Deletion of *tolA* in *Salmonella* Typhimurium generates an attenuated strain with vaccine potential

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The Gram-negative Tol-Pal system of envelope proteins plays a key role in maintaining outer membrane integrity and contributes to the virulence of several pathogens. We have investigated the role of one of these proteins, TolA, in the biology of *Salmonella enterica* serovar Typhimurium. Deletion of *tolA* rendered strain SL1344 more susceptible to killing by bile and human serum. In addition the mutant had impaired membrane integrity and displayed alterations in LPS production. The *tolA* mutant was highly attenuated in mouse infections via the oral and intravenous routes. Importantly, each phenotype displayed by the mutant was complemented by provision of *tolA* *in trans*. The *tolA* gene therefore contributes to virulence, membrane integrity, LPS production and bile and serum resistance in *S. enterica* serovar Typhimurium SL1344. Finally, immunization with the *tolA* mutant provided significant protection against subsequent challenge with wild-type SL1344. The Tol-Pal system is therefore a potential target in the development of novel attenuated live vaccines against *Salmonella* and other Gram-negative pathogens.

INTRODUCTION

Salmonella enterica is a diverse pathogen causing a range of diseases in many hosts, including humans and livestock. As the cause of typhoid fever, *S. enterica* serovar Typhi is of particular importance to human health, with an estimated annual incidence of 22 million cases and 200 000 deaths worldwide (Crump *et al.*, 2004). *S. enterica* serovar Typhimurium is an important cause of human gastroenteritis and has added significance as a model of human typhoid fever in the mouse.

The Tol-Pal system is well conserved among Gram-negative bacteria (Sturgis, 2001); it consists of (at least) five interacting envelope proteins TolQ, TolR, TolA, TolB and Pal. TolA–Q–R proteins form a complex in the inner membrane while TolB is a periplasmic protein associated with the outer-membrane protein Pal, which itself interacts with peptidoglycan (Henry *et al.*, 2004; Lazzaroni *et al.*, 2002; Lloubes *et al.*, 2001). The system plays numerous roles in the biology of Gram-negative bacteria, including LPS O-antigen surface expression, outer-membrane stability, uptake of filamentous phage DNA, resistance to detergents and virulence (Bowe *et al.*, 1998; Fortney *et al.*, 2000; Gaspar *et al.*, 2000; Heilpern & Waldor, 2000; Hellman *et al.*, 2002; Lazzaroni *et al.*, 1999; Sun & Webster, 1987; Vines *et al.*, 2005). For example, *tol-pal* mutants in the plant pathogen *Erwinia chrysanthemi* displayed reduced ability to grow in plant tissues, reduced motility, altered morphology and increased susceptibility to various anti-

microbial agents (Dubuisson *et al.*, 2005). In the case of *S. Typhimurium*, *tolB* was identified in a genome-wide screen as a virulence factor in mice contributing to resistance to deoxycholate and serum and bacterial survival in J774 cells (Bowe *et al.*, 1998). The system is also implicated in *S. Typhimurium* bile resistance following the finding that MudJ insertions in the *tolQRA* cluster result in a bile-sensitive phenotype (Prouty *et al.*, 2002). Other than these investigations, few data are available on the function(s) of this system and its individual components in *Salmonella*, including their role in virulence. A screen of a library of transposon mutants of *S. Typhimurium* in our laboratory identified a *tolA* mutant as highly attenuated in mice (unpublished observations). *tolA* was therefore selected for further study and found to be important in virulence, membrane integrity, LPS production and bile and serum resistance in *S. Typhimurium* SL1344. In addition, immunization with the *tolA* mutant offered protection against subsequent infection with wild-type SL1344.

METHODS

Generation of a *tolA* deletion mutant and its complementation.

The strains used in this study are shown in Table 1. A *S. Typhimurium* SL1344 *tolA* mutant was constructed by modification of the ET-cloning procedure (Mo *et al.*, 2006), replacing *tolA* with the kanamycin-resistance cassette from pUC4Kan (Amersham). PCR was used to amplify the antibiotic-resistance cassette with 5' and 3' 60 bp homology arms complementary to the flanking regions of *tolA* (see

Table 1. *S. Typhimurium* strains used

Name	Description	Source/reference
LB5010	Restriction-deficient derivative of LT2	Bullas & Ryu (1983)
SL1344	Wild-type for this study	Hoiseth & Stocker (1981)
SL1344 <i>tolA</i>	<i>tolA</i> deletion mutant in SL1344	This study
<i>tolA</i> pBR322- <i>tolA</i> ⁺	<i>tolA</i> mutant complemented with <i>tolA</i> cloned into pBR322	This study
<i>tolA</i> pBR322	<i>tolA</i> mutant carrying empty pBR322	This study
SL3261	<i>aroA</i> mutant of SL1344; well-characterized live attenuated vaccine strain	Hoiseth & Stocker (1981)

Table 2 for primer sequences). *S. Typhimurium* LB5010 was electroporated with the plasmid pBAD λ red and transformants selected on LB agar supplemented with 100 μ g ampicillin ml⁻¹. A single transformant was grown to an OD₅₉₅ of 0.25, then arabinose was added to 0.2% (w/v) to induce expression of the phage λ genes *exo*, *bet* and *gam* encoded by pBAD λ red and the culture grown to OD₆₀₀ 0.5. The bacteria were then electroporated with the PCR product generated above (approx. 200 pg, prepared using the Qiagen QIAquick PCR purification kit). Mutant colonies were selected on LB agar plates supplemented with 50 μ g kanamycin ml⁻¹. Allelic replacement of *tolA* was initially confirmed by PCR and sequencing of the product (Table 2).

The *tolA* mutation in *S. Typhimurium* LB5010 was transduced by P22 (Schmiegier, 1972) into strain SL1344. Transductants were selected on 50 μ g kanamycin ml⁻¹ and *tolA* deletion was confirmed by PCR and sequencing, as described above, and by Southern blotting using the kanamycin cassette as a probe. LPS serotype was confirmed by agglutination with O4 serotype antisera using O9 antisera as a negative control (Remel Europe/Oxoid).

For complementation, *tolA* from *S. Typhimurium* SL1344 was amplified by PCR (Table 2), purified, digested by *Hind*III and *Bam*HI, purified again and cloned into *Hind*III/*Bam*HI-cut pBR322 (New England Biolabs). The resultant plasmid was confirmed by sequencing and designated pBR322-*tolA*⁺. Empty pBR322 was used as a negative control and both were electroporated into the SL1344 *tolA* mutant with selection on LB plates containing ampicillin (100 μ g ml⁻¹). The presence of the correct plasmid was confirmed by PCR using primers flanking the cloning site (Table 2).

Growth *in vitro*. Overnight cultures were prepared in 10 ml LB broth by inoculation with three colonies from freshly streaked LB agar plates and incubated with shaking (180 r.p.m.) at 37 °C for 17.5 h. These cultures were diluted 1 : 100 000 into 100 ml fresh, pre-warmed

LB broth and incubated with shaking at 37 °C; samples were taken at the times indicated for OD₅₉₅ readings and viable counts on LB agar plates. Exponential generation times were calculated from growth rates between 4 and 6 h.

Motility and morphology. Overnight cultures, prepared as for growth *in vitro* above, were diluted 1 : 10 in sterile PBS and 1 μ l was inoculated into the centre of LB agar plates containing 0.4% agar.

Plates were incubated at 37 °C for 8 h and the radius of bacterial movement measured. Each experiment was performed in triplicate. For analysis of morphology, overnight cultures were scanned at 5 kV with a Phillips XL30 FEG scanning electron microscope at 5000–25 000 $\times$ magnification.

Bile resistance. Stationary-phase cultures were serially diluted and plated on LB agar supplemented with various concentrations of bovine bile (Sigma, catalogue number B3883). Colony-forming units were counted after overnight incubation at 37 °C.

Membrane integrity assay. Membrane integrity was assessed by release of intracellular RNase (Young & Silver, 1991). Cultures were streaked onto LB agar and overlaid with LB agar supplemented with 1% type VI *Torula* yeast RNA (Sigma). After overnight incubation plates were flooded with 0.5 M HCl for 10 min to precipitate the remaining RNA. Susceptibility to polymyxin B at 10 μ g ml⁻¹ (Sigma) was determined as described by Fields *et al.* (1989).

Serum resistance. Stationary-phase cultures were diluted to 10⁶ c.f.u. ml⁻¹ in LB supplemented with 50% human sera. Samples were incubated at 37 °C with shaking at 180 r.p.m. and viable counts taken at the times indicated. Untreated and heat-treated sera (45 min at 55 °C) were compared to investigate the importance of complement in antimicrobial activity. Mouse sera came from adult female BALB/c mice from Harlan.

Table 2. Primers used in this study

Name	Sequence (5'–3')	Use
<i>tolA</i> left	ctg cgt ccc gtt ggc ttg aaa gac ggc gag taa cag gcg aac agt ttt ttg gga acc gag gcc gcc gtc ccg tca agt cag <u>cg</u>	Generation of construct to delete <i>tolA</i> ; kanamycin cassette sequence underlined
<i>tolA</i> right	aca caa aac cac cag gac cag taa caa ctc aac tgc tgt aac ttc cat aaa gaa aag tat <u>ggg aaa gcc acg ttg tgt ctc</u>	Generation of construct to delete <i>tolA</i> ; kanamycin cassette sequence underlined
<i>tolA</i> test left	tga tcg cag aag taa agc gtc atc t	Confirmation of <i>tolA</i> deletion
<i>tolA</i> test right	ctg ggt gat ctg cat acg gac ttc t	Confirmation of <i>tolA</i> deletion
<i>tolAP</i> F2	ccc ggg <u>aag ctt</u> agt ctg cgt ccc gtt ggc ttg aaa gac ggc g	Complementation of <i>tolA</i> ; <i>Hind</i> III site underlined
<i>tolAP</i> R2	atc gat <u>gga tcc</u> cta cag ttt aaa gtc tag ttt ggc at	Complementation of <i>tolA</i> ; <i>Bam</i> HI site underlined
pBRseqIII R	gat ctt ccc cat cgg tga tgt cgg c	Confirmation of complementation construct
pBRseqIII L	acc att att atc atg aca tta acc t	Confirmation of complementation construct

LPS analysis. Stationary-phase cultures were harvested and resuspended in 1 ml PBS and 250 µl LPS buffer 1 (0.1875 M Tris/HCl pH 6.8; 6%, w/v, SDS; 30%, w/v, glycerol) and 20 µl of this resuspended cell pellet was boiled for 5 min. Samples were allowed to cool and 70 µl LPS buffer 2 (0.0625 M Tris/HCl pH 6.8; 0.1%, w/v, SDS; 10%, w/v, glycerol; 0.1%, w/v, bromophenol blue) was added along with proteinase K to a final concentration of 1.8 mg ml⁻¹. Samples were digested at 55 °C overnight, separated by electrophoresis using Novex 16% Tricine gels (Invitrogen) and visualized by silver staining. Equal loading was based on the viable count of the original culture.

Mouse infection models. Bacteria from fresh LB plates were grown statically in LB broth at 37 °C overnight. Cultures were then centrifuged at 4300 RCF for 10 min at 15 °C, resuspended in PBS and adjusted to the required concentration. Female BALB/c mice, 6–8 weeks old (Harlan), were inoculated intravenously with 10⁴ c.f.u. or by oral gavage under mild anaesthesia with 10⁸ c.f.u.. The dose was confirmed by viable count in LB agar. At the time points indicated, mice were sacrificed and the spleen, liver, Peyer's patches (oral infection only) and mesenteric lymph nodes (oral infection only) were removed into sterile water and homogenized using a Stomacher 80 Lab System (Seward). Bacteria were enumerated by viable counts in LB agar.

For protection studies mice were immunized intravenously with 10⁶ c.f.u.. At 13–14 weeks post-infection the primary infection was confirmed to have been cleared from the spleen and liver by viable counting (detection limit 1 c.f.u. per organ, *n*=2). Mice were subsequently challenged with wild-type SL1344 by the oral (10⁸ c.f.u.) and intravenous (10⁴ c.f.u.) routes. Viable counts in the spleen and liver were enumerated as described above. *tolA*-vaccinated mice were compared to unvaccinated age-matched control mice and mice immunized with the *aroA* mutant, SL3261.

All work was performed in compliance with UK Home Office regulations under licence.

Statistical analysis. Data were analysed by unpaired Student's *t*-test or one-way ANOVA using GraphPad Version 4, with *P*<0.05 considered as significant.

RESULTS

Growth *in vitro*, motility and morphology of SL1344 *tolA*

The growth of the *tolA* mutant in LB broth was compared to wild-type SL1344 as measured by viable count and OD₅₉₅ (Fig. 1). Growth of the *tolA* mutant was slightly impaired, as shown by a mean ($\pm$ SEM) exponential

generation time of 27.6 ($\pm$ 1.5) min compared to the wild-type generation time of 23.1 ($\pm$ 0.5) min (*P*=0.046, *n*=3 for both).

With regard to motility, there was no significant difference between SL1344 and SL1344 *tolA*. From one representative experiment the mean ($\pm$ SEM) radius of motility of the wild-type bacteria was 5.4 ($\pm$ 0.2) cm while that of the *tolA* mutant bacteria was 5.1 ($\pm$ 0.2) cm (*n*=3 for both).

Overnight cultures when examined by scanning electron microscopy showed no gross difference in morphology between SL1344 and SL1344 *tolA* (data not shown). Likewise a Gram stain of colonies from LB plates revealed no gross differences (data not shown).

tolA contributes to bile resistance in *S. Typhimurium*

MudJ transposon insertions in the *orfX-oft1-tolQRA* operon led to reduced resistance to bile in *S. Typhimurium* (Prouty *et al.*, 2002). However, none of these insertions were in *tolA* and so it is unclear if *tolA* itself contributes to bile resistance. To test directly if *tolA* does indeed influence bile resistance, cultures were plated onto LB agar supplemented with different concentrations of bovine bile. The *tolA* mutant was significantly more sensitive to killing by bile compared to the wild-type at bile concentrations of $\geq$ 2.5% (w/v) (Fig. 2). Confirming that this effect was due to deletion of *tolA*, this phenotype was complemented by introduction of pBR322-*tolA*⁺ but not by empty pBR322 alone (Fig. 2).

S. Typhimurium tolA has impaired membrane integrity

To test if *tolA* contributes to the membrane integrity of *S. Typhimurium* the release of intracellular RNase was assessed (Fig. 3a). In this qualitative assay more RNase was released by the *tolA* mutant than by wild-type SL1344 as shown by the zones of clearance surrounding colonies. Again, the wild-type phenotype was restored in the mutant by introduction of pBR322-*tolA*⁺ but not by the empty vector (Fig. 3a). To test the membrane integrity of the *tolA* mutant further, strains were exposed to the outer-membrane-damaging antimicrobial peptide polymyxin B. Compatible with impaired membrane integrity the *tolA*

Fig. 1. Growth of SL1344 *tolA* in LB broth. Overnight cultures of SL1344 wild-type (■) and SL1344 *tolA* (□) were inoculated into fresh pre-warmed LB and grown with shaking at 37 °C at 180 r.p.m. Growth was measured at the times indicated by viable counts (a) and OD₅₉₅ (b). The data are representative of three experiments giving similar results.

Fig. 2. Bile resistance in *S. Typhimurium* SL1344 wild-type and its *tolA* mutant. After overnight growth, LB broth cultures were diluted onto LB agar plates supplemented with bovine bile (Sigma) at different concentrations and the viable counts determined. The data (means $\pm$ SEM) are representative of three experiments each performed in duplicate. * $P < 0.05$ compared to wild-type. The dashed line indicates the limit of detection.

mutant was significantly more susceptible than wild-type SL1344 to killing by polymyxin B (Fig. 3b). Introduction of pBR322-*tolA*⁺ restored a wild-type phenotype to the *tolA* mutant, while the empty plasmid had no effect (Fig. 3b).

tolA enhances serum resistance in *S. Typhimurium*

Wild-type and *tolA* mutant bacteria were grown in 50 % human serum to investigate if *tolA* contributes to serum resistance (Fig. 4a). The *tolA* mutant was significantly more sensitive than the wild-type to human serum and this phenotype was complemented by pBR322-*tolA*⁺. All strains grew equally well in heat-treated serum, confirming the role of complement in serum killing (Fig. 4b). When human serum was replaced by mouse serum all strains grew equally well, even in untreated serum (data not shown), which is consistent with the fact that *S. Typhimurium* is highly resistant to mouse serum.

tolA contributes to LPS biosynthesis

Surface expression of *Escherichia coli* LPS O-antigen was significantly impaired in the absence of *tolA* (Gaspar *et al.*, 2000; Vines *et al.*, 2005). To visualize the effect of *tolA* deletion on LPS production in *S. Typhimurium*, proteinase K-digested bacterial lysates were separated by SDS-PAGE and silver stained (Fig. 5). In the *tolA* mutant the LPS molecule was slightly altered, with a reduction in the abundance of medium-length LPS polymers (Fig. 5a) and a concomitant increase in low-molecular-mass species most apparent in underdeveloped gels (Fig. 5b), i.e. when gels

Fig. 3. (a) Membrane leakage in *S. Typhimurium tolA*. Cultures were streaked onto LB agar and overlain with LB agar supplemented with 1 % type VI *Torula* yeast RNA (Sigma). After overnight incubation plates were flooded with 0.5 M HCl to precipitate the remaining RNA. Release of RNase is shown by a zone of clearance in the precipitation, indicated by white arrows. (b) Susceptibility to polymyxin B killing. Strains were grown in the presence of polymyxin B (10 μ g ml⁻¹) and viable counts after 1 h expressed as a percentage of the count in the absence of polymyxin B. The data are representative of two experiments giving similar results, each performed in duplicate. * $P < 0.05$ compared to wild-type.

were developed only long enough for the more abundant species to be apparent. Again, this phenotype was reversed by introduction of pBR322-*tolA*⁺ but not by the empty vector (Fig. 5).

tolA contributes to S. Typhimurium virulence in the mouse

The multiple effects of *tolA* deletion on the phenotype of SL1344 suggested that *tolA* might contribute to the virulence of *S. Typhimurium*. To test this, BALB/c mice were infected intravenously with 10⁴ c.f.u. of wild-type or *tolA* mutant bacteria and bacterial loads in the spleen and liver enumerated over time (Fig. 6). In both organs the bacterial count was significantly lower for mice infected with the *tolA* mutant versus those infected with wild-type SL1344. Unlike wild-type bacteria, the *tolA* mutant showed no net growth between days 1 and 3 post-infection and at

Fig. 4. Serum resistance of *S. Typhimurium* SL1344 wild-type and its *tolA* mutant. (a) Strains were incubated in 50% fresh human serum diluted in LB broth and viable counts taken at the times indicated. (b) Heat treatment to abolish complement activity was performed at 55 °C for 45 min. The data are means $\pm$ SEM of a representative experiment from three experiments each using different donor serum, each performed in duplicate and giving similar results. * $P < 0.05$ compared to wild-type.

day 3 post-infection wild-type counts were approximately 10^4 -fold greater than the mutant counts. The mutant phenotype was complemented by pBR322-*tolA*⁺ while the empty pBR322 vector had no effect (Fig. 6).

The contribution of *tolA* to virulence was further assessed following oral inoculation, the natural route of infection. Four days after oral infection, bacterial loads in the spleen, liver, Peyer's patches and mesenteric lymph nodes were enumerated (Fig. 7). In all four organs bacterial counts were significantly lower in the mice infected with the *tolA* mutant compared to those infected with wild-type bacteria. Just as with all the other *tolA* mutant phenotypes described here the defect was restored by pBR322-*tolA*⁺ but not by the empty pBR322 vector.

Immunization with SL1344 *tolA* protects against subsequent wild-type challenge

Following immunization with the *tolA* mutant, mice were rechallenged with wild-type SL1344 via the oral and intravenous routes (Fig. 8). In comparison with unimmunized age-matched control mice, the mice immunized with the *tolA* mutant had significantly reduced

Fig. 5. Analysis of LPS from *S. Typhimurium* SL1344 wild-type and its *tolA* mutant. Proteinase K-digested cell lysates from approximately 5×10^7 c.f.u. were separated by SDS-PAGE and silver stained. (a) Fully developed (no more bands likely to become apparent) and (b) underdeveloped (only the most abundant bands visible) gels show subtle alterations in LPS between wild-type and SL1344 *tolA*, indicated by brackets. Lanes: 1, SL1344; 2, SL1344 *tolA*; 3, SL1344 *tolA* pBR322-*tolA*⁺; 4, SL1344 *tolA* pBR322.

bacterial counts in the spleen and liver. The *tolA* mutant is therefore immunogenic and protective against subsequent challenge. However, immunization with the *tolA* mutant did not reduce bacterial counts as effectively as immunization with the *aroA* mutant SL3261 (Fig. 8).

DISCUSSION

The *tol-pal* system is relatively uncharacterized in *S. Typhimurium* but is likely to play key roles in the biology of this important pathogen given the functions of this complex in other Gram-negative bacteria. The *tolA* gene was selected for study because it was identified by us as a candidate gene contributing to bacterial virulence in a large-scale transposon mutagenesis screen in mice (unpublished observations). Therefore, to investigate the roles of the Gram-negative envelope protein TolA in the biology of

Fig. 6. Bacterial counts in the spleen (a) and liver (b) following intravenous infection with 10^4 c.f.u. *S. Typhimurium* SL1344 wild-type and its *tolA* mutant. The data are means $\pm$ SEM ($n=6-7$) pooled from two experiments giving similar results. * $P<0.05$ compared to wild-type.

Salmonella we generated a defined *tolA* deletion in *S. Typhimurium* SL1344. Various *tol-pal* gene deletions in *E. chrysanthemi*, including a *tolA* mutant, displayed altered morphology and decreased motility (Dubuisson *et al.*, 2005). Neither phenotype was shared by our *tolA* mutant in *S. Typhimurium*, suggesting species-specific effects for the *tol-pal* system. Previously transposon insertions in the *orfX-oft1-tolQRA* operon reduced resistance to bile in *S. Typhimurium*; however, none of these was in *tolA* (Prouty

et al., 2002). Our *S. Typhimurium tolA* mutant was more susceptible to bile than its wild-type counterpart; this appears to be the first demonstration of a role in bile resistance for the *tolA* gene. Resistance to bile is an important trait for enteric bacteria and the Tol-Pal system is clearly a vital component of this resistance in *S. Typhimurium*. This bile-sensitive phenotype may have contributed to the attenuation shown by the *tolA* mutant via the oral, but not the intravenous, route of infection.

Fig. 7. Bacterial counts in the spleen (a), liver (b), Peyer's patches (PP, c) and mesenteric lymph nodes (MLN, d) 4 days after oral infection with 10^8 c.f.u. *S. Typhimurium* SL1344 wild-type and its *tolA* mutant. The data are means $\pm$ SEM ($n=9-10$) pooled from two experiments giving similar results. * $P<0.05$ compared to wild-type.

Fig. 8. Immunization with SL1344 *tolA* protects against subsequent wild-type infection. Mice were immunized intravenously with 10^6 c.f.u. SL1344 *tolA* (□) or SL3261 (×). Following clearance of the immunizing strain, mice, including un-immunized age-matched controls (■), were subsequently challenged with wild-type SL1344 via the intravenous (10^4 c.f.u.) (a, b) and oral (10^8 c.f.u.) (c, d) routes. Bacterial counts in the spleen (a, c) and liver (b, d) were determined. Immunization with SL1344 *tolA* and SL3261 offered significant protection compared to un-immunized controls ($P < 0.05$). Data (means $\pm$ SEM, $n = 3-4$) are representative of two experiments giving similar results. Data for un-immunized mice at day 7 were not collected for ethical reasons, as the mice would not be expected to survive until this time point.

SL1344 *tolA* displayed reduced membrane integrity as demonstrated by release of intracellular RNase. This phenotype is shared with *tol-pal* mutants in *E. coli* and was seen following *tolB* deletion in *S. Typhimurium* (Bowe *et al.*, 1998). Bowe *et al.* (1998) suggested that this membrane phenotype could render *tolB* mutants more susceptible to harsh conditions in the host and thus contribute to reduced virulence via both the oral and intravenous routes. This is likely to apply to *tolA* mutants too. In support of this we found that deletion of *tolA* increased susceptibility to the antimicrobial peptide polymyxin B.

The *tolA* mutant was also more susceptible to killing by human serum. This effect was lost upon heating, showing that complement is required for this killing. No microbicidal effect was seen when using mouse serum; thus the complement-mediated serum killing of *S. Typhimurium* SL1344 depends on the source of serum. This agrees with previous data showing that mouse serum had little killing activity (Jones *et al.*, 1997; Nagy *et al.*, 2004; Ohno *et al.*, 1995). Indeed, even deep rough *Salmonella* mutants are not susceptible to killing by mouse serum (Ohno *et al.*, 1995).

It therefore seems that increased killing by serum does not contribute to the attenuation of the *tolA* mutant in the mouse.

In *E. coli*, *tolA* mutation reduces O-antigen surface expression through defective Wzy polymerization (Gaspar *et al.*, 2000; Vines *et al.*, 2005). We show here that *S. Typhimurium tolA* also displays a difference in LPS production but this is much more subtle than in *E. coli*, with only a small reduction in medium-length polymers and an associated increase in short-chain species. The latter effect was only apparent on underdeveloped gels, probably due to the abundance of these species causing rapid saturation of signal. LPS protects *Salmonella* against environmental assaults such as bile and complement and contributes to virulence (Miller *et al.*, 1989; Murray *et al.*, 2003, 2006; Ohno *et al.*, 1995; Prouty *et al.*, 2002; Thomsen *et al.*, 2003). Therefore, although subtle, this alteration of LPS in *tolA* may contribute to the other phenotypes described here. This may especially be the case with regard to resistance to human serum, which is associated with medium-length O-antigen containing between 4 and 15 repeat units (Murray *et al.*, 2006).

The SL344 *tolA* mutant was highly attenuated in mice following intravenous and oral inoculation, most likely as a result of the phenotypes we describe relating to impaired membrane integrity and altered LPS production. Attenuation was assessed based on viable counts in the spleen and liver following intravenous infection, and in the spleen, liver, Peyer's patches and mesenteric lymph nodes following oral inoculation. In each case the *tolA* mutant counts were significantly lower than those seen for wild-type SL1344, demonstrating the requirement for *tolA* for full virulence in these models. Work is now under way to compare the different Tol-Pal system components for the phenotypes described here. For example, will all the *Salmonella tol-pal* gene products be equally important for membrane integrity, LPS biosynthesis and virulence? Data from *E. chrysanthemi* suggests that they have differing influences (Dubuisson *et al.*, 2005).

Finally, immunization with SL1344 *tolA* offered significant protection against subsequent SL1344 wild-type challenge. This is believed to be the first demonstration that mutation of the *tol-pal* system has potential utility in the development of live attenuated vaccine strains in *Salmonella* and other Gram-negative pathogens.

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