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

G. K. Paterson, H. Northen, D. B. Cone, C. Willers, S. E. Peters and D. J. Maskell

Department of Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge CB3 0ES, UK

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

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

Table 2 for primer sequences. S. Typhimurium LB5010 was electroporated with the plasmid pBAD:red and transformants selected on LB agar supplemented with 100 μg ampicillin ml⁻¹. A single transformant was grown to an OD₆₀₀ 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: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 μg 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 (Schmieder, 1972) into strain SL1344. Transductants were selected on 50 μg 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 HindIII and BamHI, purified again and cloned into HindIII/BamHI-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 μg 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 OD₆₀₀ 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 μl 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 × 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 μg 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

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’-3’)</th>
<th>Use</th>
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</thead>
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<td>tolA left</td>
<td>ctt gct ccc gtt ggc tgg aaa gag gag tag cac gcg aac</td>
<td>Generation of construct to delete tolA; kanamycin cassette sequence underlined</td>
</tr>
<tr>
<td></td>
<td>agt ttt tgt gga acc gag gcc gcc gtc cgg tca aag gac cg</td>
<td>Generation of construct to delete tolA; kanamycin cassette sequence underlined</td>
</tr>
<tr>
<td>tolA right</td>
<td>aca caa aac cac cag cag cag taa cca ctc aac tgc tgt aag ctc ttc cat</td>
<td>Confirmation of tolA deletion</td>
</tr>
<tr>
<td></td>
<td>aaa gaa aag tat ggg aaa ggc acq tgg tgg ctc</td>
<td>Confirmation of tolA deletion</td>
</tr>
<tr>
<td>tolA test left</td>
<td>tga tcg cag aag taa agc gtc atc t</td>
<td>Confirmation of tolA deletion</td>
</tr>
<tr>
<td>tolA test right</td>
<td>ctt ggt gag ctc cat acq gac ttc</td>
<td>Confirmation of tolA deletion</td>
</tr>
<tr>
<td>tolAP F2</td>
<td>ccc ggg aag ctt aag ctt ccg gtt gcg tgg aag gag gag g</td>
<td>Combination of tolA; HindIII site underlined</td>
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<tr>
<td>tolAP R2</td>
<td>atc gga tgg cta cta cag ttt aaa ggt tgg at</td>
<td>Combination of tolA; HindIII site underlined</td>
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<tr>
<td>pBRseqIII R</td>
<td>gat ctt ccc cat cgg tga tgt cgg c</td>
<td>Confirmation of complementation construct</td>
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<tr>
<td>pBRseqIII L</td>
<td>acc att atc atg aca aca acc t</td>
<td>Confirmation of complementation construct</td>
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**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 OD$_{595}$ (Fig. 1). Growth of the tolA mutant was slightly impaired, as shown by a mean (±SEM) exponential generation time of 27.6 (±1.5) min compared to the wild-type generation time of 23.1 (±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 (±SEM) radius of motility of the wild-type bacteria was 5.4 (±0.2) cm while that of the tolA mutant bacteria was 5.1 (±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-of1-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 ≥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...
mutant was significantly more susceptible than wild-type SL1344 to killing by polymyxin B (Fig. 3b). Introduction of pBR322-\textit{tolA}$^+$ restored a wild-type phenotype to the \textit{tolA} mutant, while the empty plasmid had no effect (Fig. 3b).

**\textit{tolA} enhances serum resistance in \textit{S. Typhimurium}**

Wild-type and \textit{tolA} mutant bacteria were grown in 50% human serum to investigate if \textit{tolA} contributes to serum resistance (Fig. 4a). The \textit{tolA} mutant was significantly more sensitive than the wild-type to human serum and this phenotype was complemented by pBR322-\textit{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 \textit{S. Typhimurium} is highly resistant to mouse serum.

**\textit{tolA} contributes to LPS biosynthesis**

Surface expression of \textit{Escherichia coli} LPS O-antigen was significantly impaired in the absence of \textit{tolA} (Gaspar \textit{et al.}, 2000; Vines \textit{et al.}, 2005). To visualize the effect of \textit{tolA} deletion on LPS production in \textit{S. Typhimurium}, proteinase K-digested bacterial lysates were separated by SDS-PAGE and silver stained (Fig. 5). In the \textit{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 were developed only long enough for the more abundant species to be apparent. Again, this phenotype was reversed by introduction of pBR322-\textit{tolA}$^+$ but not by the empty vector (Fig. 5).

**\textit{tolA} contributes to \textit{S. Typhimurium} virulence in the mouse**

The multiple effects of \textit{tolA} deletion on the phenotype of SL1344 suggested that \textit{tolA} might contribute to the virulence of \textit{S. Typhimurium}. To test this, BALB/c mice were infected intravenously with $10^4$ c.f.u. of wild-type or \textit{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 \textit{tolA} mutant versus those infected with wild-type SL1344. Unlike wild-type bacteria, the \textit{tolA} mutant showed no net growth between days 1 and 3 post-infection and at
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 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
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-of1-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. 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 ± SEM (n=6–7) pooled from two experiments giving similar results. *P<0.05 compared to wild-type.

**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 ± SEM (n=9–10) pooled from two experiments giving similar results. *P<0.05 compared to wild-type.
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 bactericidal 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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