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Effect of *ompR* Gene Mutation in Expression of *ompC* and *ompF* of *Salmonella typhi*

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Abstract: In the present investigation, a total of 50 stool samples were collected from the food handlers to screen the typhoid asymptomatic carriers, positive result was yielded for 2 out of the 50 samples. *Salmonella typhi* was isolated and identified based on the cultural characteristics on BSA, Macconkey agar, XLD and phylogenetic analysis. The *ompR* region of these two strains was amplified, sequenced and a Neighbor-Joining algorithm tree of *ompR* was constructed. The isolates were designated as (Salmonella Strain) SS-3 and SS-5 respectively. The isolates were subjected to mutation using sodium chloride at various osmolarity concentrations in LB broth. Both the wild and mutant *Salmonella typhi* were used for the isolation of outer membrane protein. The outer membrane protein was isolated and compared with both the wild and mutated *Salmonella typhi*. The expression of outer membrane protein was showing the variation in the expression which were noticed by using SDS-PAGE. On the basis of the results, it was concluded that the *ompR-envZ* two component regulatory systems play an important role on the regulation of Vi polysaccharide synthesis in *S. typhi*, and that one of the environmental signals for this regulation may be osmolarity.

Key words: asymptomatic carriers, typhoid, *ompR* gene, sodium chloride, mutation, expression, phylogeny.

1 Introduction

Salmonella typhi causes a severe systemic illness which is communicable via ingestion of contaminated water or food containing the bacterium. It is an invasive bacterium capable of entering several types of host cells, including epithelial cells of the ileal mucosa, macrophages, and other cells and tissues of the reticuloendothelial system (Pickard *et al.*, 1994). *S. typhi* synthesizes three major outer membrane proteins (OMPs) that are highly abundant upon growth in standard laboratory media: the *ompC*, *ompF* porins and *ompA*, a structural protein (Puente *et al.*, 1991; Forst and Inouye, 1988; Mizuno and Mizushima, 1990; Pratt *et al.*, 1996). *S. typhi*, like other enteric pathogens, has to respond quickly to these changing host microenvironments encountered in vivo, which will exert different demands and stresses (i.e., osmolarity, pH, oxygen tension, and nutrient starvation) on the bacterial cell. Bacteria possess systems for sensing these external environments, responding by coordinately controlling the expression of genes whose products are employed to assist survival under different conditions (Pickard *et al.*, 1994; Dorman, 1991; Graeme-Cook *et al.*, 1989). The two-component regulatory systems are of importance in this sensory response (Albright *et al.*, 1989; Parkinson and Kofoid, 1992). Members of this family of regulatory genes comprise a histidine kinase sensor protein gene and a transcriptional activator gene (Stock *et al.*, 1989). One such regulatory system, the *ompR-envZ* regulon, has recently been identified as being of importance for *Salmonella* virulence (Chatfield *et al.*, 1991). Part of the adaptive response of bacterial pathogens such as *S. typhimurium* to conditions of high osmolarity is to preferentially express one type of porin, *ompC*, over another type, *ompF*. It has been demonstrated that mutations in *ompR* and in the porin genes that it regulates (*ompC* and *ompF*) can attenuate virulent *S. typhimurium* (Pickard *et al.*, 1994; Dorman *et al.*, 1989).

The *ompR* regulon is also involved in the expression of virulence of other organisms. *Shigella flexneri ompR* mutants are severely impaired in their ability to invade

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epithelial cells, an effect thought to be mediated by the regulation of *ompC* synthesis (Bernardini *et al.*, 1990). Interestingly, *ompC* is regulated differently in *S. flexneri*, in which *ompC* is expressed constitutively under conditions of high and low osmolarity, than in *Escherichia coli*, in which it is preferentially expressed under conditions of high osmolarity (Bernardini *et al.*, 1993). This has also been found to be the case for *S. typhi* (Pickard *et al.*, 1994; Puente *et al.*, 1991). Thereby, the present investigation aimed at screening and identifying the *Salmonella typhi* from typhoid asymptomatic carriers by biochemical studies and phylogenetic analysis and also at investigating the effect of the *ompR* mutation on the expression of *ompC* and *ompF* genes by comparing outer membrane protein from the mutant and wild strains of *S. typhi*.

2 Materials and methods

2.1 Sample collection

Samples were collected from different age groups of typhoid asymptomatic carriers. A total of 50 stool samples were collected for isolation of typhoid bacilli. Stools samples were collected in early morning. The collected samples were transported to the laboratory using screw – a capped tube contains cary blair medium (Cary *et al.*, 1965). The samples were transferred to the serenity – F (Hi media) broth to enhance the growth of the organism and it was incubated at 37 ℃ for 24 hours. The positive results show red deposit with turbidity of broth. The growth was observed in the enrichment media, then it was inoculated into the macconkey agar media (Hi-media). It was incubated at 37 ℃ for 24 hours. Then the growth was observed in the enrichment broth, it was inoculated into the selective medium such as bismuth sulfite agar (BSA) (Himedia), agarose lysine deoxycholate agar (XLD) (Himedia) and *Salmonella* diffential agar (SDA) (HiMedia). These entire medium were used for confirmation of *Salmonella* species.

2.2 Gram's staining

The isolated colonies from the selective and differential media were used for the gram staining and results were noted under the light microscope. In catalase test, about 1 mL of 3% of H_2O_2 (Hi-media) was taken in a test tube and organism was obtained with the help of glass rod and dipped into the tube. Yielding of gas bubbles was observed and the results were noted. In oxidase test, the standard oxidase disc was taken in a clean petridishes and the suspected cultures were placed in disc with the help of glass rod. Positive reaction showed purple colors and negative reaction showed no color changes.

2.3 Biochemical tests - Sugar fermentation

The fermentation capability of the organisms in different sugars, were found by inoculating the organism into sugar broth tubes containing Durham's tube. The different sugar used included glucose, sucrose, lactose, mannitol and maltose. The tubes were incubated at 37 ℃ for 24 hours, it were showing acid and gas formation. Tubes showing negative results were incubated for another 24 hours to confirm the result.

2.4 Urease Test

The culture was streaked on the urease agar slant and incubated at 37 ℃ for 24 hours. The positive reaction showed pink colors and negative reaction showed no color changes.

2.5 IMVIC test

The IMVIC series comprises of four tests such as Iodole production, Methyl Red test (MR), Vogues Proskauer test (VP) and Citrate test. In Indole test, a tube of tryptophan rich medium was selected, and then it was aseptically inoculated with a loop full of bacteria and incubated for 24 hours at 37 ℃ (a control tube was included). After incubation a few drops of fresh kava's reagent was added to the tube. Tapping the bottom of the tube mixed the contents. It was allowed to stand and was observed for the occurrence of red ring at upper layer of the broth. The ring indicated that iodole was produced in the tube and tryptophan was digested. If the ring remains yellow or light brown iodole was not produced and tryptophan digestion failed to occur.

In MR test, the glucose phosphate peptone water was inoculated with pure culture and incubated at 37 ℃ for 24 hours. Five drops of methyl red reagent was added and mixed immediately. The appearance of bright red color indicates positive test and yellow color indicates negative test.

VP test depends on the digestion of the glucose to acetylmethylcarbinol. Acetylmethylcarbonyl reacted with α-naphthal and potassium hydroxide, yielding a kind of red chemical compound. The glucose phosphate peptone water was inoculated with the selected species of bacteria. More than 0.5 mL of α-naphthal and potassium hydroxide was added and the development of red color indicates production of acetylmethylcarbinol and hence, a positive test. Absence of color changes reflects negative test. Citrate utilization test was based on the ability of certain bacteria to utilize citrate, a salt of citric acid, as a sole carbon source in growth. When citrate utilization occurs, pH of the medium rises and the indicator is bromophenol blue. If citrate utilization cannot occur the original green color of the medium remains.

2.6 Amplification and phylogenetic analysis of *ompR* gene

Salmonella typhi strains SS3 and SS5 genomic DNA was isolated by using phenol-chloroform method (Sambrooke *et al.*, 1989). The integrity of the obtained genomic DNA was detected by electrophoresis in 1% agarose gel (Sigma Aldrich, India) stained with ethidium bromide. PCR primers MGR 06 (F) and MGR 07

(R) (First Base, Singapore) were used in corresponding to the region of *ompR* to directly amplify the chromosomal DNA of *Salmonella typhi* isolates (Pickard *et al.*, 1994). The reaction mixture for PCR amplification was prepared in a total volume of 50 μL with 5 μL of 10xPCR buffer, 10 mM of dNTPs, 1.0 μL of thermo stable DNA polymerase and 2 μL of forward and reverse primers. The amplification was performed in a DNA thermal cycler (Techgene, Techne (Cambridge) Limited, UK). The PCR reaction details were as follows: initial denaturation at 94 ℃ for 2 min, cycle denaturation at 94 ℃ for 30 s, annealing at 49 ℃ for 1 min, extension at 72 ℃ for 1 min, 34 cycles in total and final extension at 72 ℃ for 7 min. The *ompR* was purified using GenElute^{TM} Gel Extraction Kit (Sigma Aldrich, USA) and sequencing of *ompR* gene was done in an automated ABI-3100 Genetic Analyser (GeNei, India).

The corresponding sequences of representative species *S. typhimurium* (NC_003197), *S. typhi* (NC_004631), *S. paratyphi* A (FM200053), *S. paratyphi* B (NC_010102) and *S. paratyphi* C (CP000857) were used for phylogenetic analyses. MEGA 4.1 software program starts with a set of aligned sequences using Clustal W, and searches for phylogenetic trees that are optimal according to Neighbor-Joining (NJ) algorithms (Tamura *et al.*, 2007). The GenBank accession numbers of sequences reported in this paper are EU834745 and EU849617.

2.7 Induction of mutation of *S.typhi* isolates

LB broth was prepared using the various osmolar concentration of sodium chloride viz. 0.1 to 0.6 M. The isolated *Salmonella typhi* strain were inoculated in LB broth and incubated for 12 hrs.

2.8 Precipitation test

The precipitation test was used to confirm the absence or presence of an intra cellular accumulation of Vi polysaccharide (Rajasekarapandian and senthilkumar, 2007). 1% agarose in phosphate buffered saline containing polyethylene glycol 6000 at a final concentration of 2% (wt/vol.) was used. Bacteria grew on LB agar plates containing aromatic compounds overnight at 37 ℃. Cells were harvested, suspended in PBS, adjusted to an optical density at 600 nm of 20 or 60 and disrupted by sonication in 30 S bursts for 2.5 min in total. This procedure was carried out at 4 ℃ with 30 S between each burst to allow the samples to cool (Pickard *et al.*, 1994).

2.9 Isolation of outer membrane protein

The entire cell envelopes were first obtained by sonication of aerobically growing LB agar cultures. The sonication was carried out with 5 mL aliquots of cells (with OD at 650 nm of 15) suspended in 10 mM sodium phosphate buffer (pH 7.2). The resulting sonicated material was subjected to low speed centrifugation to remove cell debris and spun at 100 000× g for 45 min for pellet the cell envelopes. In the end, the inner membrane was solubilized by 1% sodium lauryl sarcosinate treatment, and outer membrane enriched fraction was collected by a final centrifugation of 100 000× g for 1.5 hours (Pickard *et al.*, 1994). The pellets were resuspended in phosphate buffer and it was analyzed by SDS-PAGE on 10% polyacrylamide gels.

3 Results and discussion

3.1 Isolation and identification of *S. typhi* isolates

A total of 50 stool samples were subjected to screen typhoid asymptomatic carrier, of which only two samples yielded positive result. Both of the screened isolates were found to be *S. typhi*. The isolates were identified based on the cultural characteristics on enriched, selective and differential medium. The production of red color deposits in the selective broth, jet black colonies on BSA, red smooth colonies on Macconkey agar by the isolates indicates the presence of *Salmonella typhi* (Figures not shown). In addition biochemical characteristics were adapted to identify the isolates at the species level (Table 1). The present isolates were designated as SS3 and SS5.

3.2 Phylogenetic analysis

A 538 bp of strain SS3 and a 518 bp sequences of strain SS5 were amplified from the genomic DNA with the MGR 06 (F) and MGR 07 (R) primers (Pickard *et al.*, 1994). In this study, *ompR* gene of different *Salmonella* species (different strains of a species) was obtained for phylogenetic analysis. The cumulative results from a limited number of studies to date suggest that 16S rRNA gene sequencing provides genus identification in most cases (>90%) but less so with regard to species (65-83%), with regard to species from 1-14% of the isolates remaining unidentified after testing. Michael and Sharon (2007) reported that the minimum of 500-525 bp was essential to phylogenetic analysis. According to Michael and Sharon's (2007) report, in this study, isolate SS3 (538 bp) had 85% (ClustalW2) and SS5 (518 bp) had 86% similarity with *S. typhi*. The results of isolates SS3 and SS5 had more than 500 bp which is highly supported to the proposal of Michael and Sharon (2007) and the sequence similarity results were higher than 83% which is sufficient to confirm that both of the strains belong to *S. typhi*.

As the number of sequences available for analysis continues to grow, the structure of phylogenetic trees derived from 16S rRNA sequences becomes more intricate and more accurate. Pernodet *et al.* (1989) reported that 16S rRNA and 23S rRNA of various bacterial species were partially sequenced and used for defining all members of the genus, groups of species or individual species. As shown in Fig. 2(a) (NJ Algorithm) strain SS3 (EU834745) fell in the branch

Table 1 Biochemical characteristics of *S. typhi* isolates

Isolate	Preliminary test				Biochemical test										
					Sugar fermentation						IMVIC				
S.typhi	G	M	C	O	G	L	MAL	Man	S	I	MR	VP	C	TSI	U
	-ve rod	+	+	–	+	–	+	+	–	–	+	–	+	AK/AH$_2$S	–

Abbreviations: G—Gram staining, M—Motility, C—Catalase, O—Oxidase, G—Glucose, L—Lactose, MAL—Maltose, MAN—Mannitol, S—Sucrose, I—Indole, MR—Methyl red test, VP—Voges proskauer test, C—Citrate utilization, TSI—Triple sugar iron, U—Urease, Ak—Alkaline, A H_2S—Acid H_2S, '+'—Positive result, '–'—Negative result.

with *S. typhi* (NC_004631) and Fig. 2(b) showed that strain SS5 (EU849617) fell in the branch with *S. typhi* (NC_004631). These results supported that the phylogenic position of strain SS3 and SS5 in the genus *Salmonella* might belong to the species *S. typhi*. The results of the phylogenetic analysis based on the *ompR* sequences which were newly used for species confirmation and phylogenetic construction suggested that strains SS3 and SS5 positioned in the species *Salmonella typhi*. It clearly reveals that the results of biochemical analyses, sequence comparison and phylogenetic analyses identified strains SS3 and SS5 as *Salmonella typhi* strains.

3.3 Induction of mutation and expression of the porins *ompC* and *ompF* in the *S. typhi* isolates

S. typhi employs the same regulatory proteins to regulate Vi synthesis as *E. coli* to regulate colonic acid synthesis. The results reported here indicate that another two component systems, *ompR-envZ* possibly responding to a different environment signal is also involved in the regulation of Vi synthesis in *S. typhi*. Vi polysaccharide is considered to be important in virulence of *S. typhi,* since the majority of *S. typhi* strains isolated from the blood of patients with typhoid possess Vi antigen (Robbins and Robbins, 1984). The *in-vivo* relevance of these findings to the mechanisms of host defense and pathogenesis of *S. typhi* still remains speculative and nothing is known about the regulation of Vi synthesis *in-vivo*. However, the finding that Vi expression in *S. typhi* is regulated by members of the family of two component systems which are known to be important in controlling gene expression *in-vivo* coupled with what is known about its role in virulence suggests that there is a need to regulate Vi antigen in different host microenvironment so that *S. typhi* expresses full virulence.

Expression of the *ompF* genes in *S. typhi* is under the control of *envZ* and *ompR*, a two-component signal transduction system encoded by the *ompB* (*ompR-envZ*) locus. Interestingly, a shift in osmolarity only affects *ompF* expression, and *ompC* levels remain constant (Majumder *et al.*, 1999). The outer membrane of *S. enteritidis* generally contains three major outer membrane proteins *ompC* (36 KDa), *ompF* (35 KDa) and *ompA* (33 KDa). *OmpC* and *ompF* are encoded for porin formation, whereas *ompA* has no pore forming properties (Lugtenberg and Van-Alphen, 1983). In the present investigation focused on the effect of the *ompR* mutation on the expression of *ompC* and *ompF* by comparing outer membrane proteins obtained from the mutant and wild strains of *S. typhi*, further more the *ompR* expression was studied and analyzed by SDS-PAGE. The isolate SS3 grew on cultures with osmolar concentrations of NaCl of 0.1 M, 0.2 M, 0.3 M, and 0.4 M, but in case of SS5 isolate growing in 0.1 to 0.3 M osmolar concentration the resulting outer membrane proteins *ompC*, *ompF* and *ompA* were found to be ∼36 KDa, ∼35 KDa and ∼33 KDa respectively (Fig. 1(a)

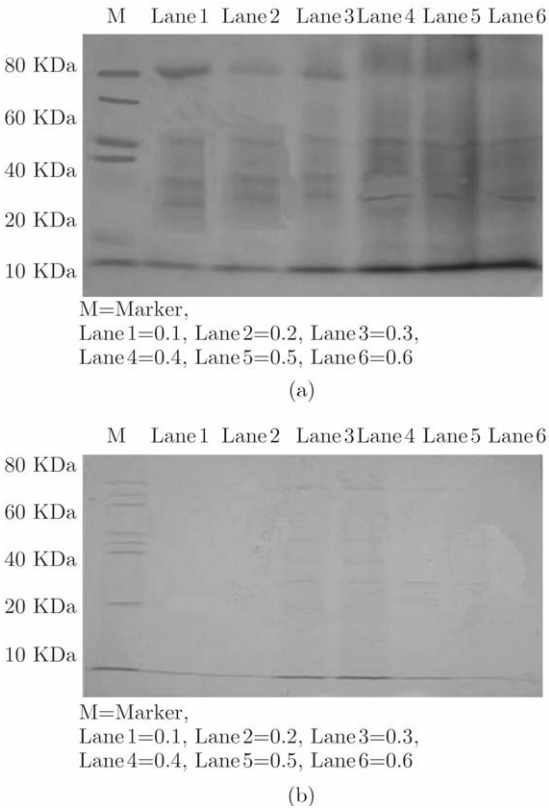

Fig. 1 Expression of porins *ompC* and *ompF* in *S. typhi*. (a) Expression of porins *ompC* and *ompF* in *S. typhi* SS3. (b) Expression of porins *ompC* and *ompF* in *S. typhi* SS5

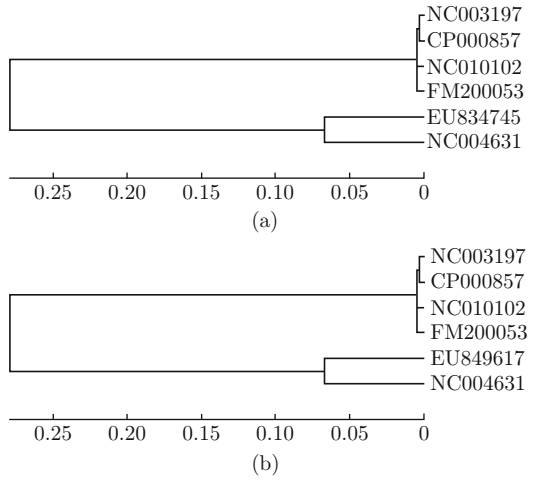

Fig. 2 Phylogenetic trees are based on the nucleotide sequences of *ompR* genes. The trees were constructed by using MEGA 4.1 software. (a) Neighbor-Joining algorithm was used for SS3 tree construction. (b) Neighbor-Joining algorithm was used for SS5 tree construction.

and 1(b)). So the low osmolar concentration of NaCl did not yield the mutant isolates. But mutant (SS-3) attenuated strain was yielded in the 0.5 M and 0.6 M NaCl solutions, and mutant (SS-5) attenuated strain was yielded in 0.4 M, 0.5 M and 0.6 M NaCl solutions, which was indicated by a single band of *ompA*. Thereby, resulting attenuated strains due to mutation were indicated by the expression of *ompC* and *ompF* proteins in the *S. typhi* SS-3 and SS-5 strains.

The wild isolates showed that the outer membrane proteins viz. *ompC*, *ompF* and *ompA* were found to be ∼36 KDa, ∼35 KDa, and ∼33 KDa respectively (Fig. 1(a) and 1(b)). However, the mutant strains showed only a single band of *ompA* which has no pore forming properties. Thus, *ompC* and *ompF* expression of the mutants was down regulated. Similar results were observed in the wild and mutant strains of *S. typhi* in the studies of Pickard *et al.* (1994). It is therefore concluded that when the isolate grows in higher than 0.4 M osmolar concentrations, it yields mutant strains and the *ompR-envZ* two component regulatory systems plays an important role in the regulation of Vi polysaccharide synthesis in *Salmonella typhi*, and one of the environmental signals for this regulation may be osmolarity. Therefore we widen the study to express Vi antigen of attenuated strains in mice and construct the attenuated typhoid vaccine.

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