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Full Length Research Paper

The impact of the *Listeria monocytogenes* large plasmid on its interaction with HeLa cells and *Acanthamoeba polyphaga* trophozoites

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Plasmids have been studied in *Listeria monocytogenes*, and plasmids with different sizes and functions have been reported. However, the role of large plasmids in the ecology of *L. monocytogenes,* in particular their impact on bacterial interactions with eukaryotes, has not been fully understood. In this study, the potential role of *L. monocytogenes* large plasmid DNA for invasion and intra-cellular growth in eukaryotic cells was determined by comparing the growth of the plasmid-cured versus wild type in HeLa cells and *Acanthamoeba polyphaga* trophozoites. Forty nine bacterial isolates were tested and 55% of environmental isolates contained plasmids. However, plasmid DNA was not found in clinical isolates. Both the plasmid-cured and the wild type bacteria showed similar behaviors in co-culture with HeLa cells and *A. polyphaga* trophozoites. After 10 successive passages through HeLa cells, the majority of bacteria recovered lost plasmid DNA. Our findings suggested that *L. monocytogenes* may lose large plasmids while growing in the enriched intra-cellular environment in eukaryotic cells. The result of this study indicated that plasmid-associated determinants have no significant impact on bacterial survival during co-culture with *A. polyphaga* and HeLa cells under conditions tested.

Key words: *Listeria monocytogenes*, *Acanthamoeba polyphaga*, HeLa cells, plasmid.

INTRODUCTION

As a pathogen, *Listeria monocytogenes* has some unusual phenotypic characteristics. For example, it is able to grow and multiply over a wide range of temperatures (1– 45°C) and pH (4.3–9.6) (Roberts and Wiedmann, 2003). These characteristics contribute to the ability of *L. monocytogenes* to cause food-borne infections under a wide range of environmental conditions (Roberts and Wiedmann, 2003). Given its ability to survive in different niches, *L. monocytogenes* may have a natural ability to acquire genes to adapt to different environmental niches. This could enable *L. monocytogenes* to appropriately respond to environmental stimuli, grow at optimum rates and conserve energy, or adjust its requirements to survive in extreme niches. One of the common ways that bacteria acquire auxiliary genetic information is through the acquisition of mobile extrachromosomal DNA, such as plasmids (Poyart-Salmeron et al., 1990; Vazquez-Boland et al., 2001).

Plasmids in *Listeria* species have been studied extensively (Lebrun et al., 1992; Perez-Diaz et al., 1982) and plasmids of different size classes, that encode a few or many genes, have been isolated from various *Listeria* species (Perez-Diaz et al., 1982; Peterkin et al., 1992;

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Slade and Collins-Thompson, 1990). The distribution of large plasmids among *L. monocytogenes* isolates is variable and environmental isolates are more likely to carry large plasmids than clinical isolates (Harvey and Gilmour, 2001; Lebrun et al., 1992). However, the role of large plasmids in the ecology of *L. monocytogenes,* and in particular their impact on interactions with eukaryotic cells remains to be investigated. For example, it is not clear whether plasmid-associated determinants confer any significant advantage for *L. monocytogenes* during infection of mammals or invasion and maintenance of an intra-cellular life-style. Furthermore, the stability of large plasmids in intra-cellular multiplication of *L. monocytogenes* remains unknown. Consequently, an examination of the role of plasmid-associated functions in interactions with eukaryotic cells may result in a better understanding of the role of large plasmids in this bacterium. This study aimed to examine the potential role of large plasmids in the interaction of *L. monocytogenes* with free- living amoebae and HeLa cells.

MATERIALS AND METHODS

Plasmid DNA was isolated from 49 isolates of *L. monocytogenes* collected from widely differing geographical locations in Australia (Table 1). Thirty one (31) were environmental isolates from food and food preparation environments such as dairy products, chicken and turkey meat. The remaining, 18 isolates, were from clinical specimens. All isolates were stored at –80°C as glycerol cultures following initial isolation. Glycerol cultures were sub-cultured on BHI agar followed by inoculation in BHI broth. Plasmid DNA was extracted from each *Listeria* isolate using the method described by Anderson and McKay (1983).

L. monocytogenes DRDC8 contained a large plasmid and strain DSE201 was an isogenic derivative of DRDC8 carried an erythromycin resistance cassette inserted into its large plasmid (Francis and Thomas, 1997). The stability of the large plasmid of *L. monocytogenes* DSE201 during intra-cellular invasion was determined by monitoring the percentage of plasmid containing bacterial cells following serial passage through HeLa cells. BHI cultures of DSE201 were grown overnight at 37°C in the presence of erythromycin. The bacterial cells were harvested by centri-fugation and washed in PBS buffer. Washed cell suspensions were then used to infect monolayers of HeLa cells at 37°C prepared in 24-well tissue culture trays. Infected HeLa cell monolayers were incubated for 8 h post infection and numbers of bacteria were then determined by lysing infected HeLa cells with Triton X-100. Recovered bacteria in the lysate were plated on BHI media with or without erythromycin to estimate the proportion of bacteria carrying plasmids. The erythromycin sensitivity was used to selected plasmid loss isolates. The loss of plasmid was confirmed using plasmid DNA extraction and PCR to amplify of two previously described plasmid associated genes, *ctpA* (Francis and Thomas, 1996) and *cadA* (Lebrun et al., 1994) as well as the *L. monocytogenes* specific chromosomal marker, *hly.* PCR of *ctpA*, *cadA* and *hly* used to confirm loss of plasmid in randomly selected.

For plasmid curing, DSE201 was serially cultured in Brain Heart Infusion Broth containing sub-inhibitory concentrations of acridine Orange (150 $\mu g.mL^{-1}$) and bacterial cells were subsequently cultured on BHI plates containing erythromycin. The loss of erythromycin resistance allowed isolation of variants cured of large plasmid DNA. Plasmid loss was confirmed in cured colonies as described earlier. A single erythromycin sensitive, plasmid loss, *hly* positive, *ctpA* and *cadA* negative isolate was selected as a plasmid-cured strain (AAC1).

The role of large plasmid DNA on bacterial interaction with eukaryotic cells was assessed using co-culture assay technique as previously described (Akya et al., 2009a, 2010). Briefly, the strain DRDC8 and its isogenic plasmid-cured variant, AAC1, were used to infect monolayers of HeLa cell cultures and *A. polyphaga* trophozoits monolayers under identical experimental conditions. HeLa cells grown in DMEM (Dulbecco's Modified Eagle medium) in 25 cm^2 flasks to semi-confluent monolayers were washed three times with PBS followed by addition of 7 mL of fresh DMEM medium. Bacteria were added to the HeLa cell monolayer preparations to achieve a MOI (multiplicity of infection) of 50 to 100 bacteria per HeLa cell. The culture flasks were centrifuged (1100×g, 15 min) to sediment the bacterial cells onto the HeLa cell surfaces, followed by incubation at 37°C for 2 h. The monolayer was then washed 4 times in PBS to remove extra-cellular bacteria, followed by addition of 7 mL of DMEM free of antibiotics. The flasks were incubated over night at 37°C in an atmosphere containing 5% CO_2. The extracellular bacteria were eliminated by washing in PBS buffer. The number of intra-cellular bacterial cells was determined using CFU (colony forming unit) counts at several time points post infection. Bacteria were also co-cultured with amoeba trophozoites in 75 cm^2 flasks contained AS buffer (Modified Neff's Amoeba Saline) for 20 days at 30°C as described before (Akya et al., 2009b). After removing extracellular bacteria by washing in AS buffer, infected amoebae were incubated at 22°C and the total viable counts (CFU) of *L. monocytogenes* were determined by plating on BHI. All experiments were repeated three times.

Statistical analysis

Results presented were the mean of at least three independent repeats of tests. Data was analyzed using t-test and two way of variance of $\log_{10}$ to find *P*- value. *P*-values of $P<0.05$ were considered statistically significant. All graphs were produced using GraphPad Prism version 4.03.

RESULTS

DNA typical of large plasmids (*ca*. 100 Kbp) was isolated from 17 out of 31 (55%) environmental isolates. Amongst environmental isolates carried large plasmid, 2 isolates also carried an additional small plasmid with sizes of 1 and 10 Kbp. However, no plasmid was isolated from the clinical isolates. Plasmid carriage was independent of bacterial serotype.

Both the plasmid-cured and the wild type *L. monocytogenes* were equally able to invade HeLa cells and maintain an intra-cellular infection. Over a period of 8 h post infection, the number of intra-cellular DRDC8 and AAC1 bacteria gradually increased during the first 6 h post infection (Figure 1). From 6 to 8 h post infection, the number of both strains of bacteria increased markedly. After 8 h post infection, the number of intra-cellular bacteria had increased by 5 fold compared to the number of bacteria at the beginning of the experiment. Importantly, when DSE201 was passaged through HeLa cells, a large proportion of intra-cellular bacteria recovered from cell culture monolayers were found to have lost plasmid DNA. The percentage of bacteria that lost large plasmids increased by the number of passages;

Table 1. Bacteria and amoebae.

Isolate	Origin	Source
6	Dairy	Dairy Technical Services, Victoria, Australia
8	Dairy	NSW Dairy Corporation, Sydney, Australia
9	Poultry	Inghams Chickens, Queensland, Australia
8	Poultry	APL
18	Clinical human	King Edward Hospital, Perth, Western Australia
Acanthamoeba polyphaga AC012	Water	South Australian Water Quality Centre, GenBank Accession number DQ490964

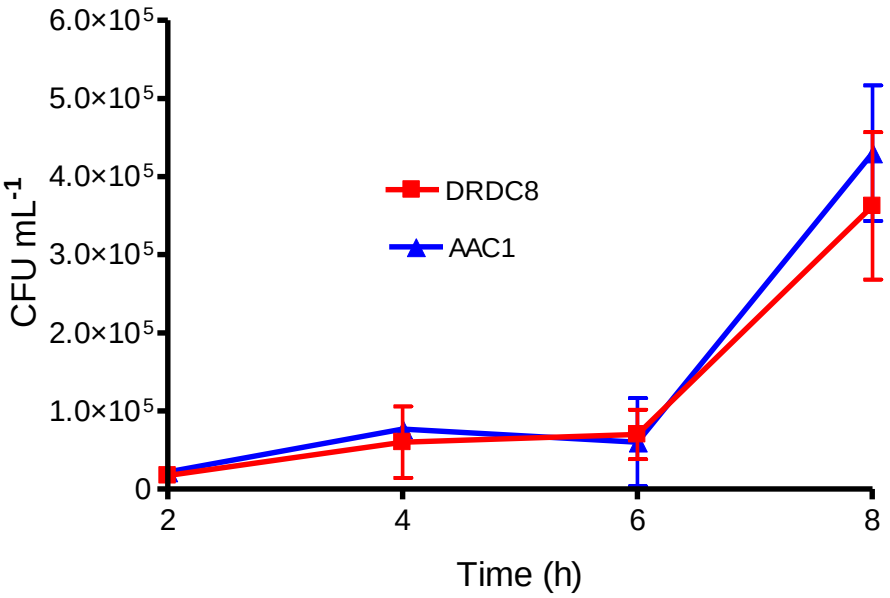

Figure 1. Growth of DRDC8 and AAC1 in HeLa cells. HeLa cells were infected with bacteria. Extra-cellular bacteria were removed and the number of intra-cellular bacteria was determined. Data represents the mean numbers of bacteria per mL for 3 replicates. Bars represent the standard deviation about the mean.

consequently, after 10 passages about 98% of bacteria recovered had lost plasmid DNA (Figure 2).

Both plasmid-cured and wild type isotype of bacteria used to co-culture with *A. polyphaga* trophozoites showed similar results. The total numbers of bacteria co-cultured with amoebae, for both plasmid-cured and wild type were dramatically reduced over the period of experiment (Figure 3).

DISCUSSION

This study represents the first attempt to test the role of plasmid associated genes carried by *L. monocytogenes* for interactions with eukaryotic cells. This was achieved by firstly establishing the instability of large plasmid DNA in *L. monocytogenes* during growth in intra-cellular compartments of HeLa cells, and, secondly, by comparison of the intra-cellular growth potential of wild type strain and the plasmid cured variant, in HeLa cells and *A. polyphaga* trophozoits.

The large plasmid of DRDC8 was readily lost during serial passage through the HeLa monolayer cell culture model. This indicated that the plasmid is inherently unstable in the absence of some selective pressure. Importantly, this result may explain the fact that large plasmid was isolated from a selection of environmental isolates of *L. monocytogenes,* but not from clinical isolates. This observation correlated with reports that the plasmid encoded *ctpA* gene is also restricted to environmental isolates (Kuenne et al., 2010; Lebrun et al., 1992; Bell, 2002; Webster, 2001). Thus, it is possible that strains of *L. monocytogenes* that lead to clinically significant infections in humans lose large plasmid DNA during infection.

The fact that clinical isolates were found not to carry

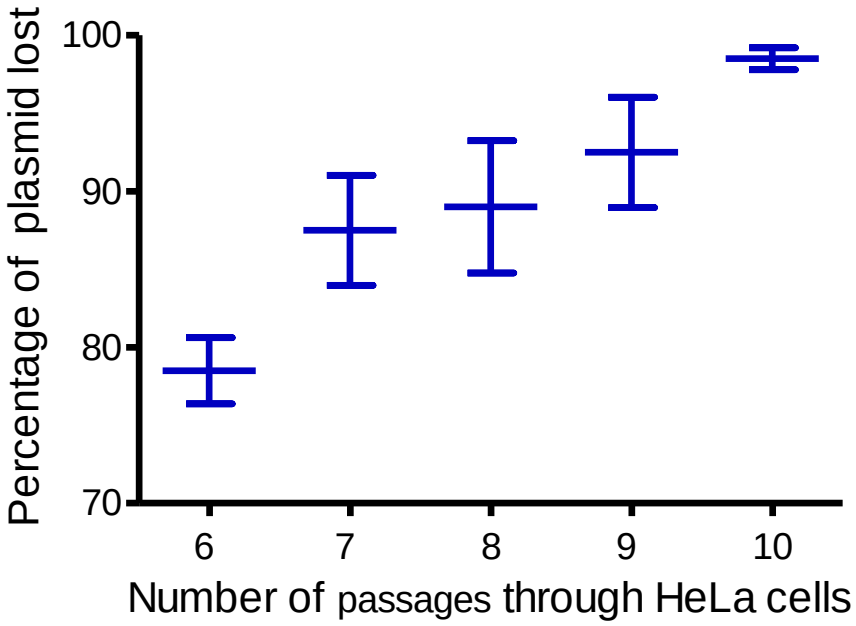

Figure 2. Loss of plasmid by *L. monocytogenes* during HeLa cell passage. Loss of plasmid was determined by the proportion of erythromycin sensitive bacteria recovered from HeLa cells following serial passages. Bars represent the standard deviation about the mean.

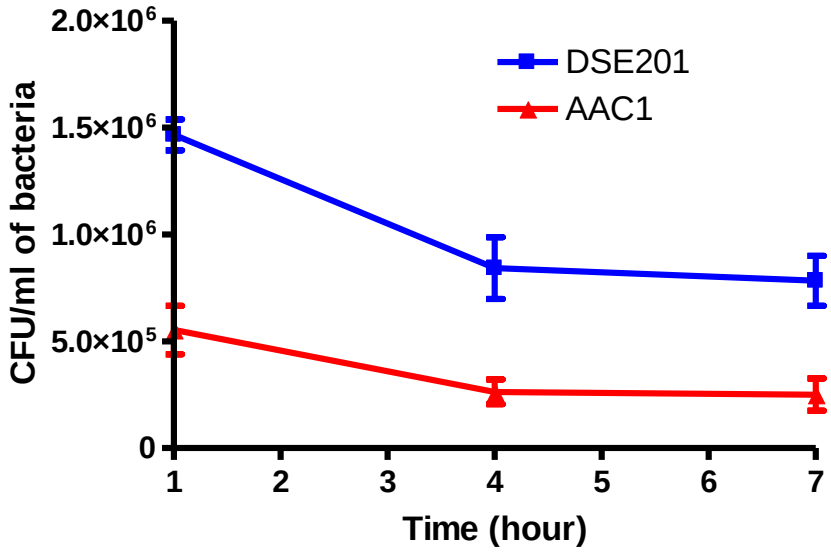

Figure 3. The growth comparison of plasmid-cured and wild type *L. monocytogenes* during co-culture with *A. polyphaga* at 22°C. Monolayers of amoebae were infected with bacteria. Infected amoebae were incubated at 22°C. Total viable counts of intra amoebic *L. monocytogenes* were determined. Error bars represent the standard deviation about the mean counts of bacteria.

large plasmids, suggests that the large plasmid may not be important for virulence and persistent infection. Certainly, no discernable difference was observed in the rate of growth, or the growth yield of the wild type strain DRDC8 and the plasmid-cured variant AAC1 in the intra-cellular compartment of HeLa cells. This indicates that large plasmid encodes genes are not absolutely required for colonization of the intra-cellular cytoplasmic compartment of *in vitro* cultured HeLa cells at least. Whether this outcome applies to *in vivo* models of infection has yet to

be tested.

The results of this study also indicated that plasmid associated genes did not have any significant effect on the interaction between *L. monocytogenes* and *A. polyphaga* tophozoites. As previously reported *A. polyphaga* was able to eliminate *L. monocytogenes* cells and bacteria actively digested by the amoebic trophozoites (Akya et al., 2009a, 2010). Certainly, no discernable difference was observed in the rate of growth, or the growth yield of the wild type strain DRDC8 and the plasmid cured variant AAC1 in the intra-cellular compartment of amoebic cells.

One explanation is that, plasmid genes are probably implicated in activities necessary for survival of bacteria while they are in extreme environmental niches. For example, cadmium efflux genes found on plasmids in *L. monocytogenes* may protect these bacteria from toxic concentrations of cadmium in the environment (Lebrun et al., 1994). However, when grown in enriched media including eukaryotic cells, adequate supplies of nutrients are present and the plasmid associated genes involved in cation transport, for example, may not be required. Consequently, these plasmid encoded genes would offer no advantage and therefore could be eliminated over time, at least under condition described.

In conclusion, our findings suggest *L. monocytogenes* cells may lose large plasmids while growing in the enriched intra-cellular environment of eukaryotic cells. This data has also indicated that plasmid-associated determinants have no significant impact on interactions of *L. monocytogenes* with *A. polyphaga* and HeLa cells.

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