fliP influences *Citrobacter koseri* macrophage uptake, cytokine expression and brain abscess formation in the neonatal rat

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Citrobacter koseri causes neonatal meningitis frequently complicated with multiple brain abscesses. During *C. koseri* central nervous system infection in the neonatal rat model, previous studies have documented many bacteria-filled macrophages within the neonatal rat brain and abscesses. Previous studies have also shown that *C. koseri* is taken up by, survives phagolysosomal fusion and replicates in macrophages *in vitro* and *in vivo*. In this study, in order to elucidate genetic and cellular factors contributing to *C. koseri* persistence, a combinatory technique of differential fluorescence induction and transposon mutagenesis was employed to isolate *C. koseri* genes induced while inside macrophages. Several banks of mutants were subjected to a series of enrichments to select for *gfp* :: transposon fusion into genes that are turned off *in vitro* but expressed when intracellular within macrophages. Further screening identified several mutants attenuated in their recovery from macrophages compared with the wild-type. A mutation within an *Escherichia coli* *fliP* homologue caused significant attenuation in uptake and hypervirulence *in vivo*, resulting in death within 24 h. Furthermore, analysis of the immunoregulatory interleukin (IL)-10/IL-12 cytokine response during infection suggested that *C. koseri* *fliP* expression may alter this response. A better understanding of the bacteria–macrophage interaction at the molecular level and its contribution to brain abscess formation will assist in developing preventative and therapeutic strategies.

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INTRODUCTION

Citrobacter spp. are Gram-negative, opportunistic bacterial pathogens of neonates and the immunocompromised, responsible for sepsis, ventriculitis and meningitis with brain abscess. Once infection has occurred, *Citrobacter* spp. exhibit an incredible tropism for the central nervous system (CNS). An intense host inflammatory response ensues, which is characterized by deterioration of the ventricular lining and extension of the infection into the white brain matter, ultimately resulting in brain abscesses (Kline *et al.*, 1988). *Citrobacter* spp. demonstrate vertical transmission and horizontal acquisition from the environment (Agrawal & Mahapatra, 2005; Doran, 1999; Feferbaum *et al.*, 2000). Several nosocomial outbreaks have occurred; however,

infections resulting in abscess formation among immuno-competent adults are extremely rare (Prais *et al.*, 2003; Tang *et al.*, 1994; Williams *et al.*, 1984). Infants with *Citrobacter koseri* meningitis develop brain abscesses at an alarming frequency of 77 %, a far higher rate than any other bacterial agent of neonatal or infant meningitis. One-third of infected infants die and 75 % of survivors suffer severe neurological impairment (Saez-Llorens & McCracken, 1998). Once the brain abscess has been established, it can persist with little regard for antibiotic treatment, often requiring surgical intervention (Kline, 1988; Renier *et al.*, 1988). Treatment limitations are further frustrated as little is known about the pathogenesis of the disease. However, recent data from our laboratory have shown that *C. koseri* is able to invade and replicate inside human U937 macrophages and human brain microvascular endothelial cells *in vitro* (Badger *et al.*, 1999; Townsend *et al.*, 2003). In addition, our studies and others utilizing the neonatal rat model of infection have demonstrated that this model closely mimics human disease in terms of age dependency, course of infection and brain abscess formation (Kline *et al.*, 1988; Townsend *et al.*, 2003). Furthermore, these studies have revealed that within the CNS (particularly within brain abscesses), *C. koseri* is intracellular, predominantly within macrophages

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Abbreviations: CNS, central nervous system; CSF, cerebrospinal fluid; FACS, fluorescence activated cell sort; i.c., intracranial; IL, interleukin; i.p., intraperitoneal; PMA, phorbol 12-myristate 13-acetate.

The GenBank/EMBL/DDBJ accession number for the *fliP* sequence of *Citrobacter koseri* mutant SMT350 determined in this study is DQ233671.

(Townsend *et al.*, 2003). Macrophages are the primary source of interleukin (IL)-12, which induces resistance to intracellular infection mediated by the type 1 cellular immune response (Trinchieri, 1997).

For this study, our objective was to identify and characterize *C. koseri* mutants demonstrating attenuated recovery from macrophages, indicating a reduction in uptake or persistence. Several such mutants were identified, including a *fliP* mutant, SMT350, which was non-motile with reduced uptake by macrophages but hypervirulent *in vivo*. In addition, we showed that flagellin influences the IL-10/IL-12 cytokine response during infection and may be an important factor contributing to the chronic nature of this disease.

METHODS

Bacterial strains and culture. *C. koseri* strains used in this study were derived from a spontaneous nalidixic acid-resistant mutant (SMT319) (Townsend *et al.*, 2003), strain JLB62, that was isolated from the cerebrospinal fluid (CSF) of an infant with *C. koseri* meningitis and a brain abscess (obtained from Joseph St Geme III, Washington University School of Medicine, USA). Mutant strain SMT320 constitutively expressed the green fluorescent protein gene (*gfp*) as a result of a random transposition insertion of promoterless *mTn10gfp* (Stretton *et al.*, 1998), downstream from a constitutive promoter on the *C. koseri* chromosome (unpublished data). Both SMT319 and SMT320 retained wild-type morphology, growth characteristics and invasive phenotypes (Townsend *et al.*, 2003; data not shown). *Escherichia coli* K1 E44, a spontaneous rifampicin-resistant mutant of meningitic *E. coli* K1 CSF isolate RS218 (O18:K1:H7) and non-pathogenic *E. coli* K-12 HB101 were used as controls (Weiser & Gotschlich, 1991). For gentamicin protection assays, bacteria were grown aerobically at 37 °C to mid-exponential phase in brain heart infusion broth with 15 µg nalidixic acid ml⁻¹ and/or 50 µg kanamycin ml⁻¹ selection. For animal studies, *C. koseri* strains were grown to mid-exponential phase and washed twice with cold PBS. Concentrations of bacterial inoculations were approximated by OD₆₂₀ and confirmed by viable counts.

U937 macrophage cultivation and gentamicin protection assay. U937 macrophages were obtained from the ATCC (CRL-1593.2) and seeded into 75 ml tissue culture flasks (Sundström & Nilsson, 1976). Cells were cultivated in RPMI 1640 with 2 mM L-glutamine, modified to contain 10 mM HEPES, 1 mM sodium pyruvate, 2.5 mM glucose and 1.8 mM sodium bicarbonate, and supplemented with 10 % fetal bovine serum (Sundström & Nilsson, 1976). At least 24 h prior to infection, cells were treated with 0.1 µg phorbol 12-myristate 13-acetate (PMA; Sigma) ml⁻¹, placed in tissue culture plates and left at 37 °C, 5 % CO₂ to adhere and become activated. Cells were gently washed with RPMI 1640 to remove residual PMA and fresh medium was added prior to infection. U937 human macrophages were infected at an m.o.i of 10 for 45 min at 37 °C in 5 % CO₂. After a 45 min incubation period, macrophages were resuspended in U937 medium supplemented with 100 µg gentamicin ml⁻¹ and incubated for an additional 45 min at 37 °C in 5 % CO₂. Macrophages were then washed twice, lysed with 0.5 % Triton X-100, serially diluted and plated to determine the number of intracellular bacteria at various time points (Zaidi *et al.*, 1996). For extended assays (persistence assays), cells were replenished with fresh medium containing 10 µg gentamicin ml⁻¹ (above the MIC). Trypan blue exclusion staining indicated that macrophage viability ranged from 80 to 95 % and was maintained for at least

72 h. For persistence assays, results for each time point are presented as the log of the percentage of inoculum that was intracellular. All assays were performed in triplicate at least two times.

Construction of *C. koseri gfp* fusion mutant bank. To construct banks of random gene fusion mutants within *C. koseri*, three independent tri-parental matings containing *C. koseri* strain SMT319 (recipient strain), SM10λ-pir pLOFKmgfp (transposon donor strain) (Stretton *et al.*, 1998) and DH5α with helper plasmid pRK2013 were performed, which gave rise to exconjugates (Afendra & Drinas, 1987). Briefly, approximately 10⁸ cells of each bacterial strain were added to 5 ml 10 mM MgSO₄ and filtered through a Millipore 25 mm diameter type HAWP 0.45 µm sterile membrane. After filtering, the membrane was placed (cell side up) on the surface of a Luria-Bertani (LB) agar plate and incubated for 5 h, transferred to an LB agar plate containing 100 µM IPTG (to increase expression of the transposase) and incubated overnight (Stretton *et al.*, 1998). The filter was then resuspended in 5 ml 10 mM MgSO₄ and 200 µl was plated on to LB agar supplemented with 50 µg kanamycin ml⁻¹ and 15 µg nalidixic acid ml⁻¹ to select for exconjugates.

Differential fluorescence induction screening and characterization of *C. koseri gfp* fusion mutants. Exconjugates were isolated from selective agar plates and grown to exponential phase in experimental media. Bacteria were subjected to an initial fluorescence activated cell sort (FACS) from which the bottom 10 % of non-fluorescent bacteria was collected, thus removing constitutively fluorescent mutants. As described above, bacteria were added to monolayers of PMA-stimulated U937 human macrophages and incubated for 1 h. The macrophages were washed twice and incubated with experimental medium supplemented with 100 µg gentamicin ml⁻¹ for 1 h. The medium was then replaced with medium supplemented with 10 µg gentamicin ml⁻¹ and incubated for an additional 3 h. Macrophages were washed extensively with RPMI 1640 and lysed with 0.5 % Triton X-100. The resultant bacterial suspension was subjected to a second FACS from which the top 10 % of fluorescent cells were selected for further characterization.

To determine the mean fluorescence induction (MFI) of individual mutants, bacteria were incubated with macrophages as described above and subjected to FACS analysis. MFI was calculated as the ratio of fluorescence of U937-associated bacteria compared with bacteria grown in experimental medium alone.

FACS analysis was performed using the FACSDiVa and the FACSDiVa software program (Becton Dickinson). FACS parameters were set using SMT320 as a positive control. Sort gates were set for each mutant bank by applying region markers on the histogram of the logarithmic green fluorescence channel and sorting the top 10 % and bottom 10 % of the fluorescent populations.

Qualitative screening to identify mutants with attenuated uptake and persistence. Qualitative assessment of uptake and persistence was performed as follows. Mutant (*C. koseri*::*Tn10gfp*) and control (*E. coli* K1 E44 and K-12, and *C. koseri* SMT319) strains to be analysed were grown in 100 µl U937 medium in 96-well plates in duplicate and incubated at 37 °C overnight. PMA-stimulated macrophages (~3.0 × 10⁵ cells per well) were prepared in 96-well plates using only rows 1 and 5 of the 96-well plates. A multichannel pipette was used to inoculate 1 µl bacterial cultures on to macrophages. Uptake of bacteria was allowed to occur for 45 min at 37 °C with 5 % CO₂. The medium was replaced with 200 µl U937 medium supplemented with 100 µg gentamicin ml⁻¹ and incubated for 1 h. The medium was then replaced with 200 µl U937 medium supplemented with 10 µg gentamicin ml⁻¹ and incubated overnight. Macrophages were washed twice with Hanks' buffered salt solution, lysed with 40 µl 0.5 % Triton X-100 for 5 min and 20 µl dH₂O was added. Serial dilutions were performed by transferring 15 µl

bacterial solution to the lower wells containing saline (i.e. 135 µl to rows 2, 3 and 4) and 200 µl of soft agar was then placed into each well (avoiding the production of bubbles). The plate was incubated overnight at room temperature. Visual inspection of bacterial growth readily determined potential 10-fold differences among strains.

Animal infections. Timed pregnant (E14) Sprague–Dawley rats (Charles River Laboratories, Raleigh, NC, USA) were obtained and gave birth in our vivarium after a 21-day gestation period. Litters averaged 12 pups and were kept with the mother in an opaque, polypropylene cage under a small animal isolator. Two- to 3-day-old rat pups were anaesthetized with isoflurane and inoculated with either 10^7 c.f.u. (10^6 c.f.u. SMT350 for 24 h serum studies) in PBS via an intraperitoneal (i.p., 0.1 ml) or 10^3 c.f.u. in PBS via an intracranial (i.c., 0.002 ml) route. Purified flagellin was obtained by acid denaturation and 300 ng was injected i.p. for rat serum studies. Briefly, the pellet from a small volume of an overnight culture of wild-type *C. koseri* was resuspended in 150 mM NaCl and 10 mM HCl and incubated at room temperature for 30 min to depolymerize the flagellin. The resulting supernatant was further clarified by centrifugation at 100 000 g for 90 min and concentrated via acetone precipitation. A bicinchoninic acid protein assay kit was used to quantify the flagellin concentration. i.c. inoculations were administered through a burr hole produced using a 26-gauge needle at coordinates approximately 5 mm caudal to the right eye and 2 mm right of the sagittal suture. A 33-gauge single internal cannula attached to a Hamilton 1801RN 10 µl syringe, with a 22-gauge needle via 24-gauge standard wall tubing, was used to administer the dose. The needle was inserted perpendicularly into the right parietal area, at a depth of approximately 2 mm from the external surface, and 2 µl was injected over 1 min. The needle was then carefully retracted. Rat pups were anaesthetized at the indicated time points and blood and CSF samples were collected aseptically via intracardiac and cisterna magnum punctures, respectively, as described previously (Kim *et al.*, 1992). CSF and blood samples (10 µl) were inoculated in LB and plated on to agar plates with appropriate antibiotic selection. Rats were then euthanized and whole brains were removed. All animal experiments were performed according to protocols approved by the Childrens Hospital Los Angeles Institutional Animal Care and Use Committee.

Histopathology. Whole brains of infected neonatal rats were fixed in 10% buffered formalin, routinely processed and paraffin embedded. Coronal sections of 4–5 µm were cut and stained with haematoxylin and eosin (H&E). Histological analysis was completed by a pathologist (I.G.-G.) who was blinded to the inoculums and time post-infection of each rat at the time of sacrifice. Animals infected with PBS were analysed as controls.

Sequence analysis. A Southern blot of SMT350 *EcoRI*-digested chromosomal DNA was hybridized with a non-radioactive *gfp* probe generated using the Prime-It Fluor Fluorescence Labelling kit (Stratagene). A 5 kb fragment was identified and cloned into pUC19. Sequencing was performed using the inverse PCR primers KNout2-F (5'-GGTATTGATAATCCTGATATG-3') and *gfp*1.5-IC (5'-CTTCTCCTTTACTCATATGTATATC-3') and primer walking. Searches for sequence homology were performed using BLASTX.

Quantification of rat IL-10 and IL-12 serum cytokine levels following i.p. inoculation. Commercial ELISA kits (Biosource) were used to measure rat pup IL-10 (sensitivity <5 pg ml⁻¹) and IL-12 p40 (sensitivity <3 pg ml⁻¹) concentrations in rat serum isolated 12 and 24 h after i.p. injection of *C. koseri* wild-type or mutant strain. *E. coli* 0111:B4 LPS (Sigma) was used as a positive control. The manufacturer's instructions for each ELISA were followed. However, the amount of serum was diluted 1:10 and this was taken into account during the final calculations. Each sample was run in

duplicate wells and the optical density values were averaged. Cytokine standards supplied by the manufacturers were used to generate standard curves.

RESULTS AND DISCUSSION

Isolation of mutants deficient in macrophage uptake and persistence

In order to identify genes influencing *C. koseri* uptake and persistence within macrophages, we utilized a combinatory transposon mutagenesis and differential fluorescence induction technique. As described in Methods, transposon mutagenesis was performed to randomly insert promoterless *gfp* within the *C. koseri* chromosome. Three independent banks of mTn10*gfp* mutants were screened for induced *gfp* expression after several hours within human U937 macrophages. Under these experimental conditions, ~2000 *C. koseri* transposon insertion mutants were isolated that showed induction of *gfp* expression when the bacteria were within macrophages. These mutants were then screened for their ability to persist within macrophages. Upon qualitative analysis, seven of these mutants were found to be attenuated for uptake and/or persistence within macrophages. However, mutants that absolutely could not be taken up or survive within macrophages were not identified in this study.

Quantitative gentamicin protection assays were performed on the seven mutants to characterize their ability to be taken up by and persist within human macrophages. Results from these assays revealed that there were various *in vitro* mutant phenotypes (Fig. 1). Mutant strains SMT324, SMT325 and SMT356 maintained wild-type uptake levels but were attenuated for intracellular replication (Table 1). Mutant strain SMT332 was attenuated for uptake and intracellular replication within macrophages; SMT336 and SMT350 were attenuated for uptake alone (Table 1). Mutant strain SMT335 showed reduced uptake and was unable to persist within macrophages (Table 1). All mutants were susceptible to gentamicin. Southern blot analysis (using the *gfp* gene as a probe) ruled out siblings and confirmed that single transposon insertions were responsible for the observed phenotypes (data not shown).

In vitro characterization of mutants deficient in macrophage uptake and persistence

To understand better the relative importance of genes associated with uptake and intracellular persistence, we compared their macrophage-dependent expression. MFI was measured by the fold increase in *gfp* expression after 2 h within macrophages compared with growth *in vitro* in tissue culture medium alone. We found that *gfp* expression in the seven mutant fusions was induced 1.5–3.1-fold when they were intracellular (Table 1). SMT332 showed the highest increase in MFI. The MFI was low for SMT335 (1.6) and moderate for SMT356 (2.3); however, both had fusions interrupting genes necessary for intracellular replication as these strains did not replicate within macrophages

Fig. 1. Intracellular uptake and persistence phenotypes of *C. koseri* transposon mutants. PMA-stimulated U937 macrophages were infected with mid-exponential phase bacteria (m.o.i. of 10) for 45 min with subsequent gentamicin treatment for 45 min (T_0). Macrophages were lysed at the indicated time points up to 72 h to quantify the number of intracellular bacteria. For each indicated time point, results are presented as the log of the percentage of the inoculum that was intracellular. Data are shown as the mean of at least two independent experiments performed in triplicate.

(Table 1). In contrast, SMT350 consistently had the lowest MFI with an intracellular generation time of 8.9 ± 1.5 h (Table 1). SMT350 was also non-motile. Previous flagellar mutants of *Salmonella enterica* serovar Typhimurium have exhibited faster net growth intracellularly, although invasiveness was attenuated; however, our rate was not significantly different to that of wild-type (Schmitt *et al.*, 2001). SMT324, SMT325 and SMT332 had intracellular generation times significantly higher than the wild-type

(Table 1). Furthermore, mutant strains SMT332, SMT335, SMT336 and SMT350 were taken up between 6- and 526-fold less than the wild-type (Table 1). All strains except SMT335 were able to persist for up to 72 h within macrophages. Persistence was maintained when % survival $\pm$ SEM was within 100 % at 48 h. SMT335 failed to persist and suffered an 83 ± 6 % reduction in intracellular c.f.u. after 24 h, which was further reduced to 96 ± 1 % at 48 h, comparable to that observed for the negative control

Table 1. Summary of phenotypic characterization of transposon mutants used in this study

Motility was assessed by 0.3 % agar stabs. MFI was determined as the ratio of mutant strain fluorescence within macrophages after 24 h to fluorescence of the mutant grown in media alone. Uptake was reported as the percentage within macrophages relative to uptake of wild-type at T_0 arbitrarily set at 100 %. Persistence data were represented by the mean intracellular generation time of at least two independent assays $\pm$ SD. The notation 'killed' indicates more than a 90 % reduction in c.f.u. at 48 h, whilst 'persists' indicates that the % survival $\pm$ SEM was within 100 % at 48 h. Rat pup serum was collected at 12 and 24 h after i.p. inoculation with *C. koseri* strains and IL-10 and IL-12 were analysed by ELISA. Kaplan–Meier survival times (days) were generated with an SPSS statistics package (SPSS 8.0). Histopathological analysis was performed to determine the nature and degree of inflammation and was graded using an arbitrary scale: 0, no inflammation; +, focal, ventricle inflamed and dilated; ++, ventricular wall destruction; + + +, cysts and extension into white matter; + + + +, brain abscess.

Strain	Motility	MFI	Macrophage uptake	Macrophage persistence	Rat IL-12		Rat IL-10		Mean survival	Brain inflammation
					12 h	24 h	12 h	24 h		
WT	+	0.0	100	11.4 ± 2.7	115 ± 16	258 ± 43	281 ± 18	0	7.8 ± 1.0	++++
SMT324	+	1.9	52 ± 8	65.2 ± 8.9					5.3 ± 0.6	++
SMT325	+	1.8	53 ± 8	21.7 ± 1.6					6.3 ± 1.0	++
SMT332	+	3.1	2.3 ± 0.7	29.3 ± 1.6					6.0 ± 1.0	++++
SMT335	+	1.6	16 ± 1	0 (persists)					7.4 ± 0.7	++++
SMT336	+	2.0	0.19 ± 0.04	13.4 ± 4.8					7.5 ± 1.2	++++
SMT350	–	1.5	4.2 ± 0.8	8.9 ± 1.5	116 ± 22	229 ± 53	0	90 ± 81	2.3 ± 0.4	+++
SMT356	+	2.3	178 ± 27	0 (killed)	163 ± 15	292 ± 48	0	0	No death	0

E. coli K-12, which gave values of 80 and 98 % reduction, respectively (data not shown).

In vivo characterization of mutants deficient in macrophage uptake and persistence

C. koseri is specifically noted for its unique ability to cause brain abscesses in neonates (Kline & Kaplan, 1987; Townsend *et al.*, 2003). To determine the consequence of deficient macrophage uptake and persistence on brain abscess formation, the ability of each strain to induce brain infection and/or abscess formation following i.c. inoculation was tested. At least six 2–3-day-old rat pups were injected i.c. with 10^3 c.f.u. of each strain and independently analysed histologically for brain abscess formation. A Kaplan–Meier survival curve was used to analyse survival time (SPSS). The mean survival time of 2-day-old rats injected i.c. with 10^3 c.f.u. of wild-type was 7.8 ± 1.0 days (Fig. 2). Interestingly, SMT350 had a mean survival time of 2.33 ± 0.42 days, whereas the survival time for SMT356 was indefinite because all rats survived the i.c. challenge ($n=11$). All other mutant survival times were similar to that of the wild-type (Table 1).

Histological analysis of neonatal rat brain following i.c. infection

SMT350 (hypervirulent mutant), SMT356 (hypovirulent mutant) and SMT336 (virulent mutant) had vastly different survival times. Therefore, infected rat pup brain tissue sections were examined to ascertain the histological basis for the observed differences. Fig. 3 depicts H&E-stained sections from neonatal rats infected by i.c. inoculation of wild-type *C. koseri* (Fig. 3a–c), SMT350 (Fig. 3d–i), SMT356 (Fig. 3j) and SMT336 (Fig. 3k). Severe bilateral ventriculitis and ventriculomegaly ensued 5 days after i.c. infection with wild-type *C. koseri*. Extension of the

inflammation into the white matter directly preceded brain abscess formation (Fig. 3a). Brain abscess formation coincided with the accumulation of inflammatory cells such as macrophages and neutrophils and the destruction of the ventricular wall (Fig. 3b). In addition, *C. koseri*-filled macrophage and inflammatory cells within the white matter adjacent to the ventricle wall may have disseminated the infection to the surrounding tissue (Fig. 3c). In contrast, ventriculitis and ventriculomegaly ensued 3 days after i.c. infection with SMT350. Inflammation within the ventricle and white matter included macrophages, neutrophils, lymphocytes, plasma cells, Russell bodies and free bacteria (Fig. 3d–i). However, the inflammation seemed less intense than that following wild-type infection and consisted of different cell types, predominantly lymphocytes and neutrophils, as opposed to macrophages, which usually respond later during infection (Fig. 3d–i). The Russell bodies indicated a massive reaction to an antigen such that the antibodies produced accumulate in the cytoplasm of the plasma cells forming Russell bodies (Fig. 3e–h). SMT356 infections did not develop brain abscesses or disease, with the only traces of insult indicated by pigmented macrophages located in the ventricle, suggesting that haemorrhage had occurred within the previous 4–5 days (Fig. 3j). In addition, free and intracellular bacteria were observed markedly less frequently than with either wild-type or the other mutants at the corresponding day post-infection. The c.f.u. recovered from i.c.-infected pups (3 days old) at 7 days post-infection averaged 4.9×10^{10} c.f.u. (g brain tissue) $^{-1}$ ($n=5$), whilst only 45 c.f.u. (g brain tissue) $^{-1}$ ($n=6$) was recovered from SMT356-infected pups (data not shown). SMT356 exhibited a drastically reduced mortality rate associated with reduced recovery of c.f.u. in the brain tissue and the absence of brain abscess formation. SMT335 was attenuated in intracellular replication within human macrophages, although it maintained full virulence *in vivo* (Table 1) and maintained wild-type brain pathology (data not shown). Other mutants (such as SMT336) also had a brain pathology similar to that of the wild-type following i.c. injection, regardless of *in vitro* phenotype (Fig. 3k), suggesting that the presence of the transposon itself did not directly affect brain pathology. Consistently, the most comparable aspect of the histology, causing a considerable amount of tissue damage in addition to periventricular brain abscesses and ventriculitis, was the development of oedema and consistent formation of perivascular fluid cysts within the white matter not adjacent to abscess material.

Identification of *fliP*

To obtain an understanding of the genetic basis for the phenotype of the hypervirulent SMT350 mutant, the disrupted gene was identified. A non-radioactive *gfp* probe hybridized to a 4 kb *EcoRI* fragment of mutant SMT350 chromosomal DNA (data not shown). This fragment was cloned and sequenced. BLASTX analysis showed that the transposon insertion occurred at aa 181 (245 aa total) within an *E. coli* *FliP* orthologue with 94 %

Fig. 2. Kaplan–Meier survival curve of *C. koseri*-infected rats. Two- to 3-day-old rat pups ($n \geq 6$) were inoculated i.c. with 10^3 c.f.u. of each strain and monitored daily for clinical signs of disease or death for 11 days. Rats inoculated with the mutant strain SMT356 or PBS ($n=4$) had no fatalities.

Fig. 3. Histological examination of *C. koseri* CNS infection in the neonatal rat. H&E-stained sections from neonatal rats infected by i.c. inoculation of wild-type *C. koseri* (a–c), SMT350 (d–i), SMT356 (j) and SMT336 (k). Bars: 2 mm (a, d, j, k), 200 μ m (b), 100 μ m (e), 50 μ m (c, f) and 20 μ m (g, h, i). (a) Severe bilateral ventriculitis and ventriculomegaly at 5 days after infection with wild-type *C. koseri*. The arrow indicates the extension of inflammation into the white matter directly preceding brain abscess formation. (b) Brain abscess formation coincided with the accumulation of inflammatory cells such as macrophages and neutrophils and the destruction of the ventricular wall as depicted here. (c) Brain abscess formation and breaching of the ventricular wall at the cellular level is shown. The arrow indicates a *C. koseri*-filled macrophage and inflammatory cells within the white matter adjacent to the ventricle wall. (d) Ventriculitis and ventriculomegaly ensued 3 days after infection with SMT350. The arrow indicates an area of inflammation that is examined in the following panels. (e) Russell bodies were present throughout the ventricle inflammation (arrow). (f) Inflammation at the ventricle wall showing Russell bodies in white matter and ventricles, free bacteria and inflammatory cells. (g) Inflammation within the ventricle showing macrophages, neutrophils, lymphocytes, plasma cells, Russell bodies and free bacteria. (h) The arrow indicates a macrophage adjacent to the ventricular wall that has engulfed Russell bodies, several bacteria and is surrounded by free bacteria. (i) *C. koseri* was observed free within the white matter (arrow). (j) Normal brain histology 9 days after SMT356 inoculation. (k) Typical brain lesions induced by intracellular replication mutants 9 days post-inoculation (SMT336).

Fig. 4. Identification of the *C. koseri* *fliP* homologue. The 5 kb chromosomal–transposon junction from SMT350 was cloned (open arrows) and sequenced. The *gfp* transposon was inserted within a gene encoding a protein with 94 % amino acid identity to *E. coli* FliP. The genetic organization of a putative class II *Citrobacter* flagella operon is indicated.

amino acid identity (Fig. 4). Weaker amino acid identity was observed to enteropathogenic *E. coli* EscR (39 %) and *Salmonella* Typhimurium SpaP (34 %) and SsaR (38 %), which are type III secretion proteins important for the translocation of virulence factors (Galán & Collmer, 1999). Further sequence analysis revealed that *fliP* was one of five contiguous genes cloned having homology to *E. coli* *fliNOPQR* (Malakooti *et al.*, 1994). The genetic organization and open reading frame orientation of these genes were identical to the *E. coli* *fli* flagella operon, which is important in the complete biosynthesis of functional flagella (Fig. 4). The *fliP* gene encodes an integral membrane protein associated with the basal body of the flagella structure. As the *fli* operon is coordinately regulated by a promoter upstream of *fliL*, expression of *fliR* and *fliQ* is lost via polar effects, as the transposon insertion occurs upstream in *fliP* (Malakooti *et al.*, 1989). As SMT350 was the result of *fliP* disruption, a gene encoding an essential component of a type III secretion system for flagellin secretion, we examined the motility phenotypes of each mutant. Only SMT350 lacked motility, as assessed by a 0.3 % soft agar motility assay (Table 1 and Fig. 5a).

To confirm that the *fliP* locus was necessary for *C. koseri* uptake within macrophages, revertants derived from SMT350 were isolated and their phenotypes assessed. Lawns of SMT350 were stabbed and screened for recovery of motility in 0.3 % soft agar stabs, taking advantage of the readily screenable phenotype to isolate strains having undergone the rare event of reversion (Fig. 5b). Using this approach, two revertant motile strains (SMTR1 and SMTR2) were isolated. Furthermore, gentamicin protection assays confirmed that the ability of these revertant strains to be taken up and replicate within macrophages had been restored to wild-type levels (data not shown).

Interestingly, SMT350 caused mortality in infant rats before brain abscesses could occur (within 3 days), with a marked reduction in the presence of infiltrating macrophages, and a notable increase in extracellular bacteria compared with wild-type was observed in this study. Similarly, others have documented reduced polymorphonuclear influx in response to non-flagellated mutants of *Salmonella* (Schmitt *et al.*, 2001). Although the migration of macrophages to the site of inflammation is understood to be non-antigen specific, these observations indicate that the expression of flagella or motility contributes to the establishment of chronic

infection. We observed that non-motile (non-flagellated) SMT350 exhibited significantly reduced uptake *in vitro* and *in vivo*, resulting in accumulation of extracellular bacteria. Similarly, *Salmonella* Enteritidis lacking flagella is significantly attenuated in its ability to invade murine, avian and human cell lines (Lockman & Curtiss, 1990; Van Asten *et al.*, 2000). Our data lend additional support to the hypothesis that flagella play a role in the process of invasion. It is also possible that flagella may mask a more toxic factor that is exposed when the flagellum structure is not expressed. Reduced uptake by macrophages would amplify this by increasing the exposure of the antigen, with the majority of the bacterial population remaining extracellular. This was supported by the observation that Russell bodies (accumulation of immunoglobulin proteins due to overexpression within plasma cells) were abundant following SMT350

Fig. 5. Motility phenotype of *C. koseri*. Each strain was stab-inoculated into 0.3 % soft agar and incubated at room temperature for 24 h. (a) *E. coli* K-12 (left) and wild-type *C. koseri* (middle) were used as negative and positive controls, respectively. SMT350 (right) was non-motile. (b) From left to right: non-motile SMT350 and motile wild-type, revertant motile mutant SMTR1 and revertant motile mutant SMTR2 strains. The figure was cropped using Photoshop version 6.0.

infection and absent during wild-type infection. Moreover, the histology revealed a pattern of inflammation (Russell bodies) that was inconsistent with what is observed during LPS-induced shock.

Serum cytokine levels in rat pups following exposure to *C. koseri*

To determine whether mutant strains deficient in macrophage uptake and persistence differentially affected cytokine expression influencing the development of type 1 and type 2 immune responses, serum IL-10 and IL-12 levels were analysed at 12 and 24 h after i.p. infection of rat pups. At 12 h post-inoculation, only the wild-type induced IL-10 expression (281 ± 18 pg ml⁻¹) compared with mutants SMT350 and SMT356, where no expression was detected (Fig. 6a). IL-10 expression in response to mutant SMT350 was delayed and reduced threefold but detected at 24 h (90 ± 81 pg ml⁻¹) (Fig. 6a). Purified flagellin (300 ng) was also injected i.p. to ascertain the influence of flagellin on cytokine expression *in vivo* and IL-10 serum levels were

found to increase significantly (4401 ± 1068 pg ml⁻¹) at 12 h post-treatment and then dropped to 302 ± 21 pg ml⁻¹ at 24 h post-treatment (Fig. 6a). LPS is also known to induce IL-10 and IL-12 expression and was tested as a positive control. Although IL-10 expression was dominant at 12 h post-inoculation, IL-12 expression increased twofold by 24 h and was favoured in comparison with IL-10 expression in all strains.

It is possible that flagella may influence the host cytokine response as a mechanism of survival (Liaudet *et al.*, 2002). Cytokines produced by macrophages, NK cells, T cells and mast cells are important mediators of an effective immune response during infection. A type 1 response induces cellular immunity, promotes cytotoxic T-lymphocyte response, activates macrophages and is critical in the development of resistance to intracellular pathogens (Flynn & Chan, 2001; Ottenhoff *et al.*, 2002). A type 2 response helps establish humoral immunity, activates the allergy response mediated by eosinophils and mast cells and actively fights helminthic infections. Induction of a type 1-specific response precludes the induction of the type 2 response and vice versa (Schmitz *et al.*, 1993; Trinchieri, 1997). The macrophage is at the centre of this dichotomy, capable of expressing both the immunoregulatory cytokine IL-12 and the immunosuppressive cytokine IL-10. When IL-12 is induced, it favours type 1-specific clonal expansion and gamma interferon production, which activates phagocytic cells and inhibits the type 2 response (Sher & Coffman, 1992). When IL-10 is induced, it inhibits IL-12 expression (and gamma interferon indirectly), which favours type 2 cell proliferation (Flesch *et al.*, 1994; Hirsch *et al.*, 1996).

The current cytokine studies showed that IL-10 expression occurred early during infection (within the first 12 h), which may initially favour a type 2 immune response to wild-type *C. koseri* infection. Comparatively, mutant strains SMT350 and SMT356 displayed differential IL-10 expression, which was delayed and reduced or absent, respectively. Further, *C. koseri* flagellin was shown to induce robust IL-10 expression in neonatal rats 12 h after i.p. injection that subsided within 24 h. In this study, it has been shown that mutant strain SMT350 exhibits delayed and reduced IL-10 expression and a hypervirulent phenotype *in vivo*. This indicates that flagella may possess immunosuppressive properties such that initial recognition of flagella alters the conventional mechanism for resolving *C. koseri* infection. These studies show that *Citrobacter* flagellin induces early IL-10 expression and may alter the host response to favour chronic infection, resulting in brain abscess formation. Previous studies have shown that various pathogens can deregulate the host cytokine response as a survival mechanism during infection (Lucey *et al.*, 1996; Wilson *et al.*, 1998). For example, when human macrophages are infected with *Mycobacterium avium*, expression of the immunoregulatory cytokine IL-12 (type 1 cytokine) is suppressed, impairing the immune response that is pivotal in resistance to intracellular infections (Wagner *et al.*, 2002).

Fig. 6. Rat serum levels of IL-10 (a) and IL-12 (b) cytokines in response to *C. koseri*. Neonatal rat plasma was collected at 12 and 24 h after i.p. challenge with *C. koseri* or purified flagellin and measured by ELISA. Neonatal rat pups received i.p. administration of *E. coli* LPS (positive control, $n=2$), purified flagellin (FLG, $n=4$), heat-killed *C. koseri* (Δ WT, $n=2$), wild-type *C. koseri* (WT, $n=6$), mutant *C. koseri* SMT350 ($n=7$) or SMT356 ($n=7$). Rats administered saline (negative control) did not produce detectable levels (not shown). Data represent the mean $\pm$ SEM of duplicate samples.

Furthermore, it has been suggested that other highly adapted intracellular-replicating organisms inhibit the type 1-specific response by suppressing expression of its mediating cytokines (i.e. IL-12) and/or inducing cytokines that mediate a type 2-specific response (i.e. IL-10) (Grazia Cappiello *et al.*, 2001; Wagner *et al.*, 2002; Wilson *et al.*, 1998). Supporting studies have used lysates from *E. coli* and *Citrobacter* spp. and demonstrated inhibition of IL-12 and IL-4 production by lymphoid cells (Malstrom & James, 1998). This suggests that bacterial factors may be responsible for the induction or suppression of cytokine expression. Furthermore, it has been shown by others that Fc γ receptor ligation induces IL-10 upregulation, precluding IL-12 expression (Grazia Cappiello *et al.*, 2001; Sutterwala *et al.*, 1998). Our previous studies have documented that Fc γ RI is the major facilitator of opsonized *C. koseri* uptake in macrophages (Townsend *et al.*, 2003). This suggests a possible mechanism enabling an intracellular signal to induce a cytokine response by cell contact alone.

The ability of *C. koseri* to invade and persist within host cells is an important step in the development of chronic CNS infection. In this study, we have shown that the *fliP* mutation causes a significant change in pathogenesis and in the host immune response during *C. koseri* infection and reduces the uptake of *C. koseri* into human U937 macrophages *in vitro*. However, the *fliP* mutant strain SMT350 is hypervirulent, essentially converting a chronic disease into an acute infection. Moreover, we have established that *C. koseri* intracellular replication within macrophages is not sufficient for brain abscess formation to occur, as the attenuated intracellular replication phenotype was separable from brain abscess formation. Thus, mutants with attenuated abilities to replicate within macrophages were still able to cause brain abscess formation during experimental i.c. infection of neonatal rats (i.e. SMT332 and SMT335). Cytokine studies have shown that the immunosuppressive cytokine IL-10 is expressed during the first 12 h of serum exposure to purified flagellin, whilst this expression was delayed and reduced in the *fliP* mutant SMT350. Taken together, flagella expression may play a pivotal role in influencing the host immune response to *C. koseri* infection by inducing an environment conducive to maintenance of chronic infection. The specific role(s) of flagella during the pathogenesis of *C. koseri* CNS infection is multifaceted and requires further study.

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