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The role of the twin-arginine translocation pathway in Escherichia coli K1 pathogenicity in the African migratory locust, Locusta migratoria

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Abstract

Escherichia coli K1 infection is a major cause of neonatal meningitis, with high rates of mortality and disability. Despite years of research, only a small number of factors contributing to E. coli K1 virulence have been identified. The Tat (twin-arginine translocation) protein export system is found in the cytoplasmic membrane of E. coli and is involved in the transport of folded proteins. In vivo and ex vivo models using the African migratory locust, Locusta migratoria, were employed to explore the role of Tat pathway in E. coli K1 virulence using tat-deletion mutants. Groups of locusts were infected and mortality was recorded at 24-h intervals. The findings revealed that ΔtatA, ΔtatAC and Δtat produced levels of mortality similar to wild-type E. coli K1, with >78% mortality recorded within 72 h. Bacteraemia was determined from haemolymph obtained 3 and 24 h postinfection. Again, wild-type and ΔtatA produced similar levels of bacteraemia. In contrast, ΔtatAC and Δtat produced lower levels of bacteraemia. Following injection of bacteria into isolated head capsules ex vivo, all mutants invaded the CNS. Overall, these studies showed no evidence of involvement of the Tat pathway in locust mortality but suggest its possible role in bacteraemia.

Introduction

Bacterial meningitis is the leading cause of morbidity and mortality in neonates worldwide. In developing countries, it is estimated that some 50 000 infants die of bacterial meningitis every year, with a mortality rate of c. 40% (Bonacorsi & Bingen, 2005). In the developed world, the incidence rate of the disease remained at c. 1 per 1000 live births until the 1980s. This rate was shown to fall to between 0.22 and 0.37 per 1000 live births throughout the 1990s, with a mortality rate of 20–30% (Bonacorsi & Bingen, 2005).

The anaerobic Gram-negative bacterium Escherichia coli is the second leading causative agent of neonatal meningitis, responsible for c. 28.5% of cases of neonatal meningitis worldwide, with an average mortality rate of 8% (Mylonakis, 2007). Studies by Mulder et al. (1984) and de Louvois et al. (1991) show an incidence rate of between 0.06 and 0.12 cases per 10 000 live births. In the developed world, this rate appears to be stabilized at 0.1 per 10 000 live births, although in developing countries the number of infections is far higher (Bonacorsi & Bingen, 2005). Most cases of E. coli meningitis occur within the first few months of life, with 10% of infections occurring between 1 and 3 months of age (Bonacorsi & Bingen, 2005). Infants born prematurely or with a low birth weight appear to be at the highest risk of developing neonatal meningitis and account for one-third of all cases. Indeed, within these groups, E. coli is the leading causative agent (Bonacorsi & Bingen, 2005). Despite major advances in diagnostics, microbial therapy and care, the mechanisms involved in the pathogenesis and the pathophysiology of neonatal meningitis are poorly understood. The disease still has high rates of mortality and morbidity, and between 33% and 50% of survivors experience subsequent neurologic or developmental abnormalities (Bonacorsi et al., 2000).

The pathogenesis of E. coli neonatal meningitis is a complex process comprising a series of interactions...
between invading bacteria and the host and begins with the translocation of bacteria into the bloodstream. Infection predominantly occurs via the intestinal tract, following acquisition of the bacteria from the mother's flora during delivery, or from the environment, but may also occur via the urinary tract (20% of cases), or in utero following maternal bacteraemia (Bonacorsi & Bingen, 2005). It has been well established that in order for bacteria to cross the blood–brain barrier (BBB), it is first necessary for a threshold level of bacteraemia to be achieved via bacterial survival and multiplication within the host bloodstream (Kim, 2001; Xie et al., 2004). Invasion of the brain microvascular endothelial cells (BMEC) and penetration of the BBB then occur following a series of microbial and host interactions. Despite ongoing research, only a small number of E. coli K1 structures responsible for allowing bacterial survival within the host and traversal of the BBB have been identified. These include fimbrial protein, FimH, outer membrane protein A (OmpA), cytoxic necrotizing factor-1 (CNF-1), Ibe proteins, AslA and TraI, which allow for BMEC invasion, and the K1 capsular polysaccharide that enables bacterial survival within the host (Kim, 2001; Xie et al., 2004).

It is highly probable that other as yet unidentified factors are likely to be involved in the pathogenesis of E. coli neonatal meningitis. Genomic analysis of the E. coli K1 strain RS218 shows that it differs by as many as 500 kbp from the nonpathogenic E. coli K-12 strain MG1655 (Kim, 2001; Mokri-Moayyed et al., 2008), suggesting that these areas of genome might hold the key to further understanding E. coli K1 pathogenicity. The involvement of bacterial protein transport systems in E. coli virulence is a little-researched area, with major discoveries being related to β-lactam resistance and curli production (Dufour et al., 2000). Recent studies concerning the requirement of several known virulence determinants strongly support the proposition that E. coli infection can be modelled in locusts (Khan & Goldsworthy, 2007; Mokri-Moayyed et al., 2008). Although vertebrate model systems are seen as immediately more relevant, the use of an invertebrate model at an early stage can offer several advantages in terms of speed, cost, technical convenience and ethical acceptance. Previous studies concerning the requirement for infectivity of African green monkey kidney cells, in vitro (Pradel et al., 2003), here we determined its role in vivo. The aim of this study was to investigate the pathogenicity of three E. coli K1 strain RS228 mutants lacking genes encoding for the protein components of the Tat translocase using in vivo and ex vivo invertebrate models of African migratory locust, Locusta migratoria (Khan & Goldsworthy, 2007; Mokri-Moayyed et al., 2008). Although vertebrate model systems are seen as immediately more relevant, the use of an invertebrate model at an early stage can offer several advantages in terms of speed, cost, technical convenience and ethical acceptance. Previous studies concerning the requirement of several known virulence determinants strongly support the proposition that E. coli K1 meningitis, the control of which has significant dependency on the innate immune system.

### Materials and methods

#### Locusts

African migratory locusts, L. migratoria, were bred and reared on a diet of wheat seedlings, grass and bran. Assays to determine the invasion of the brain by E. coli were conducted using isolated head capsules of adult male L. migratoria between 21 and 42 days of age. Adult female L. migratoria of between 25 and 32 days of age were used in the mortality and bacteraemia assays.

#### Bacterial strains

Three mutant strains of E. coli K1 were used, lacking genes coding for proteins of the Tat pathway. These included ΔtatABC (lacking the Tat translocase and referred to as Δtat in this study). Both tat and the wild
type K1 (referred in this study as RS228) were kindly provided by T. Palmer, University of Dundee, UK. In addition, ΔtatA and ΔtatAC were constructed by deleting the genes encoding TatA and TatAC (McDevitt et al., 2006; Penfold et al., 2006). The tatA deletion mutant was constructed by deleting the tatA gene and replacing it with a chloramphenicol-resistance cassette using the protocol described by Datsenko & Wanner (2000). Briefly, E. coli K1 (RS228) was transformed with plasmid pKD46, which encodes the arabinose-inducible lambda Red recombinase that promotes gene recombination between linear DNA and the host chromosome based on short stretches of homology. PCR primers containing 5´ flanking portions exactly homologous to the 5´ and 3´ ends of the tatA gene, and 3´ portions (capitalised) homologous to the chloramphenicol resistance cassette from pKD3 (Datsenko & Wanner, 2000), were synthesized (5´-ggt ggt atc agt att tgg cag tta ttg att att gcc gtc atc gca TAT GAA TAT CCT CCT TA-3´ and 5´- tta cac ctg ctc ttt atc ggt gcc ctt cgc gtc ttc tgt ttt agG TGT AGG CTG GAG CTG-3´). The resultant 1.1-kb PCR product was gel purified and transformed into RS228 (pKD46), and plated onto LB agar containing ampicillin, chloramphenicol and 50 mM arabinose at 42 °C to select for antibiotic-resistant transformants. For ΔtatAC, first a tatC mutant was constructed. Briefly, PCR primers containing 5´ flanking portions exactly homologous to the 5´ and 3´ ends of the tatC gene, and 3´ portions (capitalised) homologous to the kanamycin resistance cassette from pREP4, were synthesized (5´- atg tct gta gaa gat act caa ccg ctt atc acg cat ctt ggt ggt GT GCT GAC CCC GGA TGA ATG TCA G-3´ and 5´- cgc ttt ctg ctt cag cgt cgt ttt cct ctt ccc gat ttc gcc CGT CGG TTG GTG GGT CAT TTC GAA C-3´). The resultant 1.2-kb PCR product was gel purified and transformed into ΔtatA harbouring pKD46, and plated onto LB agar containing ampicillin, chloramphenicol, kanamycin and 50 mM arabinose at 42 °C to select for the antibiotic-resistant double-knockout mutant. In addition, E. coli K1 RS218 (O118:K1:H7), an isolate from the cerebrospinal fluid of a neonate with meningitis, was used as a positive control with E. coli K-12 strain HB101, a non-invasive isolate, as a negative control (K-12). All bacterial strains were aerobically grown for approximately 14 h in Luria-Bertani (LB) broth at 37 °C.

Mortality and bacteraemia assays

For each E. coli strain assayed, 30 randomly selected locusts were injected with 20 μL of LB broth containing 2 × 10⁶ CFU of bacteria, using an automatic pipette with a plastic tip modified to hold a stainless steel hypodermic needle. Injections were performed into the haemocoel of the locust abdomen, accessed via the intersegmental membrane between two abdominal terga as described previously (Khan & Goldsworthy, 2007). Locusts were housed in plastic ‘critter cages’ at 30 °C and supplied with fresh bran, water and wheat seedlings as required. Mortality was monitored every 24 h and any dead animals were removed.

Locusts in each group were also tested to determine bacteraemia within the haemolymph at 3 and 24 h post-injection. A sterile needle was used to puncture the arthrodial membrane as described previously (Khan & Goldsworthy, 2007), and 5 μL of haemolymph was collected using a glass capillary tube. Each sample of haemolymph was added to 450 μL phosphate-buffered saline (PBS); 10-, 100-, and 1000-fold dilutions were then performed, and 10 μL of each dilution, including the original sample, was plated onto nutrient agar to determine bacterial counts.

CNS invasion assay ex vivo

Isolation of the head capsule

To determine the ability of the bacteria to invade the CNS ex vivo, it was necessary to isolate the head capsule whilst retaining the brain and BBB. This was achieved using a ligation technique previously described (Mokri-Moayyed et al., 2008). A fine thread was passed around the neck region, between the head and thorax of the animal, and tightly tied. The head capsule was then separated from the body just below the ligature using scissors and sealed using low melting point wax to prevent any leakage of haemolymph.

Injection of the head capsule

Isolated head capsules were randomly allocated into groups of equal numbers. Injections of 10 μL of LB broth containing 10⁶ CFU of bacteria were performed using the modified automatic pipette previously described (Mokri-Moayyed et al., 2008). The hypodermic needle was inserted just below the surface of the cuticle, in a position several millimetres to the side of the top of the compound eye. It was vital to avoid injecting directly into the brain or foregut. Head capsules were then incubated at a temperature of 37 °C for 2 h.

Dissection and plating of the brain lysates

The brain was removed from each head capsule by first performing a sagittal cut to remove the left-hand side of the head. This was achieved using a sterile blade, with the cut running through the base of the left antenna. The right-hand side of the head capsule was then held in
place using a sterile pin and the brain identified at an intermediate magnification. The optic tract leading to the right-hand compound eye and the circumoesophageal connectives were first severed, and the brain was dissected out using fine forceps. All air sacs and fatty tissues were then removed, and the brain was placed into a 1-mL Eppendorf tube containing 100 lL PBS and 100 lg/mL gentamicin (stock solution 50 mg/mL; Gibco). Brains were then incubated for 1 h at a 37°C to kill any extracellular bacteria. Following incubation, brains were washed three times in 100 lL PBS, with sodium dodecyl sulphate (SDS) subsequently added to a final concentration of 0.5%. Brains were then vortexed for 10 s and left for between 10 and 30 min to lyse the brain tissue and release any contents; 10-, 100- and 1000-fold dilutions were then performed, and 20 lL of each dilution, as well as the original lysates, were plated onto nutrient agar to determine bacterial counts.

**Results**

**All Tat mutant strains tested produced locust mortality at levels similar to the wild-type E. coli K1**

Mortality assays were used to determine the ability of the three E. coli K1 Tat translocase mutants to produce locust death compared with wild-type E. coli K1 strain RS228. Wild-type E. coli K1 strain RS218 and nonpathogenic K-12 were used as controls. The findings revealed that all three mutants tested were found to produce similar patterns of locust death as wild-type K1 strain RS228.

Approximately 7% of insects infected with RS228, ΔtatA and Δtat died within 24 h (Fig. 1). With further incubation, this figure rose to c. 85% after 48 h and 100% after 72 h (Fig. 1). The Δtat mutant was found to produce almost identical levels of locust death as the wild-type control strain RS228 throughout each time period (P > 0.05 using paired t-test, one-tailed distribution). Both strains were found to cause up to c. 94% mortality within 96 h. In contrast, the nonpathogenic control E. coli K-12 was the least virulent strain (P < 0.05 using paired t-test, one-tailed distribution). Infection resulted in only c. 13% mortality after 96 h, with c. 87% of locusts still alive up to 6 days after infection.

**Escherichia coli K1 Tat mutants invaded the locust brain to differing degrees**

Isolated locust head capsules were randomly divided into groups and injected with wild-type E. coli K1 strain RS228 and the three K1 Tat mutants. Wild-type E. coli K1 strain RS218 and nonpathogenic E. coli K-12 were used as controls. Following treatment with gentamicin to kill extracellular bacteria, brain lysates showed that all mutant strains were able to invade the CNS within 2 h; however, levels of invasiveness differed dramatically, as shown in Fig. 2. The ΔtatA mutant was found to show greater levels of brain invasion compared with K1. On average, c. 61 colonies were isolated from each brain, which was some 20% higher than the c. 16 colonies isolated from insects infected with K1. In fact, ΔtatA
Groups of randomly selected locusts were injected with wild-type *E. coli* K1 strain RS228 and its Tat pathway mutant strains as described for mortality assays. In addition, wild-type *E. coli* K1 strain RS218 and nonpathogenic *E. coli* K-12 were used as controls. Five-microlitre samples of haemolymph were obtained 3 and 24 h after infection to determine the levels of bacteraemia within the locust. Figure 3 shows that infection with each strain resulted in an increase in bacterial levels in the haemolymph within 24 h. After 3 h, the level of bacteraemia was found to be highest in locusts injected with RS228, with 78 CFUs recovered per 5 μL of haemolymph. However, this strain demonstrated the limited increase in bacteraemia between 3 and 24 h, with levels rising to 110 CFU per 5 μL of haemolymph.

Infection with the three mutant strains, ΔtatA, ΔtatAC and Δtat, appeared to generate similar levels of bacteraemia in locusts after 3 h, with an average of 57.8, 58.4 and 57 colonies recovered per 5 μL of haemolymph, respectively. After 3 h, these levels were lower than in locusts infected with wild-type RS228. However, after 24 h, levels of ΔtatA (but not Δtat or ΔtatAC) increased dramatically, with 171.4 CFUs of ΔtatA isolated per 5 μL of haemolymph. These levels exceeded those produced by RS228 after 24 h. Interestingly, the pattern of bacteraemia produced by ΔtatA was strikingly similar to that produced by the wild-type RS218 strain. With the exception of the nonpathogenic K-12, infection with Δtat and ΔtatAC produced the lowest levels of bacteraemia after 24 h, with an average of 85 and 79 colonies recovered per 5 μL of haemolymph, respectively.

**Discussion**

It has long been established that the development of a threshold level of bacteraemia is a key in allowing bacteria to cross the BBB and invade the central nervous system. Research over the last few decades has identified bacterial virulence determinants (such as CNF1, FimH, OmpA, Ibe proteins, TraJ and As1A located at different regions on the *E. coli* chromosome) that are associated with *E. coli* K1 invasion of the CNS. There are, however, many other virulence factors yet to be tested. Interestingly, our recent work (Khan & Goldsworthy, 2007; Mokri-Moayyed *et al.*, 2008) has established that a locust model of bacterial meningitis conforms to what is known about *E. coli* pathogenesis in mammals. This together with the fact that there are substantial similarities in the innate immune responses of mammals and insects (Salzet, 2001; Garcia-Lara *et al.*, 2005) suggests that insects are useful models to study the pathogenesis of *E. coli* K1 (Khan & Goldsworthy, 2007; Mokri-Moayyed *et al.*, 2008). Thus, it is not surprising that *E. coli* K1 pathogenesis within both locust and mammalian systems has been shown to be dependent upon several common established virulence factors (Khan & Goldsworthy, 2007; Mokri-Moayyed *et al.*, 2008). Such data support the use of a locust system to successfully model *E. coli*
infection, and it is anticipated that the likely existence of additional virulence determinants can be investigated in vivo using this insect system that has no associated legislative regulations and ethical concerns.

Compared with the Sec translocase that facilitates the export of loosely folded proteins, the twin-arginine translocation (Tat) pathway is known to be involved in the translocation of folded proteins across the bacterial cell membrane, with substrate proteins targeted to the signal peptide–dependent protein Tat translocase (Ize et al., 2003). Major components of Tat translocase include the integral membrane proteins TatA, TatB, and TatC. The protein TatE forms a minor component and is a poorly expressed orthologue of TatA (Hicks et al., 2006). Using gene deletion mutant strains lacking tatA, tatAC and tat, the present findings revealed no evidence of the involvement of Tat pathway in E. coli K1-mediated locust mortality. This is in contrast to previous studies which showed that the cytotoxicity of Tat mutant was attenuated compared with the wild-type enterohemorrhagic E. coli O157: H7 in vitro using African green monkey kidney cells (Pradel et al., 2003). These differences may be attributed to in vitro assay using cell cultures vs. in vivo system using whole insects that heavily rely on innate immune responses to counter infection. Although the Tat system is known to be essential for many bacterial processes including energy metabolism, cell wall biosynthesis and the nitrogen-fixing symbiosis (Berks et al., 2005), the degree to which organisms exploit the Tat machinery varies greatly with only a handful of proteins for some organisms (Jongbloed et al., 2004). Furthermore, Tat system is present in both pathogenic and nonpathogenic E. coli strains (Berks et al., 2005). A functional Tat system is not required for the growth of E. coli under most condition (Dilks et al., 2005), and its components are expressed at very low levels in wild-type E. coli and almost all biochemical studies of the E. coli Tat system have utilized strains overexpressing the Tat components (Yahr & Wickner, 2001; Alami et al., 2002). Of interest, Δtat or ΔtatAC showed reduced levels of bacteremia as compared to the parent strain at 24 h, although the mortality was not affected. Surprisingly, ΔtatA showed increased levels of bacteremia and locust brain invasion as compared to Δtat or ΔtatAC. As indicated above, Tat system consists of a fourth gene, tatE that codes for a redundant but functionally similar TatA protein (Stanley et al., 2001). TatA and TatE are equivalent to each other as demonstrated by expression studies that showed tatE is a cryptic gene duplication of tatA (Jack et al., 2001). Thus, it is plausible that the deletion of tatA was compensated by overexpression of tatE, which may explain the unexpected findings.

In conclusion, the locust mortality data showed that all mutant strains demonstrated a level of virulence similar to that of wild-type E. coli K1; however, Δtat showed reduced levels of bacteremia and CNS invasion than the wild-type strain using in vivo and ex vivo assays in invertebrate. Future studies should determine the role of TatA in E. coli K1-mediated bacteremia and the potential role of Tat system in E. coli K1 virulence using vertebrate models of bacterial meningitis. A deeper understanding is essential to fully understand the role of the Tat pathway in the ability of E. coli to cause meningitis, making it an exciting and interesting subject for future research.

References


