

ToIA mediates the differential detergent resistance pattern between the *Salmonella enterica* subsp. *enterica* serovars Typhi and Typhimurium

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The *tol-pal* genes are essential for maintaining the outer membrane integrity and detergent resistance in various Gram-negative bacteria, including *Salmonella*. The role of ToIA has been well established for the bile resistance of *Salmonella enterica* subsp. *enterica* serovar Typhimurium. We compared the bile resistance pattern between the *S. enterica* serovars Typhi and Typhimurium and observed that Typhi is more resistant to bile-mediated damage. A closer look revealed a significant difference in the ToIA sequence between the two serovars which contributes to the differential detergent resistance. The *tolA* knockout of both the serovars behaves completely differently in terms of membrane organization and morphology. The role of the Pal proteins and difference in LPS organization between the two serovars were verified and were found to have no direct connection with the altered bile resistance. In normal Luria broth (LB), *S. Typhi* $\Delta tolA$ is filamentous while *S. Typhimurium* $\Delta tolA$ grows as single cells, similar to the wild-type. In low osmolarity LB, however, *S. Typhimurium* $\Delta tolA$ started chaining and *S. Typhi* $\Delta tolA$ showed no growth. Further investigation revealed that the chaining phenomenon observed was the result of failure of the outer membrane to separate in the dividing cells. Taken together, the results substantiate the evolution of a shorter ToIA in *S. Typhi* to counteract high bile concentrations, at the cost of lower osmotic tolerance.

Received 2 November 2010

Revised 6 January 2011

Accepted 14 January 2011

INTRODUCTION

Members of the genus *Salmonella* are Gram-negative rod-shaped facultative anaerobic bacteria that can survive inside the host and cause persistent infection. The two important serovars of *Salmonella enterica* affecting humans are *Salmonella enterica* subsp. *enterica* serovar Typhi (*S. Typhi*) and *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*S. Typhimurium*). *S. Typhi* is a single host-adapted pathogen and infects only humans. It causes an acute systemic disease in humans with 1% of the cases becoming asymptomatic carriers, where the organism resides in the gall bladder and is excreted in stools (Lahiri *et al.*, 2010). *S. Typhimurium* causes gastroenteritis in humans and is able to infect a wide range of hosts including farm animals and birds (Lahiri *et al.*, 2009). Bile resistance is conferred through LPS and its modifications, the efflux pump genes *acr* and *emr*, the *tol* genes *tolQ*, *R*, *A* and *B* and *pal* genes (Gunn, 2000). The Tol–Pal complex of Gram-negative bacteria includes three cytoplasmic membrane proteins ToIA, TolQ and TolR, which interact with

each other through their transmembrane segments (Derouiche *et al.*, 1995; Lazzaroni & Portalier, 1981). The outer-membrane-anchored Pal lipoprotein interacts with the periplasmic TolB protein and the C-terminal domain of ToIA to form a complex (Bouveret *et al.*, 1995). Deletion or mutation of any of the *tol* or *pal* genes results in numerous defects, such as periplasmic leakage, increased susceptibility to many toxic compounds and formation of outer membrane vesicles (Bernadac *et al.*, 1998; Webster, 1991). ToIA contains three domains (Levengood *et al.*, 1991). Domain I is the N-terminal domain which anchors the protein to the inner membrane. Domain II or the central domain consists of an extended α -helix. Domain III is the C-terminal end, which binds to the Pal and TolB proteins (Walburger *et al.*, 2002).

The main targets of detergent action are the lipoproteins and phospholipids present in the outer membrane of bacteria. The lipoproteins are generally present in the inner leaflet of the outer membrane, facing the periplasm (Bos & Tommassen, 2004). The anchorage of the outer membrane is done by many components of the outer membrane, including porins, flagellar apparatus, type three secretion systems, the Tol–Pal complex and Bruan's lipoprotein (Lpp). Among these, the most important are the Tol–Pal

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Abbreviations: CLSM, confocal laser-scanning microscopy; DiBAC₄, bis-(1,3-dibutylbarbituric acid)-trimethine oxonol.

complex and the covalent linkage of Lpp and the diaminopimelic acid molecules of the peptidoglycan layer (Clavel *et al.*, 1998).

In a seminal finding, the role of the *tol* and *tol*-associated genes has been shown to be essential in the bile resistance in *S. Typhimurium* (Prouty *et al.*, 2002). In a subsequent elegant study, *tolA* was found to be important for virulence, membrane integrity and serum resistance as well (Paterson *et al.*, 2009). In this study, a marked difference was observed in the *tolA* sequence of the *S. Typhimurium* and *S. Typhi*. It was hypothesized that this difference could contribute to an altered detergent sensitivity of the serovars and comparative studies were carried out to understand the function of *tol* and its associated genes.

METHODS

Bacterial strains. *S. Typhimurium* strain NCTC 12023 and its gene knockouts, and *S. Typhi* CT18 WT and its gene knockouts were used in all the experiments. Bacteria were routinely cultured in Luria broth (LB) at 37 °C. Antibiotics were used as indicated in Table 1. Carbenicillin, nalidixic acid and kanamycin were used at 50 µg ml⁻¹ and chloramphenicol at 10 µg ml⁻¹.

Construction of gene deletions. Knockouts were constructed as described previously (Datsenko & Wanner, 2000). *Salmonella* transformants carrying a Red helper plasmid (pKD46) were grown in LB with carbenicillin and 10 mM L-arabinose at 30 °C to OD₆₀₀ 0.35–0.4 and then made electrocompetent by washing three times with ice-cold 10% glycerol and MilliQ water. A PCR product containing the chloramphenicol resistance gene (from pKD3) or kanamycin resistance gene (from pKD4) flanked by sequences upstream and downstream of the target gene(s) was obtained by using specific primers (Table 2). Electroporation was done according to the manufacturer's instructions using 500 ng PCR product (Bio-Rad). Transformants were selected on LB agar containing chloramphenicol or kanamycin. The knockouts were confirmed using specific primers. The single gene knockouts were also additionally confirmed with a primer specific for the *CAT* gene of pKD3 or *kan* gene of pKD4 as reverse primer. Primers used are listed in Table 2.

Construction of the double mutants. pKD46 was transformed into the *S. Typhimurium* Δ *dacD* strain and made electrocompetent as above. The PCR product containing the chloramphenicol resistance gene (from pKD3) flanked by sequences upstream and downstream of the *pbpG* or *ynhG* gene was electroporated into this strain. Transformants were selected on LB agar containing kanamycin and chloramphenicol. The double knockouts generated were confirmed using specific primers designed for both the *dacD* and/or *pbpG* or *ynhG* genes (Table 2).

Complementation of the *tolA* knockouts. *tolA* was amplified by PCR from the genomic DNA of *S. Typhi* and *S. Typhimurium* using a common set of primers, as the end sequences of *tolA* of both the serovars are similar. The gene was cloned into the *NcoI* and *BamHI* sites of the pBAD plasmid which has the tightly regulated *ara* promoter. The construct was transformed into *Escherichia coli* DH5 α and selected on an ampicillin plate. The transformants were confirmed by restriction digestion using *NcoI* and *BamHI*. From the true transformants, the plasmids were isolated and transformed into both the *tolA* knockout strains for self-complementation and cross complementation.

Bile and Triton X-100 sensitivity assay. The assay was carried out in 96-well plate in duplicates. Decreasing concentrations of bile in LB was added, starting with 20% and decreasing gradually to 0%. An overnight culture was adjusted to OD₆₀₀~0.3 and diluted to 10⁴ c.f.u. ml⁻¹. An aliquot (10 µl) of the diluted culture was added to each well. Plain LB with inoculum acted as the control for bacterial growth. The plate was then incubated for 12 h at 37 °C. The OD₆₀₀ readings were taken in an ELISA reader (Elix). To make the assay objective, OD₆₀₀ 0.1 was taken as the visible growth limit. The MIC for both the wild-types and the mutants was recorded as the minimum concentration of bile (Hi-media, bile salt mixture) with no visible growth. The same procedure was repeated for Triton X-100 (Sigma). These experiments were repeated at least three times.

Triton X-100 sensitivity assay under low osmolarity conditions. The assay was carried out in 96-well plates in duplicate. Overnight cultures in LB were adjusted to OD₆₀₀~0.3 and diluted to 10⁴ c.f.u. ml⁻¹. Aliquots (10 µl) of the diluted culture were added to different concentrations of Triton X-100 ranging from 16 to 2% in 0% NaCl-LB. So, in the 96-well plate the bacteria were added in an NaCl free medium. The plate was then incubated for 12 h at 37 °C and OD₆₀₀ was recorded. To make the assay objective, OD₆₀₀ 0.1 was taken as the visible growth limit. The MIC for both the

Table 1. List of bacterial strains used in this study

Bacterial strain	Antibiotic resistance	Source
<i>S. enterica</i> subsp. <i>enterica</i> serovar Typhimurium NCTC 12023 wild-type	Nalidixic acid	M. Hensel (Hölzer <i>et al.</i> , 2009)
<i>S. enterica</i> subsp. <i>enterica</i> serovar Typhi CT18 wild-type	None	PGIMER, Chandigarh
<i>S. Typhimurium</i> Δ <i>tolA</i>	Kanamycin	This study
<i>S. Typhi</i> Δ <i>tolA</i>	Kanamycin	This study
<i>S. Typhimurium</i> Δ <i>pal</i>	Kanamycin	This study
<i>S. Typhi</i> Δ <i>pal</i>	Kanamycin	This study
<i>S. Typhimurium</i> Δ <i>wzz</i>	Kanamycin	M. Hensel (Hölzer <i>et al.</i> , 2009)
<i>S. Typhimurium</i> Δ <i>fepE</i>	Kanamycin	M. Hensel (Hölzer <i>et al.</i> , 2009)
<i>S. Typhimurium</i> Δ <i>fepE</i> Δ <i>tolA</i>	Kanamycin, chloramphenicol	This study
<i>S. Typhimurium</i> Δ <i>dacD</i> Δ <i>pbpG</i>	Kanamycin, chloramphenicol	This study
<i>S. Typhimurium</i> Δ <i>dacD</i> Δ <i>ynhG</i>	Kanamycin, chloramphenicol	This study

Table 2. Compilation of deletion primers used in this study

Primer	Sequence (5'–3')
<i>tolA</i> deletion	Forward: gtgtcaaaggaaccgaacaaaacacaagctcaaacgggtgtaggcgggtgctgcttc Reverse: cagtttaaagctagtttggcatcctttatcttctacacatagaatatcctccttag
<i>tolA</i> deletion confirmation	Forward: atgcgatccgtgtcaaaggaaccgaacaa Reverse: atgcaagcttctacagtttaaagctagttt
<i>pal</i> deletion	Forward: atgcaactgaacaaagtgtgaaggcctgatgattgccctgtaggctggagctgcttc Reverse: tttagtaaacagctacagcgacggttcttagcgttagcccatagaatatcctccttag
<i>pal</i> deletion confirmation	Forward: agtcccatggccaactgaacaaagtgtgaag Reverse: agtcgatccgtaaacagctacagcgacgg
<i>dacD</i> deletion	Forward: atgctgttgaacccgtttgtttatgccgttctttatgtgtaggctggagctgcttc Reverse: ttacgctttatgctgaaaatagctcctcagacgagagaacacatagaatatcctccttag
<i>dacD</i> confirmation	Forward: agtcccatgggactgttgaacgccttg Reverse: agtcaagctttacgctttatgctgaa
CAT gene (pKD3) internal primer	cagaccgttcagctggat (used as reverse primer)
<i>pbpG</i> deletion	Forward: atgcttaatgccggaggtgtttacgatgatatacaagctgtaggctggagctgcttc Reverse: ttaacgggctccgctttgccgctttttcagccaccagcatagaatatcctccttag
<i>pbpG</i> deletion confirmation	Forward: agtcccatgggtgagaaaaaggctggcgca Reverse: agtcaagcttatcgttctgtgctggttgcg
pKD46 plasmid confirmation	Forward: atgacaccggacattatcctg Reverse: ttcgttctatggctcagc
<i>ynhG</i> deletion	Forward: ttaacgggctccgctttgccgctttttcagccaccagcatagaatatcctccttag Reverse: ctactgctgcgctgctgtttgccctctcccgcacaaacatagaatatcctccttag
<i>ynhG</i> deletion confirmation	Forward: agtcccatgggaaaacgtgctgttcttatt Reverse: agtcaagcttctactcgtcgcgctgctg

wild-types and the mutants was recorded as the minimum concentration of bile with no visible growth. This experiment was repeated at least three times.

Membrane damage assay using the bis-(1,3-dibutylbarbituric acid)-trimethine oxonol (DiBAC₄) system. Stationary phase bacteria (10^6 c.f.u. ml⁻¹) were pelleted, washed with PBS and treated with 14 or 2% bile salts in LB for 10 min. The bacteria were then washed with PBS and incubated with 1 µg DiBAC₄ ml⁻¹ (Invitrogen) for 10 min followed by two washes with PBS. The DiBAC₄-treated samples were further analysed in a FACS scanner (BD Biosciences), at an excitation of 470–490 nm, to check for the change in membrane permeability. This experiment was repeated three times and similar results were recorded.

Phase-contrast microscopy. An overnight culture of the bacteria was spread as a thin layer and a coverslip was placed over it. The bacteria were viewed at 40× magnification to identify the chaining phenotype of the strains and suitable fields were recorded by using the built-in camera (Leica). The experiment was repeated three times and representative images are shown.

Confocal laser-scanning microscopy (CLSM). Bacterial cells were grown in normal or 0% NaCl-LB. These bacterial cells were heat-fixed on a glass slide and stained with anti-LPS antibody (BD Biosciences, 1:100), diluted in blocking buffer (0.1% saponin, 2% BSA and 2% goat serum in PBS) for 1 h. Cells were then washed twice with PBS and incubated with the appropriate secondary antibody conjugated with Cy2 (Jackson's Lab, 1:200) in the blocking buffer. Then the cells were washed three times with PBS and stained with propidium iodide for 10 min. Samples were visualized using a Carl Zeiss confocal laser scanning microscope and over 20 fields were captured. The experiment was repeated at least three times for consistency. LSM image browser was used to analyse the images.

Murein sacculi isolation for electron microscopy. A bacterial pellet from overnight culture (5 ml) was dropped in test tubes containing 1 ml boiling 8% SDS, capped and boiled for 15 min and then transferred to a boiling water bath for 6 h. It was then kept overnight with gentle shaking at 30 °C. After 1 h in a boiling water bath, the sample was cooled to 40 °C. Sacculi suspension was spun at 900 000 g for 30 min at 25 °C in an ultracentrifuge (Beckman Coulter). The sacculi were resuspended in 2.5 ml 4% (w/v) SDS and incubated for 4 h in boiling water bath. The pelleted sacculi were sedimented as above, resuspended in 1% SDS and left overnight. They were then incubated for 2 h in a boiling water bath and pelleted by centrifugation. The pellet was resuspended in 5 ml distilled water. For storage at -20 °C, the sacculi were mixed with 1:1 ethylene glycol. The sample was applied over Formvar-coated copper grids and stained with 2% uranyl acetate. Images were captured using a transmission electron microscope (Joel cXII). The experiment was carried out three times and representative images are shown.

Statistical analysis and software. Each assay was repeated at least three times. *In vitro* data were analysed by paired *t*-test (two sample, equal variance) and *P*-values below 0.05 were considered significant. FACS data were plotted and analysed using WinMDI software. Confocal microscopy images were analysed using LSM imager software. SigmaPlot and Graph pad prism were used to plot all the results.

RESULTS AND DISCUSSION

ToIA of *S. Typhi* is shorter than *S. Typhimurium* by 27 amino acids

Both *S. Typhi* and *S. Typhimurium* are enteric pathogens and encounter bile in the intestinal lumen, but *S. Typhi* is

able to survive inside the gall bladder in pure bile. This phenomenon led to the hypothesis that the bile resistance between these two serovars might be different. *tolA* is an important gene that confers bile resistance (Prouty *et al.*, 2002), suggesting that the observed phenotypes may be governed by ToIA and could be modified by altering that particular protein.

The sequences of *S. Typhimurium* and *S. Typhi* ToIA available at GenBank were compared by using sequence alignment tools. When the ToIA sequences of *S. Typhimurium*, *S. Dublin*, *S. Paratyphi* and *S. Typhi* (two strains, CT18 and TY2) were aligned using CLUSTAL W, the *S. Typhi* ToIA was found to be 27 amino acids shorter (Fig. 1). The shortening occurs in domain II of the protein where an 11 amino acid tandem repeat appears 10 times in *S. Typhimurium* and only seven times in *S. Typhi*. Domain II joins the C-terminal membrane-binding region which is inserted into the cytoplasmic membrane and the N-terminal domain which is found in the periplasm. This N-terminal domain binds to the conserved domain of the Pal protein which is embedded in the inner leaflet of the outer membrane. Domain II has no known function except

as the structural feature which traverses the murein layer (Levengood *et al.*, 1991).

S. Typhi Δ tolA and S. Typhimurium Δ tolA are both highly sensitive to bile salts but differ in their sensitivity to Triton X-100

Previous reports suggest that *S. Typhimurium tolA* is important in bile resistance, membrane integrity, serum resistance and LPS biosynthesis. The *tolA* mutant is highly attenuated in a mouse model of infection and immunization with the mutant protecting the mice from subsequent lethal challenge with the wild-type *Salmonella* (Paterson *et al.*, 2009). In order to study the role of ToIA in bile salt resistance and the significance of shortening of domain II of ToIA in *S. Typhi*, the *tolA* of both *S. Typhimurium* and *S. Typhi* were deleted using the lamda-red recombinase system.

When both the wild-type *S. Typhi* and *S. Typhimurium* were grown in varying concentrations of bile salts in high NaCl concentration (normal LB), it was observed that *S. Typhi* had greater resistance to bile than *S. Typhimurium*. While *S. Typhimurium* showed retarded growth at >5%

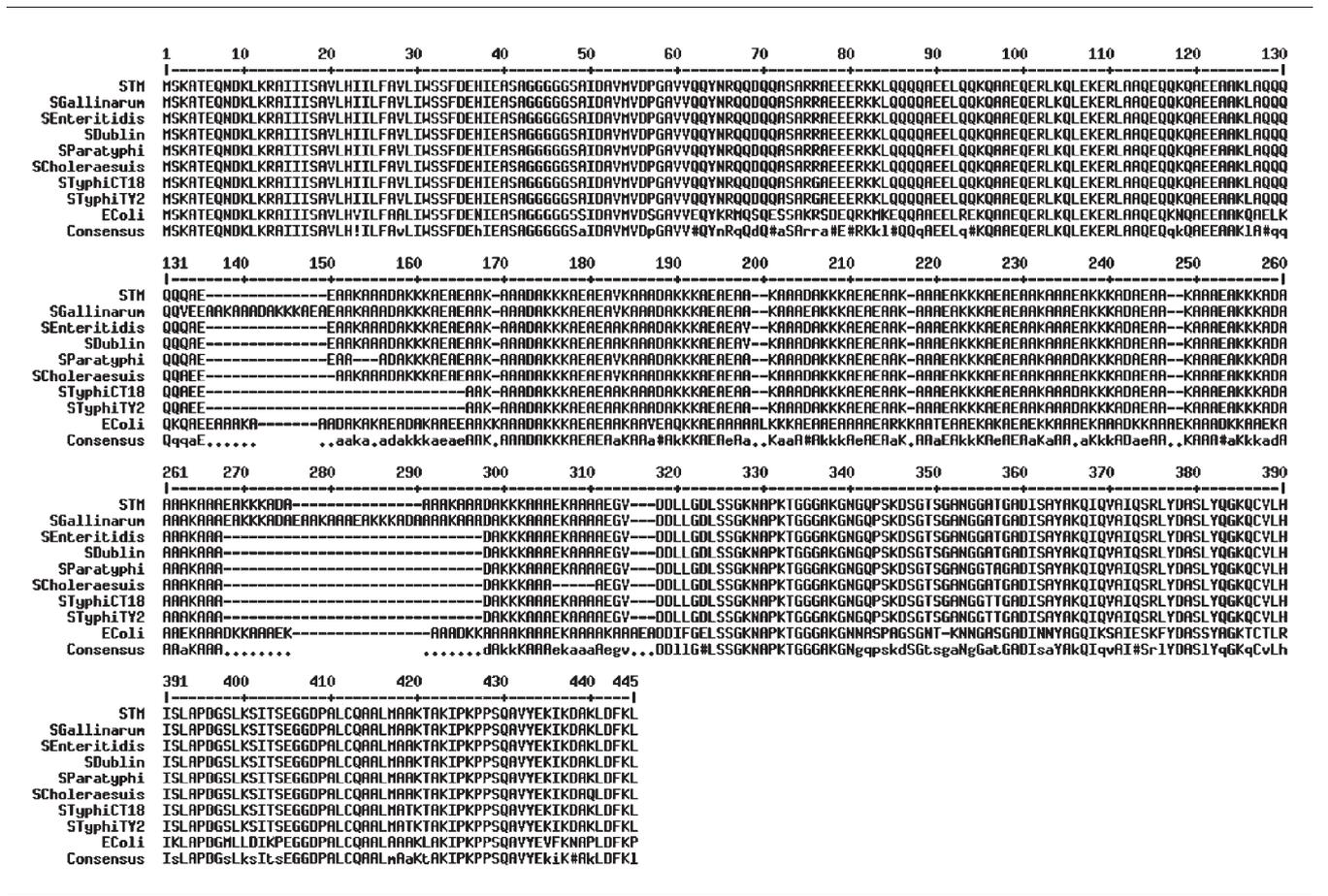


Fig. 1. ToIA of *S. Typhi* is shorter than that of *S. Typhimurium*. The amino acid sequences of ToIA were obtained from the GenBank database for *S. Typhimurium* (STM), *S. Gallinarum*, *S. Enteritidis*, *S. Dublin*, *S. Paratyphi*, *S. Choleraesuis*, *S. Typhi* (two strains, CT18 and TY2) and *E. coli* K-12. The sequences were aligned using CLUSTAL W software.

NaCl, *S. Typhi* grew even at concentrations as high as 10%. However, it was found that both *S. Typhimurium* $\Delta tolA$ and *S. Typhi* $\Delta tolA$ were highly sensitive to bile salts at high salt concentrations (Fig. 2a). Bile tolerance is provided by bile salt sensing mechanisms like the PhoP/Q two-component system, which signals LPS modification and through the action of *acr*, *emr* efflux pumps. The role of these sensors and efflux pumps can be ruled out as a reason for this difference in acute bile salt tolerance between the two serovars as the *tol* mutants, which have intact sensing mechanisms, display high sensitivity to bile's detergent action. Thus, we did not look into the sensors and the efflux pumps.

Triton X-100 was chosen for study as it is a non-ionic detergent. When the growth of *S. Typhimurium* and *S. Typhi* wild-types and mutants in the presence of Triton-X was analysed, no significant difference was found between the wild-types. However, surprisingly *S. Typhimurium*

$\Delta tolA$ was found to be resistant to Triton X-100 to the level of wild-type *S. Typhimurium*, as it was able to grow even at 12% Triton X-100. On the other hand, *S. Typhi* $\Delta tolA$ was highly sensitive and failed to grow even at 2% Triton X-100 (Fig. 2b). This difference in the sensitivity pattern between the *S. Typhimurium* and *S. Typhi* *tolA* mutants suggests the probability of another additional mechanism(s) in *S. Typhimurium* that substitutes the function of TolA, which is absent in *S. Typhi*. Lpp and the Tol-Pal machinery have similar functions in terms of outer-membrane anchoring (Cascales *et al.*, 2002).

Pal mutants of *S. Typhimurium* and *S. Typhi* behave similarly to their TolA mutant counterparts

The function of the Tol-Pal complex is performed by the combined action of all the proteins in the complex, and deletion of any of the TolQ, R, A, B or Pal proteins led to the loss of membrane integrity (Godlewska *et al.*, 2009). However, some functions of these proteins can be performed by individual member proteins in the absence of interacting partners. We then checked if these defects, caused by knockout of *tolA*, are because of the membrane tethering function of the TolQ, R, A, B and Pal proteins or are a specific function of the TolA protein. The Pal protein, which is present in the outer membrane and binds to the N-terminus of TolA, was knocked out. When the *pal* mutant was checked for both bile salt and Triton-X resistance it was found to behave similarly to the *tolA* mutants. Both *S. Typhimurium* and *S. Typhi* *palA* mutants were highly sensitive to bile salts (Fig. 3a). The Triton X-100 sensitivity was also similar to the *tolA* mutant where *S. Typhimurium* $\Delta palA$ was found to be as resistant as the wild-types (Fig. 3b).

The damage caused by bile salts is primarily because of their detergent action on the lipids of the outer membrane which leads to membrane damage. The damage to the outer membrane can be quantified by measuring the outer membrane potential of the bacteria. DiBAC₄ dye only enters the cells that have lost outer membrane integrity. A measure of the entry of this dye would estimate the level of membrane damage. *S. Typhimurium* and *S. Typhi* wild-types and their *tolA* mutants were exposed to bile salts followed by DiBAC₄ staining. The cells were then analysed by FACS for uptake of the dye. After 10 min exposure to 14% bile, *S. Typhimurium* wild-type showed more membrane damage than *S. Typhi* (Fig. 4). The number of bacterial cells with membrane damage increased by 50% in *S. Typhimurium*, whereas there was no change in membrane damage in *S. Typhi* following bile salt exposure. *S. Typhi* $\Delta tolA$ and *S. Typhimurium* $\Delta tolA$ were highly sensitive and even 2% bile induced extensive damage in both the mutants (data not shown). This increased damage to the wild-type *S. Typhimurium* following acute bile exposure shows that it is because of improper barrier function to the entry of bile salts at the outer membrane.

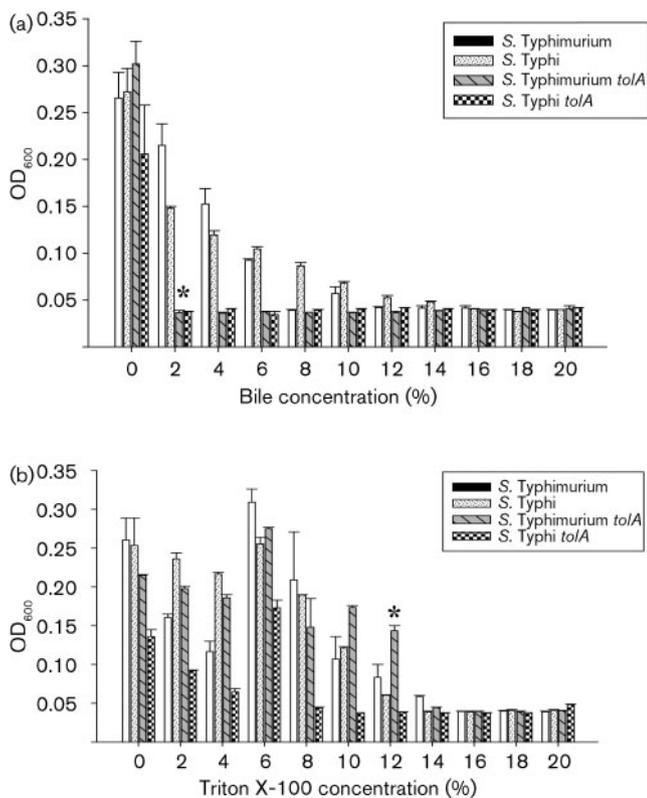


Fig. 2. Bile and detergent sensitivity of *S. Typhi* $\Delta tolA$ and *S. Typhimurium* $\Delta tolA$. *S. Typhimurium* wild-type, *S. Typhimurium* $\Delta tolA$, *S. Typhi* wild-type and *S. Typhimurium* $\Delta tolA$ (100 c.f.u. ml⁻¹) were inoculated in LB containing different concentrations of (a) bile salts or (b) Triton X-100 ranging from 0 to 20%. The assay was carried out in duplicate in 100 μ l volumes in a 96-well plate. The plates were incubated at 37 °C for 12 h and the bacterial growth was assayed by measuring OD₆₀₀. Experiments were performed in triplicate on three different occasions and means \pm SD are shown. Asterisks, **P* < 0.05 (Student's *t*-test).

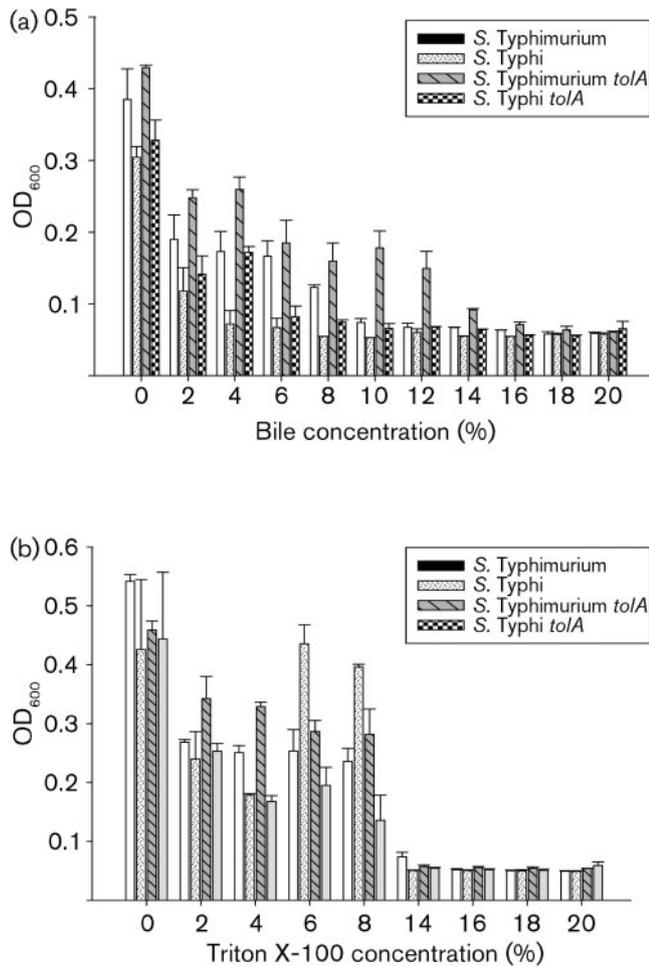


Fig. 3. *pal* knockouts of *S. Typhimurium* and *S. Typhi* behave similarly to their *toIA* knockout counterparts. *S. Typhimurium* wild-type, *S. Typhimurium* Δpal , *S. Typhi* wild-type and *S. Typhi* Δpal ($100 \text{ c.f.u. ml}^{-1}$) were inoculated in LB containing different concentrations of (a) bile salts and (b) Triton X-100 ranging from 0 to 20%. The assay was carried out in duplicate in 100 μl volumes in a 96-well plate. The plates were incubated at 37 °C for 12 h and the bacterial growth was assayed by measuring OD₆₀₀. Experiments were performed three times and representative data are shown. Bars show means \pm SD.

In LB medium, *S. Typhi* $\Delta toIA$ is filamentous while *S. Typhimurium* $\Delta toIA$ grows as single cells similar to wild-type

It has been shown previously that in the *Erwinia* species, the *tol* mutants had a severe growth and motility defect (Dubuisson *et al.*, 2005). It was also observed that unlike the wild-type species, the mutants had a very short and twisted flagellar morphology. In the case of *Salmonella*, a recent report indicates similar findings, where the growth of the *S. Typhimurium toIA* mutant was slightly impaired with no difference in motility and morphology (Paterson *et al.*, 2009). It was observed that *S. Typhi* $\Delta toIA$ always grew poorly compared with *S. Typhimurium* $\Delta toIA$ in LB.

This is also consistent with the fact that the wild-type *S. Typhi* also grows more poorly than wild-type *S. Typhimurium* (data not shown). Surprisingly, it was seen that in LB, *S. Typhi* $\Delta toIA$ grew in chains whereas the *S. Typhimurium* and *S. Typhi* wild-types and the *S. Typhimurium* $\Delta toIA$ mutant grew as single cells. The filaments of *S. Typhi* $\Delta toIA$ consisted on average of 10–20 bacterial cells attached end-to-end along the long axis. Confocal staining of bacteria was done with anti-LPS antibody, which stains the outer membrane, and propidium iodide, which stains the bacterial chromosome. The bacterial nucleoids were arranged along the length of the filament at equal lengths, suggesting that bacterial chromosomal segregation was taking place but that the daughter cells of the bacteria were unable to separate. Also, the LPS stain was found to be intact even at the septa, suggesting that only the outer membrane fails to separate completely (Fig. 5).

Filamentation is not due to the failure of murein sacculus separation during cell division

Filamentation was seen in bacteria due to the failure of separation of the recently divided daughter cells of the bacteria. The failure of septation during cell division in a Gram-negative bacterium can take place at three different stages: at the cytoplasmic membrane, the peptidoglycan layer or the outer membrane. Invagination of the cytoplasmic membrane is helped by the contraction of the ring formed by FtsZ along with a host of other cell division proteins (Addinall & Holland, 2002). The cleavage of the peptidoglycan layer is done by a host of peptidoglycan murein hydrolases and amidases (Heidrich *et al.*, 2001, 2002), whereas the outer membrane invagination is a passive process and it generally follows the cytoplasmic membrane and the peptidoglycan layer. This outer membrane invagination is believed to be aided by the Tol protein complex which anchors the outer membrane to the inner cytoplasmic membrane (Gerding *et al.*, 2007). In order to determine the stage at which failure of the daughter cell separation has occurred in *S. Typhi* $\Delta toIA$, the murein sacculi were isolated and examined by transmission electron microscopy (TEM). The murein sacculus of the wild-types and *toIA* knockouts of the two serovars appeared as single cell sacculi and with completely separated murein (Fig. 6a). It was surprising to see that the murein sacculi of *S. Typhi* $\Delta toIA$, which grows as chains in LB, were completely separated and similar to the wild-type murein sacculus (Fig. 6b). This shows that the filamentation is due to the failure of outer membrane invagination and in *S. Typhi* $\Delta toIA$, the separation of the peptidoglycan layer is complete.

S. Typhimurium $\Delta toIA$ grows in chains in low osmolarity LB medium

Previous studies have shown that in low osmolarity conditions, *E. coli toIA* mutants show a chaining phenotype

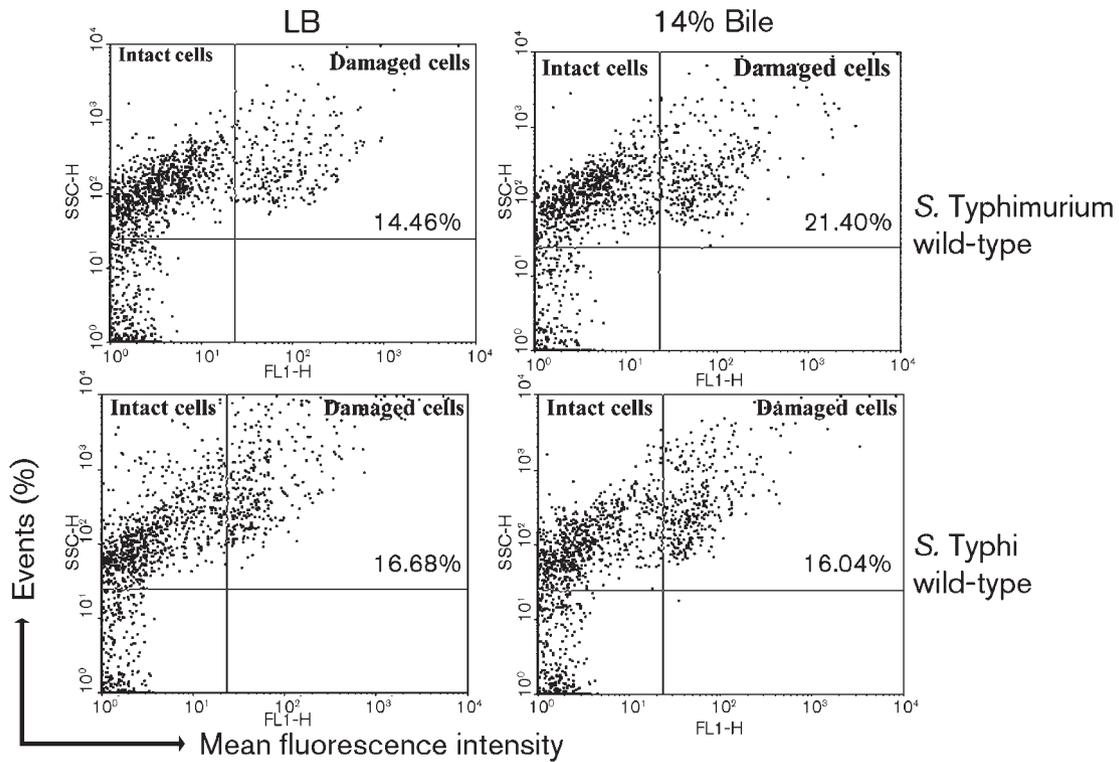


Fig. 4. *S. Typhi* is more resistant than *S. Typhimurium* to acute bile salt exposure. Bacterial strains were exposed to acute bile salts for 10 min and the extent of membrane damage was assessed by measuring the incorporation of DiBAC₄ dye using FACS. The data are representative of three independent experiments.

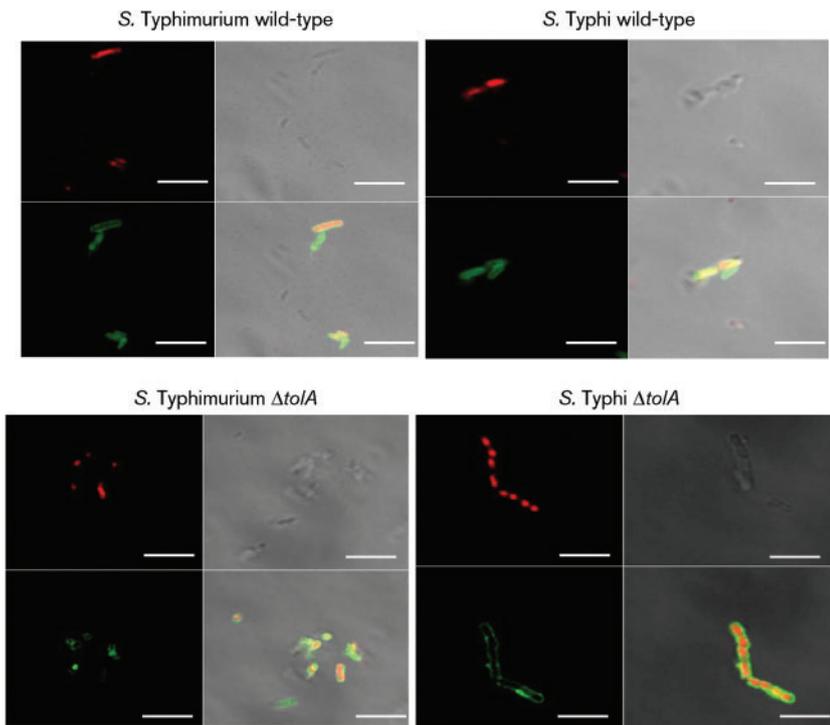


Fig. 5. *S. Typhi* $\Delta toIA$ is filamentous, while *S. Typhimurium* $\Delta toIA$ grows as single cells similar to wild-type in normal LB media. Morphology of *S. Typhimurium* wild-type, *S. Typhimurium* $\Delta toIA$, *S. Typhi* wild-type and *S. Typhi* $\Delta toIA$ grown in LB with 0.5% NaCl was observed by using CLSM. The bacteria were stained by using anti-LPS primary antibody and Cy2-conjugated secondary antibody to stain the cell surface (green) and propidium iodide to stain the bacterial nucleoid (red). The data are representative of several fields taken from three independent experiments. Scale bar, 5 μ m.

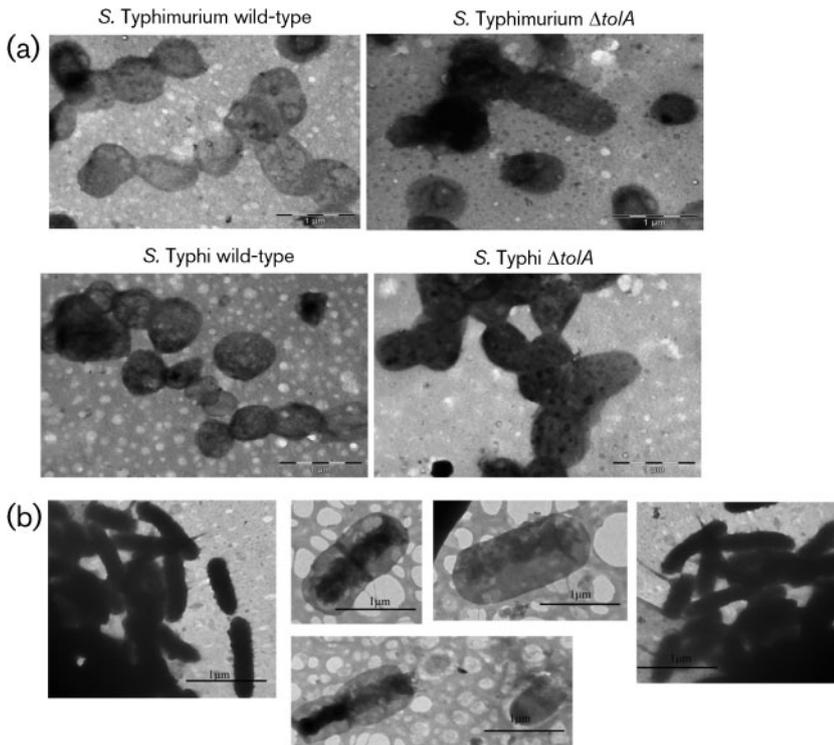


Fig. 6. Separation of the murein sacculus during cell division is not affected by *tolA* knockout. The murein sacculi were isolated from stationary phase bacteria grown in normal LB by boiling them in decreasing concentrations of SDS. The isolated murein sacculi were visualized by using TEM after uranyl acetate staining. (a) The murein sacculi of *S. Typhi* wild-type, and *S. Typhimurium* wild-type and $\Delta tolA$ appeared as single isolated murein sacs. *S. Typhimurium* $\Delta tolA$ is also shown for comparison. (b) The murein sacculi of *S. Typhimurium* $\Delta tolA$ appears mostly as single isolated sacs or rarely as doublets with visible septal rings indicating dividing cells. There was no filamentous sacculus observed even though the bacteria grew in chains in normal LB. The data are representative of many fields taken from three independent experiments. Bars, 1 μ m.

(Meury & Devilliers, 1999). To check if the effect on *Salmonella* was similar, *S. Typhimurium* $\Delta tolA$ and *S. Typhi* $\Delta tolA$ were grown in LB containing 0.5% NaCl and low osmolarity LB, containing 0% NaCl. *S. Typhimurium* $\Delta tolA$, which grows as a single cell suspension in LB, starts chaining when grown in low osmolarity LB (Fig. 7a). On the other hand, *S. Typhi* $\Delta tolA$, which grows as chains in 0.5% NaCl-containing LB, failed to grow in LB with 0% NaCl. This clearly shows that *S. Typhi* has low osmolarity tolerance compared with *S. Typhimurium*. This led to the hypothesis that low osmolarity leads to the chaining phenotype which is due to failure of outer membrane invagination as shown in the TEM analysis of murein sacculi of *S. Typhi* $\Delta tolA$. Low osmolarity leads to an increase in the volume of the periplasmic space. In the absence of tethering protein TolA, the outer membrane fails to follow the invaginating peptidoglycan layer leading to chaining phenotype. One of the other major mechanisms by which outer membrane is attached to the peptidoglycan layer is through a covalent linkage between Lpp and the diaminopimelic acid residue of the peptidoglycan. This mechanism could be disrupted in *S. Typhi* as it shows chaining in the absence of TolA even in normal LB.

***S. Typhimurium* $\Delta tolA$ fails to grow in the presence of Triton X-100 in low osmolarity conditions**

The observation that *S. Typhi* $\Delta tolA$ grows as chains in 0.5% NaCl containing LB and is sensitive to growth in

the presence of Triton X-100 hints at the possibility that *S. Typhimurium* $\Delta tolA$, which starts chaining in low osmolarity LB, might also become sensitive to Triton X-100. *S. Typhimurium* and *S. Typhi* wild-type and the *tolA* mutants were grown in the presence of various concentrations of Triton X-100 and bile salts in LB containing 0% NaCl. While growth in the presence of bile salts in low osmolarity LB (Fig. 7b) was similar to that in normal LB (Fig. 2a), growth of the *tolA* knockouts of both the serovars was sensitive to these conditions (Fig. 7b). It was observed that *S. Typhimurium* $\Delta tolA$, as expected, became sensitive to growth at 2% Triton X-100, indicating that irrespective of the salt concentration, filamentous bacteria lacking *tolA* are sensitive to growth in the presence of Triton X-100 (Fig. 7c).

To show that the sensitivity to Triton X-100 is due to low osmolarity and not due to the absence of NaCl, the experiment was repeated with varying concentrations of NaCl – an ionic osmolarity agent – and sucrose – a non-ionic osmolarity agent. The growth was restored by increasing concentrations of both NaCl (Fig. 8a) and sucrose (Fig. 8b) and the growth tapered off at high osmolarity in both cases. Growth in the presence of sucrose was lower than in the presence of NaCl in absolute terms. This is probably because of the requirement for NaCl as a nutrient rather than as an osmolarity agent. The MIC and minimum bactericidal concentration (MBC) of various detergents against the wild-types and *tolA* mutants are given in Table 3.

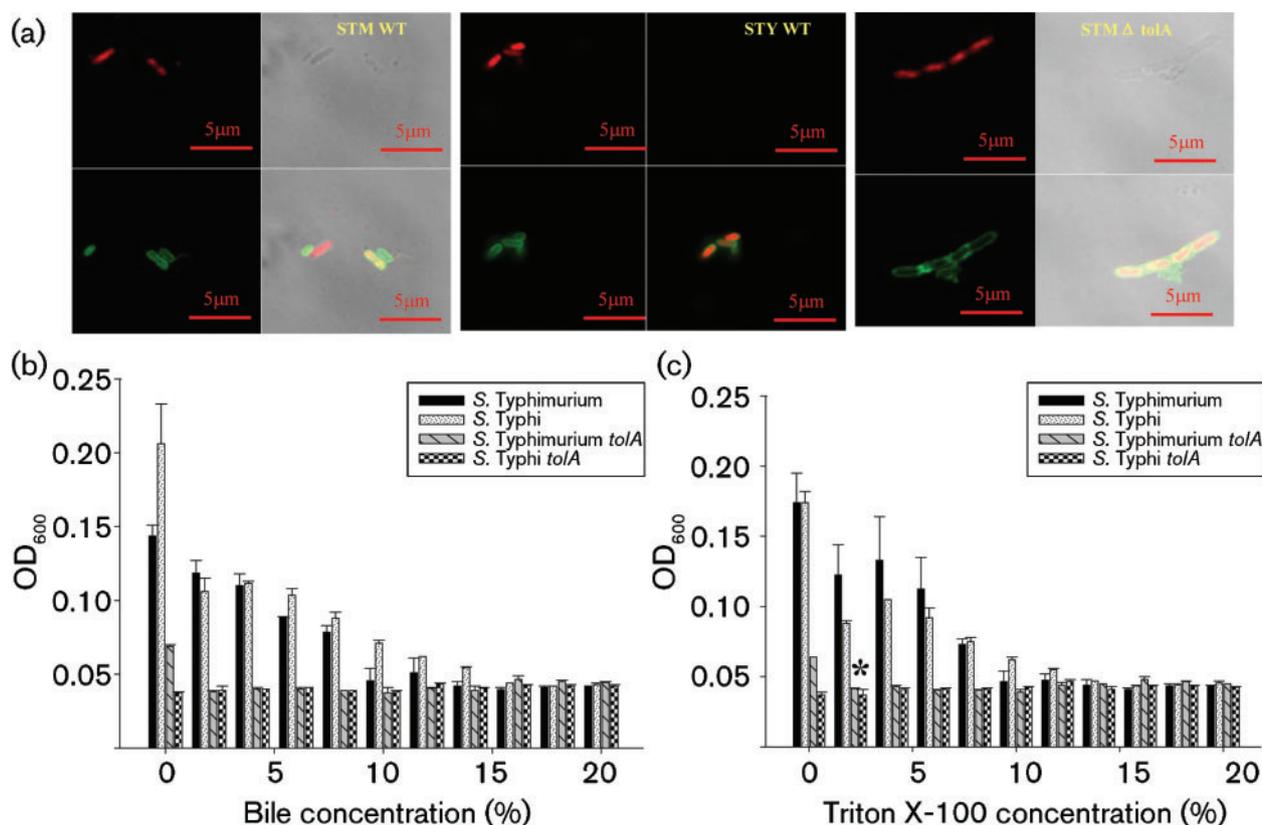


Fig. 7. *S. Typhimurium* ΔtoA starts filamenting in low osmolarity LB media. *S. Typhimurium* and *S. Typhi* ΔtoA strains were grown in normal LB with 0.5% NaCl and in low osmolarity LB with 0% NaCl. (a) The bacteria were surface stained with anti-LPS antibody (green) and the bacterial nucleoid was stained with propidium iodide (red). The cells were viewed by using CLSM with UV illumination. (b, c) *S. Typhimurium* wild-type, *S. Typhimurium* ΔtoA , *S. Typhi* wild-type and *S. Typhimurium* ΔtoA ($100 \text{ c.f.u. ml}^{-1}$) were inoculated in low osmolarity LB with 0% NaCl containing different concentrations of (b) bile salts and (c) Triton X-100 ranging from 0 to 20%. The assay was carried out in duplicate in 100 μl volumes in a 96-well plate. The plates were incubated at 37 °C for 12 h and the bacterial growth was assayed by measuring OD_{600} . Experiments were performed in triplicate on three different occasions and the means \pm SEM are shown. The data in (b) were not significant (Student's *t*-test); asterisk (c) indicates significant difference, * $P < 0.05$.

The filamentation phenotype is not due to *fepE* of *S. Typhimurium*, which is a pseudogene in *S. Typhi*

The outer membranes of the two serovars are quite different and one of the significant differences is in the LPS, which is a major outer membrane constituent. The LPS of the two serovars differ in major side chain modifications and in the presence of very long chain polysaccharide in *S. Typhimurium*, which is absent in *S. Typhi*. This is because the gene responsible for very long chain synthesis, *fepE*, is a pseudogene in *S. Typhi*. While the *fepE* gene mediates the polymerization of the very long chain, *wzz* mediates that of the long chain (Murray *et al.*, 2003). In order to rule out the role of very long chain LPS in the differential bile resistance of the two serovars, we deleted *fepE* and *wzz* from *S. Typhimurium* and studied the bile resistance pattern. There was no significant change in sensitivity to growth in the presence of bile salts in either the *fepE* or *wzz* knockout strains compared with the wild-type (data not shown). *S. Typhimurium toIA-fepE* double

knockout also showed a bile salt sensitivity pattern similar to the *S. Typhimurium toIA* single knockout (Fig. 8c). These findings suggest that shortening of *ToIA* in *S. Typhi* leads to better detergent resistance in isotonic conditions and poor detergent resistance in hypotonic conditions and does not involve the long and very long chains of LPS.

S. Typhi toIA is able to complement only *S. Typhi* $\Delta toIA$ but not *S. Typhimurium* $\Delta toIA$

To implicate a shorter *ToIA* as the reason for increased bile salt resistance in *S. Typhi*, it was necessary to trans-complement *S. Typhi toIA* in *S. Typhimurium* $\Delta toIA$ and to determine whether it imparts better bile salt resistance than its endogenous *toIA*. Both *S. Typhimurium* $\Delta toIA$ and *S. Typhi* $\Delta toIA$ were cloned under the highly stringent and inducible *ara* promoter. The two cloned *toIA* genes were introduced into both the serovars. The complementation was measured as the restoration of wild-type resistance

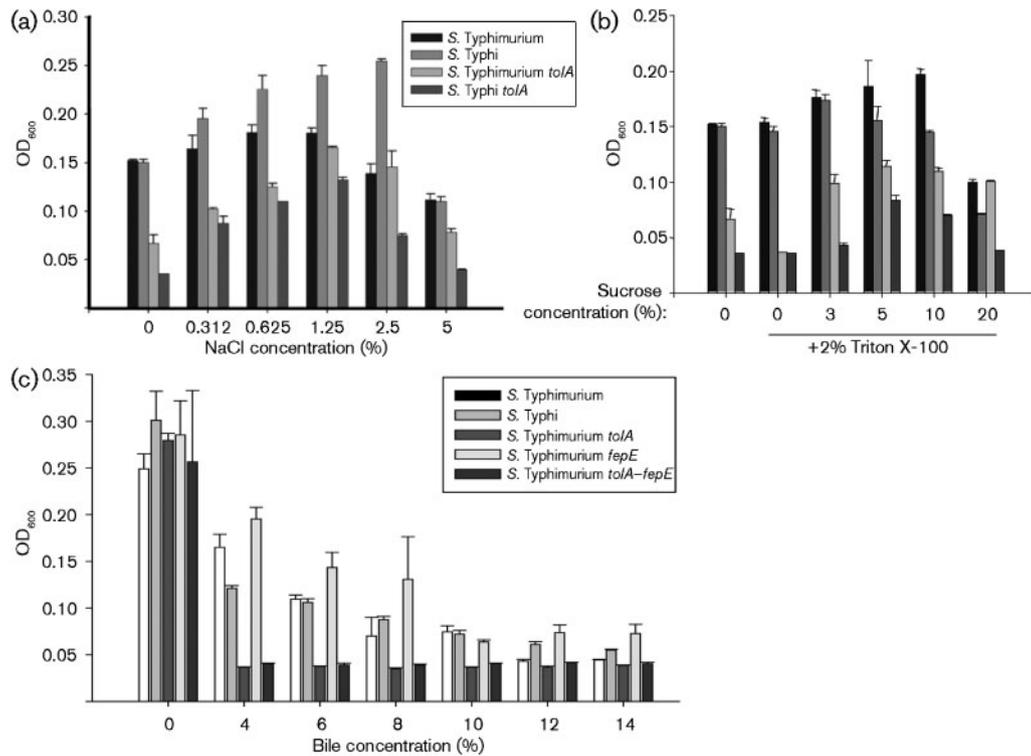


Fig. 8. Growth of the *tolA* mutants in the presence of Triton X-100 is rescued by increase in osmolarity. *S. Typhimurium* wild-type, *S. Typhimurium* Δ *tolA*, *S. Typhi* wild-type and *S. Typhimurium* Δ *tolA* (100 c.f.u. ml⁻¹) were inoculated in LB with different concentrations of osmolarity agents: (a) NaCl (ionic) ranging from 0 to 5 % and (b) sucrose (non-ionic) ranging from 0 to 20 %. All of the media wells contained 2 % Triton X-100, whereas the control wells contained LB with 0 % NaCl and no Triton X-100. (c) *S. Typhimurium* wild-type, *S. Typhimurium* Δ *tolA*, *S. Typhimurium* Δ *tolA* Δ *fepE* and *S. Typhi* wild-type (100 c.f.u. ml⁻¹) were inoculated in LB with different concentrations of bile salts ranging from 0 to 14 %. The assay was carried out in duplicate in 100 μ l volumes in a 96-well plate. The plates were incubated at 37 °C for 12 h and the bacterial growth was assayed by measuring OD₆₀₀. The data are representative of at least three independent experiments.

pattern against bile salts. However, all four complemented strains (*S. Typhimurium* Δ *tolA*::*S. Typhimurium tolA*, *S. Typhimurium* Δ *tol*::*S. Typhi tolA*, *S. Typhi* Δ *tolA*::*S. Typhi tolA* and *S. Typhi* Δ *tolA*::*S. Typhimurium tolA*) failed to grow even in the presence of 2 % bile salts. This failure to restore the wild-type phenotype fully can be explained by the fact that TolA is one member of

the multiprotein complex TolQ, R, A, B and Pal; the stoichiometry of interaction here is very important and unless the entire operon was cloned, full complementation would not be possible. However, partial complementation was observed as *S. Typhimurium* Δ *tolA* which grows as chains in 0 % NaCl-LB, started growing as single cells similar to wild-type *S. Typhimurium* when complemented

Table 3. MIC and MBC of various detergents against *S. Typhimurium* and *S. Typhi* wild-type and *tolA* mutants

STM, *S. Typhimurium*; STY, *S. Typhi*.

	MIC			MBC	
	Bile (%) 0.5 % NaCl-LB	Triton X (%) 0.5 % NaCl-LB	Triton X (%) 0 % NaCl-LB	Bile (%)	Triton X (%)
STM	12	12	12	<10	>20
STY	14	12	12	14	>20
STM Δ <i>tolA</i>	<2	12	<2	<8	12
STY Δ <i>tolA</i>	<2	<2	<2	<8	8

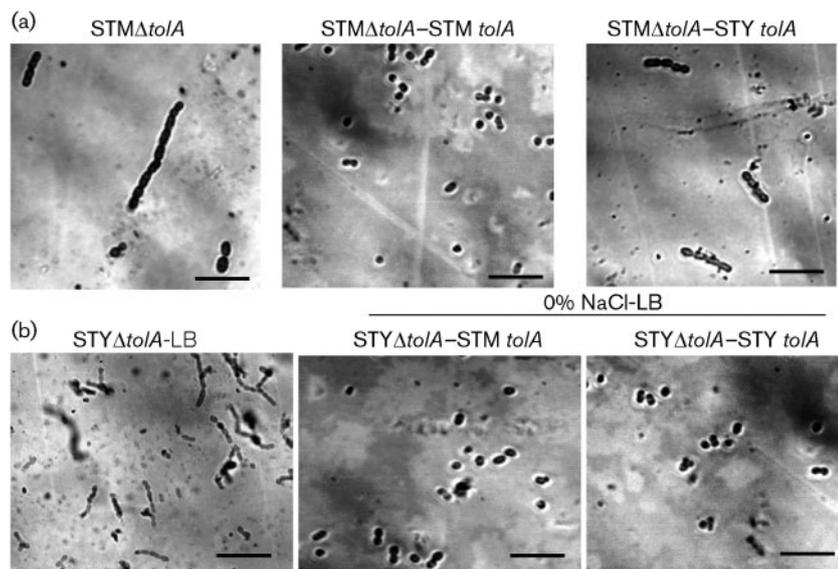


Fig. 9. *S. Typhi tolA* is unable to complement *S. Typhimurium ΔtolA*. (a) *S. Typhimurium ΔtolA*, *S. Typhimurium ΔtolA::S. Typhimurium tolA* and *S. Typhimurium ΔtolA::S. Typhi tolA* were grown overnight in low osmolarity LB media containing 0% NaCl. The bacteria were applied to a glass slide and observed under a phase-contrast microscope. (b) *S. Typhi tolA* and *S. Typhimurium tolA* are able to complement *S. Typhi ΔtolA*. *S. Typhi ΔtolA* was inoculated in LB media with 0.5% NaCl, *S. Typhi ΔtolA::S. Typhimurium tolA* and *S. Typhi ΔtolA::S. Typhi tolA* were grown overnight in low osmolarity LB media containing 0% NaCl. The bacteria were applied to a glass slide and observed under phase-contrast microscope. The data are representative of various fields taken from three independent experiments. Bar, 5 μm.

with *S. Typhimurium tolA* but failed to grow as single cells when complemented with *S. Typhi tolA* under the same growth conditions (Fig. 9a).

S. Typhi ΔtolA, which generally grows as chains in normal LB, started growing as single cells similar to the wild-type bacteria when complemented with either of the *tolA* genes. *S. Typhi ΔtolA* failed to grow in LB containing 0% NaCl. However, when complemented with either *S. Typhimurium tolA* or *S. Typhi tolA*, it started growing normally as single cells in 0% NaCl-LB (Fig. 9b). The 'longer' *S. Typhimurium TolA* was able to complement both *S. Typhimurium ΔtolA* and *S. Typhi ΔtolA* but the 'shorter' *S. Typhi TolA* was able to complement only *S. Typhi ΔtolA* but not *S. Typhimurium ΔtolA*. We conclude that the shortening of TolA is a specific adaptation of *S. Typhi* and this adaptation has been accompanied by compensatory adaptations, which have not happened in *S. Typhimurium*. The fact that shortening has taken place in domain II of TolA which traverses the peptidoglycan layer suggests the possibility that the peptidoglycan layer of *S. Typhimurium* is thicker than that of *S. Typhi*. This being true, the 'shorter' *S. Typhi TolA* will be unable to function in *S. Typhimurium ΔtolA* which has a thicker peptidoglycan layer. The 'longer' *S. Typhimurium TolA* is able to complement *S. Typhi ΔtolA*, which has a thinner peptidoglycan layer.

Analysis of peptidoglycan synthesis and modifying genes reveals that *dacD* and *pbpG* are pseudogenes in *S. Typhi*, and a large segment of *ynhG* of *S. Typhi* is deleted

The shortening of TolA in *S. Typhi* is part of a larger adaptation which involves large scale changes in the peptidoglycan layer. Peptidoglycan is a complex polymer that involves many genes in its synthesis and modification. From the published literature, a list of all the genes involved in peptidoglycan synthesis and modification was

compiled. The amino acid sequences for all these genes were retrieved from NCBI GenBank from the complete genome of the two serovars. Major structural differences between the homologous genes from the two serovars were identified by using alignment tools. In this analysis it was found that two of the peptidoglycan-modifying genes, namely *dacD* whose protein product is also known as penicillin-binding protein (PBP) 6b and *pbpG* whose protein product is also known as PBP 7, were pseudogenes in *S. Typhi*. *dacD* has D,D-carboxypeptidase activity (Baquero *et al.*, 1996). *pbpG* has D,D-endopeptidase activity (Henderson *et al.*, 1995). Further, another gene *ynhG* appears to have a major deletion in *S. Typhi*. YcbB and YnhG are the only L,D-transpeptidases for synthesis of meso-DAP³-meso-DAP³ peptidoglycan cross-links in *E. coli* (Magnet *et al.*, 2008).

S. Typhimurium pbpG–dacD double mutant and *pbpG* single mutant show increased sensitivity to Triton X-100 under low osmolarity, similar to wild-type *S. Typhi*

The three genes that were found to be either pseudogenes (*pbpG* and *dacD*) or highly truncated with doubtful functionality (*ynhG*) in *S. Typhi* were deleted using the red recombinase system from *S. Typhimurium*. Further double knockouts of *pbpG–dacD* and *ynhG–dacD* were created. The knockouts were expected to have less Lpp-diaminopimelic acid covalent linkages, thereby making them sensitive to detergent action under hypotonic conditions. The growth of these mutants in the presence of 8% Triton-X in low osmolarity LB containing 0% NaCl was tested. The *pbpG–dacD* double mutant was found to be deficient in growth compared with the wild-type *S. Typhimurium*. The growth deficiency was similar to that of *S. Typhi* wild-type strain (Fig. 10). The *pbpG* single mutant also showed similar decreased growth to the double

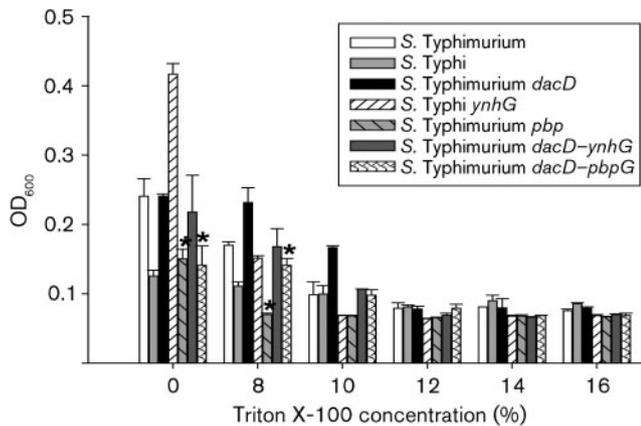


Fig. 10. *pbpG* single mutant and *pbpG-dacD* double mutant in *S. Typhimurium* show increased sensitivity to Triton-X under low osmolarity. *S. Typhimurium*, *S. Typhi* wild-type, *S. Typhimurium* $\Delta dacD$, *S. Typhimurium* $\Delta ynhG$, *S. Typhimurium* $\Delta pbpG$, *S. Typhimurium* $\Delta dacD \Delta pbpG$ and *S. Typhimurium* $\Delta dacD \Delta ynhG$ (100 c.f.u. ml⁻¹) were inoculated into low osmolarity LB containing 0% NaCl and different concentrations of Triton-X ranging from 8 to 16%. The assay was carried out in duplicate in 100 μ l volumes in a 96-well plate. The plates were incubated at 37 °C for 12 h and the bacterial growth was assayed by measuring OD₆₀₀. Experiments were performed in triplicate on three different occasions and means \pm SEM are shown. * $P < 0.05$ (Student's *t*-test).

mutant. However, the single mutants of *dacD* and *ynhG* in *S. Typhimurium* exhibited little enhanced growth, which strongly suggests the importance of *pbpG* in *S. Typhimurium*. The role of *dacD* and *ynhG* needs to be further investigated in this regard. This study shows that the *pbpG* and *dacD* double and the *pbpG* single knockout makes the bacteria sensitive to detergent action under low osmotic conditions. This finding, in light of the established fact that the *pbpG-dacA* double knockout has lowered Lpp-peptidoglycan covalent binding, shows that the *pbpG* knockout can also reduce the Lpp and peptidoglycan covalent binding. Low osmotic conditions cause swelling of the periplasmic space. The absence of Lpp-peptidoglycan covalent bonding leads to separation of the outer membrane from the peptidoglycan layer. As a result, the peptidoglycan embedded lipoproteins are exposed to the action of Triton X-100.

The maintenance of outer membrane integrity by the *tol-pal* system can also be substituted by Lpp to some extent (Cascales *et al.*, 2002). The major lipoprotein Lpp is one of the most abundant outer-membrane proteins, and about one-third of this is covalently bound to murein (Braun, 1975). In the absence of Lpp, or with *lpp* mutations that affect the covalent attachment to the murein layer, the outer membrane forms blebs and cells become hypersensitive to various toxic compounds and release periplasmic proteins to the extracellular medium (Suzuki *et al.*, 1978; Yem & Wu, 1978). Lpp plays a major role in maintaining the outer membrane peptidoglycan contact. Lpp, despite its

involvement in outer membrane invagination, is evenly distributed along the outer membrane (Hiemstra *et al.*, 1987). On the other hand, the Tol-Pal complex has been shown to be a part of the cell division machinery, it concentrates at the cell division site and is involved in proper invagination of the outer membrane (Gerding *et al.*, 2007).

Concluding remarks

S. Typhimurium infects many hosts including rodents, domestic animals, humans and birds. *S. Typhi*, on the other hand, is a single-host-restricted pathogen that affects only humans. Because of this highly restricted host specificity, *S. Typhi* has become a specialized pathogen. In this process, *S. Typhi* has lost many genes that are essential in a wide variety of environments and hence accumulated a number of pseudogenes (Holt *et al.*, 2009). In this study, we unveil a novel mechanism which reveals that shortening of TolA pulls the outer membrane in closer association to the peptidoglycan layer. This close association of the lipoproteins and phospholipids of the inner leaflet of the outer membrane protects it from the effect of detergents. LPS, which is the first barrier against bile, would have faced the same selection pressure to resist bile. The modifications to LPS would have been a trade-off between its bile resistance and its other functions. One of the visible differences between *S. Typhimurium* and *S. Typhi* LPS is the absence of very long chain in *S. Typhi*, but this did not affect bile resistance. In this study, we show that shortening of TolA in *S. Typhi* has resulted in increased bile salt resistance by bringing the outer membrane in close contact with the peptidoglycan layer.

Overall, it can be hypothesized that loss of peptidoglycan-modifying genes in *S. Typhi* led to thinning of the peptidoglycan layer and also probably reduced the Lpp-peptidoglycan covalent binding which is compensated by the shortening of the TolA protein. This shortening prevented perturbation of the periplasmic space by bringing the outer membrane in close proximity to the peptidoglycan layer, which also made it more resistant to detergent action. This close association reduced the exposure of the lipoproteins of the inner leaflet of the outer membrane to detergent action. This proposed model of detergent resistance in *S. Typhi* arose as a result of optimization of the periplasmic space structures as a means of achieving better detergent resistance at the cost of reduced low osmotic tolerance. The loss of the genes involved in peptidoglycan modification was probably essential to enable the reduction in length of the TolA protein. The sequence in which the genetic changes must have occurred is difficult to predict. The other typhoidal *Salmonella* serovar, *S. Paratyphi*, which has a similar lifestyle to *S. Typhi*, has an 'intermediate' length TolA between those of *S. Typhimurium* and *S. Typhi*. Its peptidoglycan modification genes are intact. This could indicate a convergent evolution aiming to maximize the detergent resistance.

In summary, periplasmic space remodelling in *S. Typhi* has led to better detergent resistance. TolA of *S. Typhi* is shorter than that of *S. Typhimurium* which in turn pulls the outer membrane in closer proximity to the peptidoglycan layer. Due to this, the detergent resistance is improved as the lipoproteins become unavailable for detergent action. *S. Typhi* also has a thinner peptidoglycan layer, which is caused by the multiple mutations in *dacD*, *ynhG* and *pbpG* genes; however, the most important gene is *pbpG*. These mutations may also reduce the Lpp-peptidoglycan covalent bonding leading to detergent susceptibility in low osmolarity conditions, and this study will be a fruitful avenue of further research.

ACKNOWLEDGEMENTS

We would like to thank Dr Omana Joy for helping us with the FACS analysis, V. B. Sudhagar for the Bioinformatics and Minakshi Sen for the confocal studies. This work was supported by the Director of Indian Institute of Science, Bangalore, India; [grant Provision (2A) Tenth Plan (191/MCB)] and the Department of Biotechnology (DBT 197 and DBT 172) to D.C. Infrastructure support from ICMR (Center for Advanced Study in Molecular Medicine), DST (FIST) and UGC (special assistance) is acknowledged.

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Edited by: R. J. Maier