Role of TolC in Virulence of Salmonella enterica Serovar Typhi

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Salmonella enterica serovar Typhi (S. Typhi) is the causative agent of typhoid fever. TolC, an efflux pump protein, is reported to have a role in the virulence of S. Typhimurium, which does not cause typhoid fever or carriers in humans. The role of TolC in the virulence of S. Typhi is unknown. This study addresses this question by utilizing gene knock-out technique to produce \( \Delta \text{tolC} \) deletion mutants and plasmid recombination technique to produce complementation mutants \( \Delta \text{tolC}^+ \) from S. Typhi strains cultured from both Ty21a (live vaccine strain) and from an acute typhoid fever patient (acute strain). The results showed that TolC deletion mutants derived from both vaccine and acute strains of S. Typhi showed a significant reduction in: 1) SDS tolerance, 2) biofilm formation ability, 3) macrophage invasion ability, and 4) cell membrane integrity, as compared with their parent (wild-type) strains. Complementation strains reverted the above phenotype completely except for biofilm formation and membrane integrity of the \( \Delta \text{tolC} \) derived from the acute strain only. These results suggest that TolC has a direct role as an efflux pump, host cell invasion, and maintenance of cell membrane integrity.

Keywords: tolC mutant; Salmonella; Invasion; Biofilm; Membrane Integrity.

Typhoid fever is a life-threatening febrile illness caused by the bacterium S. Typhi. Typhoid disease is a serious public health problem in developing countries, Typhoid fever remains a significant health burden; the estimated total number of typhoid fever episodes in 2010 was 13.5 million (Buckle et al., 2012). Pathogenic bacteria have evolved various escape mechanisms and strategies, one is efflux pump to survive and propagate in the hostile environment of the host. The gall bladder of the host is inhospitable to most microorganisms due to the presence of bile (Antunes et al., 2011; Hernández et al., 2012). However, S. Typhi, the causative agent of typhoid fever is able to survive and persist in the gallbladder of typhoid carriers (Crawford et al., 2010; Gonzalez-Escobedo et al., 2011). Bile has been reported to regulate the production of virulence factors, motility, adhesion, and bile resistance, efflux pump expression, and biofilm formation (Crawford et al., 2008). Biofilm formation which prevents the bacteria from being exposed and the efflux systems help the bacteria to grow in adverse conditions by pumping out toxic substances which enable the bacteria to persist even in the macrophage.

TolC is a protein found in all Gram-negative bacteria that forms a channel through the outer membrane while interacting with the AcrAB periplasmic protein (Koronakis et al., 2004). The AcrAB-TolC system is the most prominent of all the multidrug resistance efflux systems. (Koronakis et al., 2004). Apart from its role as an efflux pump,
TolC has recently been reported to play multifarious roles ranging from adhesion and invasion of epithelial cells and macrophages to virulence, persistence and stress resistance of Gram-negative bacteria, such as *Francisella tularensis, Brucella suis, Vibrio cholera, Salmonella enterica* serovar Typhimurium and *Salmonella* enteritidis (Bina and Mekalanos, 2001; Buckley et al., 2006; Gil et al., 2006; Posadas et al., 2007; Santos et al., 2010). Piddock and co-workers indicated the requirement of tolC as a general requirement for colonization and persistence of bacteria in the host includes plants, animals, and humans (Piddock, 2006b; Virlogeux-Payant et al., 2008). In addition, tolC also plays an important role to export of chromosomal and plasmid-encoded toxins such as microcins and hemolysin (Delgado et al., 1999; Wandersman and Delepelaire, 1990). Variants of the *E. coli* tolC have been described in a number of bacterial systems including *S. Typhimurium* (Cosme et al., 2008; Eaves et al., 2004; Ferhat et al., 2009; Karatzas et al., 2008; Nishino and Yamaguchi, 2001; Posadas et al., 2007) with varying functions. In spite of many reports of the importance of TolC in various systems and its associated with survival in the host, its function has not been studied in detail in *S. Typhi*.

The aim of this study was to delineate the role of the *S. Typhi* TolC as an important bacterial virulence mechanism such as the invasion of human macrophages, biofilm formation, and maintenance of the membrane integrity of the bacteria in comparison with that the wild type. Two kanamycin resistant gene knockout tolC mutants (ΔtolC) of *S. Typhi* were constructed for this purpose. Deletion of tolC gene was observed to have profound effects on the various aspects studied, suggesting that the TolC is an important protein in the maintenance of virulence and pathogenicity of *S. Typhi*.

**MATERIALS AND METHODS**

**Bacterial Strains, Plasmids, and Growth Conditions**

The *S. Typhi* mutant strains were derived from a wild-type strain of a local isolate from an acute fever typhoid patient (acute strain) and from Ty21a vaccine strain (ATCC, USA). Bacterial strains were grown at 37°C in Luria–Bertani broth (LB) (1.0% tryptone, 0.5% yeast extract, 0.5% NaCl).

**Deletion of tolC gene in S. Typhi strains (ΔtolC)**

The tolC mutants (ΔtolC) were constructed from two of *S. Typhi* strains (local clinical isolate from an acute typhoid patient also call acute strain and Ty21a vaccine strain) using the method described by Datsenko and Wanner (2000). The target tolC gene was replaced by a kanamycin resistant gene which also serves as a marker for the gene knock-out procedure. Briefly, PCR was used to prepare the replacement gene cassette contained the antibiotic resistance gene, flanked at both ends by 50 bp DNA sequences that were identical to sequences flanking the open reading frame of the target gene, tolC, according to Datsenko and Wanner (2000).

**Construction of complementation strain (ΔtolC+)**

Complementation plasmid pKK-tolC, derived from the low copy number plasmid pKK-tolC. For tolC complementation, tolC was amplified from the Ty21a strain with primers using (primers tolC_F, 5'-TTAATGGAAATCTTACCAGCTTG TGCTGCC and tolC_R, 5'-GACGGAAAGCTTTGCAAGGAATGGATT; underlined sequences indicate restriction enzyme recognition sites). These primers were designed to amplify the entire coding sequence of tolC, including the native stop codon from the Ty21a strain. The amplified PCR product was purified, digested with EcoRI and HindIII and ligated into plasmid pKK that was digested with the same enzymes, and the ligation mixture was transformed into DH5α. Plasmid pKK-tolC was isolated from the Amp' transformants, and the presence of a tolC was verified by agarose gel electrophoresis, restriction analysis. Plasmid pKK-tolC was transformed into tolC mutant (ΔtolC) to create a tolC complemented strain (ΔtolC+). After plasmid pKK-tolC had been introduced into the ΔtolC and selected by growth on ampicillin (50 mg/L) and 0.01% SDS containing agar plates a selective medium on which ΔtolC mutants are not able to grow.

**Determination of growth kinetics**

The growth kinetics of all *S. Typhi* strains was determined by bacterial culture by measuring the optical density (OD) at 595 nm as previously described (Sheridan et al., 2013). Growth characteristics of the reference wild-type (WT), complemented (ΔtolC+) and mutant (ΔtolC) strains were assessed by growth kinematics studies at
37°C. A single colony was picked and placed into a tube containing 10 ml LB broth and incubated for overnight at 37°C without shaking. Five microliters from the overnight culture were inoculated into 200 μl of LB broth. Samples were placed in a microtiter plate and bacterial growth was assessed by measuring the OD of the microtiter plate wells at scheduled 2 h time intervals at 595 nm (Thermo Scientific, Multiskan Spectrum). All quantitative growth assays were performed in six technical replicates with three independent experiments. They were compared with wild type by one-way analysis of variance (ANOVA).

**Cell invasion assay**

THP-1 derived macrophages were used for invasion assay (Buckley et al., 2006). The THP-1 line is a human monocyte cell line which differentiates into macrophage-like cells when treated with phorbol 12-myristate 13-acetate (PMA), (Sigma). THP-1 derived macrophage cells were grown in RPMI-1640 tissue culture medium containing 10% fetal calf serum (Gibco-BRL). Invasion assays were performed with gentamycin protection assay originally described by Isberg and Falkow, (1985). THP-1 macrophages (1x10^6/ml in 1 ml complete tissue culture media) were allowed to attach to the surface of 6-well tissue culture plates by incubation at 37°C in 5.0% CO₂ for overnight, following which the media was discarded and the plates washed with PBS three times to remove the non-adherent cells. Bacterial cells were grown to mid-log phase (O.D at 595 nm of 0.6), washed with tissue culture medium and 5x10^7 CFU inoculums in a total of 1 ml volume of tissue culture media was used to infect confluent macrophage monolayers. The bacterial inoculation was followed by incubation of the plates at 37°C in 5.0% CO₂ for 2 hours to allow bacterial entry. The monolayers after the incubation were washed with phosphate-buffered saline (PBS; pH 7.0) and tissue culture medium containing 100 μg/ml gentamycin and were subjected to a further incubation for 1 h to kill the extracellular bacteria. After removal of any remaining extracellular bacteria by the washing of the monolayers with PBS 1 ml, ice cold distilled water at 4°C was added to each well and incubated further for 20 min for macrophage cell lysis. The intracellular bacteria were quantitated by plating serial dilutions of the macrophage cell lysate. Experiments were performed to ensure that the data obtained were genuine and not experimental artifacts. All quantitative invasion assays were performed in triplicates with three independent experiments. They were compared with wild type by one-way analysis of variance (ANOVA).

**Crystal violet biofilm assay**

Biofilm assay was established on 96-well polystyrene microtiter plates from a previously described method with some modifications (Sheridan et al., 2013). A single colony was selected and inoculated into 5 ml of LB media and incubated at 37°C for 18 h. Two μl aliquots from the overnight culture were diluted in 1 ml of PBS. 1:100 diluted bacteria in fresh media antibiotic-free LB broth containing 3.0% bile (Begley et al., 2009). The ninety-six-well polystyrene microtiter plate each well was inoculated with 200 μl of this suspension and incubated at 37°C for 48 h with gentle agitation. After 48-hour incubation the medium was removed from the wells and the microtiter plate was washed five times with sterile distilled water to remove unbound bacteria and media. The plate was air dried for 45 min and each well was stained with 1.0% (w/v) crystal violet solution in distilled water for 45 min. After staining, the wells of the plate were washed twice with sterile distilled water. The amount of bacterial biofilm produced was estimated by determining the amount of dye restrained in the microtiter plate wells by the addition of 95% ethanol to the wells, and the dye solution was measured using a microtiter plate reader at 595 nm. All biofilm assays were performed three times with three biological and twelve technical replicates per study. One-way analysis of variance (ANOVA) was used to compare the statistical significance of results each mutant to wild-type and complemented strains.

**Scanning electron microscopy of bacterial cells**

Cell from all three strains, i.e. ΔtolC, wild-type, and ΔtolC+ were processed and observed via scanning electron microscopy (SEM) (Yuen et al., 2012). Briefly, bacteria were grown to the exponential phase in LB broth and centrifuged at 2,000 ×g for 15 min. The pellet was fixed overnight with fixing solution and centrifuged at 2,000×g for 15 min, dehydrated through a graded ethanol series (20%, 40%, 60%, 80%, 95%, 100% ethanol) and subjected to gold coating. The cells were viewed using a Leo Supra 50 VP field emission scanning electron microscope (Carl-Zeiss SMT, Oberkochen, 2012).
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RESULTS

Verification of tolC gene deletion in S. Typhi

Deletion of the tolC gene was confirmed by colony PCR. Based on the results of these assays and sequencing (sequencing result is not shown), we concluded that tolC was successfully deleted from the wild-type strains, resulting in strain ΔtolC strains were unable to grow on 0.01% SDS Luria agar (LA) plate but wild-type and ΔtolC+ strains could grow on 0.01% SDS LA (result is not shown).

Growth Curve

Growth characteristics of ΔtolC strain derived from both vaccine and acute strains, acute wild-type reference strains were examined to measure bacterial fitness (Fig. 1A). The ability of the ΔtolC strain to growth was compared with that of the wild-type and ΔtolC+. The results showed that ΔtolC strain displayed similar growth kinetics to the wild-type parent strain over the before 8 h duration of the experiment. After 8h, the growth of ΔtolC significantly lower than wild-type and ΔtolC+ (p<0.05). The result has shown here acute strains because the ΔtolC of vaccine strain Ty21a was given similar results like ΔtolC of acute strain.

In vitro invasion assay

The invasion ability of the ΔtolC in THP-1 derived macrophages was significantly reduced when compared to the wild-type strain and ΔtolC+ strain of acute strains (p<0.0001, Fig. 1B). The defect was, however, restored in the ΔtolC+ strain (Fig. 1B), even to a higher degree than with the wild type strains. ΔtolC+ corresponds to ΔtolC in which a TolC-positive phenotype was reintroduced. Results correspond to the mean of three independent experiments carried out in three replicates. Statistical significance: p <0.0001 wild-type vs. ΔtolC/ΔtolC+. An approximate similar result was observed for ΔtolC of vaccine strain result is not shown here.

Crystal violet biofilm assay

ΔtolC strain is unable to produce biofilm on biofilm inducing media

The role of tolC in biofilm formation was investigated using a standard microtiter plate assay. We compared biofilm formation by the wild-type, ΔtolC and ΔtolC+ of both, Ty21a and acute strains of S. Typhi on LB media containing bile. The ability of wild-type, ΔtolC and ΔtolC+ strains to attach to the walls of polystyrene microtiter plates was assessed in biofilm inducing media. Results of the assays revealed that wild-type and ΔtolC+ strains were able to attach to polystyrene and showed strong biofilm formation. However,
the ΔtolC strain was unable to attach to polystyrene wells and lost ability to form biofilm in the crystal violet assays (Fig. 2D,3D). The formations of biofilm by the different strains were compared by one-way analysis of variance (ANOVA). The results show a significant difference ($p < 0.0001$) in the degree of biofilm formation in the ΔtolC strains. Restored expression of the deleted tolC by using complementation vectors contain tolC, resulted in the strain returning to biofilm phenotype.

Cell membrane integrity

The scanning electron microscopy comparison of cell surfaces of the wild-type, ΔtolC and ΔtolC+ S. Typhi strains of both strains, Ty21a and acute of S. Typhi showed observable differences affecting cell membrane integrity in the ΔtolC strains (Fig. 2A, 2B, 2C and 3A, 3B, 3C).

**DISCUSSION**

The study of functions of tolC in S. Typhi, two mutants was generated by deleting tolC from different strains of S. Typhi. In this study, these ΔtolC showed significant reduction in: 1) SDS tolerance, 2) biofilm formation ability, 3) macrophage invasion ability, and 4) cell membrane integrity, as compared with their parent (wild-type) strains.

Demonstrating that, tolC, contributed to in vitro fitness and that deletion of tolC gene resulted in additive effects during the stationary phase of growth. Proving that growth delays were directly due to lack of tolC, growth during the stationary phase of the genetically complemented mutant (ΔtolC+) were approximately similar to that of the wild-type reference proving that growth delays were directly due to absence of tolC. The ability of the mutant lacking tolC to grow anaerobically was not affected in log phase of growth (Webber et al., 2009) according to our result. Also our result similar to the previous report on the study on Sinorhizobium meliloti (Santos et al., 2010). Here, growth rates of wild-type and the ΔtolC of Sinorhizobium meliloti growth were determined. During the first 8 hours the growth

![Fig. 2. Ty21a S. Typhi recombinant and wild strains - Scanning electron micrographs and biofilm formation](image)

Scanning electron micrographs of three strains: 2A - Ty21a S. Typhi wild strain, 2B - ΔtolC and 2C - ΔtolC+ strain. Arrows indicate altered surfaces of ΔtolC bacterial cells. The cells were observed at 100,000X magnification. 2D - Biofilm formation by S. Typhi Ty21a wild-type reference strain, ΔtolC and ΔtolC+ on polystyrene in LB containing bile. Each bar represents the mean optical density measured at 595 nm of 12 replicates from a single sample taken from each of three independent trials for all strains.
rate was similar to both strains; afterwards the ΔtolC presented a lower growth rate and reduced biomass formation. It is probable that under the cytoplasmic and extra cytoplasmic stress conditions suffered by the ΔtolC, many proteins and cofactors become defective and essential to be synthesized de novo or defended from denaturation. (Santos et al., 2010). if. Extra ATP would be spent to maintain cell homeostasis and not to produce biomass. It is also a formal possibility that uncertainties to the cell envelope may decrease the proton electrochemical gradient, negatively affecting ATP synthesis and, therefore, generating the need to increase the expression of genes related to energy metabolism not related to growth (Santos et al., 2010; Zgurskaya et al., 2011).

The study of the “tolC” showed that these mutant exhibit reduced invasion ability compared to their wild-type parental strains. Complementation of this ΔtolC restored to ability to invade human macrophage cell similar to their wild-type parents’ strains this result conformed that tolC play role in during invasion of S. Typhi to human host. It can therefore be consider having impact on host-bacteria interaction. This result is in line with previous research on other bacteria which demonstrated that these ΔtolC were loss ability to invade host cell and reduction in adherence to host epithelial cells when compared to parental wild type bacteria was a feature observed in studies with S. Typhimurium. This study was further supported by similar observations with reduction in ability to adhere, invade and survive in human embryonic intestine cells (INT-407, RAW 264.7 cell lines) and mouse monocyte macrophages virulence using ΔtolC strains of S. Typhimurium and L. pneumophila (Buckley et al., 2006; Ferhat et al., 2009). In this context, the work of Stone and Miller, 1995 who observed that S. Typhimurium strain that was generated using a transposon mutagenesis system was less virulent than wild-type holds interest. Not much is known on the role

Fig. 3. Acute S. Typhi recombinant and wild strains - Scanning electron micrographs and biofilm formation

Scanning electron micrographs of three strains: 3A - Acute S. Typhi wild strain, 3B - “tolC”, and 3 C - “tolC+” strain. Arrows indicate change surfaces of “tolC” bacterial cells. The cells were observed at 100,000X magnification. 3D - Biofilm formation of S. Typhi acute reference strain, “tolC” and “tolC+” on polystyrene in LB containing bile. Each bar represents the mean optical density measured at 595 nm of 12 replicates from a single sample taken from each of three independent trials for all strains.
of the tolC in S. Typhi infection. Since epithelial adherence and penetration are the most important pathogenicity factors in S. Typhi infection, this aspect was investigated with the help of the invasion assay using the THP-1 derived macrophage cell line. The results of this study was in agreement with the earlier observations in other systems supporting that tolC is an important player in the pathogenicity of S. Typhi. In addition Intracellular yield of wild-type S. Typhi in invasion assays were in the range of $3.5 \times 10^5$ to $5.1 \times 10^5$ cfu/ml. similar to the observations of Buckley et al., 2006; Dibb-Fuller et al., 1999 and Virlogeux-Payant et al., 2008, with wild-type S. Typhimurium. There is no clear mechanism for function of TolC in invasion but report from Virlogeux-Payant et al. (2008); Webber et al. (2009) have shown tolC deletion decrease expression of SPI-1 genes, which involved in invasion of bacteria to host cell. Other possibility, TolC have role in secretion of virulent factor which may be control invasion toward host cell (Song et al., 2015). Complementation with the tolC-bearing plasmid had significant effect on increase invasion of ΔtolC+, this is because may be multiple copy of tolC present at cytoplasmic level as compared to single tolC present in wild-type strain.

We demonstrate the in vitro biofilm formation assay it was observed significantly reduced biofilm production in ΔtolC as compared to their wild-type parental strains. Complementation of this ΔtolC restored ability to form biofilm similar to their wild-type parents strains this result conformed that tolC of S. Typhi have role in biofilm formation in human host. This biofilm defect was might be due to decrease in growth rates after 8h of growth. In our study ΔtolC+ of acute strain unable to produce biofilm similar to ΔtolC+ of Ty21a strain, possible reasons for this, might be over expression of AcrAB-TolC in ΔtolC+ acute strain, previous report shown that MexEF-OprM over expressing strains of P. aeruginosa exhibit compromised biofilm formation ability (Fernando and Kumar, 2013), OprM which is a homologous of TolC. We expecting some other possible reasons for this may be for this difference are (1) SEM picture of ΔtolC+ of Ty21a strain has shown membrane similar to wild type Ty21a strain (Fig.2A, 2C) but ΔtolC+ acute strain unable to repair membrane as in acute wild type (Fig.3A, 3C). (2) An intact, functional AcrAB-TolC efflux system is crucial for S. Typhi to form a biofilm. Defects in this system blocks biofilm formation. The role of AcrAB-TolC in biofilm formation appears to be related to regulation of matrix formation by activation of transcription of biofilm matrix components (Baugh et al., 2014). ΔtolC, ΔacrB, S. Typhimurium are unable to form biofilm, unlike ΔacrA, which was able to compromise to form biofilm (Baugh et al., 2014). Chemical inhibition and genetic inactivation of efflux pumps results in transcriptional repression of biofilm matrix components and a lack of biofilm formation (Baugh et al., 2014), therefore we used tolC from Ty21a strain to develop ΔtolC+ of acute strain. This may be possible as tolC from Ty21a strains unable to dock with AcrAB in acute strain. Since this defect in acute strain unable to form biofilm, this might be attributed to transcriptional repression of biofilm matrix components. Our results are in line with previous research on S. Typhimurium and other bacterial pathogens play role in many persistent human infections, are observed to be associated with biofilm growth (Crawford et al., 2010). It has been known for decades that bacterial biofilm can tolerate antibacterial agents like biocides, detergents, and antibiotics far better than planktonic cells (Anderl et al., 2000) and possible roles of AcrAB-TolC channel in biofilm formation has been stressed upon. Studies in E. coli, and other bacteria observed tolC to be active in promoting cell aggregation between bacteria, biofilm formation, adhesion to cells and AcrAB-TolC efflux pumps functioning (Imuta et al., 2008; Kvist et al., 2008; Costerton et al., 1999; Roberts, 1996). Bacterial biofilm, in turn, aids the bacteria to colonize and develop resistance towards antimicrobial agents and host immune responses (Costerton et al., 1999).

The results of the present study highlight the effect of tolC on cell membrane integrity in both Ty21a and acute S. Typhi strains. ΔtolC have some phenotypic difference on cell membranes from wild type strain and ΔtolC+ strain (Fig.2B 3B). No previous reports have documented this morphological difference in ΔtolC of S. Typhi before. This difference suggests that disruption of tolC, which encodes TolC outer membrane channel protein, an integral part of AcrAB-TolC channel, may affect the structural integrity of the outer membrane of S. Typhi cells. Our results on
structural integrity of the outer membrane have some degree of relationship on previous research on role of tolC deletion on integrity of the outer membrane defects such as depletion of essential metabolites, cytoplasmic membrane stress and alteration in NAD+/NADH ratios have been reported in literature (Dhamdhere and Zgurskaya, 2010). Studies available on ΔtolC of E. coli have observed that the loss of tolC leads to metabolic shut down of E. coli grown in a minimal medium with glucose. ΔtolC is reported to compromise the integrity of the bacterial inner membrane leading to inactivation of NADH oxidases and overconsumption of proton motive force (Dhamdhere and Zgurskaya, 2010). Rosner and Martin, 2009 suggested that the up regulation of MarA, SoxS and Rob activity in ΔtolC could be due to the response of membrane stress and also metabolic imbalance, this may be also possible reason for growth defect after 8h (fig 1A). These observations point towards the role of TolC in the maintenance of membrane integrity in survival process of the bacteria. However, the role of tolC in relation to infection process remains obscure.

CONCLUSION

In conclusion, the ΔtolC strain exhibited differences in phenotype and morphology compared to the wild type. tolC may operate as part of the multiple regulatory mechanisms governing external stress response and is involved in membrane integrity in S. Typhi, although tolC was found in our study to be involved in biofilm formation and involved in invasion into macrophage cell. It would also be interesting to study the effect of tolC deletion on other functions such as signal transduction and quorum sensing.

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REFERENCES

microbe interactions, 2010, 21, 947-957.


18. Dhamdhere, G., Zgurskaya, H.I., Metabolic shutdown in Escherichia coli cells lacking the outer membrane channel ToLC. Molecular microbiology, 2010; 77: 743-754.


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