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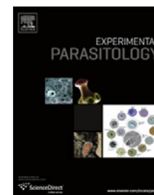

The type III secretion system is involved in *Escherichia coli* K1 interactions with *Acanthamoeba*

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ABSTRACT

The type III secretion system among Gram-negative bacteria is known to deliver effectors into host cell to interfere with host cellular processes. The type III secretion system in *Yersina*, *Pseudomonas* and Enterohemorrhagic *Escherichia coli* have been well documented to be involved in the bacterial pathogenicity. The existence of type III secretion system has been demonstrated in neuropathogenic *E. coli* K1 strains. Here, it is observed that the deletion mutant of type III secretion system in *E. coli* strain EC10 exhibited defects in the invasion and intracellular survival in *Acanthamoeba castellanii* (a keratitis isolate) compared to its parent strain. Next, it was determined whether type III secretion system plays a role in *E. coli* K1 survival inside *Acanthamoeba* during the encystment process. Using encystment assays, our findings revealed that the type III secretion system-deletion mutant exhibited significantly reduced survival inside *Acanthamoeba* cysts compared with its parent strain, EC10 ($P < 0.01$). This is the first demonstration that the type III secretion system plays an important role in *E. coli* interactions with *Acanthamoeba*. A complete understanding of how amoebae harbor bacterial pathogens will help design strategies against *E. coli* transmission to the susceptible hosts.

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1. Introduction

Acanthamoeba is a free-living protozoan pathogen capable of producing blinding keratitis and fatal granulomatous encephalitis. *Acanthamoeba* has been isolated from diverse environments including air, soil, tap water, swimming pools and is known to be one of the most ubiquitous protists (reviewed in Marciano-Cabral and Cabral, 2003; Schuster and Visvesvara, 2004; Khan, 2009). With the wide environmental distribution, it is not surprising that *Acanthamoeba* have been shown to interact with viruses, bacteria, algae, yeast and other protists (Allen and Dawidowicz, 1990; Weekers et al., 1993; La Scola et al., 2003; La Scola and Raoult, 2001; Ghedin and Fraser, 2005; Khan, 2009). From public health perspective, the ability of *Acanthamoeba* to harbor bacterial pathogens, protect them in hostile environments, and assist in their environmental distribution and transmission to susceptible individuals has gained particular attention. The Trojan horse property of amoebae may contribute indirectly to human and animal infections caused by pathogenic bacteria (Greub and Raoult, 2004; Khan, 2009).

Our previous studies have shown that *Acanthamoeba* act as a reservoir for neuropathogenic *Escherichia coli*, a causative agent of neonatal meningitis (Alsam et al., 2006). Several virulence factors such as outer membrane protein A (OmpA), lipopolysaccharide (LPS) and K1 capsule were identified as important bacterial determinants required for *E. coli* interactions with *Acanthamoeba* (Alsam et al., 2006; Jung et al., 2007).

The type III secretion system in *Yersina*, *Pseudomonas* and Enterohemorrhagic *Escherichia coli* has been well documented to be involved in the bacterial pathogenicity. It allows some Gram-negative bacteria to adhere to the surface of eukaryotic cells by injecting bacterial proteins across the two bacterial membranes and the eukaryotic cell membrane to destroy or subvert the target cell (Lee, 1997; Cornelis, 2002). These systems consist of a secretion apparatus, made of over 25 different proteins, and an array of proteins released by this apparatus termed as "effectors" (Ehrbar et al., 2002; Matsumoto and Young, 2009; Shrivastava and Miller, 2009). The effectors are delivered into the host cell to interfere with host cellular processes. It has been implicated for different purposes in different bacteria. For instance, intracellular pathogens such as species of *Chlamydia*, *Salmonella* and *Shigella* use the type III secretion system for invasion of and/or multiplication within host cells (Menard et al., 1996; Galan, 2001; Fields et al., 2003), while *Yersinia* spp. use the type III secretion system to resist the uptake of bacteria by phagocytic cells in the later stages of pathogenesis (Cornelis and Van Gijsegem, 2000). Pathogenic *E. coli* strains that cause diarrheal disease use the type III secretion system to deliver

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effector proteins that result in intimate bacterial attachment to and effacement of the microvilli from intestinal epithelial cells, eventually leading to the formation of attaching/effacing lesions (Celli et al., 2002). The overall aim of the present study was to determine the role of type III secretion system in *E. coli* K1 interactions with *Acanthamoeba castellanii* (a keratitis isolate belonging to the T4 genotype).

2. Materials and methods

2.1. Culture of A. castellanii

All chemicals were purchased from Sigma Laboratories (Poole, Dorset, England), unless otherwise stated. A clinical isolate of *A. castellanii* belonging to T4 genotype, isolated from a keratitis patient (American Type Culture Collection, ATCC 50492) was used in the present study. Amoebae were grown without shaking in 15-ml of PYG medium [0.75% (w/v) proteose peptone, 0.75% (w/v) yeast extract, and 1.5% (w/v) glucose] in T-75 tissue culture flasks at 30 °C as previously described (Bornes et al., 1970; Khan and Siddiqui, 2009). To obtain vegetative trophozoites, the media were refreshed 17–20 h prior to experimentation, which resulted in more than 99% amoebae in the trophozoite forms.

2.2. E. coli strains and growth conditions

E. coli K1, used in this study, is a streptomycin-resistant mutant of strain EC10 (O7:K1). This strain is a clinical isolate from the CSF of a neonate with meningitis. A non-invasive *E. coli* K-12 laboratory strain, HB101, was used as a non-pathogen.

A type III secretion system-deletion mutant generated from EC10 strain was used (Yao et al., 2009). This strain lacks ETT2 genes cluster that are necessary for the type III secretion system and were replaced with kanamycin-resistance cassette as previously described (Yao et al., 2009). For simplicity, invasive *E. coli* isolate and its type III secretion system-deletion mutant are referred to as K1 and TTKO, respectively, while the non-invasive *E. coli* (strain HB101) is indicated as K-12. All bacteria were grown in Luria–Bertani (LB) broth overnight with appropriate antibiotics: kanamycin (50 μg/ml) or streptomycin (50 μg/ml).

2.3. Association assays

E. coli association assays were performed as described previously (Jung et al., 2007). Briefly, *A. castellanii* was grown in 24-well plates in PYG medium (5×10^5 amoebae/ml per well) until confluence. The cells were washed once with PBS. Next, *E. coli* strains [2×10^6 CFU in 0.5 ml PBS per well] were added, and the plates incubated for 1 h at room temperature. After this incubation, amoebae were washed with PBS three times, and counted using a haemocytometer. Finally, amoebae were lysed by adding SDS at 0.5% final concentration to each well for 20 min, and bacterial CFU were enumerated by plating on nutrient agar plates. The bacterial CFU associated with *A. castellanii* were calculated as follows: (number of bacterial CFU/number of amoebae) × 100 = bacteria associated with *A. castellanii* (percentage).

2.4. Invasion assays

E. coli invasion assays were performed similarly to the above-mentioned association assays. Briefly, amoebae were grown until confluence in 24-well plates followed by the addition of 2×10^6 *E. coli* as described above. After 1 h incubation, the wells were washed three times with PBS, followed by the addition of gentamicin (100 μg/ml final concentration, for 45 min) to kill extracellular bacteria. Finally, amoebae were counted, and the intracellular bacterial CFU enumerated as described above. The intracellular bacterial CFU were calculated as follows: (number of bacterial CFU/number of amoebae) × 100 = bacteria invasion of *A. castellanii* (percentage).

2.5. Intracellular survival assays

To determine the long-term effects of *A. castellanii* interactions with *E. coli*, intracellular survival assays were performed. Briefly, amoebae were incubated with *E. coli*, followed by the addition of gentamicin (100 μg/ml) 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 bacterial CFU/number of amoebae) × 100 = bacteria intracellular survival of *A. castellanii* (percentage).

2.6. E. coli survival intracellular of Acanthamoeba cysts

To determine the ability of *E. coli* to survive inside *Acanthamoeba* cysts, encystment assays were performed. Briefly, following invasion assays, the mixtures were transferred onto non-nutrient agar plates [prepared using 3% (w/v) purified agar, Oxoid limited, Basingstoke, UK]. The plates were incubated at 30 °C for up to several days. This allowed the complete encystment of *Acanthamoeba trophozoites* as observed visually under a phase-contrast microscope. Cysts on the non-nutrient agar plates were then gently scraped-off the agar surface using cell scraper by adding 10 ml of PBS. Cysts were collected by centrifugation at 1200×*g* for 10 min and resuspended in PBS. Cysts were counted using a haemocytometer and subsequently treated with SDS (0.5% final conc.) and the associated numbers of bacterial CFU were enumerated by plating on nutrient agar plates. The bacterial CFU counts were calculated as follows: (number of bacterial CFU/number of amoebae cysts) × 100 = bacteria intracellular of *A. castellanii* cysts (percentage).

3. Results

3.1. The type III secretion system-deletion mutant of E. coli exhibited reduced association with A. castellanii keratitis isolate belonging to T4 genotype compared with the wild type strain EC10

To determine the role of the type III secretion system in *E. coli* association with *A. castellanii*, assays were performed using type III secretion system-deletion mutant (referred to as TTKO) and its parent strain EC10 isolated from the cerebrospinal fluid of a meningitis patient (referred to as K1). The growth rate of the TTKO *in vitro* is comparable to that of the parental strain (data not shown). Our findings revealed that deletion of the type III secretion system had significant effect on *E. coli* association with *A. castellanii* compared with the wild type strain EC10 (Fig. 1) ($P = 0.0067$, using paired T-test, one-tail distribution). Nearly 70% *E. coli* K1 were associated with *A. castellanii*, compared with approximately 36% of TTKO (Fig. 1). Also, TTKO showed comparable association to the non-invasive *E. coli* K-12 strain, HB101 (Fig. 1) ($P > 0.05$), suggesting that the type III secretion system plays a role in *E. coli* K1 association with *A. castellanii*.

3.2. The type III secretion system-deletion mutant exhibited significantly reduced invasion into and/or uptake by A. castellanii

We next determined whether the type III secretion system affected the invasive ability of *E. coli* in amoebae. As shown in Fig. 2, the invasive ability of the parent *E. coli* K1 strain EC10 was approximately 10-fold greater than that of strain HB101. The TTKO

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