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Regulatory Role of *fnr* Gene in Growth and *tolA* Gene Expression in *Salmonella* Typhimurium

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ABSTRACT

Background: *Salmonella* Typhimurium (*S*.Typhimurium) adapts to the broad fluctuations of oxygen concentrations encountered in the host. The transition from aerobic to microaerobic/anaerobic condition encountered in the intestine is mainly regulated by fumarate and nitrate reductase (*fnr*) regulatory gene and aerobic respiratory control A (*arcA*) gene. Aim is to appraise the role of *fnr* gene under anaerobic conditions.

Methods: In this study, we deleted *fnr* gene from *S*.Typhimurium using lambda red-recombinase mediated gene knockout protocol. Further carried out *in vitro* characterization and analyzed the differential protein expression in wild type (WT) and isogenic Δ*fnr* null mutant (Δ*fnr*) using SDS-PAGE and MALDI-TOF mass spectrometry under anaerobic conditions.

Result: In growth competition, WT strain outcompeted the Δ*fnr* and biofilm-forming ability of Δ*fnr* was significantly reduced compared to WT strain. Swimming motility was reduced in Δ*fnr* strain. Besides, differential protein expression revealed the global changes in the expression of many proteins in *fnr* strain. One differentially expressed protein was identified as TolA, an inner membrane envelope protein. It points out that *fnr* may regulate the genes responsible for motility and biofilm formation. FNR protein positively regulates TolA, which is important for bacterial virulence, maintenance of membrane integrity, LPS production and replication of bacteria.

Key words: *fnr*, Motility, Virulence.

INTRODUCTION

Salmonella enterica serovar Typhimurium (*S*.Typhimurium) is a broad host range pathogen that causes acute self-limiting gastroenteritis in humans, cattle, swine, poultry, other large vertebrates. It can cause bacteraemia and systemic infection in immunosuppressed hosts, occasionally in healthy adult humans and animals (Pegues, 2005). Bacteria adapt to the various adverse conditions in the environment or in the host, which increases the persistency of infection (Chakroun *et al.*, 2017). It causes non-typhoidal Salmonellosis (NTS) and infection occurs mainly through the ingestion of contaminated food or water. Bacteria survive in the acidic pH of the stomach and enter the intestine (HARRIS *et al.*, 1972; Chopra *et al.*, 1994). The organism generally targets and colonizes in the intestinal epithelium of the host and causes gastroenteritis (Finlay and Falkow, 1990). *Salmonella* pathogenicity island-1 (SPI-1) type III secretion system (T3SS) is important for the bacterial invasion of intestinal epithelial cells and its virulence (Miki *et al.*, 2004). Bacterial adhesion and invasion to the epithelial cells occur more in anaerobic condition than in the aerobically grown cells (Lee and Falkow, 1990). The mechanisms or genes controlling the adaptation to the anaerobic conditions may play an important role in the virulence of this species (Fink *et al.*, 2007; Fernández *et al.*, 2018). The changes in the cellular process during the transition from aerobic to anaerobic environment have been studied in detail in *E.coli*. More than 70 genes, important for the adaptation to the anaerobic conditions are under the control of the DNA-binding protein FNR (Fumarate and Nitrate Reduction regulatory protein), a cytoplasmic sensor of oxygen. The active form of FNR contains one $[4Fe\text{-}4S]^{2+}$ cluster per protein monomer and it gets converted to $[2Fe\text{-}2S]^{2+}$, an inactive form along with other less defined iron species, following exposure to oxygen in both *in vitro* and *in vivo* condition (Park and Gunsalus, 1995; Khoroshilova *et al.*, 1997; Crack *et al.*, 2004). FNR binds to promoter sequences usually at position -41 relative to the start of transcription and may also bind at position -61,-71,-81, -91 (Wing *et al.*, 1995). The sequence recognized by FNR is palindromic (TTGATN4ATCAA) and when bound to this sequence, it interacts with the RpoA subunit of RNA polymerase and increases the efficiency of transcription (Lombardo *et al.*, 1991). A reduced level of oxygen in the

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intestine during the bacterial colonization forces the organism to opt for anaerobic respiration. Adaptation to anaerobic conditions is controlled either alone by FNR protein or by inducing/repressing various other regulators such as ArcA, RpoS, NarL, Fur, or SoxR (Spiro and Guest, 1990; Hassan and Sun, 1992; Gunsalus and Park, 1994). The aim of our experiment was to generate *fnr* null mutant of *S*. Typhimurium through lambda red-recombinase mediated gene knock out, to assess the role of *fnr* gene through *in vitro* experiments and proteomic analysis of WT and Δ*fnr* strains grown under anaerobic condition.

MATERIALS AND METHODS

All experiments were carried out in the Division of Animal Biochemistry, ICAR-IVRI, Izzatnagar, Bareilly in the time period of 2017-18. For MALDI-TOF, the sample was sent to IISc, Bengaluru.

Bacterial strains and culture

Salmonella Typhimurium (*S*. Typhimurium) strain PM45 (poultry isolate) was used. The cultures were streaked on Hektoen Enteric (HE) agar (HiMedia). Isolated colonies were characterized by biochemical tests and further confirmed by amplification of *inv*A, a *salmonella* specific gene (Park *et al.*, 2008). DH5α strain of *E.coli* was obtained from Stratagene.

Vectors

A helper plasmid pKD46, donor plasmid pKD4 and pCP20 were procured from Addgene. Oligonucleotide Primers were Procured from Xceleris Labs limited, Ahmedabad, India and sequence details are given in Table 1.

Construction of *fnr* gene deletion mutant *S*.Typhimurium

fnr gene from *S*. Typhimurium was deleted by one step gene inactivation protocol (Datsenko and Wanner, 2000).

PCR reaction conditions

The confirmation of Δ*fnr* strain was done in 50µL reaction volume containing 1x Taq DNA polymerase buffer, 1.5mM $MgCl_2$, 20 pmol of each primer (FNR outer primers), 200µM dNTPs, 1µL genomic DNA, 2U Taq DNA Polymerase (Thermo scientific) and nuclease free water to 50µL. After initial denaturation at 94°C for 5 min, the amplification was carried out for 34 cycles each of 94°C-30 s, 52°C-45 s, 72°C-2 min with a final extension of 10 min at 72°C. Further confirmation was done using *fnr* inner primers, it was carried out in 50µL reaction volume containing 1x Taq DNA polymerase buffer, 1.5mM $MgCl_2$, 20 pmol of each primer (FNR inner primers), 200µM dNTPs, 1µL genomic DNA, 2U Taq DNA Polymerase (Thermo scientific) and nuclease free water to 50µL. After initial denaturation at 94°C for 5 min, the amplification was carried out for 34 cycles each of 94°C-30 s, 52°C-30 s, 72°C-30 s with a final extension of 10 min at 72°C.

In vitro characterization of *fnr* gene deletion mutant of *S*.Typhimurium (Δ*fnr*)

In vitro characterization of Δ*fnr* was carried out in an anaerobic jar.

Growth competition assay

The WT and the Δ*fnr* strains were grown anaerobically at 37°C in MOPS (morpholinepropanesulfonic acid)-buffered (100mM, pH 7.4) Luria Bertani (LB) broth supplemented with 20mM D-xylose (LB-MOPS-X). Anaerobic chamber (HiMedia) and anaerobic gas pack (BD BBL™ GasPak™ anaerobic and CO_2 indicators) were used. All solutions were pre-equilibrated for 48 hours in an anaerobic chamber. A single isolated colony of both WT and Δ*fnr* were inoculated into LB-MOPS-X broth for 16 hrs in anaerobic chambers and later from these fresh cultures were again inoculated in the same media till saturation. After saturation, both cultures were mixed in equal volume (1.0 ml each) and grown in 100 ml of MOPS buffered LB medium and growth competition was monitored at different time intervals (20, 40 and 60 hrs) (Samhita *et al.*, 2014). The numbers of Δ*fnr* and WT strains were enumerated randomly by colony PCR.

Table 1: List of primers used.

Primers	Sequence	Length (bp)	Tm (°C)	GC (%)	Product length (bp)	Accession number and position
FNR del FP	5′ GTTAAAATTGACAAATATCAATTACGGCT	61	70	57		CP019649.1
FNR del RP	TGAGCAGACCTTGTGTAGGCTGGAGCTGCTT 3′				1100	(1724373-1725205)
	5′ ACGATATGGCAGAAGATAACATCAATGGT TTAGCTGACGTCATATGAATATCCTCCTTA 3′	59	51	32		
FNR outer FP	5′ AAATTGACAAATATCAATTACGGCT 3′	25	52	28	825	CP019649.1
FNR outer RP	5′ TATGGCAGAAGATAACATCAATGGT 3′	25	55	36		(1724377-1725201)
FNR inner FP	5′ AGCTTTGCATCCCGTTCACACTT 3′	23	62	48	213	CP019649.1
FNR inner RP	5′ GGTCGCCTGCTAAATGGAATCC 3′	22	61	55		(1724492-1724704)
InvA FP	5′ TCATCGCACCGTCAAAGGAACC 3′	22	62	55	284	CP019649.1
InvA RP	5′ GTGAAATTATCGCCACGTTCGGGCAA 3′	26	64	50		(3030243-3030527)
TolA RT FP	5′ AGGCGTCGACGATCTGCTTG 3′	20	63	60	185	CP043400.1
TolA RT RP (qRT-PCR)	5′ CGCCATTCAGAGCCGTCTGT 3′	20	63	60		(815945-816129)
16s r RNA FP	5′ AGGCCTTCGGGTTGTAAAGT 3′	20	59	50	97	CP043400.1
16s rRNA RP (qRT-PCR)	5′ GTTAGCCGGTGCTTCTTCTG 3′	20	59	55		(2855069-2855165)

Biofilm formation assay

Biofilm formation by *Salmonella* isolates was assessed using microtitre plate assay. The assay was performed in sterile 96-well flat-bottom polystyrene microplates filled with 180µL of LB-MOPS-X media. The negative control wells contained 200µL of media only. 20µL of overnight grown cultures of both WT and Δ*fnr* were dispensed to the wells in triplicate. The inoculated plates were then incubated anaerobically at 37°C for 72 hours. After incubation, the contents of the plates were poured off and the wells were thoroughly washed thrice with PBS (pH 7.2). The adherent bacterial cells were then stained with 200 µL of 0.5% (w/v) crystal violet stain per well for 10 min. After staining, plates were washed thrice with sterile distilled water. The plates were air-dried and 250µL of 33% glacial acetic acid was added to each well and mixed properly with gentle shaking. The optical density (O.D.) of each well was measured at 590nm using an automated ELISA reader (Nair *et al.*, 2015; Shukla and Rao, 2017).

Swimming motility assay

Assay was performed as per the standard protocol described (Monteiro *et al.*, 2012). Both WT and Δ*fnr* bacteria were grown overnight at 37°C on an HEA plate and stab inoculated on 0.3% LB without salt agar plate with a toothpick. The plates were incubated at 28°C for 7hrs in an anaerobic chamber. The diameters of migrating bacteria from the point of inoculation (turbid zone) were measured. The results shown are representative of at least three independent experiments.

Sample preparation for proteomic study

WT and Δ*fnr* strains were grown in LB MOPS-Xylose broth in an anaerobic environment. Overnight grown cultures were pelleted by centrifugation at 4500 rpm for 15 minutes. Pellets were suspended in lysis solution (7M urea, 2M thiourea, 4% CHAPS, 20mM Tris) and the sample was sonicated and centrifuged for 30 minutes at 7000 rpm, supernatants were collected and stored. The protein concentrations of lysates were determined by the Lowry method (Lowry *et al.*, 1951).

Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out in midigel (Tarson, India) apparatus with 5% stacking and 12% separating gel as per protocol (Sambrook, 2001). Equal amounts (concentration) of samples were loaded with a 5x Laemmli sample loading buffer along with the prestained protein marker. Electrophoresis was carried out at 100 volts.

Peptide mass fingerprinting

Differentially expressed proteins were cut, sent for MALDI-TOF mass spectrometry and obtained mass to charge ratios of peptides were analyzed using Mascot search engine software (Peptide mass fingerprinting).

Real– time quantitative reverse transcription PCR (qRT-PCR)

This technique was used to analyze the expression level of TolA protein at the RNA level and to validate the result of SDS-PAGE and mass spectrometry. Total RNA isolated from anaerobically grown overnight cultures of WT and Δ*fnr* strains using Trizol reagent. Isolated RNA samples were treated with Dnase I and dissolved in nuclease free water. RT-PCR was performed as per the Thermo Scientific RevertAid First Strand cDNA Synthesis Kit protocol. qRT-PCR was carried out using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific), AriaMx Real-Time PCR System (Agilent Technologies) and data were analyzed by Agilent Aria Software Setup 1.6. TolA RT and 16s rRNA primers used for qRT-PCR were given in Table 1 (Gupta *et al.*, 2014; Behera *et al.*, 2020). All the procedure was carried out according to the MIQE guidelines.

Statistical method

One-way ANOVA was carried out using SPSS version 20 to test whether absorbance was significantly variable among different groups.

RESULTS AND DISCUSSION

Confirmation of *fnr* gene deletion mutant (Δ*fnr*)

Δ*fnr* was confirmed by FNR outer primers which resulted in a product size of 120 bp size whereas WT gave a product of 825 bp (Fig 1) and with FNR inner primers WT gave 213 bp size whereas only primer dimers were seen in Δ*fnr*, there was no PCR product (Fig 2). Primers were presented in Table 1.

Growth competition assay

At 20, 40 and 60 hrs interval, WT and Δ*fnr* bacterial growth patterns were observed. WT strain outcompeted the growth of Δ*fnr* strain at these time intervals. Strain abundance of Δ*fnr* was found to be 38%, 16% and 16% at 20, 40 and 60 hrs respectively (Fig 3). In a growth competition assay, a considerable difference was observed in relative growth between WT and Δ*fnr* strains. The strain abundance of Δ*fnr* started decreasing from 38% to 16% from 20-60 hrs intervals. This indicates that *fnr* gene deletion reduced the ability to compete with WT strain in nutrient deficient conditions and thus resulted in some degree of growth attenuation.

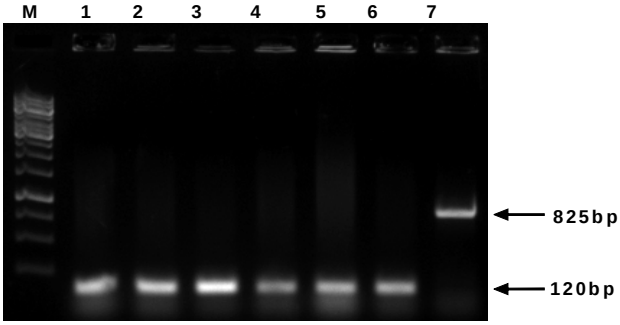

Fig 1: Confirmation of *fnr* gene deletion mutant (Δ*fnr*) by FNR outer primers.

Lane M: 1kb DNA ladder.

Lane 1-6: *fnr* gene deletion mutant (Δ*fnr*).

Lane 7: WT.

fnr gene contribute to the biofilm formation

Biofilm formation ability was checked in 96-well plate as it is a measure of the persistence of bacteria in the environment and in the host by colonization through forming an exopolymer matrix (Grantcharova *et al.*, 2010). The mean absorbance of wild strain (0.562±0.025) was significantly higher than that of Δ*fnr* strain (0.346±0.018) (Fig 4). Statistical analysis: One way Anova, Post hock test Tukey's, P*<0.05 P**<0.01, P***<0.001, N.S.-Not significant. Thus, *fnr* gene played important role in the attachment of bacteria and so it may affect the colonizing ability of bacteria in the intestinal epithelium, essential for bacterial pathogenesis (Grantcharova *et al.*, 2010; Chelvam *et al.*, 2014; Chakroun *et al.*, 2018). *fnr* may also control the genes responsible for biofilm formation (Chakraborty *et al.*, 2020).

Δ*fnr* strain has reduced Swimming motility

The effect of the deletion of *fnr* gene on the motility of *Salmonella* Typhimurium was analyzed on 0.3% LB agar plate without salt. Δ*fnr* had shown significant reduction (P<0.001) in motility as compared to WT strain**.** A swimming motility test, a marker of bacterial invasion through the flagellar movement was conducted (Iyoda *et al.*, 2001). Motility of both WT and Δ*fnr* strains was assessed by taking the diameter of the turbid zone around the stab inoculated region. The diameter of the migrating bacteria on semisolid media of Δ*fnr* strain was profoundly less (2.032±0.0578) compared to the WT strain (2.967±0.152). It may indicate that *fnr* gene regulates flagellar genes and motility. It is important for the bacterial invasion to the host cells (Khoramian-Falsafi *et al.*, 1990; Morimoto *et al.*, 2017) (Fig 5). Statistical analysis: T-test, P*<0.05 P**<0.01, P***<0.001, N.S.-Not significant. Our findings are in correlation with the transcriptomic and proteomic analysis of Δ*fnr* strain of *S.* Typhimurium under anaerobic condition, that flagellar genes are activated by *fnr* (Fink *et al.*, 2007; Behera *et al.*, 2020).

One dimension-PAGE analysis of WT and Δ*fnr* showed differential expression of many proteins

An equal amount of proteins from both WT and Δ*fnr* cell lysates (100µg) each was loaded onto SDS-PAGE and gel was stained with 0.25% Coomassie brilliant blue (CBB-R 250). There were significant changes in the expression of many proteins and differentially expressed protein in the 45 kDa region from WT lane was cut and sent for mass spectrometry analysis (MALDI-TOF) (Fig 6). Analyzed protein sequence through peptide mass fingerprinting and identified as TolA (Supplementary material, S.5). we are first to demonstrate that *tolA* gene is regulated by *fnr* under anaerobic condition.

Real –time quantitative reverse transcription PCR (qRT-PCR) demonstrate that Δ*fnr* has reduced expression of *tolA* gene

Measured mRNA levels were normalized to the expression of housekeeping gene 16s rRNA. These normalized values are used for the calculation of fold expression. *tolA* gene

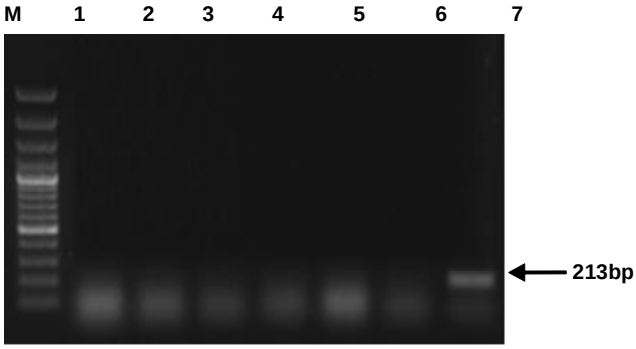

Fig 2: Confirmation of gene deletion mutant (Δ*fnr*) by FNR inner primers.
Lane M: 100bp DNA ladder.
Lane 1-6: *fnr* gene deletion mutants (Δ*fnr*).
Lane 7: WT.

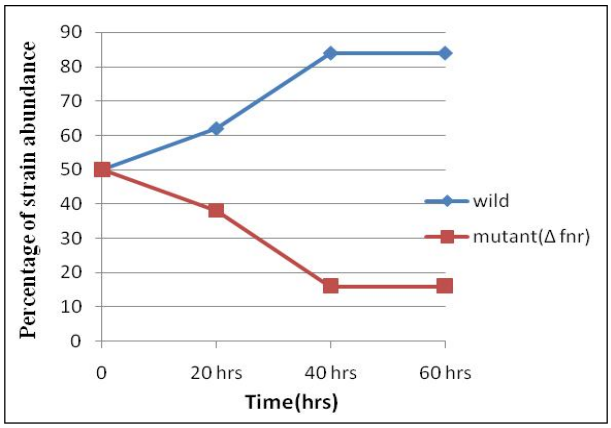

Fig 3: Growth competition assay. Growth competition assay was carried out at different time intervals of 20, 40 and 60 hrs and strain abundance was done by random PCR amplification.

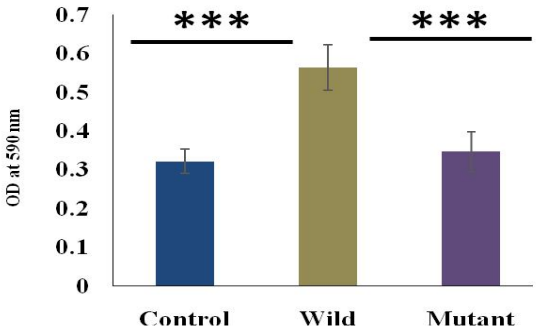

Fig 4: Biofilm formation assay.
Biofilm formation ability of both WT and Δ*fnr* strains were carried out in microtitre plate method. Data is represented as mean±S.D of two individual experiments (n=3) (***denotes p<0.001).

was 27 fold down-regulated in Δ*fnr* compared to WT. Which supports our proteomic study that *fnr* gene activates *tolA* gene under anaerobic condition. Further validation was done by qRT-PCR technique, *tolA* gene was 27 fold down-regulated Δ*fnr* compare to WT strain at the RNA level (Fig 7). First time we are reporting that *tolA* gene is positively regulated by *fnr* gene in an anaerobic environment that is

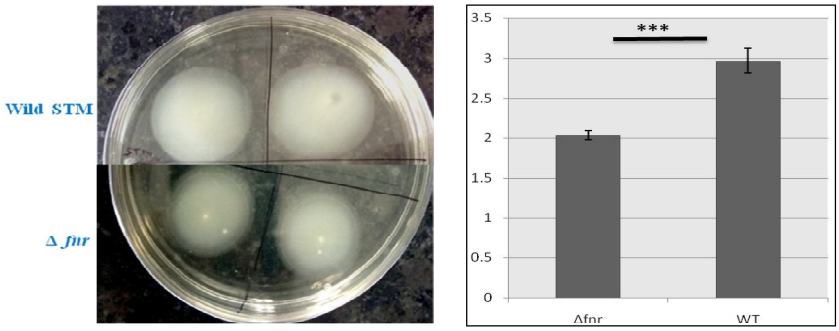

Fig 5: Swimming motility test: Data is represented as mean ± S.D of two individual experiments (n=3) (***denotes p<0.001).

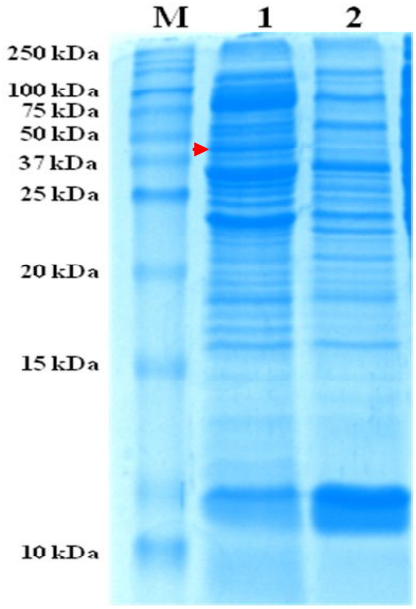

Fig 6: SDS-PAGE analysis of whole protein lysates of WT and Δ*fnr* strain.

Equal amount of proteins from both WT and Δ*fnr* cell lysates were loaded on to SDS-PAGE and gel was stained with 0.25% comassie brilliant blue (CBB-R 250). There were significant changes in expression of many proteins. Arrow indicated band was cut and sent for MALDI-TOF Analysis.

Lane M- Prestained Protein Marker.

Lane 1- WT lysate.

Lane 2- Δ*fnr* lysate.

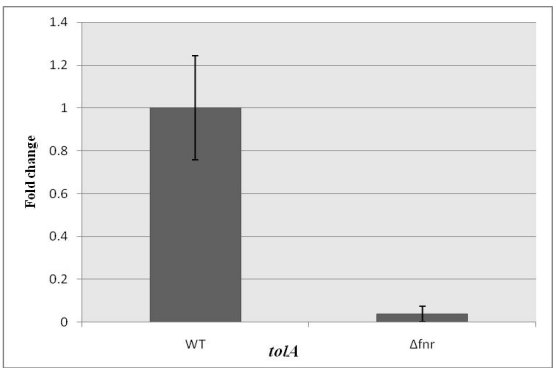

Fig 7: qRT- PCR analysis of *tolA* gene: Data is represented as mean ± S.E of three individual experiments in triplicates.

responsible for bacterial growth and pathogenesis. TolA is an inner membrane integrity protein, important for bacterial virulence, maintenance of membrane integrity, LPS production and bacterial replication of *Salmonella* Typhimurium (Paterson *et al.*, 2009; Masilamani *et al.*, 2018).

CONCLUSION

In the current study, we reported that *fnr* gene plays an essential role in regulating the bacterial growth, motility and virulence of *S.* Typhimurium by controlling the expression of proteins like TolA and other proteins.

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REFERENCES

Behera, P., Nikhil, K.C., Kumar, A., Gali, J.M., De, A., Mohanty, A.K., Ali, M.A. and Sharma, B. (2020). Comparative proteomic analysis of Salmonella Typhimurium wild type and its isogenic fnr null mutant during anaerobiosis reveals new insight into bacterial metabolism and virulence. Microb. Pathog. 140: 103936.

Chakraborty, S., Roychoudhury, P., Samanta, I., Subudhi, P.K., Das, M., De, A., Bandyopadhayay, S., Joardar, S.N., Mandal, M. and Qureshi, A. (2020). Molecular detection of biofilm, virulence and antimicrobial resistance associated genes of Salmonella serovars isolated from pig and chicken of Mizoram, India. Indian J. Anim. Res. 54(5).

Chakroun, I., Cordero, H., Mahdhi, A., Morcillo, P., Fedhila, K., Cuesta, A., Bakhrouf, A., Mahdouani, K. and Esteban, M.Á. (2017). Adhesion, invasion, cytotoxic effect and cytokine production in response to atypical Salmonella Typhimurium infection. Microb. Pathog. 106: 40-49.

Chakroun, I., Mahdhi, A., Morcillo, P., Cordero, H., Cuesta, A., Bakhrouf, A., Mahdouani, K. and Esteban, M.Ị. (2018). Motility, biofilm formation, apoptotic effect and virulence gene expression of atypical Salmonella Typhimurium outside and inside Caco-2 cells. Microb. Pathog. 114: 153-162.

Chelvam, K.K., Chai, L.C. and Thong, K.L. (2014). Variations in motility and biofilm formation of Salmonella enterica serovar Typhi. Gut Pathog. 6(1): 2.

Chopra, A.K., Peterson, J.W., Chary, P. and Prasad, R. (1994). Molecular characterization of an enterotoxin from Salmonella typhimurium. Microb. Pathog. 16(2): 85-98.

Crack, J., Green, J. and Thomson, A.J. (2004). Mechanism of oxygen sensing by the bacterial transcription factor fumarate-nitrate reduction (FNR). J. Biol. Chem. 279(10): 9278-9286.

Datsenko, K.A. and Wanner, B.L. (2000). One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc. Natl. Acad. Sci. 97(12): 6640-6645.

Fernández, P., Velásquez, F., Garcias-Papayani, H., Amaya, F.A., Ortega, J., Gómez, S., Santiviago, C.A. and Álvarez, S.A. (2018). Fnr and ArcA regulate lipid a hydroxylation in Salmonella enteritidis by controlling lpxO expression in response to oxygen availability. Front. Microbiol. 9: 1220.

Fink, R.C., Evans, M.R., Porwollik, S., Vazquez-Torres, A., Jones-Carson, J., Troxell, B., Libby, S.J., McClelland, M. and Hassan, H.M. (2007). FNR is a global regulator of virulence and anaerobic metabolism in Salmonella enterica serovar Typhimurium (ATCC 14028s). J. Bacteriol. 189(6): 2262-2273.

Finlay, B.B. and Falkow, S. (1990). Salmonella interactions with polarized human intestinal Caco-2 epithelial cells. J. Infect. Dis. 162(5): 1096-1106.

Grantcharova, N., Peters, V., Monteiro, C., Zakikhany, K. and Römling, U. (2010). Bistable expression of CsgD in biofilm development of Salmonella enterica serovar typhimurium. J. Bacteriol. 192(2): 456-466.

Gunsalus, R.P. and Park, S.-J. (1994). Aerobic-anaerobic gene regulation in Escherichia coli: control by the ArcAB and Fnr regulons. Res. Microbiol. 145(5-6): 437-450.

Gupta, A.R., Dey, S., Saini, M. and Swarup, D. 2014. Down-regulation of expression of type 1 collagen gene in dentin of fluoride exposed rats. Indian J. Anim. Res. 48(6): 601-604.

Harris, J.C., Dupont, H.L. and Hornick, R.B. (1972). Fecal leukocytes in diarrheal illness. Ann. Intern. Med. 76(5): 697-703.

Hassan, H.M. and Sun, H.C. (1992). Regulatory roles of Fnr, Fur and Arc in expression of manganese-containing superoxide dismutase in Escherichia coli. Proc. Natl. Acad. Sci. 89(8): 3217-3221.

Iyoda, S., Kamidoi, T., Hirose, K., Kutsukake, K. and Watanabe, H. (2001). A flagellar gene fliZ regulates the expression of invasion genes and virulence phenotype in Salmonella enterica serovar Typhimurium. Microb. Pathog. 30(2): 81-90.

Khoramian-Falsafi, T., Harayama, S., Kutsukake, K. and Pechere, J.C. (1990). Effect of motility and chemotaxis on the invasion of Salmonella typhimurium into HeLa cells. Microb. Pathog. 9(1): 47-53.

Khoroshilova, N., Popescu, C., Münck, E., Beinert, H. and Kiley, P.J. (1997). Iron-sulfur cluster disassembly in the FNR protein of *Escherichia coli* by O2:[4Fe-4S] to [2Fe-2S] conversion with loss of biological activity. Proc. Natl. Acad. Sci. 94(12): 6087-6092.

Lee, C.A. and Falkow, S. (1990). The ability of Salmonella to enter mammalian cells is affected by bacterial growth state. Proc. Natl. Acad. Sci. 87(11): 4304-4308.

Lombardo, M.J., Bagga, D. and Miller, C.G. (1991). Mutations in rpoA affect expression of anaerobically regulated genes in Salmonella typhimurium. J. Bacteriol. 173(23): 7511-7518.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275.

Masilamani, R., Cian, M.B. and Dalebroux, Z.D. (2018). Salmonella Tol-Pal reduces outer membrane glycerophospholipid levels for envelope homeostasis and survival during bacteremia. Infect. Immun. 86(7): e00173-18.

Miki, T., Okada, N., Shimada, Y. and Danbara, H. (2004). Characterization of Salmonella pathogenicity island 1 type III secretion-dependent hemolytic activity in Salmonella enterica serovar Typhimurium. Microb. Pathog. 37(2): 65-72.

Monteiro, C., Papenfort, K., Hentrich, K., Ahmad, I., Le Guyon, S., Reimann, R., Grantcharova, N. and Römling, U. (2012). Hfq and Hfq-dependent small RNAs are major contributors to multicellular development in Salmonella enterica serovar Typhimurium. RNA Biol. 9(4): 489-502.

Morimoto, Y. V, Namba, K. and Minamino, T. (2017). Measurements of free-swimming speed of motile Salmonella cells in liquid media. Bio-Protocol 7: e2093.

Nair, A., Rawool, D.B., Doijad, S., Poharkar, K., Mohan, V., Barbuddhe, S.B., Kolhe, R., Kurkure, N. V, Kumar, A. and Malik, S.V.S. (2015). Biofilm formation and genetic diversity of Salmonella isolates recovered from clinical, food, poultry and environmental sources. Infect. Genet. Evol. 36: 424-433.

Park, H.J., Kim, H.J., Park, S.H., Shin, E.G., Kim, J.H. and Kim, H.Y. (2008). Direct and quantitative analysis of Salmonella enterica serovar Typhimurium using real-time PCR from artificially contaminated chicken meat. J Microbiol Biotechnol. 18(8): 1453-1458.

Park, S.J. and Gunsalus, R.P. (1995). Oxygen, iron, carbon and superoxide control of the fumarase fumA and fumC genes of Escherichia coli: role of the arcA, fnr and soxR gene products. J. Bacteriol. 177(21): 6255-6262.

Paterson, G.K., Northen, H., Cone, D.B., Willers, C., Peters, S.E. and Maskell, D.J. (2009). Deletion of tolA in Salmonella Typhimurium generates an attenuated strain with vaccine potential. Microbiology. 155(1): 220-228.

Pegues, D.A. (2005). Salmonella species, including Salmonella typhi. Princ. Pract. Infect. Dis. 2636–2654.

Sambrook, J. (2001). Molecular cloning: a laboratory manual/Joseph Sambrook, David W. Russell.

Samhita, L., Nanjundiah, V. and Varshney, U. (2014). How many initiator tRNA genes does Escherichia coli need? J. Bacteriol. 196(14): 2607-2615.

Shukla, S.K. and Rao, T.S. (2017). An Improved Crystal Violet Assay for Biofilm Quantification in 96-Well Microtitre Plate. bioRxiv 100214.

Spiro, S. and Guest, J.R. (1990). FNR and its role in oxygen-regulated gene expression in Escherichia coli. FEMS Microbiol. Rev. 6(4): 399-428.

Wing, H.J., Williams, S.M. and Busby, S.J. (1995). Spacing requirements for transcription activation by Escherichia coli FNR protein. J. Bacteriol. 177(23): 6704-6710.