SUMMARY

The bacterial chromosomal DNA is folded into a compact structure called nucleoid. A number of proteins, named as histone-like proteins or nucleoid-associated proteins (NAP), are found to be involved in the compaction of chromosome (Dame, 2005;Dame *et al.*, 2000). A single *E. coli* has been shown to possess, 12 members of the NAP family proteins named as factor for inversion stimulation (Fis), H-NS (histone-like nucleoid structuring protein), HU (heat-unstable nucleoid protein), IHF (integration host factor), Lrp (leucine-responsive regulatory protein), CbpA (curved DNA binding protein A), CbpB (curved DNA binding protein B), DnaA (DNA-binding protein A), Dps (DNA-binding protein from starved cells) and StpA (suppressor of *Td2* phenotype A)(Azam and Ishihama, 1999). Each NAPs contain characteristic features either they have an individual expression patterns or they are often found to cross-regulate each other's genes and co-regulate other target genes, either cooperatively or antagonistically (Dorman and Deighan, 2003). Amongst NAP family of proteins Fis has been investigated in detailed.

The factor for inversion stimulation (Fis) was named for its role as cofactor in site specific recombination. It is unique among site specific DNA-binding proteins as it binds to a large number of different DNA sequences. The *E.coli* Fis crystal structure revealed that it is a small, basic, homodimeric DNA binding protein (Kostrewa *et al.*, 1992; Safo *et al.*, 1997; Yuan *et al.*, 1991; Yuan *et al.*, 1994). Each monomer contain four α helices, β turns and its N-terminal part is involved in DNA inversion reaction and C-terminal part is involved in DNA binding reaction.

Fis also plays a wide range of roles in *E.coli* and *Salmonella* beyond its relationship with the invertase enzymes (Finkel and Johnson, 1992). It contributes to the lifecycle of bacteriophage lambda by acting as a cofactor for phage integration and excision from the chromosome *in-vitro* and *in-vivo* (Esposito and Gerard, 2003; Papagiannis *et al.*, 2007; Thompson *et al.*, 1987). Fis is also involves in initiating the replication of the chromosome at oriC in *E. coli* (Filutowicz *et al.*, 1992; Gille *et al.*, 1991; Ryan *et al.*, 2004) and it regulates the transcription of a very large number of bacterial genes in *E.coli* and *Salmonella* (Grainger *et al.*, 2008; Kelly *et al.*, 2004). In this latter role, Fis can activate and repress promoters (Keane and Dorman, 2003;Ninnemann *et al.*, 1992). It functions in some cases as a conventional transcription activator, making physical contact with RNA polymerase (Bokal *et al.*, 1997;McLeod *et al.*, 2002). In other

cases, its positive effect on transcription is indirect and involves modulation of local DNA supercoiling at the target promoter in ways that enhance transcription initiation (Auner *et al.*, 2003). These widespread effects on transcription underline the importance of Fis as a governor of cellular physiology. This point is further reinforced when it consider that Fis regulates the expression of genes such as *rpoS*, *gyrA*, *gyrB*, and *topA* that in turn encode global regulators playing pivotal role in the physiology of *E.coli* and *Salmonella* (Hirsch and Elliott, 2005; Travers *et al.*, 2001; Weinstein-Fischer and Altuvia, 2007).

Additionally, DNA microarray analysis reveals a large regulon of genes that are regulated by Fis in *S. Typhimurium* and *E.coli* which are involved in carbon metabolism, propanediol utilization, ethanolamine utilization, amino acids and nucleotide biosynthesis (Bardley *et al.*, 2007;Kelly *et al.*, 2004). In essence, Fis plays a key role in coordinating expression of housekeeping genes and virulence factors thus influence the survival of the pathogen in human gut. Role of Fis is also well studied in regulation of biofilm formation of EAEC, *P. putida* and *D. dandantii* (Seikh *et al.*, 2001;Jakovleva *et al.*, 2012;Prigent-Combaret *et al.*,2012). The expression of Fis is tightly regulated by growth pattern of the cell where it is maximally present in the exponential phase followed by a decrease as the cells enter into stationary phase. Surprisingly, Fis is expressed at elevated level in *S. Typhimurium* grown to stationary phase under non-aerated condition (O Croinin and Dorman, 2007). Thus far, its role as a coordinator of diverse cellular events has been evaluated in enteric bacteria like enteropathogenic *E. coli*, *S. Typhimurium* and *S. flexneri*.

In *V. cholerae*, Fis has been found to influence the expression of quorum regulatory small RNAs in conjunction with central regulator LuxO (Lenz *et al.*, 2007). Interestingly, Fis promotes the expression of *qrr4* maximally. It remains unclear how Fis operates differently with the promoter region of various *qrr* genes. Similarly, its involvement in the regulation of *qrr* genes in other Vibrios has not been addressed. As there is a dearth of information regarding the role of Fis in biofilm development and motility in *V. cholerae,* prompted us to formulate this proposal.

In this piece of work, we analyzed the evolution of Vibrionaceae Fis. Morett and Bork have shown that Enterobacteriaceae Fis originated from C-terminal domain of an ancestral α-proteobacterial NtrC (Morett and Bork,1998). To, analyzed the evolutionary relationship between Vibrionaceae Fis and NtrC, multiple sequence alignment and phylogenetic analysis was done. Our result suggested that Fis shares a significant sequence

identity with the COOH-terminal domain of NtrC which belongs to α-proteobacteria. Thus, it seems that Fis originated from an ancestral α-proteobacterial NtrC protein and which is transferred to the Enterobacteriaceae, Vibrionaceae and other γ-proteobacteria by horizontal gene transfer.

Another part of study was focused on phylogenetic analysis of Vibrionaceae Fis. Our result from multiple sequences alignment and phylogenetic analysis suggested the conservation of Fis among the members of Vibrionaceae. Interestingly, Fis sequence of *V. fischeri* revealed 11 amino acid differences in comparison with Fis sequence of *V. cholerae*. This prompted us to select the $Fis_{V.fischeri}$ for functional characterization via evaluating its binding with promoter region of $qrr1_{V.fischeri}$. The DNA binding assay results indicated that $Fis_{V.fischeri}$ binds with promoter region of $qrr1_{V.fischeri}$. Moreover, $Fis_{V.fischeri}$ was also able to bind with promoter region of $qrr1_{N16961}$ and $qrr4_{N16961}$. In addition to this, our results also suggested that Fis_{N16961} was also able to bind with promoter region of $qrr1_{V.fischeri}$, on the contrary it was unable to bind with its own *qrr*1.

Previous studies suggest that *E.coli* Fis crystal structure is divided into two functionally distinct domains, namely a C-terminal DNA binding region and N-terminal region which is involved in Hin-mediated inversion. The mutagenesis experiments revealed the crucial residues in the C-terminal helix-turn-helix (HTH) DNA binding motif (Osuna *et al.*, 1991;Koch *et al.*, 1991). These residues are required to attain DNA binding because mutations in these residues reduced or abolished this function. The D helix residues R^{85}, T^{87}, R^{89}, K^{90}, K^{91} and K^{94} are the most critical residues in achieving highly specific DNA binding. Additionally, replacement of proline at 61position by leucine or serine severely incapacitates the mutant protein to promote DNA inversion while keeping the DNA binding property intact (Yuan *et al.*, 1991). Collectively, the mutations in helix-turn-helix motif, helices α-C and α-D, can directly influence DNA binding. Additionally, it was reported that *E.coli* Fis mutants exhibit altering affinity with different DNA binding sites. It is also documented that substitution of binding sites or presence of flanking sequences also influence the DNA binding.

As evident from preceding section that *E.coli* Fis mutants suggest different binding properties according to DNA sites. Moreover, it was also reported that Fis of *V. cholerae* binds to each of the *qrr* promoters with different affinities (*qrr*4 > *qrr*2, 3 > *qrr*1) (Lenz *et al.*, 2007). It has been evidenced that certain amino acids at the N-terminal as well as C-

terminal region of *E.coli* Fis contribute critically to mediate its interaction with various target promoters. In similar approach we wanted to probe the key amino acids of Fis (*V.cholerae* strain N16961) playing an important role in binding to promoter region of *qrr*1$_{N16961}$ and *qrr*4$_{N16961}$. Our results revealed all Fis$_{N16961}$ mutants were also unable to bind with *qrr*1$_{N16961}$ promoter region like wild type Fis$_{N16961}$. Interestingly, the substitution of **K^{90}A** is able to bind with promoter region of *qrr*1$_{N16961}$. This result suggested importance of **K^{90}** residue of Fis$_{N16961}$ to achieving the specific binding. Additionally, alanine derivatives of residues **K^{91}A** and **K^{94}A** resulted in complete loss of binding with promoter region of *qrr*4$_{N16961}$. The alanine substitution of P^{61}, R^{85}, T^{87}, R^{89}, K^{90}, K^{93} residues resulted in no significant loss in DNA binding ability with promoter region of *qrr*4$_{N16961}$. Mutations within the central helix B region of Fis exhibit fundamentally different activity such as substitution of **P^{61}L** or **P^{61}S** severely affected the ability of the mutant proteins to bind with promoter region of *qrr*4$_{N16961}$. Additionally, the substitution of **R^{89}C** resulted in loss of binding ability with promoter region of *qrr*4$_{N16961}$.

As evident from preceding section that Fis regulates the diverse cellular events such as motility, flagellar gene expression and virulence in *E.coli* and *S. Typhimurium*. In addition to this, role of Fis in biofilm regulation has been documented in pathogenic strains (Seikh *et al.*, 2001;Jakovleva *et al.*, 2012;Prigent-Combaret *et al.*,2012). To investigate the involvement of Fis in regulation of motility of *V.cholerae*, the motility assay was performed. Our motility assay results revealed that reduction in the motility of *fis* mutant unlike its wild type strain. Consecutively, Fis$_{N16961}$ has shown interaction with representative promoter regions of genes related to flagellar machinery such as *flr*A, *flr*C and *fli*A. We also evaluated the role of Fis in biofilm formation of *V. cholerae* strain N16961. Our data revealed a decrease in biofilm production by *fis* mutants in comparison with wild type strain of *V. cholerae*.

In next part of study, we investigated the utilization of ethanolamine in *V. alginolyticus*. The preliminary knowledge of ethanolamine utilization was elucidated in Enterobacteriaceae such as *S. Typhimurium* and *E.coli*. The *eutB* and *eutC*, which encode the ethanolamine ammonia lyase large and small subunits, are central genes of this process and in addition to this it contains 16 accessory genes. The sequences of genes encoding EutB and EutC have been reported in nearly 100 bacterial genomes (Tsoy *et al.*, 2009), there was no information regarding the existence of these genes in *Vibrio* genomes. This

prompted us to investigate the genes of *eut* operon in the available *Vibrio* genomes. To identify the EutB and EutC proteins and their homologs the PSI-BLAST analysis was performed. This analysis revealed the presence of homologs in several species of *Vibrio*. Additionally, we also search the presence of other *eut* operon genes in Vibrios. This analysis revealed that *V. alginolyticus* 12G01, *V. furnissi* CIP 102972, *V. sp.* EJY3 and *V. metschnikovii* CIP 69.14 contained the maximum number of *eut*-operon-related proteins.

We used *V. alginolyticus* as a model system for experimental confirmation of our prediction that some *Vibrio spp.* are likely to metabolize ethanolamine. Our results suggested that *V. alginolyticus* can use ethanolamine as a preferred nitrogen source. This is the first report describing the presence of a functional, albeit minimal, ethanolamine utilization operon in *Vibrio* species (Khatri *et al.*, 2012).

Collectively, this study suggested the evolution of Fis of Vibrionaceae from α-proteobacterial NtrC proteins and mutagenesis study revealed the presence of critical residues of Fis_{N16961} required for binding with DNA sites. Moreover, it was suggested that Fis of *V. cholerae* has found to be involved in the regulation of motility and biofilm formation. Continued work on Fis of Vibrionaceae may present a research avenue to elucidate the details surrounding of its biological function.