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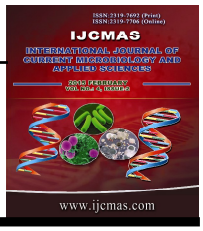

Original Research Article

Role of Bile in Regulating Small RNAs in *Salmonella typhi* – A Factor for Chronic Infection within Gallbladder?

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Keywords

Non-coding RNA in bacteria, Gallbladder disease, Bacterial carcinogenesis

A B S T R A C T

The human bile which is supposed to have bactericidal activity can regulate a number of mechanisms for bacterial survival. *Salmonella typhi* chronically persists within the gallbladder and is proposed to be a pre-disposing factor for gallbladder cancers. This study describes the ability of *S. typhi* to adapt to bile which could regulate stress responses within the organism via certain sRNAs. The organisms can be resistant even before they contact bile, and with initial adaptation the occurrence of bile resistant colonies shows a Poisson distribution with minimal variance, indicating the significance of bile in regulating survival mechanisms. One such mechanism involves the regulation of small regulatory RNA's that function during stress. Expression analysis reveals that two sRNA's MicF (targets OmpF) and DsrA (regulates RpoS, motility and drug resistance) are significantly up-regulated as a response to bile stress. This is consistent with findings that this organism down-regulates OmpF to inhibit entry of bile salts, is able to form biofilms by up-regulating RpoS and inhibiting motility and shows multiple drug resistance. The protein profiles also show alteration in intracellular and outer membrane proteins; the latter being possibly linked to major Omp's and drug efflux pumps regulated by many sRNA's.

Introduction

Small non-coding RNAs have been shown to regulate mechanisms within bacteria required for their survival and adaptation (Wassarman, 2002; Repoila *et al.*, 2003; Waters and Storz, 2009). These sRNAs interact directly with the protein or base pair with target mRNA to alter their activity (Wagner *et al.*, 2002; Tjaden *et al.*, 2006; Sharma and Vogel, 2009). The targets for many sRNAs are yet to be identified, but some function as a response to stress which alters membrane structure and general stress responses in bacteria (Gottesman *et al.*, 2006; Guillier *et al.*, 2006). The importance of understanding such a regulation is when organisms like *S. typhi* are able to survive within gallbladder-a site for storage of bile (Hazrah *et al.*, 2004; Nath *et al.*, 2008). Human bile possesses detergent like properties being bactericidal and sterile

when obtained from a healthy individual (Merritt and Donaldson, 2009). Studies have reported the chronic persistence of *S. typhi* within the gallbladder (Gonzalez-Escobedo *et al.*, 2011), being proposed as one of the predisposing factors towards gallbladder carcinogenesis (Hazrah *et al.*, 2004; Nath *et al.*, 2010a).

At epidemiological level, the incidence of gallbladder cancers has increased in Northern part of India with *S. typhi* infections being endemic within this region (Nath *et al.*, 2010a,b). *Salmonella* survival in the mammalian gallbladder seems to involve several strategies including DNA repair (Prieto *et al.*, 2006), invasion of gallbladder epithelium (Gonzalez-Escobedo *et al.*, 2011), biofilm formation on gallstones (Crawford *et al.*, 2008), regulation of drug efflux mechanisms (conferring antibiotic resistance) and alteration in virulence (regulation of pathogenicity islands) (Gonzalez-Escobedo *et al.*, 2011, Van Velkinburgh and Gunn, 1999).

Bile is shown to be one of the key mediators for these mechanisms by altering genetic material or bringing about changes in protein expressions (Begley *et al.*, 2005; Prouty *et al.*, 2004). But the effect of bile in regulating sRNA in *Salmonella typhi* is yet to be understood.

In this study we try to understand the regulation of these sRNAs during *S. typhi* adaptation to bile, and correlate them with expression of their known targets by observing changes in gene expression level and modulation in certain behavioural characters (Figure 1).

Our model envisions that such epigenetic mechanisms are an important factor for regulating distinct expressions in the bacterial world during chronic infections in extreme environments.

Materials and Methods

Bacterial strains and growth conditions:

Bacterial strains include *Salmonella typhi* clinical isolates obtained from patients. Bile was aspirated from patients undergoing cholecystectomy.

The PCR amplified flagellin gene fragment was eluted using QiaQuick Gel Extraction Kit (Qiagen) and cloned in the pGEM-T Easy vector system (Promega). Cloned PCR amplicons were sequenced by Chromus Biotech Pvt. Ltd., Bangalore, India and analysed using ChromasPro 1.7.5 provided by the vendor.

Growth characteristics and Adaptation assays for *S. typhi* isolates: The log phase cultures were challenged with bile, and assayed for MICs in microtitre well plates (Himedia, India). The MBCs were determined by plating the well exhibiting no apparent growth in the MIC assay. A fluctuation analysis was performed as described by Luria-Delbruk (Luria and Delbruck, 1943). Adaptation of *S. typhi* to bile was carried using a method described by Hernández *et al.* (2012) with few modifications, *S. typhi* isolates were grown in LB broth+ 5% bile and these were plated on LB media containing 20% bile. The isolates obtained were transferred to LB broth and grown overnight. The MIC of bile for these isolates was determined (Figure 3B). Isolates still tolerating higher concentration of bile were characterized as stable bile resistant mutants (Figure 4A and 4B). The data is representative of at least three independent replicates with standard errors for all experiments.

Biofilm quantification and antibiotic resistance assay: Bacterial attachment assays were performed in microtitre well plates adding 0.01ml of overnight grown

(OD_{600nm} ~ 0.4-0.6) culture to growth media containing increasing concentration of bile. Every well had enough glass coverslips required for time based analysis. Removed coverslips were washed in LB media and incubated at $60^{O}C$ for 45 minutes to fix the cells. The biofilms were stained using a solution of 0.1% crystal violet for 10 minutes at room temperature followed by a thorough wash using 1X PBS. The dye was extracted using 30% acetic acid and then quantified at 570nm. For estimating antibiotic resistance, antibiotic disc diffusion assay was performed by plating the organism on LB media and LB + Bile.

Quantifying gene expression using reverse-transcriptase polymerase chain reaction (RT-PCR): Total RNA was extracted with RNeasy Protect Bacteria Mini Kit (Qiagen, India) and quantified using NanoDrop Lite spectrophotometer (Thermo Scientific). RT-PCR was carried out using Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, India) and random hexamer primers as per the manufacturer's protocol. For every reaction of cDNA synthesis 1µg of purified RNA was used followed by PCR amplification with specific primers (Table 2) carried out within the linear range of amplification (30 cycles). A 10µl aliquot of the amplified product was loaded on 1.6% agarose gel and bands obtained were analysed using ImageJ 1.46r software. The normalized gene expression with a > 1.5 fold increase and a p-value of < 0.01 was considered to be significant. The fragments were cloned in pGEM-T Easy vector system and sequenced to confirm desired gene amplification.

Protein expression profiles using SDS-PAGE: The membrane proteins were isolated as described by Hamid and Jain (2008) with few modifications. For isolating membrane proteins, harvested bacterial cells were kept on ice and resuspended in 300µl of 0.1M Tris/HCL (pH 7.5). The cells were then incubated for 12 minutes in lysis solution followed by addition of the extraction buffer. The mixture was incubated at room temperature till a clear solution was obtained and dialysed against distilled water for 72 hours. The dialysed material was centrifuged at 10000rpm for 60 minutes and the supernatant containing the outer membrane protein was lyophilized to obtain the crude OMP fraction stored at -20 °C.

For intracellular protein extracts, cells were suspended in 250µl of autoclaved distilled water and sonicated on ice. The lysed mixture was centrifuged at 10000rpm for 15 minutes at 4 °C. The supernatant obtained was stored at -20 °C.

The concentration of protein in the samples was determined by using a NanoDrop lite spectrophotometer (Thermo Scientific). Samples were denatured by boiling them with the 10µl 5X loading dye for 5 minutes. A total of 5µg protein was loaded in the wells followed by SDS-PAGE on 12% separating gel. The gels were stained in silver stain and bands analysed with ImageJ 1.46r software.

Results and Discussion

Growth characteristics of *S. typhi* isolates in bile: Clinical isolates obtained were characterized as *S. typhi* by biochemical analysis and PCR specific to the H-1 d region of flagellin gene (*FliC*) (Figure 2A). The nucleotide sequences in region VI of *S. typhi* H1-d gene are substantially different from those in other *Salmonella* species (Frankel *et al.,* 1989). These pathogenic isolates which were able to establish successful infection in humans were studied for their ability to tolerate varying

concentration of bile. As shown in figure 2B, the growth analysis revealed a similar curve to that of the control grown in absence of bile. The minimum inhibitory concentration (MIC) of bile for the isolates was found to be 6% and the minimum bactericidal concentration (MBC) for isolates varied between 8%–10% (Figure 2C).

Adapting *S. typhi* to human bile

Salmonella typhi isolates shows a constant level of resistance to human bile, which can be enhanced by adapting the organism to lower concentration of bile as shown in figure 3(A) & 3(B). To determine the concentrations which permit *S. typhi* growth in high bile concentrations, organisms were grown up to their MIC (1% to 6%) of bile and then transferred to increasing concentrations of bile above MIC. As depicted by figure 3(A), *S. typhi* initially growing in 3%–5% of bile was able to tolerate higher concentrations of bile. The *S. typhi* strains that tolerated lethal bile concentration were transferred to LB media (without bile) and grown overnight. Aliquots from these were used to determine the MIC of bile. The resistance for all isolates, except for the one tolerating 5% bile initially (MIC increased to 7%) were reduced back to original characteristics of *S. typhi* isolates. The increase in MIC may possibly be due to permanent adaptation of few organisms to slightly higher concentration (6%) of bile. Thus *S. typhi* adaptation to bile is reversible and indicates ability to possess mutants which can be isolated and studied further.

Characterising inherited and induced bile resistant *S. typhi*: Bile-resistance can be acquired by mutations which may be inherited or induced. Cells with inherited mutations of spontaneous origin (those which are not involved in loss of function) can easily be recovered by plating them on lethal concentrations of bile (Luria and Delbruck, 1943). For *S. tyhpi* growing initially in LB and plated on lethal concentration of bile, the fluctuation analysis revealed a higher variance in occurrence of bile resistant colonies across different culture tubes as compared to the variance obtained within one single culture tube. When *S. typhi* was adapted to a concentration of bile determined from MIC, the bile resistant colonies appeared at a similar frequency with minimal variance between independent and single culture. The number of survivors was distributed in a way where the mean was almost equal to the variance satisfying the Poisson distribution. The frequency of occurrence of bile resistant colonies in *S. typhi* cultures was much lower (10^{-10}) when compared to the bile-adapted cultures of *S. typhi* (10^{-6}) suggesting the role of bile in modulating bacterial persistence (Table 1). Upon non selective growth of these bile resistant colonies in LB alone followed by a MIC assay to bile, it was observed that many of these were unstable mutants in case of bile adapted *S. typhi* with only a few in actual *S. typhi* cultures. Thus, inherited bile resistance may be mostly permanent (stable) whereas initial exposure to lower bile concentrations may adapt the organisms to survive in bile-rich environment only till the selective agent is present (unstable). The variance and frequency of stable bile resistant colonies also followed a similar phenomenon as described above in both cases.

Biofilm formation and antibiotic resistance of bile adapted *S. typhi*: A time dependent study for bile adapted *S. typhi* revealed that an increasing concentration of bile enhances biofilm formation. The typical features of micro-colony initiation followed by biofilm formation were seen as evident from figure 5A. A crystal violet staining of biofilms revealed an increased ability to form biofilms with increasing bile

concentration for stable bile resistant colonies (Figure 5B). The resistance shown by certain *S. typhi* isolates to specific drugs may be inherited, but almost all bile adapted *S. typhi* show resistance to ampicillin (β-lactam), ciprofloxacin (quinolone), erythromycin and clarithromycin (Macrolides), chloramphenicol, rifampicin and kanamycin (Figure 5C). The sensitivity shown towards other antibiotics was comparatively lower in the bile adapted *S. typhi*. The resistance activity or sensitivity was determined from the size of zone of inhibition seen during antibiotic disc diffusion assay (Figure 5C).

Analysing gene expression of sRNA and their targets: Since *S. typhi* is able to adapt and resist high bile concentrations we analyse expression of two candidate sRNA's *i.e., MicF* and *DsrA* along with their major targets. *MicF* located upstream of the ompC promoter, when over expressed decreases the amount of *OmpF* by direct pairing with its mRNA, which limits the entry of toxic compounds into the cell (Ramani *et al.*, 1994). *DsrA* regulates levels of two global transcription regulators *i.e. RpoS* and H-NS by sequence-specific RNA-RNA interactions (Sledjeski and Gottesman, 1995; McCullen *et al.*, 2010). Thus, *DsrA* contributes to biofilm formation indirectly by up-regulating CsgD via *RpoS* and down-regulating FlhDC via H-NS (Mika and Hengge, 2013). A semi-quantitative reverse-transcriptase PCR (Figure 6) is a cost effective way of determining gene expression of candidate sRNAs and their possible targets (Table 2). As shown in figure 6D, a significant increase in expression of *MicF* and *DsrA* (red arrows) was seen in *S. typhi* isolates exposed to a MIC of bile. Interestingly a two-fold increase was observed in these sRNAs in stable bile resistant mutants challenged with 30% bile, thus predicting their up-regulation with increasing concentration of bile upon adaptation. Bile down-regulates *OmpF* expression (blue arrow) which is the only proposed target for *MicF*, possibly inhibiting entry of bile salts. A significant increase of *RpoS* gene expression and a decrease in *FliC* expression levels (green arrows) justifies the ability of the organism to form biofilms in response to bile stress (Table 3). The enhanced antibiotic resistance shown by bile adapted mutants may not be attributed to *MicF* alone, but *DsrA* has also been shown to modulate certain drug efflux pumps (Nishino *et al.*, 2011). This is a first study reporting relevance of small non-coding RNAs in *S. typhi* during adaptation to human bile stress.

Analysis of intracellular and membrane proteins in stable bile resistant mutants

A comparison between *S. typhi* isolate and bile resistant mutants revealed certain alteration in protein expression between ~19kDa to ~66kDa. The major Omp's (*OmpF*, OmpW, OmpC OmpD and OmpA) regulated by sRNA (Guillier *et al.*, 2006) lie within this range including drug efflux components like TolC. As seen in figure 7A, the decrease in expression for certain bands may be linked to the Omp's, whereas the increased expression may be linked with components of drug efflux mechanisms. Also changes within intracellular proteins were observed which may be associated with stress responses and adaptation of the organism to bile (Figure 7B). A thorough analysis of these alterations may be further needed to confirm the changes in mentioned proteins.

Bacteria have evolved various mechanisms as a response to stress for their survival. Recent studies have reported small non coding RNAs in prokaryotes with similar functions to that of microRNA detected in

higher organisms (Gottesman, 2004; Soper *et al.,* 2010). Despite of all this information available with us, many questions still remain unresolved in case of chronic infections caused by *S. typhi* within the gall bladder. Many researchers have tried to uncover the mystery that underlies their persistence following acute phase of infection, and possible roles they may play in regulating host cell machinery (Haghjoo and Galán, 2004; Gonzalez-Escobedo *et al.,* 2011). The latter is of particular concern because *S. typhi* may be responsible for malignant transformation of the gallbladder. Again the conclusions are hypothetical not giving a clear thought to prove *S. typhi* a causative agent of gallbladder cancer. Firstly, we need to understand if the organism is able to adapt to a bile-rich environment significantly to produce a disease in course of the time. If non coding RNAs are regulating stress responses in enteric organisms what would be their possible role in *S. typhi* adaption to bile? Many in vitro studies have shown strategies employed by *S. typhi* to survive within the gallbladder which include regulating stress responses and virulence, biofilm formation and alterations in membrane architecture. But again, are these abilities inherited or acquired upon adaptation to bile.

We have shown that clinical isolates of *Salmonella typhi* contain cells which may be resistant to bile even before they come in contact with it. Though the occurrence of bile resistant colonies was extremely low, their random distribution confirmed inheritance which may be attributed to the prior drug resistance shown by the organism. We find that if *S. typhi* is adapted to sub-lethal concentration of bile, an even occurrence of bile resistant colonies was observed which explains that the lower bile concentration within the intestine may promote modulation of survival mechanisms within *S. typhi* to tolerate high bile concentration in gallbladder. A speculative explanation for such an adaptation may be mutational or non-mutational, and it was conceivable to have both stable and unstable bile resistant colonies respectively.

The stable bile resistant isolates could tolerate very high concentration of bile and were consistently able to form biofilms with increasing concentrations of human bile. The latter shows the ability of bile resistant *S. typhi* to regulate their mechanisms depending on the concentration of bile sensed within an environment. It was not surprising to see an elevated resistance to antibiotics upon adaptation of the organism to bile which may be of concern because it can lead to dissemination of multi-drug resistant *S. typhi*.

A gene expression analysis using reverse-transcriptase PCR revealed that adaptation to bile involves up-regulation of stress induced sRNAs *i.e. MicF* and *DsrA*. Both, the stable bile resistant *S. typhi* exposed to 30% bile and *S. typhi* growing in sub-lethal bile concentration significantly produces these sRNA, as seen during osmotic and cold stress (Ramani *et al.,* 1994; McCullen *et al.,* 2010). From these findings, we cannot avoid the fact that changes in *S. typhi* behaviour upon bile stress may be attributed to these sRNAs which can be important during bacterial survival and persistence within the gallbladder. Another relevant observation was the changes observed in the outer membrane protein profiles of bile resistant colonies exposed to 30% bile. We propose that these may be referred to the major outer membrane proteins and drug efflux mechanisms regulated by different sRNAs, though a further analysis is required to ascertain the exact role of each.

Table.1 Fluctuation analysis for determining occurrence of bile-resistant colonies

	***S. typhi* grown in LB**	***S. typhi* grown in LB+bile**		
	Separate culture tubes	**Single culture tube**	**Separate culture tubes**	**Single culture tube**
No. of samples	20	20	20	20
Mean	18.3	8.2	56.15	36.85
Variance	556.43	110.58	53.6	39.42
Occurrence of bile-resistance colonies	4.5×10^{-10}	3.9×10^{-10}	2.8×10^{-6}	1.7×10^{-6}

Table.2 PCR primers to candidate genes used for this study

Gene	**Description**	**Primers**	**Size (bp)**
MicF	Involved in regulating outer membrane protein by post-transcriptionally binding to mRNA in response to stress	LP: CTGGTGGACGGTCTCTCTTT RP: TGCATCATATTTCGCGCCAA	289
DsrA	Positive regulator of general stress response regulator (*RpoS*)	LP: CCTACGGGTCGGGATCAAAC RP: ACATCAGATTTCCTGGTGTAACGA	71
RpoS (σS)	Transcriptional regulator (sigma unit of RNA polymerase) that helps the cell respond to stress by controlling various gene expression levels	LP: CTCAACGAGCGCATTACCTC RP: CCGCGAACACTATCCACAAG	498
OmpF	Outer membrane protein generally down-regulated during osmotic stress to prevent entry of salts	FP: CTGGTGGACGGTCTCTCTTT RP: CCAGCAGGTTGAAACGGTAG	520
FliC	The gene encodes major component of the flagella and thus is important for motility	LP: TCGTTTGAGGATAAAAACGGTA RP: CAGTTTGAGCAACGCCAGTA	181
RpoD	Representative housekeeping gene encoding the major RNA polymerase sigma factor	LP: GTCAACAGTATGCGCGTGAT RP: TTACCCACTTCTTCCAGCGT	926

Table.3 Analysis of mRNA expression levels using RT-PCR

	Types of mRNA analysed				
	RpoS	***MicF***	***OmpF***	***DsrA***	***FliC***
Bile-resistant *S. typhi* mutant in 30% bile					
Normalized expression Mean	1.344833	1.475133	0.6306	1.4136	0.662233
t-value	5.992079	8.50989	9.726489	9.860574	6.768677
p-value	0.003901	0.001046	0.000626	0.000593	0.002486
Significance	$p < 0.01$	$p < 0.01$	$p < 0.01$	$p < 0.01$	$p < 0.01$
Fold-change	2.2344	2.3848	-1.8284	2.3168	-1.6942
***S. typhi* isolate grown in 5% bile**					
Normalized expression Mean	1.112333	1.093567	0.647	1.0844	0.633967
t-value	8.784476	12.66635	8.695087	8.844661	7.159142
p-value	0.000926	0.000224	0.000963	0.000902	0.002015
Significance	$p < 0.01$	$p < 0.01$	$p < 0.01$	$p < 0.01$	$p < 0.01$
Fold-change	1.8481	1.7686	-1.7820	1.7773	-1.7697

*The negative value indicates the decrease in expression compared with the control strain expression

Figure.1 Proposed model for *S. typhi* adaptation modulated by sRNA. sRNA, small RNA; *RpoS*, RNA polymerase subunit; *DsrA*, down-stream region A; *MicF*, mRNA interfering cRNA; *OmpF*, outer membrane protein F; Inhibition; Activation; down-regulation; up-regulation

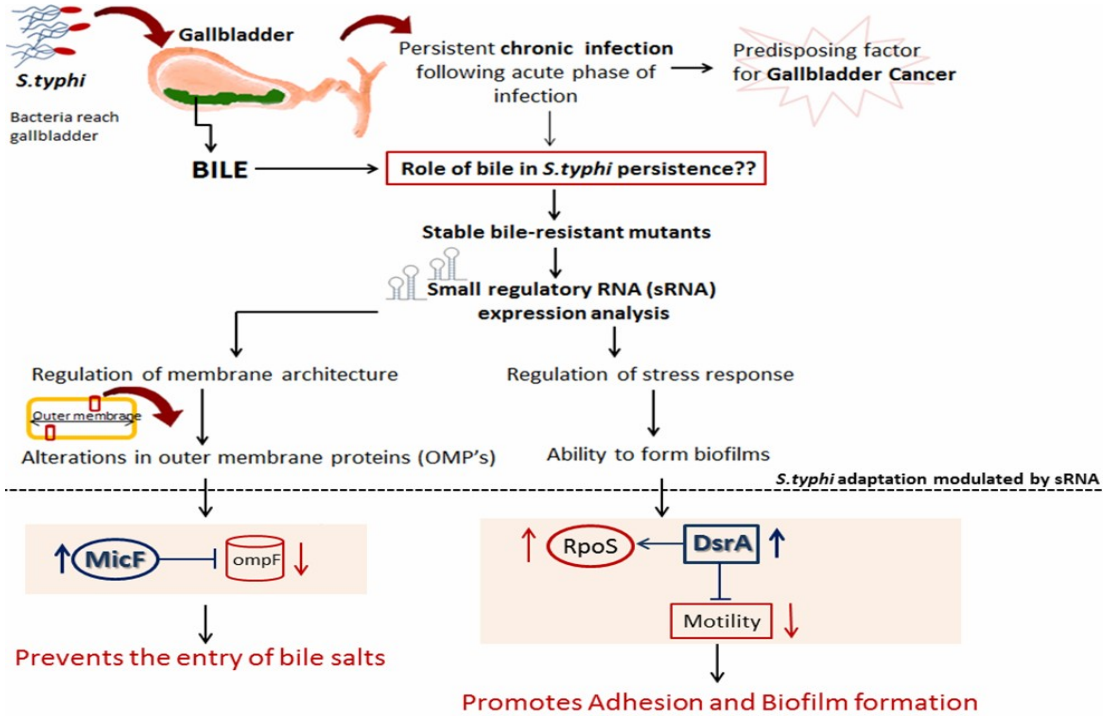


Figure.2 Growth characteristics for *S. typhi* isolates in Bile: The growth of *S. typhi* in bile was studied and their MIC and MBC to bile was determined. (A) PCR assay to the H-1 d region of flagellin gene of *S. typhi* shows the amplification of 181bp gene on 1.2% agarose gel. Lane 1: molecular weight marker; Lane 2: positive control; Lane 3-7: *S. typhi* clinical isolates; Lane 8: Negative control. (B) Growth of *S. typhi* in varying concentrations of bile for different isolates measured at 600nm (C) The MIC determined by growing organisms in increasing bile concentration followed by MBC assay for concentration above MIC which show no apparent growth in medium

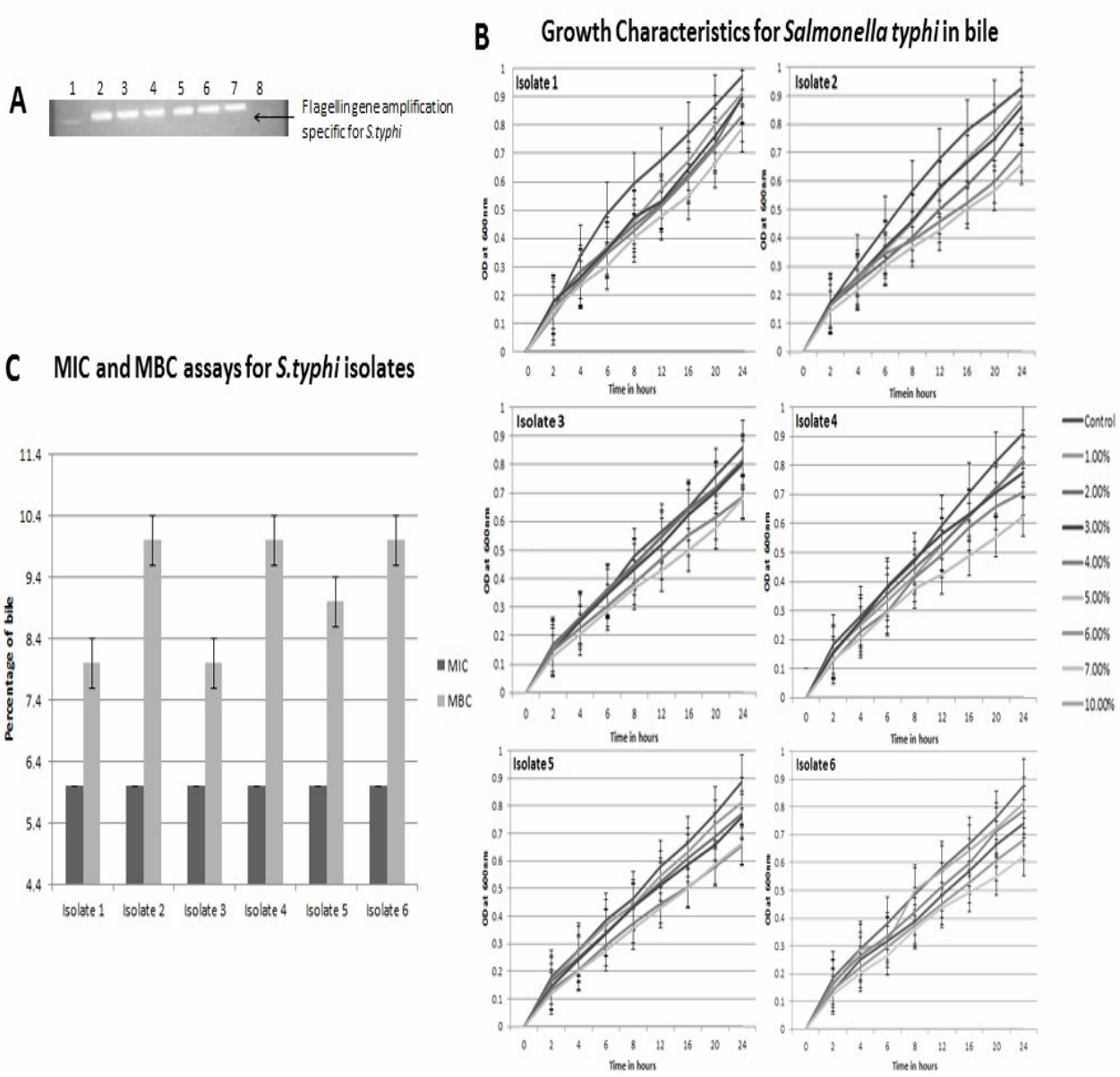


Figure.3 Adaptation of *S. typhi* to human bile. Adaptation assay was performed to isolate bile resistant colonies. (A) Determining the change in MIC for *S. typhi* pre-adapted to concentration of bile up to their MIC. The *S. typhi* cells able to tolerate high concentration of bile were checked for reversible nature by growing in LB without the selective agent and rechecking their MIC. (B) Detecting the occurrence of bile-resistant cells (Stable and unstable) using Luria-Delbruk's fluctuation test for *S. typhi* isolates. The analysis was performed by exposing *S. typhi* to lethal bile concentration with and without prior adaptation to low bile concentration

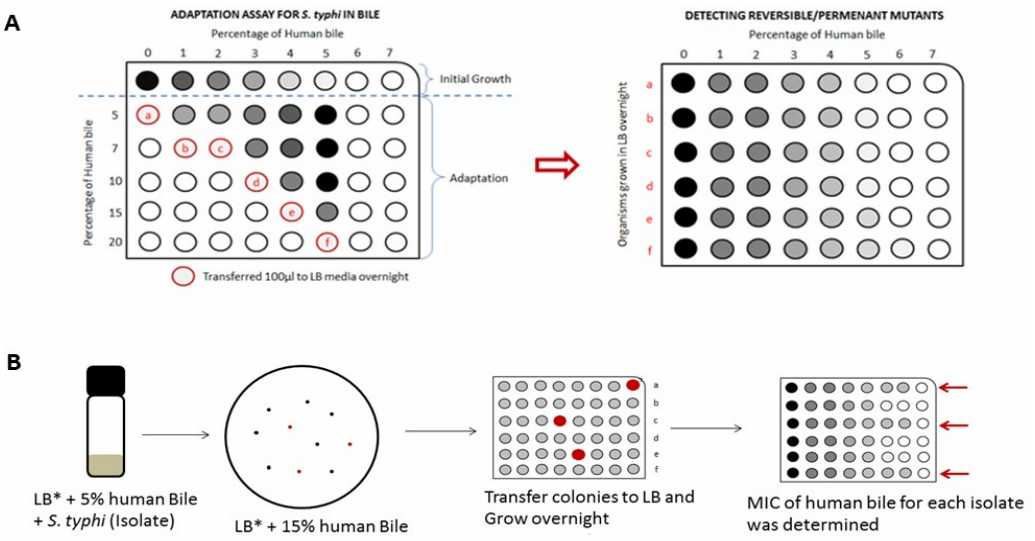


Figure.4 Characterising the stable bile resistant *S. typhi* colonies. The stable bile resistant colonies obtained for each isolate upon pre-adaptation to bile was estimated. (A) A representative of stable bile resistant colonies obtained for each isolate as determined from their MIC to bile. (B) Ability of the bile-resistant colony to tolerate varying concentration of bile

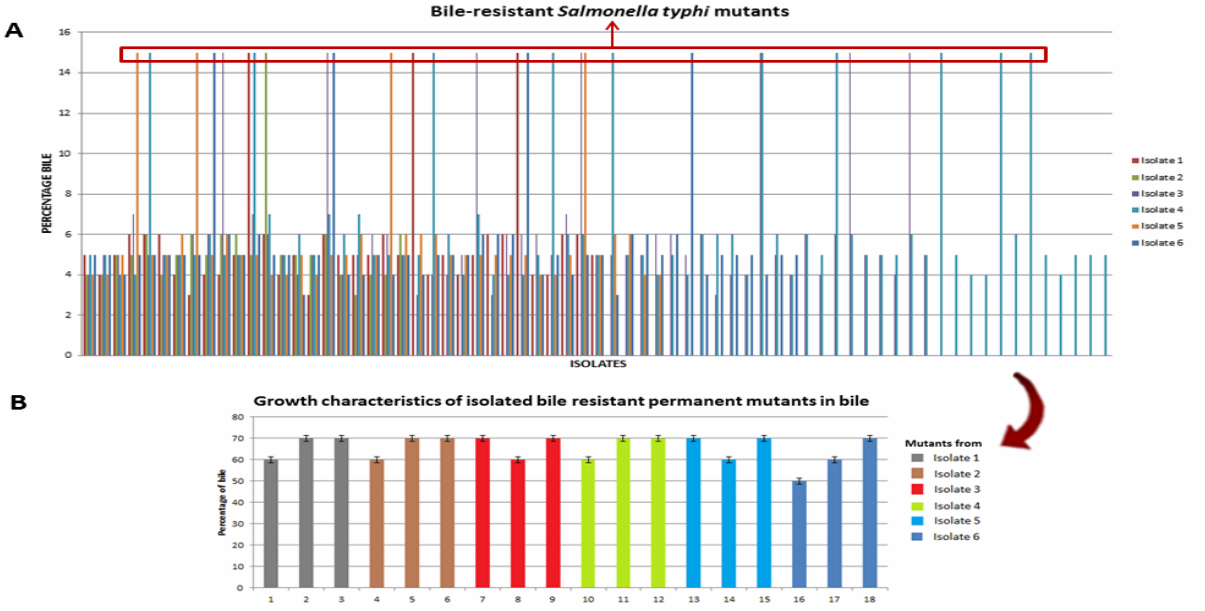


Figure.5 Biofilm quantification and antibiotic resistant assays: The ability of the stable bile resistant isolates to form biofilms and show antibiotic resistance was analysed. Single stable bile-resistant organism from every isolate was used for the study. (A) *S. typhi* showing microcolony formation and initiation of biofilm with course of time. (B) A crystal violet staining analysis for biofilm quantification in presence and absence of bile. The amount of dye retained by the cells forming biofilms was estimated spectrophotometrically at 540nm. (C) The antibiotic sensitivity was determined by measuring the zones of inhibition shown by isolates against antibiotic discs. The organism was considered to be resistant to any antibiotic showing no zones of inhibition

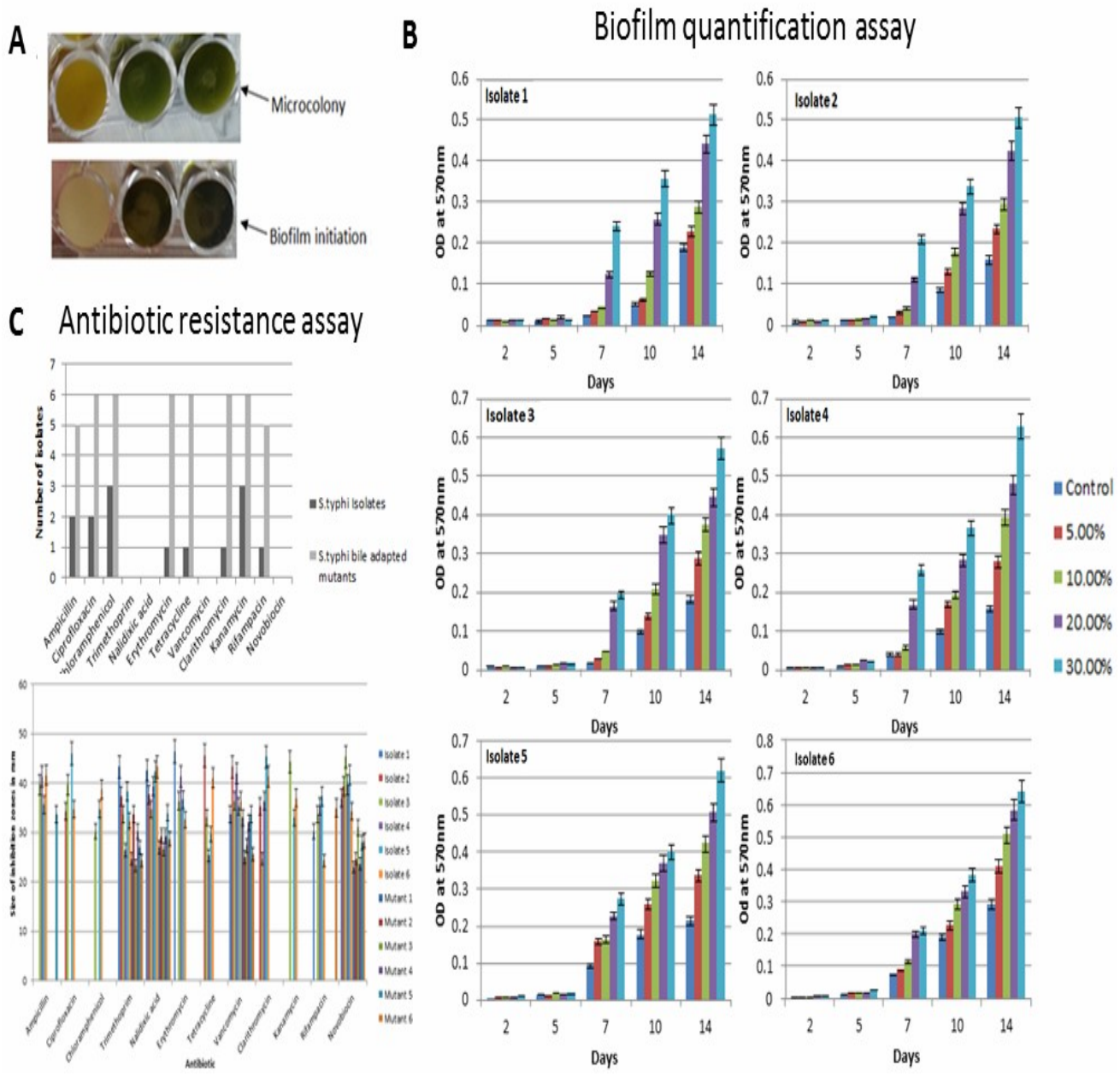


Figure.6 Reverse transcriptase PCR assay for *MicF, DsrA, rpoS and* ompF expression analysis. The mRNA levels of mentioned genes within *S. typhi* were estimated using reverse transcriptase PCR. Expression patterns for selected genes shown by (A) *S. typhi* grown in 5% bile. (B) Stable bile-resistant *S. typhi* isolates grown in 30% bile (C) *S. typhi* grown in LB without bile. The mRNA was extracted and cDNA synthesized, followed by PCR to candidate genes using specific primers. The products were run on 1.4% agarose gel and band intensity analysed by ImageJ 1.46r software. (B) Normalized mRNA expression levels used to determine the fold change. * The data was significant as the p-value obtained was $p < 0.01$ with fold changes of greater than 1.5

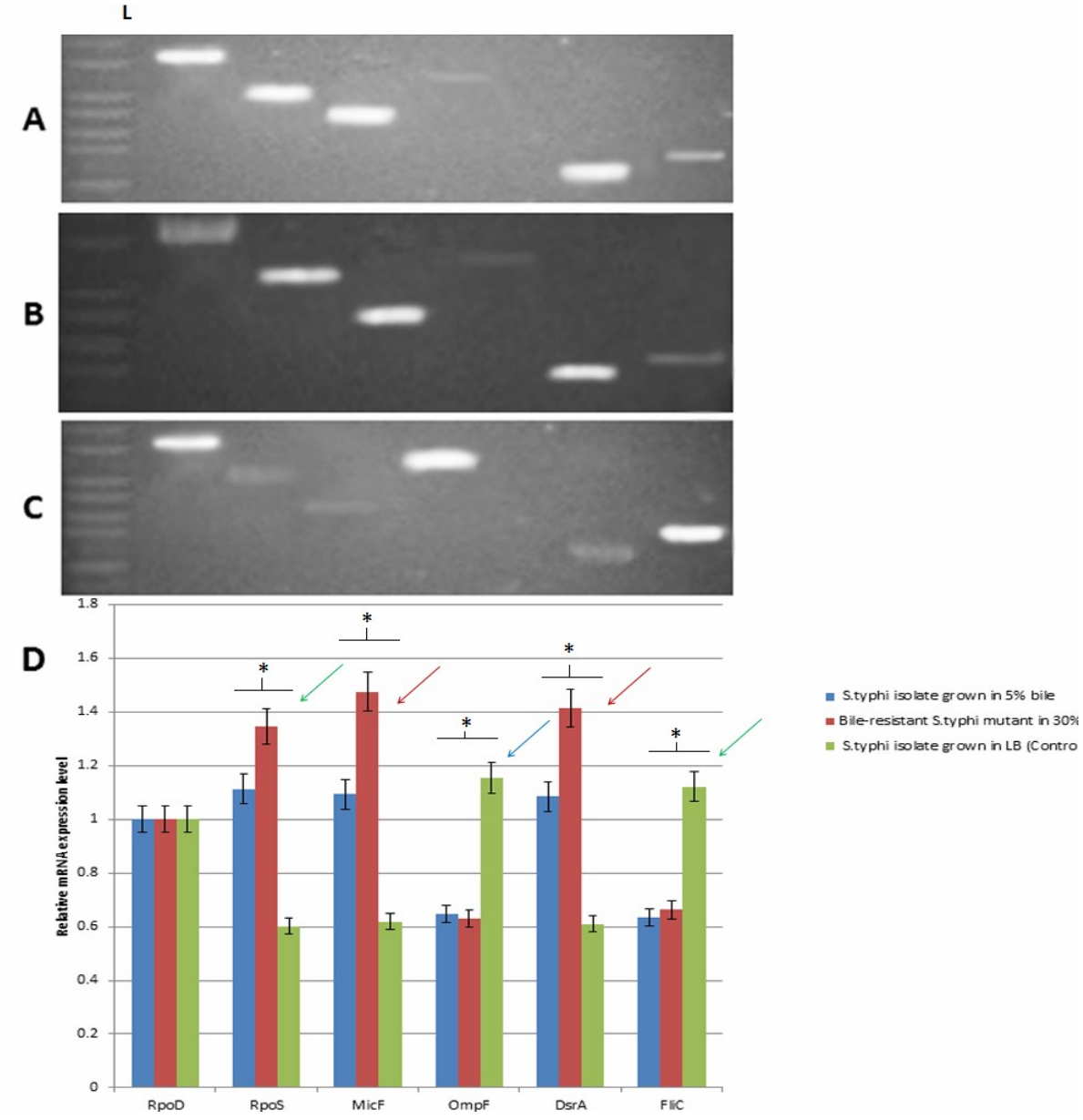
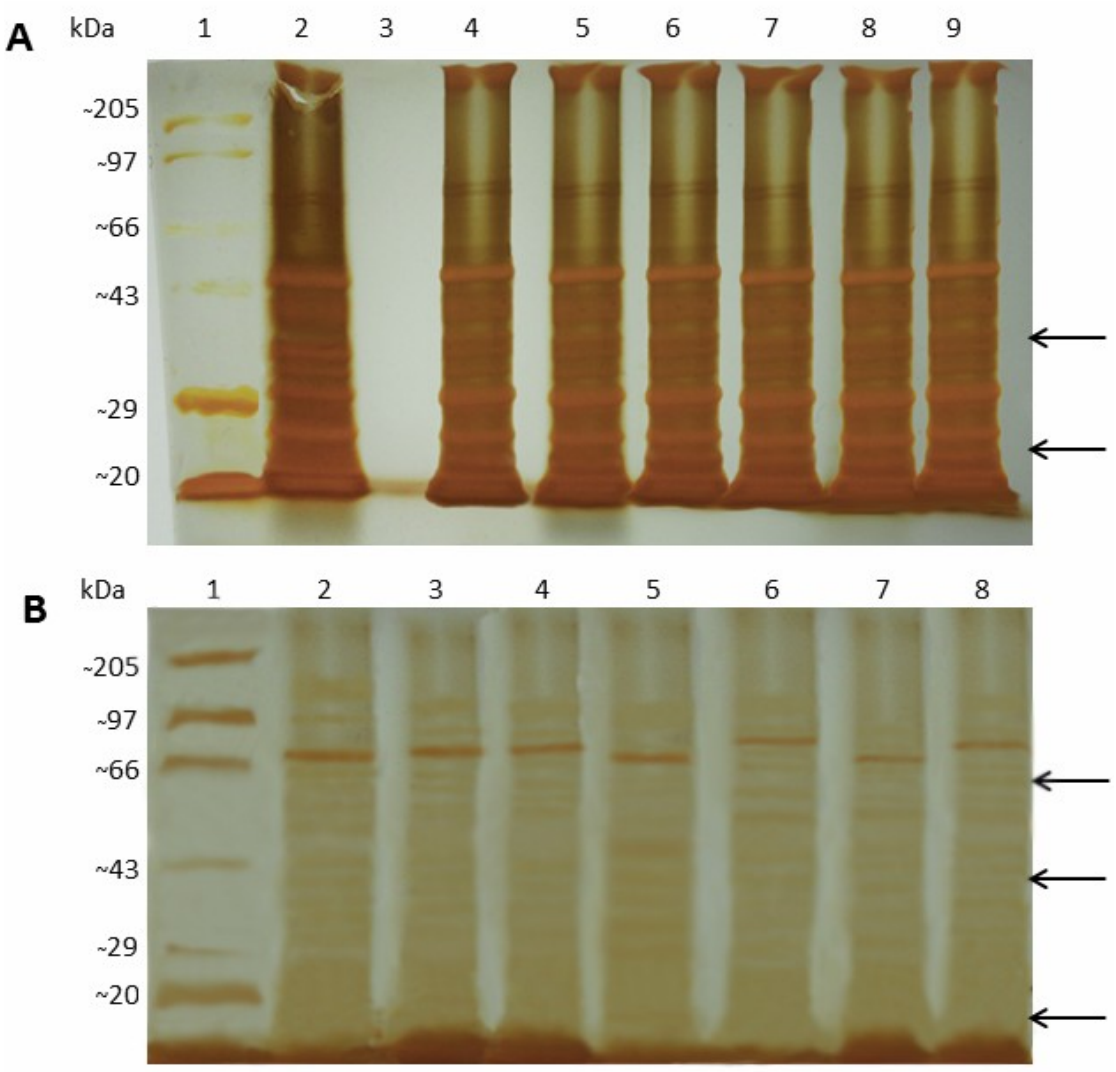


Figure.7 Protein profiles for stable bile resistant *S. typhi*: An SDS-PAGE analysis was carried out for (A) intracellular proteins. Lane 1: molecular weight marker; Lane 2: *S. typhi* grown in LB only; Lane 3: Negative control; Lane 4–9: Stable bile resistant isolates. (B) Outer membrane proteins. Lane 1: molecular weight marker; Lane 2–4 & 6–8: Stable bile resistant isolates; Lane 5: *S. typhi* grown in LB only. An aliquot containing 5µg protein sample was loaded and electrophoresed using 12% polyacrylamide gel. The bands were stained with silver stain. Arrow ( ) represents positions at which changes were observed in protein expressions



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