Absence of Nitrogen regulatory protein C affects the growth physiology of Salmonella Typhimurium by limiting glucose transport in the cell

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Abstract

The NtrC is crucial for nitrogen regulation in *S.* Typhimurium. Under nitrogen limitation, NtrC activates the set of genes involved in ameliorating the slowing of growth. Schumacher et al (2013) demonstrated that *ntr*C mutation increases intracellular concentration of α-KG in the cell. Another report explained that α-KG inhibits (Enzyme 1) E1 protein. Taking this as a clue, we studied the glucose uptake of Δ*ntr*C. Indeed, the Δ*ntr*C was slow to uptake the glucose. It also showed smaller colonies and reduced cell size in an optimum glucose medium. The transcriptome studies in carbon and nitrogen rich medium, showed suppressed nitrogen transport and metabolism genes, and induction of maltose operon genes (encoding high affinity glucose transporters) in Δ*ntr*C. Despite having suppressed nitrogen transport and metabolism genes in Δ*ntr*C, there was no significant difference in nitrogen (ammonia) utilization between WT and Δ*ntr*C. Hence, we show that, Δ*ntr*C having hampered glucose transport but normal expression of glucose metabolism genes, exhibits glucose limiting growth (intracellular glucose deficiency). Consequently, generate hunger response (small cell size, slow growth rate and induced maltose operon genes) even during growth in glucose rich medium. Therefore, the current work adds evidence for intricate overlapping control of nitrogen and carbon metabolism.

Significance

The food-borne pathogen, *S.* Typhimurium cycles between the host and external environment. While cycling, *S.* Typhimurium often encounters uneven nutrient distribution in the external environment. Carbon and nitrogen are the most common growth-limiting nutrient in the environment. Therefore, how *S.* Typhimurium balances its metabolism under patchy nutrient distribution conditions always remains a big question to be addressed. In this context, we studied the effect of knockout mutation of nitrogen regulatory gene (*ntr*C) on carbon utilization. We observed that, to balance cellular metabolism, *S.* Typhimurium in the absence of nitrogen activator protein (NtrC), reduces glucose transport in the cell. Consequently, even under optimal carbon and nitrogen concentration, the Δ*ntr*C showed carbon limiting growth i.e. small cell size, reduction in doubling time, induction of maltose operon, and virulence genes expression.

Introduction

Bacteria evolved sophisticated signal transduction systems to counter the variation in nutrient availability. These signal transduction systems help bacteria to maintain balanced metabolism by regulating nutrients uptake and utilization (Nixon et al., 1986, Merrick & Edwards, 1995, Martín & Liras, 2019). Nitrogen regulatory protein C (NtrC) is a part of the NtrBC two component signal transduction system. NtrC protein's association with Glutamine synthetase (*gln*A gene), led to the identification of the role of NtrC in nitrogen metabolism (Merrick & Edwards, 1995). Therefore, the majority of studies were conducted to explore the contribution of NtrC in regulation of nitrogen metabolism under nitrogen deficit or excess conditions (Shiau et al., 1992, Zimmer et al., 2000, Reitzer, 2003). These studies established NtrC as the global regulator of nitrogen metabolism genes. The central nitrogen metabolism is mediated

by two pathways. One pathway is mediated by GS (Glutamine synthetase) and GOGAT (Glutamate synthase). The second pathway is mediated by the GDH (Glutamate dehydrogenase). These pathways provide the nitrogen sources (glutamate and glutamine) for synthesis of nitrogen-containing compounds (Merrick & Edwards, 1995, Reitzer, 2003). The GDH has a higher K_m for ammonium (> 1mM) than GS (< 200μM). Therefore, under nitrogen rich and carbon limiting condition GDH is the predominant pathway and provides sufficient nitrogen to maintain optimal growth (Yan, 2007, Schumacher et al., 2013). Under nitrogen limiting condition, the GS-GOGAT pathway is predominant and is activated by NtrC (Miller & Stadtman, 1972, Merrick & Edwards, 1995).

Research on *ntr*C gene has identified its role in multiple metabolic pathways such as, Poly-3-Hydroxybutyrate (PHB) biosynthesis, stringent response, exopolysaccharides in biofilm formation, cell motility, invasiveness and virulence, starvation tolerance, competitive fitness etc. (Sun et al., 2000, Kim et al., 2009, Brown et al., 2014, Liu et al., 2017, Brown, 2019, Alford et al., 2020, Switzer et al., 2020, Mishra & Shashidhar, 2022). Studies have also shown the regulation of NtrC, in response to carbon and phosphorus limitation (Mao et al., 2007, Santos-Beneit, 2015). In glucose rich medium, intracellular concentration of α-ketoglutarate (α-KG) is high. The α-KG, the carbon skeleton for nitrogen assimilation, bind and promote uridylylation of PII signalling protein (sensor of α-KG) (Huergo & Dixon, 2015). Uridylylation of PII, prevents binding of PII to NtrB protein. Free NtrB phosphorylates NtrC protein, causing activation of expression of genes involved in nitrogen transport and metabolism (Merrick & Edwards, 1995). In addition to this, cAMP receptor protein (CRP) under low glucose condition, binds to the promoter region of *gln*A-*gln*L-*gln*G operon and prevent the binding of NtrC to glnAP2 promoter, resulting in low expression of nitrogen transporter and metabolism genes (Mao et al., 2007). Therefore, this is how the presence of carbon regulates nitrogen transport and metabolism in bacteria. However, how nitrogen availability coordinates with the uptake and metabolism of carbon remained unexplored. Therefore, we studied the role of NtrC in coordinating carbon-nitrogen uptake and metabolism using a Δ*ntr*C knockout mutant. Our results show that absence of NtrC limit glucose transport, which in-turn retards growth and cell physiology of *S.* Typhimurium. Besides that, limited glucose transport induces maltose operon genes (hunger response) and virulence gene expression.

Materials and methods

Bacterial strains and growth conditions

The *Salmonella enterica subsp. enterica* serovar Typhimurium strain LT2 (MTCC 98) culture was obtained from Microbial Type Culture Collection and Gene Bank (MTCC) Chandigarh, India. We used Lysogeny broth, (LB) for the overnight growth of strains in all the experiments. M9 was used as a base for minimal medium (6g L^{-1} Na_2PO_4, 3g L^{-1} KH_2PO_4, 1g L^{-1} 68 NH_4Cl, 0.5g L^{-1} NaCl, 2mM $MgSO_4$, 0.1mM $CaCl_2$) and supplemented with 0.4% glucose whenever needed. All cultures were incubated cultures at 37°C with shaking at 150 rpm min^{-1}. The LB agar plates and those supplemented with kanamycin (50μg ml^{-1}) and carbenicillin (30μg ml^{-1}) were used wherever required.

Gene knock-out mutant preparation

The *ntrC (glnG)* gene knock-out mutant (Δ*ntrC* mutant) was generated with the help of Quick and Easy *E. coli* gene deletion kit by Red®/ET® Recombination, Gene Bridges, (Heidelberg Germany). We obtained *ntrC* gene sequence of *S.* Typhimurium from NCBI (NC_003197.2). We confirmed *ntrC* mutation phenotypically, through newly acquired antibiotic (kanamycin) resistance, and genotypically, by diagnostic PCR. The mutant characteristic phenotypes, was matched with the phenotype given in literatures. We used pJET1.2/blunt cloning system (ThermoFisher Scientific, Massachusetts, USA) for complementation of *ntrC* gene with its native promoter (pntrBC *glnLG*). The primers and their sequences used in the study are given in supplementary 1.

Growth studies in different nutrient medium

We studied the growth characteristics of WT and Δ*ntr*C by adding, 198µl of fresh LB or M9 (supplemented with 0.4% glucose) in 96 well microtiter plates along with 2µl of overnight grown culture. To monitor the growth of WT and Δ*ntr*C, the plate was placed in BioTek Synergy H1 plate reader (Winooski, Germany). The reading was taken for 24 hours in form of O.D at 600 nm, with continuous shaking condition at 37°C. For growth rate calculation, the data was plotted in log plot and fit into the exponential curve/line using equation slop $Y = Ae^{Bx}$, doubling time = ln (2)/B. For lag time calculation, the time taken from the inoculation to the intercept of the tangent drawn to the exponential phase of the growth curve was considered as lag time (λ).

Microscopy

For study under light microscope, the overnight M9 medium grown WT and Δ*ntr*C strains were taken (10 µl) on slide, heat fixed and monochrome staining was done using crystal violet stain and washed with alcohol and then with water. Dry specimen was covered with coverslip and immersion oil was added before sample was analysed under microscope. The images of sample were taken at 100 X magnification. The Scanning Electron Microscopy of WT and Δ*ntr*C was done by taking one colony directly from agar plate on cover slip and followed same protocol as in case of study under light microscope (no chemical fixation and coating of specimen was done). The specimen was observed at 5000 x, pressure 65 Pa, HV 10 kV (FEI quanta 200, Hillsboro Oregon, USA).

Gene expression studies

For gene expression studies, 500µl of overnight Lysogeny broth grown culture was transferred to 50 ml fresh M9 medium supplemented with 0.4% glucose. For study of the log-phase of WT and Δ*ntr*C strains, cells were harvested after 8–10 hour of incubation (O.D 0.4) in M9 medium. The total RNA extraction was carried out by the TRIzol method using TRIzol reagent (Invitrogen). The concentration and impurity of RNA samples was determined by spectroscopy at 260/280nm and 260/230nm. The RNA integrity was analysed by running gel electrophoresis using 1% agarose gel. The cDNA synthesis was carried out using TransGen (Beijing, China) reagent and protocol. The primers were designed using 'Integrated DNA Technologies Primer Quest software' (www.idtdna.com/site). The 16s rRNA was used as the

housekeeping gene. The cDNA products were subjected to SYBR green RT-qPCR assay using primers and DyNAmo Flash SYBR Green qPCR Kit Finnzymes (Espoo, Finland) in amplification master cycler, Roche (Basel, Switzerland) at 94°C for 10min, followed by 40 cycles consisting of denaturation at 95°C for 10 s, annealing at 55°C for 10 s and extension at 72°C for 20 s. Following amplification, determination of threshold cycle (CT) values and melting curve analysis was carried out. The analysis was carried out following the MIQE guidelines for real-time PCR experiments (Bustin et al., 2009).

RNA-seq and transcriptome data analysis

The WT and Δ*ntr*C were grown until O.D reached 0.4 (log phase) in M9 glucose medium. The RNA was isolated using TRIzol method as mentioned in above section of gene expression studies. The total RNA-seq library were constructed, and sequenced in Illumina Hiseq 500 instrument using pair-end method by Genotypic technology (Genotypic Inc., Bangalore). To analyse the gene expression variation of WT and mutants, the fragments per kb of CDS per million mapped reads (FPKM) value was used to normalize the data and represent the overall gene expression. The differentially expressed genes between WT and mutants was selected according to their significance based on chi-square test ($P < 0.05$) and at least 2 fold difference. Each transcriptome experiments were repeated independently three times.

Glucose uptake assay

The glucose transport in the cell was monitored by using a fluorescent derivative of glucose, 2- [N-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl) amino]-2-deoxy-d-glucose (2-NBDG) (Sigma-Aldrich, USA). Both WT and mutants *S*. Typhimurium (10^7 cell) were treated with 40μM 2NBDG for 2 minute, to monitor substrate uptake capability. The fluorescence was measured in microplate reader at excitation/emission maxima of ~ 480/535 nm (Tao et al., 2016).

Glucose estimation assay

For glucose estimation, 10^7 cells of washed WT and Δ*ntr*C were incubated in 1 ml M9 medium, supplemented with 1mg ml^{-1} of glucose. At different time points, the unused glucose in the minimal media was estimated using Sigma Glucose (GAGO20) assay kit (New jersey, USA). To measure glucose concentration in solution, we followed the instruction given in the kit.

Ammonia estimation in spent medium

For ammonia estimation, 10^8 cells of washed WT and Δ*ntr*C were incubated in 50 ml M9 minimal media, supplemented with 18 mM ammonium chloride. At different time points, the unused ammonia in the minimal media was estimated using Nessler's reagent (Loba Chemical Mumbai, India). To measure ammonia concentration in solution, 20 μl of spent media was mixed with 50 μl of Nessler's reagent, and then diluted in 430 μl sterile water. Incubated for 10 minutes at RT. The measurement was taken at 540 nm (Absorbance at 540 nm).

Protein extraction and quantification

The WT and Δ*ntr*C were grown to O.D 0.4 in M9 medium supplemented with 0.4% glucose. 1 ml of each sample containing (10^7 cells/ml) was centrifuged and the pallet was re-suspended in 100 µl of 4X protein loading dye (0.2 M Tris-HCl, 0.4 DTT, 277 mM SDS (8.0% w/v), 6 mM Bromophenol blue, 4.3 M). The cells were disrupted by incubating at 80° C for 10 minutes. The soluble protein fraction was collected, and quantified by Bradford assay using BSA as standard. Aliquot containing 100 µg was loaded on SDS-PAGE. The protein band of desired size was cut and sent to C-CAMP (Bangalore, India) for LC/MS/MS analysis (Orbital Fusion "Tribrid" Mass Spectrometer). Protein hits with a score cut off more than 25 and significance threshold $P < 0.05$, were considered.

Statistical analysis

All the experiments were carried out in duplicates with three independent biological experiments. In all cases, population was tested as a random factor and environmental regime was tested as a fixed factor. The generation time of WT and Δ*ntr*C were calculated by using log plot. For growth curve assays and Real-Time PCR results, standard deviation (SD) was used to show the error and significance between the results. Standard deviation was performed in Microsoft Excel. The two-sample t-test was carried out to determine the significant results at $P < 0.05$.

RESULTS

Both slow doubling time and small cell size contribute to the poor growth of Δ ntr C.

In previous report we studied the role of NtrC on the growth and survival of *S*. Typhimurium under different nutrient conditions, and observed that *ntr*C mutation caused extends lag phase and poor growth in M9 minimal medium (Mishra & Shashidhar, 2022) (Fig. 1a). Next, we study the effect of NtrC on cell physiology. For that, we grow WT and Δ*ntrC* on M9 minimal medium containing agar plate. On the M9 agar plate, Δ*ntrC* formed small size colonies as compared to the WT (Fig. 1b). After 48 hours of growth on M9 agar plate, the WT and Δ*ntrC* formed 2.2 ± 0.2 mm and 1 ± 0.15 mm size colonies, respectively (Fig. 1b). The complemented strain of Δ*ntrC*, containing *ntr*C gene under the control of native promoter, rescued the poor growth phenotype (Supplementary 2, Fig. 1)). Therefore, next the factors that affect growth measurement, the doubling time and cell size, were studied (Stevenson et al., 2016). The doubling time of Δ*ntrC* and WT cells was 48 ± 4.2 and 44 ± 2.5 minutes, respectively. Under light microscope, the average size of WT was 2.0 ± 0.3 µm and average cell size of Δ*ntrC* was 1.1 ± 0.3 µm (Fig. 1c). The Δ*ntrC* was 45% smaller than the WT. For further confirmation of the cell size of WT and Δ*ntrC*, the cells were analysed under scanning electron microscope (SEM). The average cell size of WT and Δ*ntrC* under SEM were found 2.1 ± 0.5 and 1.2 ± 0.5 µm, respectively (Fig. 1d). Therefore, *ntr*C mutation not only increases the doubling time but also reduce the cell size of the *S*. Typhimurium.

ntr C mutation cause induction of maltose and virulent gene expression

Since, *ntr*C mutation impaired the growth and cell physiology of *S*. Typhimurium in a minimal medium. Therefore, to evaluate the changes occurred at gene expression level, Transcriptome analysis of log

phase cell of WT and Δ*ntr*C was carried out. The differentially expressed genes were selected based on fold change (FC) ≥ 2 for up-regulated and ≤ -2 for down-regulated DEGs (Differentially expressed genes). Based on these criteria, 1837 up –and/or downregulated transcripts as reliable DEGs were identified. Among 1837 DEGs, 617 genes were upregulated and 1220 genes were downregulated in Δ*ntr*C. While, 2676 were neutrally expressed in WT and Δ*ntr*C. The downregulated genes were involved in different pathways, such as, carbon and nitrogen source transport and metabolism, lipid metabolism, nucleotide biosynthesis, cell motility. The upregulated genes were from maltose/maltodextrin transport systems and from secretory systems (virulence genes) (Fig. 2a).

When stringent selection criteria (DEGs, $\log_2$ fold change ≥ 4 for upregulated and $\log_2$ fold change ≤ − 4 for downregulated DEGs with P value ≤ 0.001) were used, 40 genes were selected (Fig. 2b). The Δ*ntr*C showed downregulation of nitrogen metabolism genes, like *glt*J, *glt*K, *glt*L, *amt*B, *gln*H, *gln*K, *gln*Q, *gln*P, *hop*D, *yqg*C etc. The Δ*ntr*C showed upregulation of maltose/maltodextrin and virulence genes. Genes like *mal*M, *mal*P, *mal*Q, *lam*B were 25–40 fold upregulated. Genes from pathogenesis or virulence, like *sse*L, *ssa*J, *sse*A, *ssa*M, *ssa*K, *ssa*S, *ssa*G, *ssa*L, *sse*C were upregulated in Δ*ntr*C.

For further validation of the results obtained from RNA-seq studies of WT and Δ*ntr*C. The Real-Time PCR analysis of selective genes was carried out. The Real-Time PCR results were comparable with RNA-seq data (Fig. 3). All the tested maltose operon and virulence genes showed enhanced expression in Δ*ntr*C as compared to WT. The *mal*P gene showed ~ 40-fold upregulation, while the gene *mal*M, *mal*K, *mal*Q, *lam*B, *mal*E, *mal*F, showed 20 to 30 fold upregulation (Table 1, supplementary 2). The *mal*T gene showed ~ 5 fold up-regulation. The virulence genes like *sse*A, *ssa*M, *pip*B2 showed 10 to 15 fold higher expression, while *sse*L, *sse*D, *ssa*P, showed 5–10 fold upregulation. The *ssa*G and *sse*G showed over 15 fold upregulation (Fig. 3b). Few genes from carbon and nitrogen, transport and metabolism were analysed. Insignificant changes observed in the expression of glucose transporters genes. The *amt*B gene was 12 fold downregulated and *gln*A gene was 16 fold downregulated in Δ*ntr*C (Fig. 3c).

The Δ ntr C has low glucose uptake and consumption rate than WT

Previous studies have shown that the maltose operon genes expression increases in WT cells during growth in glucose limiting conditions (Notley & Ferenci, 1995, Ferenci, 2001). However, we observed high expression of maltose operons genes in Δ*ntrC* under optimal glucose concentration. Therefore, by taking clues from the WT growth studies under glucose limiting condition. We examined the possibility that *ntr*C mutation hampers the glucose transport in the cell. The glucose uptake in WT and Δ*ntr*C was compared at the log phase, in M9 minimal medium. The substrate uptake activity was determined by monitoring the accumulation of 2NBDG in the cytoplasm (4). The strain with higher transport activity shows more 2NBDG signal (Nikolic et al., 2013). The 2NBDG assay revealed that the Δ*ntrC* has 28% reduction in 2NBDG uptake as compared to WT (Fig. 3d). Therefore, the Δ*ntrC* has a slower glucose uptake than the WT.

The result of glucose uptake assay was further validated by the glucose utilization ability of WT and Δ*ntrC*, using enzyme (glucose oxidase) based method. The glucose concentration in the growth medium was detected at different time intervals. The Δ*ntrC* mutant was using glucose at a slower rate (~ 125 µg h^{-1}) than the WT *S*. Typhimurium (~ 166 µg h^{-1}). The WT cell consumed glucose of the medium within 5 hours, while Δ*ntrC* took 8 hours to consume glucose of the medium (Fig. 3d). These results suggest that the knockout mutation of the *ntrC* (*gln*G) gene has severely affected the glucose transport and metabolism in Δ*ntrC*.

Ammonia does not limit the growth of WT or Δ ntr C

The Δ*ntr*C showed suppression of a large number of nitrogen transporter genes, including ammonium transporter encoding gene, *amt*B. Therefore, ammonium transport in the WT and Δ*ntr*C was compared. The evaluation of nitrogen consumption by the WT and Δ*ntrC* revealed that there was no significant difference in ammonia consumption by these two strains. As, even after 48 hours of growth, the spent medium of both strains contained 7 mM of unused ammonia (Fig. 3f). In our experiment, we observed that the 7 mM ammonia suffices to promote optimal bacterial growth. This suggests that optimum concentration of nitrogen does not limit the growth of Δ*ntrC*.

Analysis of Δ crp, a regulator of carbon metabolism

Since, the Δ*ntr*C showed slow growth, small cell size, and poor transport and utilization of glucose, in addition, previous studies have shown that glucose availability affect the growth rate, cell size, maltose operon and virulence gene expression in bacteria (Notley & Ferenci, 1995, Yanagida et al., 2011, Ritzert & Lathem, 2018). In this context, the knockout mutant of CRP, a regulator of carbon metabolism, regulates glucose transport in the cell, was examined for the above mentioned physiological and molecular effects (Steinsiek & Bettenbrock, 2012). The Δ*crp* also showed a small colony size on M9 agar plate supplemented with 0.4% glucose. Both light and electron microscopy confirmed that Δ*crp* has a small cell size (Fig. 4 (a-b)). Further, analysis of glucose transport in the log phase (O.D 0.3–0.4) WT and Δ*crp* showed that the Δ*crp* accumulates less glucose than WT strain (Fig. 4d).

The RNA-seq of M9 medium grown log phase cells (O.D 0.3–0.4) of WT and Δ*crp* was carried out. The differentially expressed genes in Δ*crp* with respect to WT, (Fold change ≥ 2 for upregulated and ≤ -2 for downregulated DEGs). Based on these criteria, 1519 up –and/or downregulated transcripts as reliable DEGs were identified. Among 1519 DEGs, 663 genes were upregulated, 856 genes were downregulated, while 2995 were neutrally expressed in Δ*crp* as compared to WT (Fig. 5a). The Δ*crp* also showed upregulation of genes involved in invasion and pathogenesis, 32 genes belonging to virulence were found upregulated in Δ*crp* (Table 2, supplementary 2). The heat map shows the 40 highly differentially expressed genes in Δ*crp* relative to the WT *S*. Typhimurium (with adjusted $P \leq 0.001$ and $\log_2$ fold change ≥ 4) (Fig. 5b).

Effect of ntrC knockout mutation on the abundance of nitrogen assimilation proteins

The comparative transcriptome analysis and RT-PCR of WT and Δ*ntrC* showed down regulation of nitrogen assimilation genes in Δ*ntrC*. Therefore, the protein concentration of these genes were determined. The Δ*ntrC* has 4,000 copies of Glutamine Synthetase (GS) and 2, 600 copies of Glutamate synthase (GOGAT), per cell. Whereas, the WT has 24,000 copies Glutamine Synthetase (GS), and 6,400 copies Glutamate synthase (GOGAT) per cell. Therefore, *ntr*C mutation caused 6 fold reduction in the abundance of Glutamine Synthetase (GS) with P value < 0.05, and 2.4 fold reduction in abundance of Glutamate synthase (GOGAT) with P value < 0.6.

Discussion

In enteric bacteria, nitrogen uptake and metabolism are regulated by NtrC protein (4). Under carbon limitation, CRP and PII control the expression of NtrC to tailor nitrogen uptake in response to carbon availability (Ninfa & Jiang, 2005, Mao et al., 2007). However, the role of NtrC in carbon metabolism is not studied well. Therefore, we have investigated the role of NtrC in carbon utilization and cell physiology.

ntr C mutation reduces cell size of S. Typhimurium.

Previous works have extensively studied the role of NtrC in growth and survival under nitrogen sufficient and deficient conditions. These studies demonstrate that NtrC is essential for growth under nitrogen deficient condition. NtrC under N-limiting activates the set genes involved in transport and metabolism of alternate N sources, which ultimately ameliorate the slowing of growth due to N deficiency (Zimmer et al., 2000, Brown, 2019). However, during the study we observed that Δ*ntr*C grows slowly even in nitrogen rich condition. Indicating that, NtrC is required during growth in nitrogen rich condition, also. Therefore, factors affecting the growth measurement through optical density, doubling time and cell size, were determined (Stevenson et al., 2016). We observed that Δ*ntr*C has longer doubling time and smaller cell size than WT. Previous studies on Δ*ntr*C of *E. coli* have shown that the slower growth monitored through an optical density of the growth medium is because of longer doubling time (Schumacher et al., 2013, Brown et al., 2014). However, they did not observe the cell size of Δ*ntr*C. Here, we examined both doubling time and cell size. Therefore, in agreement with previous reports, we conclude that the *ntr*C mutation affects both the growth rate and cell size of the *S.* Typhimurium, and the retarded growth observed through optical density, is a combined effect of slower growth and small cell size of Δ*ntr*C.

Δ ntr C grows as hungry bacteria.

The RNA-seq analysis of WT and Δ*ntr*C revealed that the Δ*ntr*C has downregulation of nitrogen transport and metabolism genes, like *gln*A gene (*gln*ALG operon), which is regulated by NtrC protein. The *gln*A encodes Glutamine Synthetase, responsible for assimilation of ammonia into glutamine. The Δ*ntr*C showed upregulation of maltose operon genes and virulence genes. There are few reports on

transcriptome studies of Δ*ntr*C (Zimmer et al., 2000, Alford et al., 2020, Naren & Zhang, 2021). However, these transcriptome studies were performed using microarray with a limited set of genes, or using RNA-seq but in different physiological conditions. Zimmer et al. (2000) studied the microarray of *ntr*C(up) and *ntr*C(null) mutant and compared the expression of a limited set of genes (99 genes of 43 operons). Alford et al. (2020) studied the transcriptome of WT and Δ*ntr*C in swarming condition. Naren *et al*. (2021) performed RNA-seq of WT and Δ*ntr*C of *Pseudomonas* fluorescens in M9 minimal medium, having succinate and histidine as carbon and nitrogen sources, respectively. There are no reports explaining the correlation between NtrC and maltose gene expression. The maltose operon system controls uptake and metabolism of glucose polymers (maltodextrins). The earlier studies on differential expression of maltose operon genes have shown that *mal*EFG and *mal*K*lam*B are expressed under glucose limiting conditions (Notley & Ferenci, 1995, Ferenci, 2001). The *mal*EFGK encode maltose/maltodextrins transport protein, and *lam*B encode for LamB (outer membrane maltoporin), having a role in outer membrane permeability of carbohydrates and induced during growth under glucose limiting condition (Death et al., 1993). Therefore, we monitored glucose uptake in the WT and Δ*ntr*C, using a fluorescent analog of glucose, 2NBDG. Compared to the WT, Δ*ntr*C showed less glucose accumulation, suggesting that Δ*ntr*C is receiving less glucose than WT. For further confirmation, the glucose utilization by WT and Δ*ntr*C was compared by measuring the unused glucose present in the growth medium. The Δ*ntr*C consumed glucose more slowly than the WT. The transcriptome and glucose uptake and utilization studies suggest that Δ*ntr*C has lower glucose intake than required (glucose limited growth). Furthermore, the maltose operon expression was compared among the WT cells, WT grown in glucose (50 µM) limiting condition Vs WT grown in glucose (2 mM) sufficient condition. We observed that the WT growing on 50 µM glucose showed upregulation of maltose operon genes as compare to WT growing on 2 mM glucose. Therefore, similarity in the molecular response of Δ*ntr*C growing in glucose rich medium with the molecular response of WT cells growing on glucose limiting medium suggest that, the upregulation of maltose operons genes in Δ*ntr*C might be because of glucose limiting growth. Therefore, despite of having sufficient glucose in the surrounding, the Δ*ntr*C exhibits glucose limiting growth. The low glucose absorption leads to generation of hunger response; reduction in growth rate ($\mu max > \mu \neq 0$) and activation of maltose operons (*lam*B expression) (Ferenci, 2001). Hence, our result suggests that NtrC is required during growth in nutrient rich medium also. Presence of NtrC ensures optimal glucose uptake.

Impaired cell physiology of Δ ntr C is due to glucose limitation.

Deficiency of carbon or nitrogen often limits the growth rate and cell size. Therefore, next we studied the nitrogen utilization by the WT and Δ*ntr*C. We observed that nitrogen was not limiting the growth of Δ*ntr*C in adequate nitrogen medium. After 48 h of growth, 7 mM unused ammonia was present in the spent medium of WT and Δ*ntr*C. Since, previous studies on ammonia transport in bacteria revealed that ammonia concentration higher than 30 µM is sufficient for diffusion uptake (Kim et al., 2012, Schumacher et al., 2013). Besides this, Yang et al. (2021) monitored nitrogen uptake in WT and Δ*ntr*C of *P. stutzeri* and showed although Δ*ntr*C hampers uptake of nitrogen sources like glutamine, histidine, arginine, nitrate and nitrite. However, there was no significant difference in ammonia uptake capabilities between these two strains (Yang et al., 2021). Therefore, current and previous observations showed that,

despite downregulation of ammonium transporter gene (*amt*B), the ammonia does not limit the growth of Δ*ntr*C. However, when we observed the growth of WT cells on M9 agar plate supplemented with 50 µM glucose. The WT cells showed very small size colonies. The SEM analysis revealed that WT cells grown on 50 µM glucose, have smaller cell size than WT cells grown on 2 mM glucose (supplementary 2). Therefore, from these experiments we concluded that, instead of ammonia, glucose is the limiting factor for Δ*ntr*C growth and cell physiology. Moreover, Similarity in cell physiology of WT growing on glucose limited plate with the Δ*ntr*C growing on plate containing adequate glucose, suggests that the Δ*ntr*C small cell size might be due to low glucose transport in the cell (creating glucose limitation inside the cell).

Limited glucose in Δ ntr C removes repression from the virulent gene.

The expression of virulence genes is tightly regulated in *S.* Typhimurium. Therefore, *S.* Typhimurium does not express virulent genes in the exponential phase of the growth (El Mouali et al., 2018). However, the factors that suppress the expression of virulence genes under normal growth conditions remained unexplored. Interestingly, during the study of differentially expressed genes in WT and Δ*ntr*C, we observed that Δ*ntr*C, at log phase, has upregulation of many genes (26 genes) involved in virulence. Valdes et al. (2018) working on pathogen *Streptococcus*, showed that the glucose limited condition induces expression of genes. Out of those induced genes, 10% are involved in virulence (Valdes et al., 2018). Ritzer *et al*. (2018) working on another pathogen, showed that *Yersinia pestis* rapidly consume glucose, to make glucose depleted surrounding that in-turn activate virulence genes. They observed that deletion of *pts*G gene encodes for glucose transporter (EIIBC), rescue glucose level result in reduced expression of virulence genes (Ritzert & Lathem, 2018). Therefore, for further validation of glucose dependent effect on cell physiology and gene expression, we studied Δ*crp* under the same condition. The CRP regulates glucose transport by controlling the expression of the glucose transporter (*pts*G) gene expression (Zheng et al., 2004, Yao et al., 2011). The Δ*crp* showed similar changes as we observed in Δ*ntrC*, except upregulation of the maltose operon system. The Δ*crp* has small cell size, low glucose uptake and upregulated virulent gene expression, at log phase. Under glucose limiting condition the maltose operons are induced by CRP and MalT proteins. Therefore, Δ*crp* did not show the upregulation of maltose operon genes. Taken together, the knockout mutant of the global regulator of nitrogen and carbon metabolism, the Δ*ntr*C and Δ*crp*, respectively, showed similarity in the effect of mutation. Both, Δ*ntr*C and Δ*crp* showed low glucose intake, small cell size and upregulation of virulence genes. Therefore, it seems that the tight regulation on virulence gene expression during exponential growth in WT cells might be due to intracellular abundance of glucose. As, the growth of WT cells under glucose limiting condition, and the growth of Δ*ntr*C and Δ*crp* in optimal glucose condition (exhibit glucose limiting growth) induced expression of virulence genes.

Proposed model

Nitrogen is assimilated by two pathways, via the GS-GOGAT and GDH mediated pathway, in enteric bacteria, (Fig. 6a) (Merrick & Edwards, 1995). Previous reports and our experiment showed that the *ntr*C mutation suppresses nitrogen transporters and metabolism genes, like *gln*A, encoding Glutamine

synthetase. Glutamine synthetase catalyses assimilation of ammonia into glutamine by utilizing α-ketoglutarate. Hence, in *Δntr*C, GS-GOGAT pathway is affected. However, GDH mediated pathway continues to assimilate nitrogen when nitrogen is in adequate concentration. However, dysfunctional GS-GOGAT pathway may cause a physiological imbalance in the cell. In our experiment, protein level analysis showed that the Δ*ntrC* has 6 fold lower abundance of Glutamine synthetase than WT. Schumacher et al. (2013) have shown that in nitrogen rich (10mM NH4Cl) Gutnick medium WT cells contain 18,691 copies of Glutamine synthetase while Δ*ntrC* has 4,456 copies of Glutamine synthetase. This led to a reduction in intracellular glutamine concentration in Δ*ntrC*. Besides that, Schumacher *et al* also detected intracellular concentration of α-ketoglutarate, and observed higher accumulation of α-ketoglutarate in Δ*ntrC* than WT *E. coli* (Schumacher et al., 2013). Further, Doucette et al. (2011) working on *E. coli* has shown that accumulation of α-ketoglutarate blocks glucose uptake by inhibiting Enzyme 1 (E1) of glucose transporter (Doucette et al., 2011). In our report, we have shown that Δ*ntrC* has supressed nitrogen metabolism genes, reduction in abundance of GS and GOGAT protein, low glucose uptake at the log phase, and induction of high affinity glucose transporter genes. Putting together all this information, we postulate the following model. Mutation of *ntr*C suppresses nitrogen metabolism genes. These genes in-turn slows down nitrogen metabolism i.e., assimilation of free ammonia into glutamine. The slow rate of nitrogen assimilation leads to α-ketoglutarate accumulation in the cell. The high concentrations of α-ketoglutarate block glucose transport by inhibiting Enzyme 1 (E1) of PTS glucose transport system (Fig. 6b). However, the nitrogen metabolism through the GDH mediated pathway is providing sufficient nitrogen for growth (Yan, 2007).

In conclusion, NtrC is required for optimal glucose uptake in *S.* Typhimurium. Absence of NtrC limits glucose transport in the cell; consequently, create intracellular glucose deficiency, even in glucose surplus condition (Fig. 6b). The *ntr*C mutation led glucose deficient growth in glucose rich medium results in reduced cell size, induction of alternate and high affinity glucose transporters and virulence gene expression, under non-permissible conditions.

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Figures

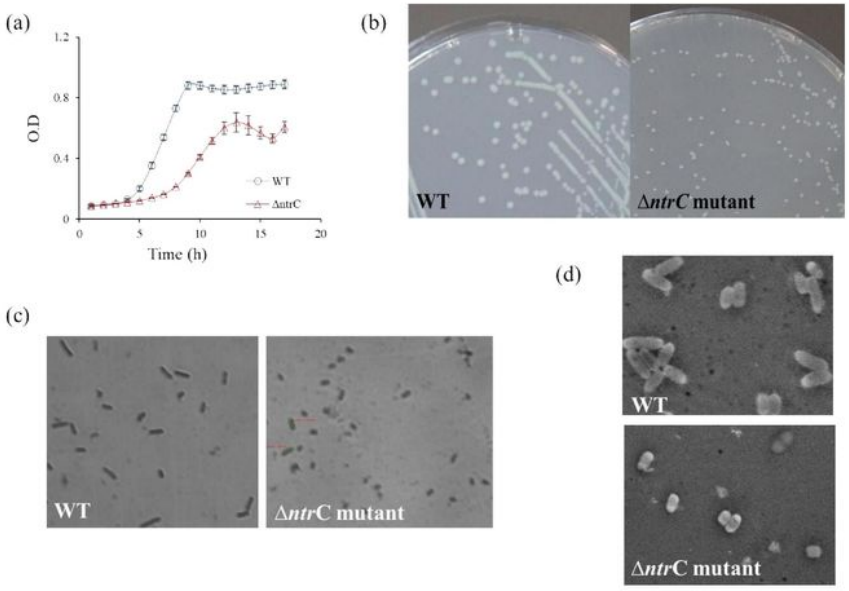

Figure 1

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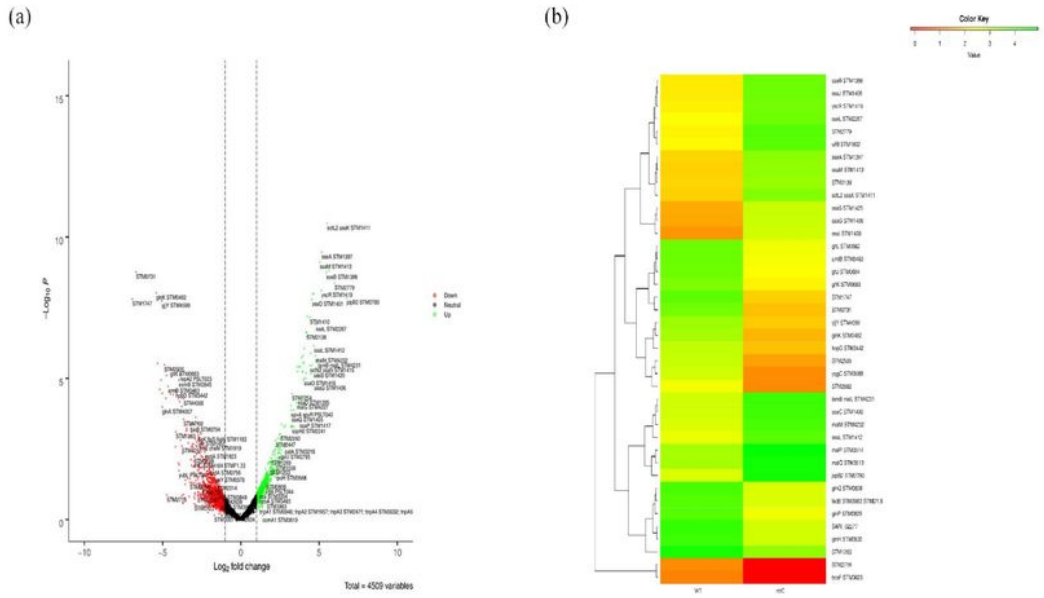

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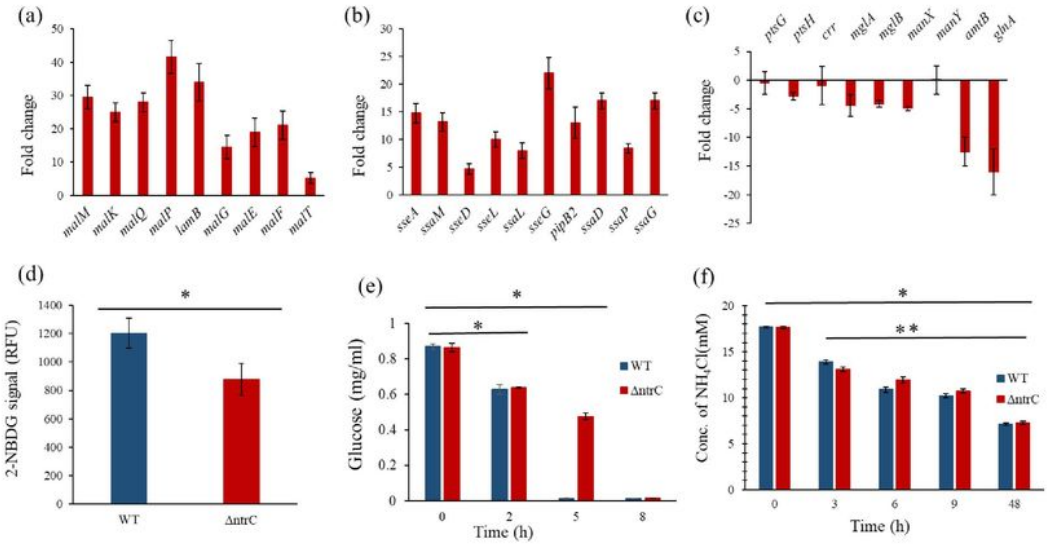

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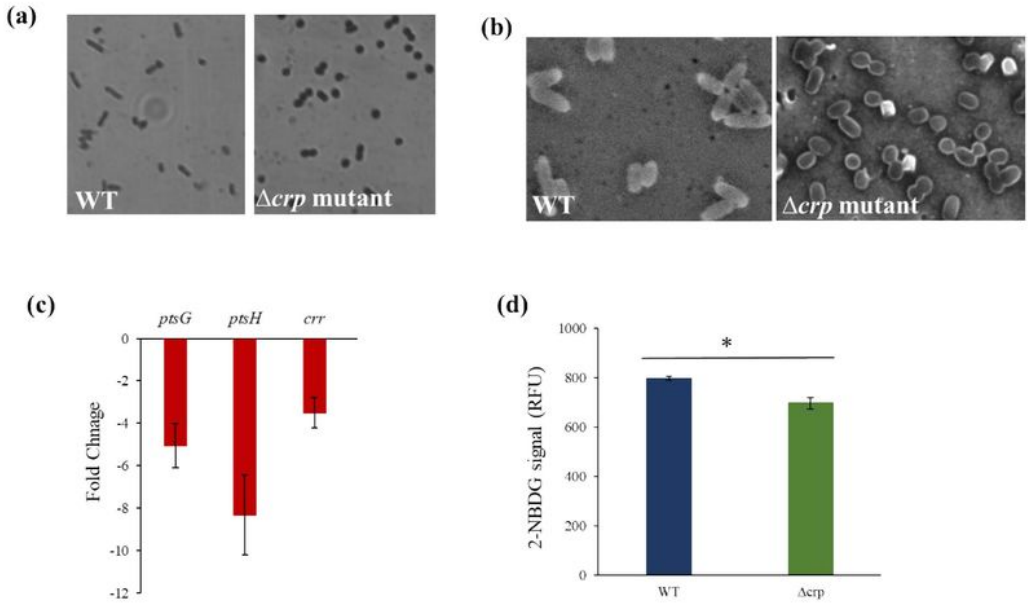

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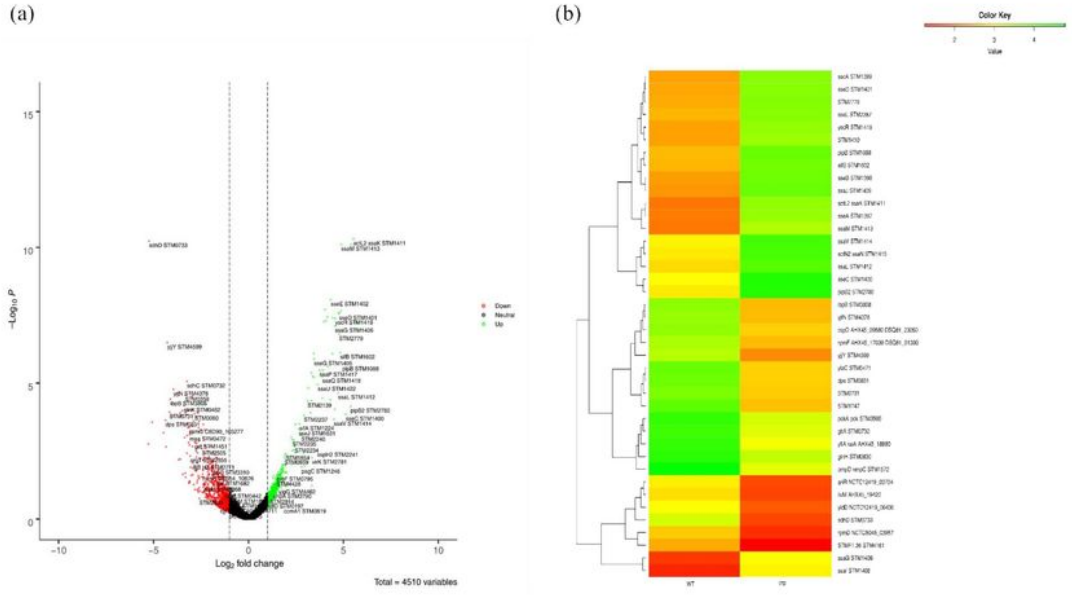

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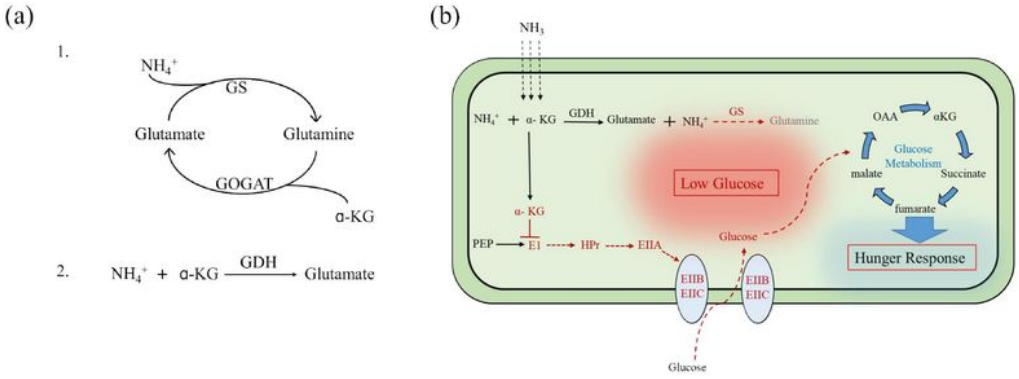

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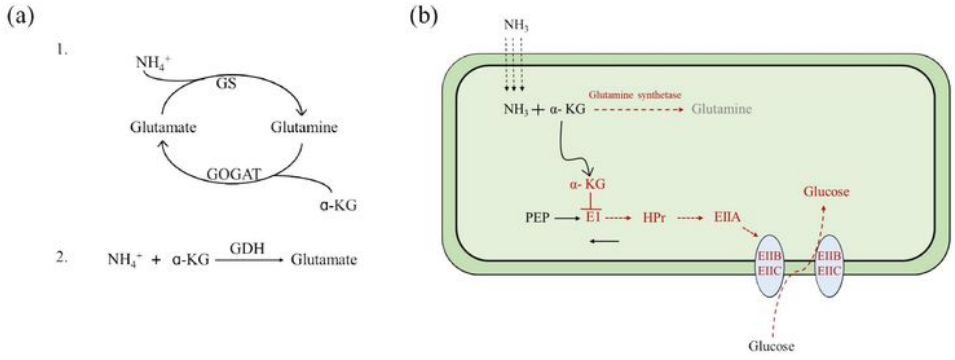

Figure 7

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