Escherichia coli interactions with *Acanthamoeba*: a symbiosis with environmental and clinical implications

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The ability of *Acanthamoeba* to feed on Gram-negative bacteria, as well as to harbour potential pathogens, such as *Legionella pneumophila*, *Coxiella burnetii*, *Pseudomonas aeruginosa*, *Vibrio cholerae*, *Helicobacter pylori*, *Listeria monocytogenes* and *Mycobacterium avium*, suggest that both amoebae and bacteria are involved in complex interactions, which may play important roles in the environment and in human health. In this study, *Acanthamoeba castellanii* (a keratitis isolate belonging to the T4 genotype) was used and its interactions with *Escherichia coli* (strain K1, a cerebrospinal fluid isolate from a meningitis patient, O18 : K1 : H7, and a K-12 laboratory strain, HB101) were studied. The invasive K1 isolate exhibited a significantly higher association with *A. castellanii* than the non-invasive K-12 isolate. Similarly, K1 showed significantly increased invasion and/or uptake by *A. castellanii* in gentamicin protection assays than the non-invasive K-12. Using several mutants derived from K1, it was observed that outer-membrane protein A (OmpA) and LPS were crucial bacterial determinants responsible for *E. coli* K1 interactions with *A. castellanii*. Once inside the cell, *E. coli* K1 remained viable and multiplied within *A. castellanii*, while *E. coli* K-12 was killed. Again, OmpA and LPS were crucial for *E. coli* K1 intracellular survival in *A. castellanii*. In conclusion, these findings suggest that *E. coli* K1 interactions with *A. castellanii* are carefully regulated by the virulence of *E. coli*.

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INTRODUCTION

Acanthamoeba is a free-living protozoan pathogen that is widely distributed in the environment, including air, soil, tap water and swimming pools (Khan, 2003; Marciano-Cabral & Cabral, 2003; Schuster & Visvesvara, 2004). *Acanthamoeba* can cause *Acanthamoeba* keratitis (AK), a painful sight-threatening infection, primarily associated with contact-lens usage, and a fatal granulomatous amoebic encephalitis (GAE), mostly limited to immunocompromised patients. During the last two decades there has been an upsurge in *Acanthamoeba* infections, due to an increase in the number of people wearing contact lenses, and a rise in the number of immunocompromised patients. In addition to its direct role in causing human infections, it is now well established that *Acanthamoeba* acts as a host for various bacterial pathogens, including *Legionella pneumophila* (the causative agent of Legionnaires' disease) (Rowbotham, 1980), *Coxiella burnetii* (Q fever) (La Scola & Raoult, 2001),

Pseudomonas aeruginosa (keratitis) (Michel *et al.*, 1995), *Vibrio cholerae* (cholera) (Thom *et al.*, 1992), *Helicobacter pylori* (gastric ulcers) (Winiecka-Krusnell *et al.*, 2002), *Listeria monocytogenes* (listeriosis) (Ly & Muller, 1990) and *Mycobacterium avium* (respiratory infections) (Krishna-Prasad & Gupta, 1978; Steinert *et al.*, 1998), and may act as a vector to transmit these pathogens to susceptible hosts.

Among Gram-negative bacteria, *Escherichia coli* is the leading cause of neonatal meningitis. At least 80 % of *E. coli* strains that cause meningitis possess the capsular polysaccharide antigen K1 capsule (Xie *et al.*, 2004). The pathogenesis of *E. coli* K1 meningitis involves bacterial entry into the bloodstream and the development of bacteraemia, followed by the crossing of the blood–brain barrier to produce disease (Wang *et al.*, 2004). A high level of bacteraemia ($> 10^3$ c.f.u. bacteria per millilitre of blood) is a prerequisite of *E. coli* K1 meningitis (Xie *et al.*, 2004). However, we are only beginning to understand the mechanisms associated with *E. coli* K1 survival and multiplication in the bloodstream, *E. coli* survival of the macrophage onslaught, and

Abbreviations: CNF1, cytotoxic necrotizing factor-1; OmpA, outer-membrane protein A.

crossing of the blood–brain barrier. Here, we studied *Acanthamoeba* interactions with the invasive *E. coli* K1 and the non-invasive *E. coli* K-12 strains, and identified the bacterial determinants responsible for K1 interactions with amoebae.

METHODS

Culture of *Acanthamoeba*. All chemicals were purchased from Sigma, unless otherwise stated. A clinical isolate of *A. castellanii* belonging to the T4 genotype, isolated from a keratitis patient, was used in the study. The amoebae were grown without shaking in 15 ml PYG medium (0.75%, w/v, proteose peptone; 0.75%, w/v, yeast extract; 1.5%, w/v, glucose) in T-75 tissue-culture flasks at 30 °C, as previously described (Khan, 2001), the medium being refreshed 17–20 h prior to all experimentation. This resulted in more than 95% of the amoebae in the trophozoite form.

***E. coli* strains and growth conditions.** *E. coli* K1, used in the present study, is a rifampicin-resistant mutant of strain RS218 (serotype O18:K1:H7). This strain is a clinical isolate from the cerebrospinal fluid of a neonate with meningitis. A non-invasive *E. coli* K-12 laboratory strain, HB101, was used as a non-pathogen. In addition, we used several isogenic mutants of K1, including $\Delta fimH$, constructed by deleting the entire *fimH* gene and replacing it with an antibiotic-resistance cassette using standard molecular methods (Teng *et al.*, 2005). FimH is a 29 kDa protein and is expressed on the tip of bacterial fimbriae. Other mutants included an outer-membrane protein A (OmpA) mutant ($\Delta ompA$) (OmpA is a 35 kDa protein expressed in the outer membrane of *E. coli*; Prasadarao *et al.*, 1999), and a cytotoxic necrotizing factor-1 (CNF1) mutant ($\Delta cnf1$) (CNF1 is a 110 kDa AB type bacterial toxin; Khan *et al.*, 2002). In addition, a rough LPS mutant, constructed using chemical mutagenesis, was used (Kim *et al.*, 1992). For simplicity, the rough LPS mutant is referred to as ΔLPS , even though there were no genetic manipulations. All bacteria were grown in Luria–Bertani (LB) broth overnight with appropriate antibiotics: kanamycin (40 $\mu\text{g ml}^{-1}$) or chloramphenicol (25 $\mu\text{g ml}^{-1}$).

***E. coli* association assays.** To study *E. coli* interactions with *A. castellanii*, association assays were performed (Fig. 1). Briefly, *A. castellanii* was grown in 24-well plates in PYG medium (5×10^5 amoebae ml^{-1} per well) until confluent. The cells were washed once with PBS. Next, *E. coli* strains [2×10^6 c.f.u. per well (per 0.5 millilitre PBS)] were added, and the plates incubated for 1 h at room temperature. Following this incubation, amoebae were washed with PBS three times to remove non-adherent bacteria, and counted using a haemocytometer. Finally, amoebae were lysed by adding SDS, 0.5% final concentration, to each well for 20 min, and the number of bacteria was enumerated by plating on nutrient agar plates. The bacteria associated with *A. castellanii* were calculated as follows: number of bacteria/number of amoebae $\times 100$ = percentage bacteria associated with *A. castellanii*.

***E. coli* invasion assays.** To determine the ability of bacteria to invade or be taken up by *A. castellanii*, invasion assays were performed (Fig. 1). Briefly, amoebae were grown until confluent in 24-well plates followed by the addition of 2×10^6 *E. coli* cells, as described above. After 1 h incubation, the wells were washed three times with PBS, followed by the addition of gentamicin (100 $\mu\text{g ml}^{-1}$ final concentration, for 45 min) to kill extracellular bacteria. Finally, amoebae were counted and the intracellular bacteria enumerated as described above. The intracellular bacteria were calculated as follows: number of bacteria/number of amoebae $\times 100$ = percentage intracellular bacteria.

Intracellular survival assays. To determine the long-term effects of *A. castellanii* and *E. coli* interactions, intracellular survival assays were performed (Fig. 1). Briefly, amoebae were incubated with *E. coli*, followed by the addition of gentamicin for 45 min. After incubation, wells were washed three times with PBS and subsequently incubated in 0.5 ml PBS for 24 h at 30 °C. Finally, amoebae and *E. coli* were enumerated as described above, and intracellular bacteria after 24 h incubations were calculated as follows: number of bacteria/number of amoebae $\times 100$ = percentage bacteria after 24 h. To determine the effects of environmental conditions on *A. castellanii* and the intracellular bacteria, survival assays were performed in the presence of PYG, instead of PBS, for 24 h.

Fig. 1. Stages in *E. coli* association assays, *E. coli* invasion assays and *E. coli* intracellular survival assays.

RESULTS AND DISCUSSION

Invasive *E. coli* K1 exhibits higher association and increased invasion/uptake by *A. castellanii* than non-invasive *E. coli* K-12

To determine the ability of *E. coli* to interact with *A. castellanii*, association assays were performed. Our findings revealed that the non-invasive K-12 strain exhibited significantly less association with *A. castellanii* than the invasive K1 strain (47 ± 14 and 122 ± 2.2 %, respectively) ($P < 0.05$). Here, the term ‘association’ is used to describe both *E. coli* that were inside the amoebae and those that were attached to the amoebae. Next, to determine the numbers of intracellular *E. coli*, invasion assays were performed. We observed a higher recovery (0.5 ± 0.01 %) of intracellular *E. coli* K1 than *E. coli* K-12 (0 %).

Invasive *E. coli* K1 survive intracellularly in *A. castellanii*, while non-invasive *E. coli* K-12 are killed

To determine the fate of *E. coli* in long-term interactions with *A. castellanii*, intracellular survival assays were performed by incubating *E. coli* with *A. castellanii* in PBS for 24 h. Our findings revealed that once inside the cell, *E. coli* K1 remained viable and multiplied, while K-12 were killed (2.8 ± 0.46 and 0 %, respectively). It is important to emphasize that *A. castellanii* remained intact for this period of incubation. To determine the effects of favourable environmental conditions, *E. coli* were incubated with *A. castellanii* in PYG medium for 24 h, instead of PBS. Under these conditions, *E. coli* K1 lysed the amoebae and grew exponentially, whereas *E. coli* K-12 exhibited minimal growth (16785 ± 988 and 12 ± 1.4 %, respectively). In addition, *E. coli* K-12 had no effect on *Acanthamoeba* viability as determined by the trypan blue exclusion test (data not shown).

OmpA and LPS are important determinants required for *E. coli* K1 association with amoebae

To identify the bacterial determinants responsible for *E. coli* K1 association with *A. castellanii*, several mutants lacking known virulence determinants were used in tandem with the invasive *E. coli* K1. Firstly, we studied the role of FimH, which is expressed at the tip of fimbriae, owing to its importance as an initial point of contact with the host cells. An isogenic *fimH* deletion mutant derived from *E. coli* K1 was used. Both the wild-type and the $\Delta fimH$ mutant exhibited similar levels of association with *A. castellanii* (122 and 152.6 %, respectively) (Fig. 2).

Next, we determined the role of OmpA, which is one of the proteins embedded in the external membrane of *E. coli*. OmpA resembles one of the *Neisseria* proteins, Opa, that is implicated in the invasion of epithelial cells. Using an isogenic *ompA* deletion mutant ($\Delta ompA$), the results revealed that OmpA is a crucial determinant for *E. coli* K1 association with *A. castellanii* (Fig. 2). The $\Delta ompA$ mutant exhibited significantly decreased levels of association compared with the non-invasive K-12. Similarly, the *E. coli* K1 strain expressing rough LPS exhibited significantly less association with *A. castellanii* than its parent strain, *E. coli* K1 ($P < 0.05$) (Fig. 2). Interestingly, CNF1 (a dermanecrotic protein toxin) exhibited no effects on *E. coli* K1 association with *A. castellanii*. This was shown by the demonstration that an isogenic *cnf1* deletion mutant, $\Delta cnf1$, exhibited amoebae association similar to its parent strain, *E. coli* K1 (Fig. 2).

OmpA and LPS, but not FimH and CNF1, are important determinants required for *E. coli* K1 invasion of and/or uptake by *A. castellanii*

To study the effect of the aforementioned factors on bacterial invasion of and/or uptake by *A. castellanii*, invasion assays were performed. The results demonstrated that the $\Delta fimH$ and $\Delta cnf1$ mutants exhibited similar levels of

Fig. 2. OmpA and LPS are crucial determinants for *E. coli* K1 binding/association with *A. castellanii*. To determine the bacterial determinants responsible for *E. coli* K1 interactions with *A. castellanii*, several mutants lacking important virulence determinants were used. Briefly, amoebae were grown in 24-well plates in PYG medium until confluent. The cells were washed and incubated with *E. coli* in PBS for 1 h, and amoebae and *E. coli* enumerated as described in Methods. Note that both the rough LPS mutant and the *ompA* deletion mutant exhibited significantly decreased binding/association with *A. castellanii* compared to wild-type *E. coli* K1. Results are the mean of three independent experiments performed in duplicate. Error bars represent standard error.

Fig. 3. OmpA and LPS, but not FimH and CNF1, are important determinants required for *E. coli* K1 invasion of and/or uptake by *A. castellanii*. To determine the bacterial determinant responsible for *E. coli* K1 invasion/uptake in *A. castellanii*, several mutants lacking important virulence determinants were used. Briefly, amoebae were incubated with *E. coli* for 1 h, as described above. After this incubation, gentamicin was added to kill the extracellular *E. coli*, followed by amoebae and *E. coli* counting as described in Methods. Note that both the rough LPS mutant and the *ompA* deletion mutant exhibited significantly decreased invasion/uptake in *A. castellanii* compared to wild-type *E. coli* K1. Results are the mean of three independent experiments performed in duplicate. Error bars represent standard error.

invasion/uptake to those of the wild-type *E. coli* K1 (0.46, 0.51 and 0.49%, respectively) (Fig. 3). In contrast, the $\Delta ompA$ and rough LPS mutants exhibited significantly reduced levels of invasion/uptake by *A. castellanii* ($P < 0.05$) (Fig. 3).

OmpA is a crucial determinant required for *E. coli* K1 survival inside *A. castellanii*

To identify the bacterial determinants required for intracellular survival of *A. castellanii*, intracellular survival assays

were performed as described in Methods. Both *E. coli* and *A. castellanii* were incubated for 24 h in a non-nutrient environment, PBS. The results showed that all mutants tested exhibited a decreased ability to survive intracellularly compared with the parent K1 strain (Fig. 4a). Both the rough LPS mutant and the *cnf1* deletion mutant showed some survival ability, but significant decreases in their ability to survive within *A. castellanii* were observed (Fig. 4a). The *ompA* deletion resulted in the loss of the ability of *E. coli* K1 to survive inside *A. castellanii*. To determine the effects of favourable conditions on *E. coli* K1 interactions with *A.*

Fig. 4. OmpA is a crucial determinant required for *E. coli* K1 survival inside *A. castellanii*. (a) To identify the bacterial determinants required for intracellular survival of *A. castellanii* in the absence of nutrients, assays were performed using several isogenic deletion mutants derived from *E. coli* K1. Briefly, amoebae were incubated with *E. coli* for 1 h, followed by the addition of gentamicin to kill the extracellular bacteria. Finally, *A. castellanii* plus intracellular *E. coli* were incubated in PBS (a) or PYG (b) for 24 h. Note that in the presence of PBS, the *ompA* deletion resulted in the loss of the ability of *E. coli* K1 to survive inside *A. castellanii*. Results are the mean of three independent experiments performed in duplicate. Error bars represent standard error.

castellanii, assays were performed in the presence of PYG medium, instead of PBS. Again, the $\Delta ompA$ mutant exhibited a limited ability to survive intracellularly, even under favourable conditions, i.e. PYG medium (Fig. 4b). Overall, these findings indicate that OmpA and LPS are critical determinants for bacterial binding, invasion, and/or uptake and survival inside *A. castellanii*.

It is well known that *Acanthamoeba* acts as a host for several bacteria, and its ability to host bacterial pathogens, such as *L. pneumophila*, *C. burnetii*, *P. aeruginosa*, *V. cholerae*, *H. pylori*, *List. monocytogenes* and *M. avium*, has gained particular attention. This is a consequence of the finding that *Acanthamoeba* may host bacterial pathogens under harsh environmental conditions, and may help transmit bacterial pathogens to susceptible hosts, thus offering a potential route of entry into the human body. However, the precise mechanisms associated with bacteria–amoebae interactions remain incompletely understood. Further complexity is added by the fact that amoebae feed on bacteria. So why bacteria interact with amoebae and how pathogenic bacteria maintain survival inside amoebae, while non-pathogens are killed, is unclear. Here, we studied *E. coli* interactions with *A. castellanii*. Using clinical and non-clinical isolates of *E. coli*, we demonstrated that the outcome of *E. coli* interactions with *A. castellanii* varies, depending on the virulence of *E. coli*. The invasive *E. coli* K1 has the ability to gain entry to amoebae and remain viable, while the non-invasive *E. coli* K-12 is killed.

It is interesting to observe that both K1 and K-12 exhibited binding-association with amoebae, 122 and 47 %, respectively (although K-12 showed reduced levels of association). This is in contrast to previous findings, which have shown that *E. coli* K1 and K-12 exhibit more than 10-fold differences in binding to other cell types, such as human brain microvascular endothelial cells, and have identified FimH as an important adhesion determinant (Prasadarao *et al.*, 1999; Teng *et al.*, 2005). However, our findings are not surprising. The fact that amoebae feed on bacteria suggests that the initial interactions between *E. coli* and *Acanthamoeba* may not be totally dependent on the virulence properties of *E. coli*. In support, we observed that a *fimH* deletion mutant exhibited similar levels of association with amoebae to those of the parent strain. However, it is the post-invasion events that vary greatly between invasive and non-invasive *E. coli* strains in their interactions with *A. castellanii*. In this regard, we observed that under harsh conditions, the invasive K1 isolate remains intracellular and viable but does not kill the host, i.e. *A. castellanii*. However, under favourable conditions (presence of nutrients), the invasive K1 isolate grows exponentially and lyses the host cells. In addition, we identified OmpA as a critical bacterial determinant required for *E. coli* K1 association with, invasion/uptake by, and intracellular survival within *A. castellanii*.

One intriguing aspect is that these findings show remarkable similarities to *E. coli* K1 interactions with human macrophages. For example, recent studies have shown that *E. coli*

K1, but not K-12, is able to enter murine and human macrophages, and survive and replicate intracellularly (Sukumaran *et al.*, 2003), and this property may be crucial for *E. coli* survival in the bloodstream, a primary step in the development of meningitis. Moreover, these properties of *E. coli* K1 have been directly attributed to the expression of OmpA protein. The fact that *Acanthamoeba* resembles human macrophages in many ways, particularly in its phagocytic activity and cell surface receptors (Yan *et al.*, 2004), and that macrophages and *Acanthamoeba* exhibit parallel mechanisms in their interactions with *E. coli* K1, suggests that *Acanthamoeba* may provide an alternative model to study *E. coli* pathogenesis and to understand its immune evasion mechanisms. The implication of this observation remains unclear, but will be the subject of further studies.

Although we observed clear differences between invasive and non-invasive *E. coli* strains in their ability to survive intracellularly in *A. castellanii*, the precise mechanisms of *E. coli* K1 intracellular survival remain unknown. Interestingly, previous studies of the interactions of *L. pneumophila* with *A. castellanii* have demonstrated the ability of *L. pneumophila* to inhibit the fusion of lysosomes with phagosomes as a critical step in the intracellular survival of this bacterium (Bozue & Johnson, 1996). *E. coli* K1 may use similar mechanisms to evade the host-cell defences; however, this remains to be determined. Overall, our findings suggest that the interactions of *E. coli* and *A. castellanii* are highly complex and depend on the virulence of *E. coli*. *Acanthamoeba* may act as a bacterial predator, or as a reservoir or ‘Trojan horse’ for bacteria, with environmental and clinical implications.

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