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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 *tolC* deletion mutants (Δ*tolC*) and plasmid recombination technique to produce complementation mutants (Δ*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 Δ*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

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and macrophages to virulence, persistence and stress resistance of Gram-negative bacteria, such as *Francisella tularensis*, *Brucellasuis*, *Vibrio cholera, Salmonella enteric* 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 (Δ*tolC*) 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 37^0C in Luria–Bertani broth (LB) (1.0% tryptone, 0.5% yeast extract, 0.5% NaCl).

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

The *tolC* mutants (Δ*tolC*) were constructed from two of *S*. Typhi strains (local clinical isolate from an acute fever 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 (Δ*tolC*+)

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'-TTAATGGAATTCTTACGCATTG TGCTGCCC and *tolC*_R, 5'-GACGGAAGCTTT CAATGCCGGAATGGATT; 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 DH5á. Plasmid pKK-*tolC* was isolated from the Amp^r transformants, and the presence of a *tolC* was verified by agarose gel electrophoresis, restriction analysis. Plasmid pKK-*tolC* was transformed into *tolC* mutant (Δ*tolC* to create a *tolC* complemented strain (Δ*tolC*+). After plasmid pKK-*tolC* had been introduced into the Δ*tolC* and selected by growth on ampicillin (50 mg/L) and 0.01% SDS containing agar plates a selective medium on which Δ*tolC* 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 (Δ*tolC*+) and mutant (Δ*tolC*) 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 ìl 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 experiment. 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% CO_2 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% CO_2 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 experiment. 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

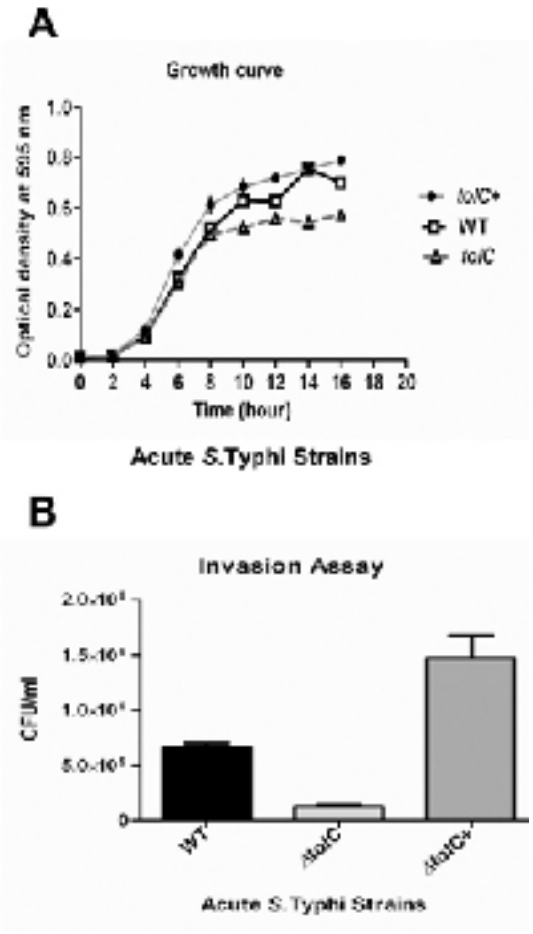
Cell from all three strains, i.e. Δ*tolC*, wild-type, and Δ*tolC*+ were processed and observed via scanning electron microscopy (SEM) (Yuen *et al*., 2012). Briefly, bacteria were grown to the exponential phase in LB broth and centrifuged at 2,000 ×g for 15 min. The pellet was fixed overnight with fixing 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, Germany) equipped with an Oxford NCA 400 energy dispersion X-ray microanalysis system (Oxford Instruments, Bucks, UK).

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 is strain Δ*tolC* strains were unable to grow on 0.01% SDS Luria agar (LA) plate but wild-type and Δ*tolC*+ strains could grow on 0.01% SDS LA (result is not shown).



1A - Growth curve. Growth was by determining absorbance at 595 nm for 16 h in LB at 37^{0}C.

1B. Histogram showing the *in-vitro* invasion of THP-1 derived macrophage cells by acute *S*. Typhi strains. CFU/ml of intracellular bacteria was determined after inoculation of THP-1 derived macrophages by acute wild- type or its *tolC* strains ("*tolC and "tolC*+) of *S*. Typhi and gentamicin treatment. Cells were infected with the acute *S*. Typhi strains for 2h (multiplicity of infection = 50).

Fig.1. Growth plot and histogram of invasion assay of the acute *S*. Typhi wild-type reference strain and its +*tolC* and +*tolC*+

Growth Curve

Growth characteristics of Δ*tolC* 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 Δ*tolC* strain to growth was compared with that of the wild-type and Δ*tolC*+. The results showed that Δ*tolC* 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 Δ*tolC* significantly lower than wild-type and Δ*tolC*+ (p<0.05). The result has shown here acute strains because the Δ*tolC* of vaccine strain Ty21a was given similar results like Δ*tolC* of acute strain.

In vitro invasion assay

The invasion ability of the Δ*tolC* in THP-1 derived macrophages was significantly reduced when compared to the wild-type strain and Δ*tolC*+ strain of acute strains (p<0.0001, Fig. 1B). The defect was, however, restored in the Δ*tolC*+ strain (Fig. 1B), even to a higher degree than with the wild type strains. Δ*tolC*+ corresponds to Δ*tolC* 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. Δ*tolC*/Δ*tolC*+. An approximate similar result was observed for Δ*tolC* of vaccine strain result is not shown here.

Crystal violet biofilm assay

Δ*tolC* 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, Δ*tolC* and Δ*tolC*+ of both, Ty21a and acute strains of *S*. Typhi on LB media containing bile. The ability of wild-type, Δ*tolC* and Δ*tolC*+ strains to attach to the wells of polystyrene microtiter plates was assessed in biofilm inducing media. Results of the assays revealed that wild-type and Δ*tolC*+ strains were able to attach to polystyrene and showed strong biofilm formation. However, the Δ*tolC* 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 Δ*tolC* 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, Δ*tolC* and Δ*tolC*+ *S*. Typhi strains of both strains, Ty21a and acute of *S*. Typhi showed observable differences affecting cell membrane integrity in the Δ*tolC* 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 Δ*tolC* 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 (Δ*tolC*+) 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 Δ*tolC* of *Sinorhizobium meliloti* growth were determined. During the first 8 hours the growth rate was similar to both strains; afterwards the Δ*tolC* presented a lower growth rate and reduced biomass formation.

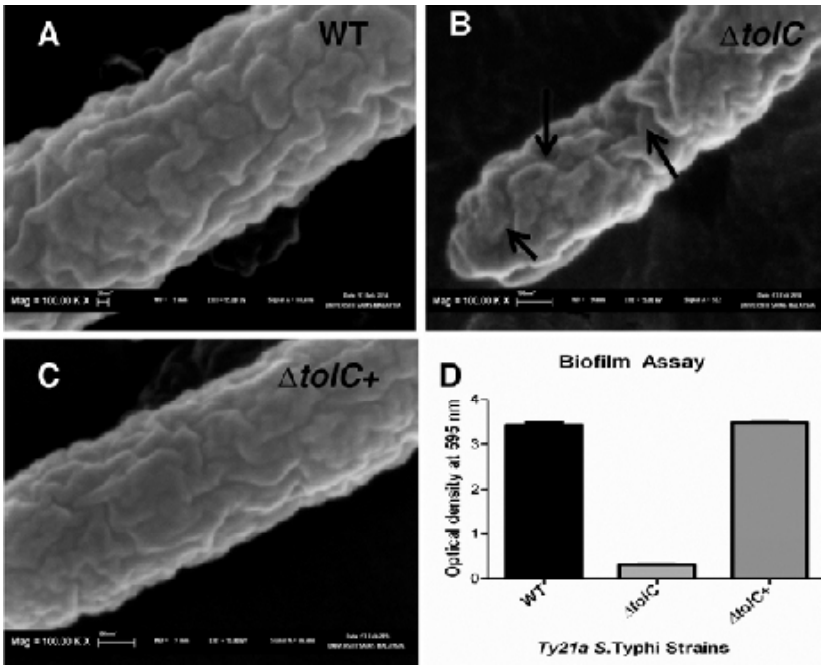


Fig. 2. Ty21a *S*. Typhi recombinant and wild strains - Scanning electron micrographs and biofilm formation

Scanning electron micrographs of three stains: 2A - Ty21a *S*. Typhi wild strain, 2B - Δ*tolC* and 2C - Δ*tolC*+ strain. Arrows indicate altered surfaces of Δ*tolC* bacterial cells. The cells were observed at 100,000X magnification**.** 2D **-** Biofilm formation by *S*. Typhi Ty21a wild-type reference strain, Δ*tolC* and Δ*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.

It is probable that under the cytoplasmic and extra cytoplasmic stress conditions suffered by the Δ*tolC*, 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 Δ*tolC* 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 Δ*tolC 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 obseved 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 Δ*tolC* 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 of the *tolC* in *S*. Typhi infection. Since epithelial adherence and penetration are the most important pathogenecity factors in *S*. Typhi infection, this aspect was

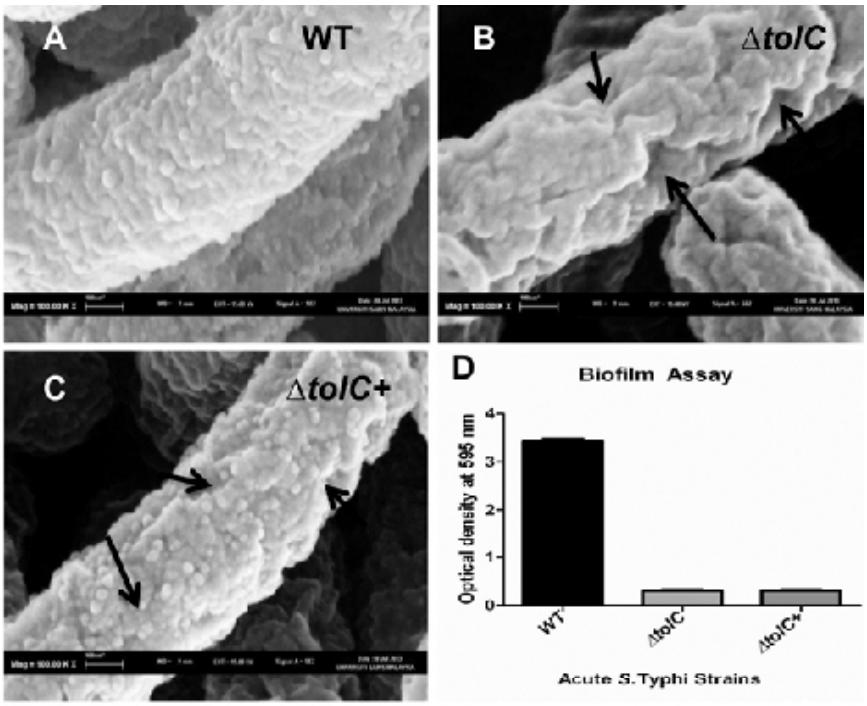


Fig. 3. Acute *S*. Typhi recombinant and wild strains - Scanning electron micrographs and biofilm formation

Scanning electron micrographs of three stains: 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.

investigated with the help of the invasion assay using the THP-1 derived macrophge 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 pathogenecity of *S*. Typhi. In addtion Intracellular yield of wild-type *S*. Typhi in invasion assays were in the range of 3.5×10^5"5.1×10^5 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 Δ*tolC*+, 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 Δ*tolC* as compared to their wild-type parental strains. Complementation of this Δ*tolC* 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 Δ*tolC*+ of acute strain unable to produce biofilm similar to Δ*tolC*+ of Ty21a strain, possible reasons for this, might be over expression of AcrAB-TolC in Δ*tolC*+ 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 Δ*tolC*+ of Ty21a strain has shown membrane similar to wild type Ty21a strain (Fig.2A, 2C) but Δ*tolC*+ 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). Δ*tolC*, Δ*acrB, S.* Typhimurium are unable to form biofilm, unlike Δ*acr*A, 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 Δ*tolC*+ 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. Δ*tolC* have some phenotypic difference on cell membranes from wild type strain and Δ*tolC*+ strain (Fig.2B 3B). No previous reports have documented this morphological difference in Δ*tolC* 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 Δ*tolC* 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. Δ*tolC* 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 Δ*tolC* 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 Δ*tolC* 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

1. Anderl, J.N., Franklin, M.J., Stewart, P.S., Role of antibiotic penetration limitation in *Klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxacin. Antimicrob Agents Chemother, 2000; **44**: 1818-1824.
2. Antunes, L.C.M., Andersen, S.K., Menendez, A., Arena, E.T., Han, J., Ferreira, R.B., Borchers, C.H., Finlay, B.B., Metabolomics reveals phospholipids as important nutrient sources during *Salmonella* growth in bile in vitro and in vivo. *Journal of bacteriology,* 2011; **193**, 4719-4725.
3. Baba, T., Mori, H., The construction of systematic in-frame, single-gene knockout mutant collection in *Escherichia coli K-12*. *Methods in molecular biology* (Clifton, N.J.) 2008; **416**, 171-181.
4. Baugh, S., Phillips, C.R., Ekanayaka, A.S., Piddock, L.J., Webber, M.A., Inhibition of multidrug efflux as a strategy to prevent biofilm formation. *The Journal of antimicrobial chemotherapy,* 2014; **69**, 673-681.
5. Begley, M., Kerr, C., Hill, C., Exposure to bile influences biofilm formation by *Listeria monocytogenes*. *Gut pathogens,* 2009; **1**: 11.
6. Benz, R., Maier, E., Gentschev, I., TolC of *Escherichia coli* functions as an outer membrane channel. Zentralblatt fur Bakteriologie : *international journal of medical microbiology,* 1993; **278**: 187-196.
7. Bina, J.E., Mekalanos, J.J., *Vibrio cholerae tolC* is required for bile resistance and colonization. *Infection and immunity*, 2001; **69**: 4681-4685.
8. Buckle, G.C., Walker, C.L., Black, R.E., Typhoid fever and paratyphoid fever: Systematic review to estimate global morbidity and mortality for 2010. *Journal of global health,* 2012; **2**: 010401.
9. Buchanan, S.K., Type I secretion and multidrug efflux: transport through the TolC channel-tunnel. *Trends in Biochemical Sciences,* 2001; **26**: 3-6.
10. Buckley, A.M., Webber, M.A., Cooles, S., Randall, L.P., La Ragione, R.M., Woodward, M.J., Piddock, L.J.V., The AcrAB–TolC efflux system of *Salmonella enterica* serovar Typhimurium plays a role in pathogenesis. *Cellular Microbiology,* 2006; **8**: 847-856.
11. Cosme, A.M., Becker, A., Santos, M.R., Sharypova, L.A., Santos, P.M., Moreira, L.M., The outer membrane protein TolC from *Sinorhizobium meliloti* affects protein secretion, polysaccharide biosynthesis, antimicrobial resistance, and symbiosis. *Molecular plant-microbe interactions*, 2010, **21**, 947-957.
12. Costerton, J.W., Stewart, P.S., Greenberg, E.P., Bacterial biofilms: a common cause of persistent infections. Science (New York, N.Y.), 1999; **284**, 1318-1322.
13. Crawford, R.W., Gibson, D.L., Kay, W.W., Gunn, J.S., Identification of a bile-induced exopolysaccharide required for *Salmonella* biofilm formation on gallstone surfaces. *Infection and immunity,* 2008; **76**: 5341-5349.

14. Crawford, R.W., Rosales-Reyes, R., de la Luz Ramírez-Aguilar, M., Chapa-Azuela, O., Alpuche-Aranda, C., Gunn, J.S., Gallstones play a significant role in *Salmonella spp.* gallbladder colonization and carriage. *Proceedings of the National Academy of Sciences,* 2010; **107**: 4353-4358.
15. Crump, J.A., Luby, S.P., Mintz, E.D., The global burden of typhoid fever. *Bulletin of the World Health Organization,* 2004; **82**: 346-353.
16. Datsenko, K.A., Wanner, B.L., One-step inactivation of chromosomal genes in *Escherichia coli K-12* using PCR products. Proceedings of the National Academy of Sciences of the United States of America, 2000; **97**: 6640-6645.
17. Delgado, M.A., Solbiati, J.O., Chiuchiolo, M.J., Farias, R.N., Salomon, R.A., *Escherichia coli* outer membrane protein TolC is involved in production of the peptide antibiotic microcin J25. *J Bacteriol,* 1999; **181**: 1968-1970.
18. Dhamdhere, G., Zgurskaya, H.I., Metabolic shutdown in *Escherichia coli* cells lacking the outer membrane channel TolC. *Molecular microbiology,* 2010; **77**: 743-754.
19. Dibb-Fuller, M.P., Allen-Vercoe, E., Thorns, C.J., Woodward, M.J., Fimbriae- and flagella-mediated association with and invasion of cultured epithelial cells by *Salmonella enteritidis*. *Microbiology,* 1999; **145**(Pt 5), 1023-1031.
20. Eaves, D.J., Ricci, V., Piddock, L.J., Expression of acrB, acrF, acrD, marA, and soxS in *Salmonella enterica serovar* Typhimurium: role in multiple antibiotic resistance. *Antimicrobial agents and chemotherapy*, 2004; **48**: 1145-1150.
21. Ferhat, M., Atlan, D., Vianney, A., Lazzaroni, J.-C., Doublet, P., Gilbert, C., The TolC protein of *Legionella pneumophila* plays a major role in multi-drug resistance and the early steps of host invasion. *PLOS one,* 2009; **4**, e7732.
22. Fernández Fuentes, M.Á., Ortega Morente, E., Abriouel, H., Pérez Pulido, R., Gálvez, A., Antimicrobial resistance determinants in antibiotic and biocide-resistant gram-negative bacteria from organic foods. *Food Control,* 2014; **37**: 9-14.
23. Fernando, D.M., Kumar, A., Resistance-nodulation-division multidrug efflux pumps in gram-negative bacteria: role in virulence. *Antibiotics*, 2013; **2**: 163-181.
24. Gerlach, R.G., Jäckel, D., Hölzer, S.U., Hensel, M., Rapid oligonucleotide-based recombineering of the chromosome of *Salmonella enterica*. *Applied and environmental microbiology*,. 2009; **75**: 1575-1580.
25. Gil, H., Platz, G.J., Forestal, C.A., Monfett, M., Bakshi, C.S., Sellati, T.J., Furie, M.B., Benach, J.L., Thanassi, D.G., Deletion of TolC orthologs in *Francisella tularensis* identifies roles in multidrug resistance and virulence. *Proceedings of the National Academy of Sciences,* 2006; **103**: 12897-12902.
26. Gonzalez-Escobedo, G., Marshall, J.M., Gunn, J.S., Chronic and acute infection of the gall bladder by *Salmonella Typhi*: understanding the carrier state. Nature reviews. *Microbiology,* 2011; **9**: 9-14.
27. Hatfaludi, T., Al-Hasani, K., Dunstone, M., Boyce, J., Adler, B., Characterization of TolC efflux pump proteins from *Pasteurella multocida*. *Antimicrobial agents and chemotherapy,* 2008; **52**: 4166-4171.
28. Hernández, S.B., Cota, I., Ducret, A., Aussel, L., Casades°s, J., Adaptation and Preadaptation of *Salmonella enterica* to Bile. *PLoS Genet 8,* 2012; e1002459.
29. Hinchliffe, P., Symmons, M.F., Hughes, C., Koronakis, V., Structure and operation of bacterial tripartite pumps. *Annual review of microbiology,* 2013; **67**: 221-242.
30. Imuta, N., Nishi, J., Tokuda, K., Fujiyama, R., Manago, K., Iwashita, M., Sarantuya, J., Kawano, Y., The *Escherichia coli* efflux pump TolC promotes aggregation of enteroaggregative *E. coli* 042. *Infection and immunity,* 2008; **76**: 1247-1256.
31. Isberg, R.R., Falkow, S., A single genetic locus encoded by Yersinia pseudotuberculosis permits invasion of cultured animal cells by *Escherichia coli K-12*. *Nature,* 1985; **317**: 262-264.
32. Karatzas, K.A., Randall, L.P., Webber, M., Piddock, L.J., Humphrey, T.J., Woodward, M.J., Coldham, N.G., Phenotypic and proteomic characterization of multiply antibiotic-resistant variants of *Salmonella enterica* serovar Typhimurium selected following exposure to disinfectants. *Applied and environmental microbiology,* 2008; **74**: 1508-1516.
33. Koronakis, V., Eswaran, J., Hughes, C., Structure and function of TolC: the bacterial exit duct for proteins and drugs. *Annual review of biochemistry*, 2004; **73**: 467-489.
34. Kvist, M., Hancock, V., Klemm, P., Inactivation of efflux pumps abolishes bacterial biofilm formation. *Applied and environmental microbiology,* 2008; **74**: 7376-7382.
35. Levy, H., Diallo, S., Tennant, S.M., Livio, S., Sow, S.O., Tapia, M., Fields, P.I., Mikoleit, M., Tamboura, B., Kotloff, K.L., Lagos, R., Nataro, J.P., Galen, J.E., Levine, M.M., PCR method to identify *Salmonella enterica* serovars Typhi, *Paratyphi* A, and *Paratyphi* B among *Salmonella* Isolates from the blood of patients with clinical

enteric fever. *Journal of clinical microbiology*, 2008; **46**: 1861-1866.
36. Mezal, E.H., Sabol, A., Khan, M.A., Ali, N., Stefanova, R., Khan, A.A., Isolation and molecular characterization of *Salmonella enterica* serovar Enteritidis from poultry house and clinical samples during 2010. *Food Microbiology*, 2014; **38**: 67-74.
37. Nishino, K., Yamaguchi, A., Analysis of a complete library of putative drug transporter genes in Escherichia coli. *Journal of bacteriology,* 2001; **183**: 5803-5812.
38. Piddock, L.J., Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. *Clinical microbiology reviews*, 2006a; **19**: 382-402.
39. Piddock, L.J., Multidrug-resistance efflux pumps? not just for resistance. *Nature Reviews Microbiology,* 2006b; **4**: 629-636.
40. Posadas, D.M., Martín, F.A., Sabio y García, J.V., Spera, J.M., Delpino, M., Baldi, P., Campos, E., Cravero, S.L., Zorreguieta, A., The TolC homologue of *Brucella suis* is involved in resistance to antimicrobial compounds and virulence. *Infection and immunity,* 2007; **75**: 379-389.
41. Roberts, I.S., The biochemistry and genetics of capsular polysaccharide production in bacteria. *Annual review of microbiology,* 1996; **50**: 285-315.
42. Rosner, J.L., Martin, R.G., An excretory function for the *Escherichia coli* outer membrane pore TolC: upregulation of marA and soxS transcription and Rob activity due to metabolites accumulated in tolC mutants. *J Bacteriol,* 2009; **191**: 5283-5292.
43. Santos, M.R., Cosme, A.M., Becker, J.D., Medeiros, J.M., Mata, M.F., Moreira, L.M., Absence of functional TolC protein causes increased stress response gene expression in *Sinorhizobium meliloti*. *BMC microbiology,* 2010; **10**: 180.
44. Sato, T., Yokota, S.-i., Uchida, I., Okubo, T., Usui, M., Kusumoto, M., Akiba, M., Fujii, N., Tamura, Y., Fluoroquinolone Resistance Mechanisms in an *Escherichia coli* Isolate, HUE1, Without Quinolone Resistance-Determining Region Mutations. *Frontiers in Microbiology,* 2013; **4**.
45. Sheridan, A., Lenahan, M., Condell, O., Bonilla-Santiago, R., Sergeant, K., Renaut, J., Duffy, G., Fanning, S., Nally, J., Burgess, C., Proteomic and phenotypic analysis of triclosan tolerant verocytotoxigenic *Escherichia coli* O157: H19. *Journal of proteomics,* 2013; **80**: 78-90.
46. Stone, B.J., Miller, V.L., *Salmonella enteritidis* has a homologue of *tolC* that is required for virulence in BALB/c mice. *Molecular microbiology,* 1995; **17**: 701-712.
47. Song, S., Lee, B., Yeom, J.-H., Hwang, S., Kang, I., Cho, J.-C., Ha, N.-C., Bae, J., Lee, K., Kim, Y.-H., MdsABC-Mediated Pathway for Pathogenicity in Salmonella enterica Serovar Typhimurium. *Infection and immunity,* 2015; **83**: 4266-4276.
48. Virlogeux-Payant, I., Baucheron, S., Pelet, J., Trotereau, J., Bottreau, E., Velge, P., Cloeckaert, A., TolC, but not AcrB, is involved in the invasiveness of multidrug-resistant *Salmonella enterica* serovar Typhimurium by increasing type III secretion system-1 expression. *International journal of medical microbiology : IJMM*, 2008; **298**: 561-569.
49. Wandersman, C., Delepelaire, P., TolC, an *Escherichia coli* outer membrane protein required for hemolysin secretion. *Proceedings of the National Academy of Sciences,* 1990; **87**: 4776-4780.
51. Webber, M.A., Bailey, A.M., Blair, J.M.A., Morgan, E., Stevens, M.P., Hinton, J.C.D., Ivens, A., Wain, J., Piddock, L.J.V., The Global Consequence of Disruption of the AcrAB-TolC Efflux Pump in Salmonella enterica Includes Reduced Expression of SPI-1 and Other Attributes Required To Infect the Host. *Journal of Bacteriology,* 2009; **191**: 4276-4285.
50. Yuen, C.W., Ong, E.B., Mohamad, S., Manaf, U.A., Najimudin, N., Construction and characterization of a *Burkholderia pseudomallei* wzm deletion mutant. *Journal of microbiology and biotechnology,* 2012; **22**: 1336-1342.
51. Zgurskaya, H.I., Krishnamoorthy, G., Ntreh, A., Lu, S., Mechanism and function of the outer membrane channel TolC in multidrug resistance and physiology of *enterobacteria*. *Frontiers in microbiology,* 2011; **2**.