-amoebal growth of bacteria may induce phenotypes that are considerably different from *in-vitro*-grown strains in terms of physiological status, survival and infectivity. The cell morphology and protein, lipopolysaccharide and fatty acid content of *Leg. pneumophila* after growth within *A. polyphaga*, was found to differ considerably from cells grown *in vitro* (Barker *et al.*, 1993). Bacteria grown on Legionella agar after cocultivation with acanthamoebae reverted to the morphological phenotype associated with *in vitro* conditions (typically nonmotile and filamentous),

whereas, intra-protozoal growth induced legionellae that are morphologically similar to those observed within macrophages, i.e. small and highly motile (King *et al.*, 1991). The changes in the molecular composition of intra-amoebal-grown bacteria could be important to infection processes in the human host because it is well established that surface molecules play a vital role in bacterial survival and virulence (Brown & Williams, 1985). Indeed, it is tempting to speculate that protozoa could be used as alternatives to macrophages and other animal cell-lines for studying the virulence characteristics of obligate intracellular parasites such as *Chlamydia*, *Coxiella* or even *M. leprae*.

Amoebae are an integral part of natural and man-made water systems and they will not be easily controlled or eradicated. Their role in the maintenance of human disease such as legionellosis has only recently been acknowledged and their role in the survival and distribution of other pathogenic bacteria has received scant attention. Yet these host/parasite interactions may be of considerable importance to the maintenance of infectious agents in the environment. Furthermore, intra-protozoal growth of bacteria may well optimize their potential for virulence: inside the protozoal 'Horse' they may be adapting to the human 'Troy'.

We would like to thank Dr T. Rowbotham for helpful discussions on this topic.

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