J. Am. Soc. Mass Spectrom. (2019) 30:1001–1012
DOI: 10.1007/s13361-019-02145-2

FOCUS: ION MOBILITY SPECTROMETRY (IMS): RESEARCH ARTICLE

Proteomic Analysis of FNR-Regulated Anaerobiosis in *Salmonella* Typhimurium

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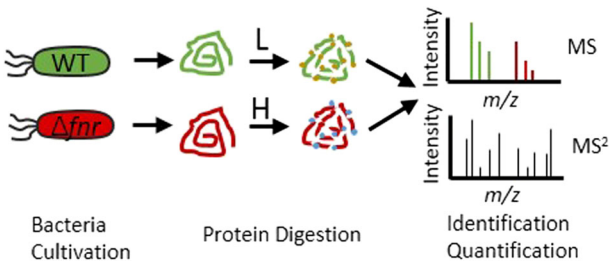

Abstract. Bacterial pathogens such as *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) have to cope with fluctuating oxygen levels during infection within host gastrointestinal tracts. The global transcription factor FNR (fumarate nitrate reduction) plays a vital role in the adaptation of enteric bacteria to the low oxygen environment. Nevertheless, a comprehensive profile of the FNR regulon on the proteome level is still lacking in *S.* Typhimurium. Herein, we quantitatively profiled *S.* Typhimurium proteome of an *fnr*-deletion mutant during anaerobiosis in comparison to its parental strain. Notably, we found that FNR represses the expression of virulence genes of *Salmonella* pathogenicity island 1 (SPI-1) and negatively regulates propanediol utilization by directly binding to the promoter region of the *pdu* operon. Importantly, we provided evidence that *S.* Typhimurium lacking *fnr* exhibited increased antibiotics susceptibility and membrane permeability as well. Furthermore, genetic deletion of *fnr* leads to decreased bacterial survival in a *Caenorhabditis elegans* infection model, highlighting an important role of this regulator in mediating host-pathogen interactions.

Keywords: *Salmonella* Typhimurium, The FNR regulon, Anaerobiosis, Quantitative proteomics

Received: 2 December 2018/Revised: 23 January 2019/Accepted: 24 January 2019/Published Online: 22 March 2019

Introduction

As a Gram-negative bacterial pathogen, *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) causes gastroenteritis by colonizing the intestinal epithelium of the host upon oral ingestion of contaminated food or water [1]. In host gastrointestinal tracts, bacteria often encounter limited supply of oxygen which governs the energy metabolism of bacterial cells. Thus, during infection *S.* Typhimurium must switch its metabolism from aerobiosis to anaerobiosis [2]. Such transition is often accompanied by differential expression of a large array of bacterial genes, which is in turn transcriptionally controlled by global regulators such as FNR (fumarate nitrate reduction). Named on the basis of the *Escherichia coli* mutants deficient in fumarate and nitrate reduction [3], FNR allows bacteria to sense oxygen levels and alter their metabolic modes accordingly. Mostly characterized in *E. coli*, the N-terminal sensory domain of FNR contains four cysteine residues required for binding of iron-sulfur clusters [4, 5]. Under oxygen-limiting conditions, binding of one molecular $[4Fe\text{-}4S]^{2+}$ promotes FNR dimerization and enhances its DNA binding to turn on/off genes transcription [6, 7]. Under aerobic

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Electronic supplementary material The online version of this article (https://doi.org/10.1007/s13361-019-02145-2) contains supplementary material, which is available to authorized users.

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conditions, oxygen triggers the conversion of $[4Fe\text{-}4S]^{2+}$ into $[2Fe\text{-}2S]^{2+}$, favoring monomer formation and protein dissociation from DNA sequences and the transcription machinery [5, 8, 9].

Although extensively studied in *E. coli* by various techniques including DNA-microarray, Chip-Seq, and RNA-Seq [10–14], the FNR regulon in bacterial pathogens such as *S.* Typhimurium remains much less characterized. In particular, a comprehensive profile of the FNR regulon is still lacking on the proteome level during *Salmonella* anaerobiosis. Herein, we conducted large-scale quantitative proteomic analyses of wild type *S.* Typhimurium SL1344 and its isogenic strain lacking *fnr* (Δ*fnr*) under anaerobic conditions. Comparative studies revealed that 259 proteins significantly altered among ~1600 total identifications. In addition to several known pathways as previously reported, we found that FNR represses the expression of a large number of virulence factors encoded by *Salmonella* pathogenicity island 1 (SPI-1). Notably, FNR represses propanediol utilization by directly binding to the promoter region of the *pdu* operon. Strikingly, both SPI-1 and propanediol utilization were adversely regulated by ArcA, another anaerobic regulator. Additionally, our data suggest profound remodeling of bacterial membranes such as down-regulation of a multidrug resistance protein EmrA (localized in the inner membrane) and an outer membrane protein OmpW in the Δ*fnr* mutants, rendering the bacterium more susceptible to kanamycin and polymyxin and increasing outer membrane permeability.

Methods

Bacterial Strains and Culture Conditions

The *Salmonella enterica* serovar Typhimurium strain SL1344 was described previously [15]. All bacterial strains were maintained at −80 °C in 2% peptone solution containing 25% glycerol. Frozen strains were routinely grown on LB plates at 37 °C with 1.5% agar and 30 μg/mL streptomycin. A single colony was picked and then inoculated into 3 mL of MOPS (morpholinepropanesulfonic acid)-buffered (100 mM, pH 7.4) LB broth supplemented with 20 mM D-xylose (LB-MOPS-X) [16]. The overnight culture was then subcultured under anaerobic conditions as previously described [16] and harvested at OD_{600} = ~0.3.

Molecular Cloning and Construction of Bacterial Mutants

The *S.* Typhimurium *fnr*-deletion mutant (Δ*fnr*) was constructed by using the standard homologous recombination method as previously described [16]. The λ-red recombination system was used to construct chromosomally 3 × FLAG-tagged SipB, SopB, PrgH, HilA, PduJ, and PduT in various genetic backgrounds (WT, Δ*fnr*, and Δ*arcA*) as previously reported [17]. Briefly, the sequences encoding the 3 × FLAG epitope were inserted in-frame at the C-termini of the genes of interest right before the stop codon. Successful deletion or tagging of the target genes was confirmed by both PCR analyses and sequencing.

Proteomic Sample Preparation and Stable Isotope Dimethyl Labeling

To uncover potential FNR-regulated proteins, we performed comparative proteomic analyses of the wild type (WT) and Δ*fnr* strains cultured under anaerobic conditions. Proteomic sample preparation and peptide dimethyl labeling were performed as described previously [18, 19]. Briefly, bacterial lysates were fractionated into eight fractions by SDS-PAGE prior to in-gel protein digestion. Extracted peptides were vacuum dried for further dimethyl labeling. The samples from the WT and *fnr* mutant strains were labeled with formaldehyde (CH_2O) and its deuterated version (CD_2O), respectively, as described [20]. Finally, light- and heavy-labeled peptides were equally mixed and vacuum dried immediately.

Nanoflow LC-MS/MS Analyses

Nanoflow reversed-phase LC separation was carried out on an EASY-nLC 1200 System (Thermo Scientific). The capillary column (75 μm × 150 mm) with a laser-pulled electrospray tip (Model P-2000, Sutter Instruments) was home-packed with 4 μm, 100 Å Magic C18AQ silica-based particles (Michrom BioResources Inc., Auburn, CA). Labeled peptides were dissolved in solvent A (described below) and approximately 200 ng of samples were loaded onto the analytical column in a single LC-MS/MS run. The mobile phase was comprised of solvent A (97% H_2O, 3% ACN, and 0.1% FA) and solvent B (80% ACN, 20% H_2O, and 0.1% FA). The following gradient was used for LC separation: solvent B was started at 7% for 3 min, and then raised to 35% over 40 min. Subsequently, solvent B was rapidly increased to 90% in 2 min and maintained for 10 min before 100% solvent A was used for column equilibration. Peptides eluted from the capillary column were electrosprayed directly onto a hybrid linear ion trap-Orbitrap mass spectrometer (LTQ Orbitrap Velos, Thermo Scientific) for MS and MS/MS analyses in a data-dependent mode. One full MS scan (m/z 350–1200) was acquired by the Orbitrap mass analyzer with R = 60,000, and subsequently, the ten most intense ions were selected for collision-induced dissociation (CID) in the ion trap with the following parameters: ≥ + 2 precursor ion charge, 2 Da precursor ion isolation window, and 35% normalized collision energy. Dynamic exclusion was set with repeat duration of 30 s and exclusion duration of 12 s. In total, we analyzed three paired biological replicates of Δ*fnr* and wild type bacterial samples in 48 LC-MS/MS experiments.

Proteomic Data Processing and Bioinformatics Analysis

The raw MS files were searched against the *S.* Typhimurium LT2 protein database (downloaded from UniProt, version 2016_11 and complemented with those unique to SL1344) by using MaxQuant 1.5.3.30. The precursor mass tolerance

was set at 20 ppm, and the fragment mass tolerance was set at 0.8 Da. The digestion enzyme was set as trypsin with a maximum of two missed cleavages. Variable modifications include dimethyl (K, N-term), dimethyl (D_4K, D_4N-term), and oxidation (M). Both peptide and protein assignments were filtered to achieve a false discovery rate (FDR) < 1%. Only proteins with at least two unique peptides were quantified. The MaxQuant software was used to calculate the intensity of H- and L-labeled proteins. The intensity values from MaxQuant were normalized and further processed by using the Perseus software (version 1.5.4.1). We removed those protein identifications that were only assigned with modifications or matched to the reverse database as well as common contaminants. Logarithmic values (log_2) of the H- and L-labeled protein intensity were calculated. The missing values (that refer to the scenario where a peptide signal is absent or not detected in one of the two-paired samples) were replaced with random numbers from a normal distribution with the default parameters (width = 0.3, shift = 1.8) by using the imputation method in Perseus. The *p* values were calculated by using the two-tailed Student's *t* test. Proteins with ratios (H/L) > 2.0 or < 0.5 and *p* values < 0.05 were considered significant difference between the WT and Δ*fnr* strains. Further, multiple hypothesis testing was conducted with the Benjamini-Hochberg method. For the analysis of protein-protein interactions and/or their functional association, differentially expressed proteins were searched against the STRING database (http://string-db.org/) with the highest confidence score (score > 0.9). In order to have more protein hits for such network analyses, we used the proteomic data prior to multiple hypotheses testing. The software Virtual Footprint was used to identify the putative FNR-binding site in the promoter region of the *pdu* operon.

Reverse Transcription-quantitative PCR

RT-qPCR (reverse transcription-quantitative PCR) measurements of gene expression were performed as previously described [21]. The total RNA was extracted by using the illustra RNAspin Mini Kit (GE Healthcare Life Sciences) following the manufacturer's instructions. The extracted RNA samples were treated with DNase I (the turbo DNA Free Kit, Ambion) to remove any genomic DNA contaminants. Reverse transcription was performed by using Super Script II reverse transcriptase (Invitrogen, Carlsbad) and random hexamer (Invitrogen, Carlsbad) to generate cDNA. Quantitative PCR was conducted by using the SYBR Green PCR master mix (Applied Biosystems) and specific primers on a StepOnePlus Real-time PCR system (ABI). The house-keeping gene *rrsA* (encoding 16S RNA) was used as an internal control. All primers used in RT-qPCR are listed in Table S1.

Immunoblotting Analysis

The 3 × FLAG-tagged *Salmonella* strains were anaerobically grown in LB-MOPS-X media to an OD_{600} of ~ 0.3. The gel-fractionated bacterial proteins were first transferred onto polyvinylidene difluoride (PVDF) membranes and then blotted with primary antibodies specific for DnaK (Enzo Life Sciences) (1:5000) or FLAG (Cwbio, China) (1:2500) and horseradish peroxidase (HRP)-conjugated secondary antibodies (Cwbio, China) (1:5000). Finally, the antibody bands were visualized with enhanced chemiluminescent (ECL) reagents by using a Tanon-5200 Imaging System (Tanon, China).

The MIC Assays

The MICs (minimal inhibitory concentrations) of antibiotics were determined following the instructions of MIC testing of the *Manual of Antimicrobial Susceptibility Testing* [22]. The *S.* Typhimurium strains were grown anaerobically to an OD_{600} of ~ 0.3 as described. The OD_{600} of all strains were normalized to the concentration of 5×10^5 cells/mL and inoculated to 96-well plates containing various concentrations of antibiotics. Subsequently, the bacteria-containing plates were incubated at 37 °C anaerobically for 20 h. The MICs were determined by OD_{600} reading.

Disk Diffusion Test

Disk diffusion test was performed following the instructions of Disk Diffusion testing of *Manual of Antimicrobial Susceptibility Testing* [22]. The *S.* Typhimurium cells were grown anaerobically to an OD_{600} of ~ 0.3 as described. The cells were then mixed with 0.6% top agar with the final cell density as 5×10^5 cells/mL and poured onto the LB agar plates. Sterilized paper disks containing various concentrations of polymyxin were applied onto the plates. The images indicating the susceptibility of *S.* Typhimurium cells to antibiotics were recorded following incubation at 37 °C anaerobically for 20 h.

Membrane Permeability Assays

Membrane permeability assays were conducted as described previously [23]. *S.* Typhimurium strains were grown anaerobically to an OD_{600} of ~ 0.3, centrifuged, and resuspended in equal volumes of 5 mM HEPES buffer (pH 7.2). These bacterial suspensions were immediately pipetted to fluorometer plate wells containing 10 μM 1-N-phenylnaphthylamine (NPN). The excitation and emission wavelengths for NPN were set at 360 and 460 nm, respectively. Fluorescence indicates the access of NPN to glycerophospholipid milieu.

Adaptation Assays in Caenorhabditis elegans

The nematode *C. elegans* WT (N2) was cultured as previously described [16]. At day 2, synchronized nematodes (L4 stage) were transferred to LBMOPSX plates containing 100 μL of *S.* Typhimurium WT and the isogenic Δ*fnr* cells (OD_{600} as 1), respectively. The worms were allowed to feed on the lawns at 25 °C. After 24 hpi or 48 hpi; 12 worms were transferred to 500 μL of M9 worm buffer (0.5% NaCl, 0.3% KH_2PO_4, 0.6% Na_2HPO_4, and 1 mM $MgSO_4$) and washed in M9 worm buffer for 6 times to remove external bacteria. The worms were finally suspended in ddH_2O and disrupted mechanically. The *S.* Typhimurium that survived in the *C. elegans* intestine was then

recovered and quantified on selective MacConkey plates following series dilutions. Upon incubation of the plates at 37 °C for 24 h, the CFUs of the survived *S.* Typhimurium were detected.

Data Availability

The proteomics data reported in this paper have been deposited to the iProx database (URL:http://www.iprox.org/page/HMV006.html) and are available under the accession number IPX0001421001.

Results

Quantitative Analyses of Protein Expression in S. Typhimurium Wide Type and Δfnr Strains During Anaerobiosis

To study the FNR-regulated cellular processes in *S.* Typhimurium, we carried out comparative profiling of protein expression between the WT strain and an *fnr*-deletion mutant (Δ*fnr*) cultured under anaerobic conditions. From three biological replicates, in total, we measured 1580 *S.* Typhimurium proteins with an average of 1489 identifications per bacterial sample (at FDR < 1%), among which 187 and 72 proteins were up and downregulated, respectively (protein ratios > 2.0 or < 0.5 and p values < 0.05). Upon correction for multiple testing, 60 bacterial proteins remain to be significantly altered with Benjamini-Hochberg FDR set at 0.05 (there are 142 and 230 proteins with FDR set at 0.1 and 0.2, respectively) (Table S2 sheet 4). A full list of all protein identifications is provided as Table S2, and a protein volcano plot with abundance ratios can be seen in Fig. S1. In addition, we performed the Pearson correlation analysis of the triplicate samples, demonstrating good reproducibility of our measurements (Fig. S2). To delineate the functional associations/interactions of those altered proteins, we conducted network analyses by utilizing the STRING database. Among the upregulated proteins in the Δ*fnr* mutant (those repressed by FNR), STRING analyses revealed at least three prominent clusters with the highest confidence (score > 0.9). A large interaction network features a number of proteins associated with *S.* Typhimurium SPI-1 type III secretion system (T3SS) (enclosed in a blue circle) (Figure 1a). In addition, two small clusters were also evident including the proteins in the tricarboxylic acid (TCA) cycle (enclosed in a black circle) and propanediol utilization (enclosed in a red circle) (Figure 1a). This result is consistent with the well-recorded function of FNR to repress enzymes in the TCA cycle under anaerobic conditions [24]. Nonetheless, the regulation of propanediol utilization by FNR has not been reported thus far in *S.* Typhimurium. In contrast to the large number of upregulated proteins, we identified fewer downregulated proteins in the Δ*fnr* strain (Figure 1b). A noticeable network in this category comprises many sulfate assimilation proteins such as CysP, CysN, CysA, CysI, and Sbp.

Other than those large clusters described above, our dataset also revealed a number of small groups of FNR-regulated proteins, which are well-recognized by previous studies. For example, FNR-repressed proteins include formate dehydrogenases (FdoG and FdoH) [24], aerobic ribonucleotide reductases (NrdA and NrdB) [11, 12], and cytochrome o/d oxidases (CyoA, CydA) [25, 26], and FNR-activated proteins encompass fumarate reductases/hydratases (FrdA, FrdB, and FumB), nitrite/nitrate reductases (NirB and NapA) [27, 28], and anaerobic dimethyl sulfoxide reductases (DmsA and DmsB) [11, 24, 29]. Importantly, our data significantly expand the current FNR regulon particularly in *S.* Typhimurium. The new FNR-repressed proteins that were identified include *Salmonella* virulence regulators (PhoP, PhoQ, and SsrB) and antioxidant factors (SodA, SodB, and KatE). Furthermore, we are the first to report the following FNR-activated proteins: peptide methionine sulfoxide reductases (MsrA and MsrB) and mannonate dehydration proteins (UxuA, UxaC, and STM3136).

Repression of Salmonella SPI-1 T3SS by FNR

Among those upregulated proteins, *Salmonella* SPI-1-encoded virulence factors comprise the largest network (Figure 1a). Further classification reveals that these FNR-repressed proteins include the structural components of the secretion apparatus, effector proteins, and SPI-1 transcription factors (Figure 2a). For example, translocon proteins (SipB, SipC, and SipD) were approximately 4–10 folds more abundant in the Δ*fnr* mutant compared to its parental strain. Other repressed structural components encompass PrgH, PrgK, InvA, and InvG (5–10 folds) that are associated with the basal body. Notably, the expression of T3SS effectors was almost unanimously elevated including SipA, SopA, SopB, SopE, SopE2, and SptP in Δ*fnr* mutants (5–20 folds). Notably, a known SPI-1 regulator, HilA, was also upregulated (4 folds), suggesting that the global repression of SPI-1 T3SS by FNR is likely to be exerted transcriptionally via HilA. Consistent with our proteomic data, immunoblotting analyses of *S.* Typhimurium cells expressing 3 × FLAG-tagged SopB, PrgH, and HilA, respectively, showed higher levels of these T3SS proteins in the Δ*fnr* mutant. As a control, their levels were decreased in the Δ*arcA* mutant, consistent with our previous findings [16] (Figure 2b and Fig. S3). To further ascertain the repression of SPI-1 by FNR on the transcript level, next we conducted RT-qPCR analyses of their mRNA levels. Indeed, we found substantially higher (> 50 folds) levels of *sopB*, *prgH*, and *hilA* transcripts in the Δ*fnr* mutant than in its parental WT strain (Figure 2c). Together, these data suggest that FNR transcriptionally represses the expression of SPI-1 T3SS, which is likely to be HilA-dependent.

Negative Regulation of the Propanediol Utilization Pathway by FNR

Intriguingly, many proteins in the propanediol utilization pathway (encoded by the *pdu* operon) were negatively regulated by FNR including PduE, PduG, PduJ, PduD, PduS, and PduT (5–48 folds more abundant in the Δ*fnr* mutant, see

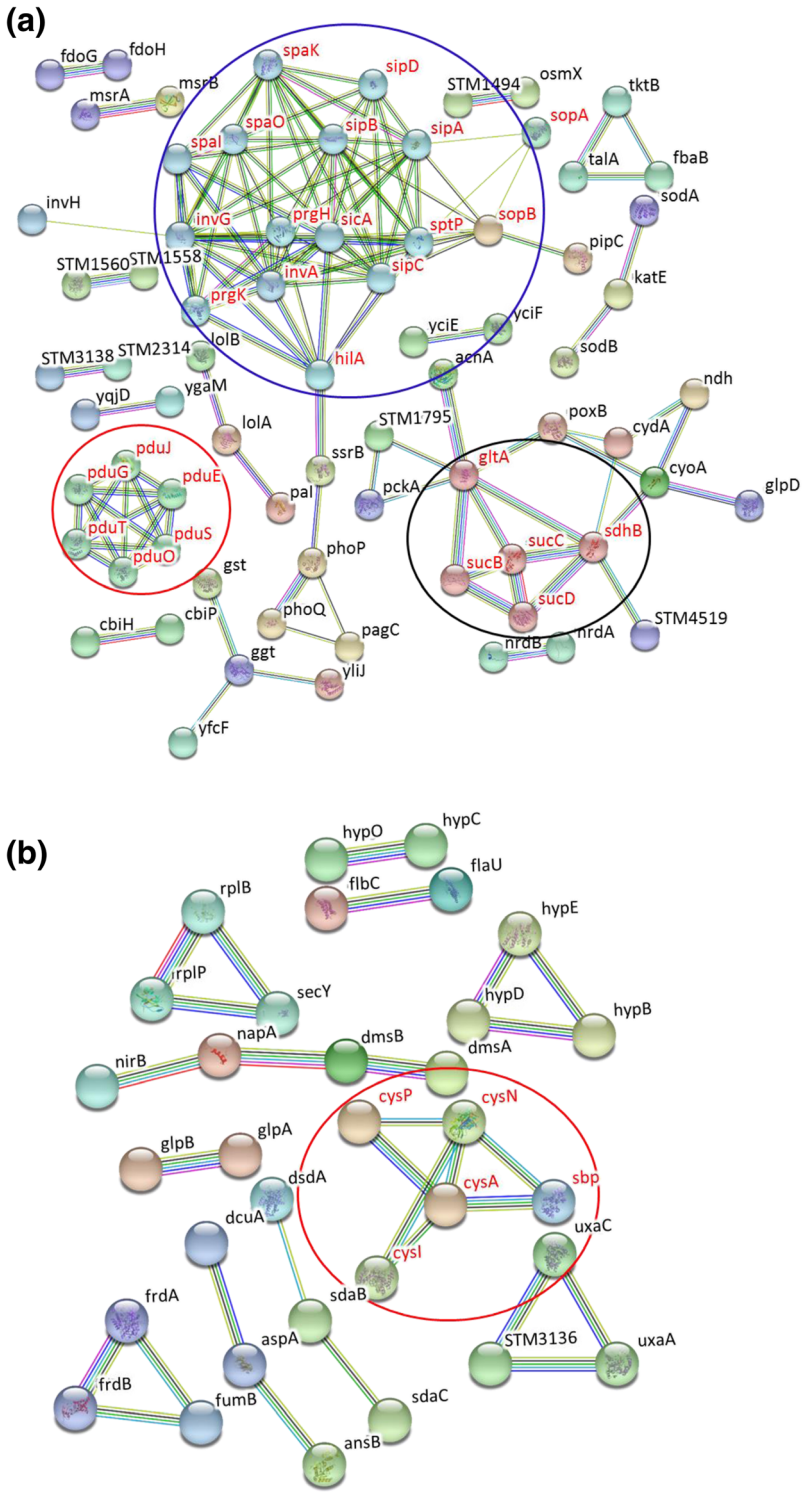


Figure 1. Network analyses of protein-protein interactions and functional associations of differentially expressed proteins in the *fnr* mutant. Different clusters of associated proteins were identified by using the STRING software with a highest confidence score (0.9). (**a**) FNR-repressed proteins. (**b**) FNR-activated proteins. Black or blue circles indicate those FNR-regulated proteins that are consistent or inconsistent with previous transcriptome data, respectively. Red circles indicate the FNR-regulated proteins that were newly identified in this study. The network nodes represent differentially expressed proteins, and color-coded lines linking different nodes represent the types of evidence used in prediction (red line, fusion evidence; green line, neighborhood evidence; blue line, co-occurrence evidence; purple line, experimental evidence; yellow line, text-mining evidence; light-blue line, database evidence; black line, co-expression evidence). The color of nodes is used only as a visual aid to identify which node goes with which description in the list of input. Empty nodes represent proteins of unknown 3D structures, and filled nodes represent those with known or predicted 3D structures. The different size of nodes indicates the availability of structural information (i.e., it is larger to fit a thumbnail picture)

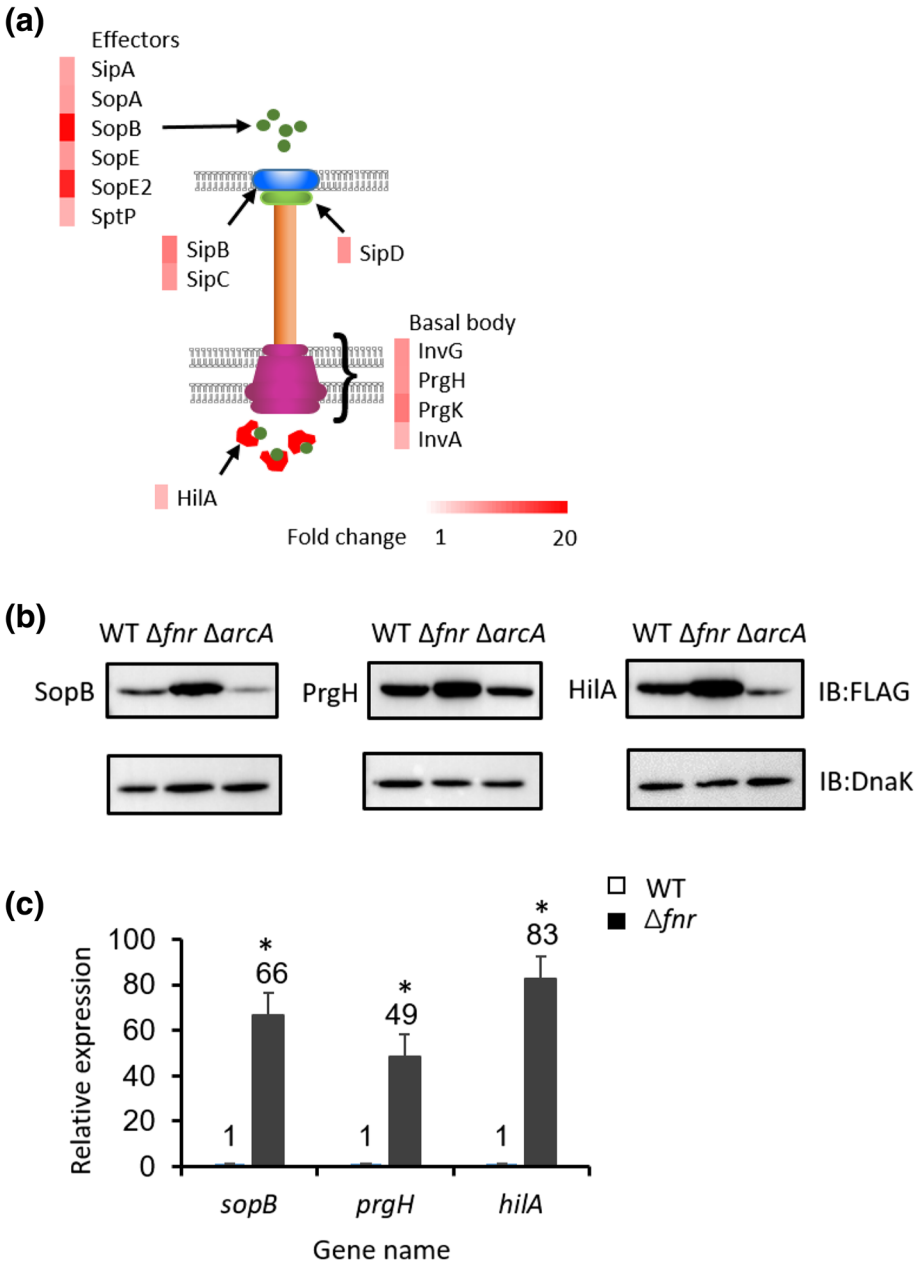


Figure 2. Repression of *S*. Typhimurium SPI-1 T3SS by FNR. (**a**) A schematic diagram of SPI-1 T3SS with fold change of virulence factors. (**b**) Immunoblotting analyses of SopB, PrgH, and HilA expression in various genetic backgrounds (WT, Δ*fnr*, Δ*arcA*) under anaerobic conditions. Asterisks indicate significant differences (*, $p < 0.05$, p values were calculated by using the Student's t test)

Figure 3a). In an opposing manner, strikingly, we previously found that ArcA positively regulated this pathway. Immunoblotting analyses with chromosomally tagged Pdu proteins further confirmed the opposing regulatory roles of FNR and ArcA on this pathway (Figure 3b and Fig. S3). Furthermore, RT-qPCR analyses revealed substantially higher (71 and 34 times) transcript levels of *pduJ* and *pduT* in the Δ*fnr* mutant relative to its parental strain (Figure 3c). As propanediol is a carbohydrate enriched in intestinal epithelial cells, propanediol assimilation is thought to confer a selective advantage for pathogenic *Salmonella* in anaerobic environments [30, 31].

Given the relevance of propanediol utilization in the host-pathogen interface, next we sought to determine if FNR exerts a direct regulatory role in *pdu* operon by using electrophoretic mobility shift assays (EMSAs). Bioinformatics analysis identified a putative FNR-binding site in the promoter region of the *pdu* operon (Figure 3d). We then utilized a previously constructed O_2-stable FNR variant, $(FNRD154A)_2$ [32], to examine its potential binding with the *pdu* promoter. After electrophoresis, a concentration-dependent mobility shift was observed corresponding to the DNA-protein complexes of FNR with P*pdu* (Figure 3e). Together, these findings reveal that FNR

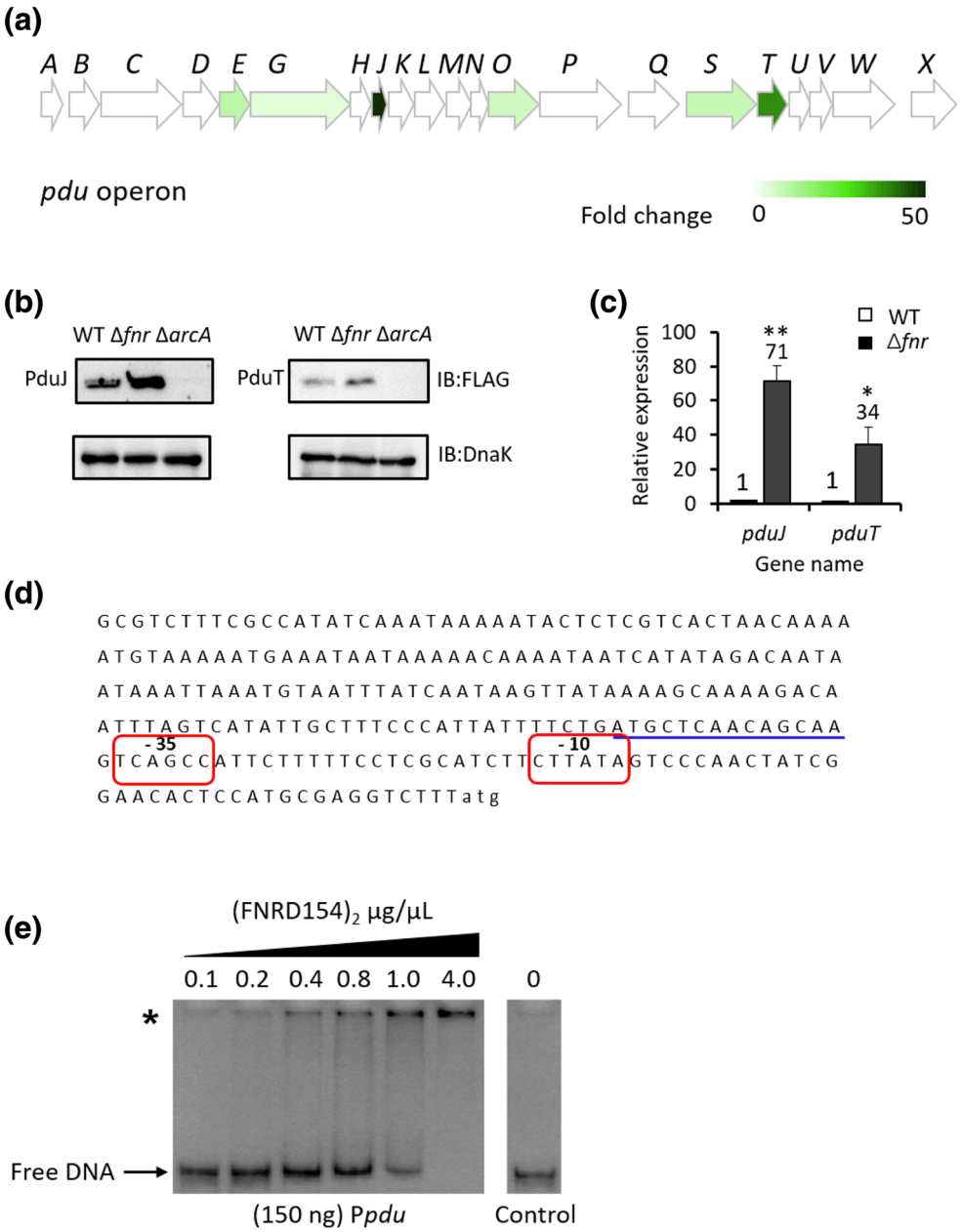


Figure 3. Repression of the propanediol utilization pathway by FNR. (**a**) A schematic diagram of the *pdu* operon. (**b**) Immunoblotting analyses of PduJ and PduT in various genetic backgrounds (WT, Δ*fnr*, Δ*arcA*) under anaerobic conditions. (**c**) RT-qPCR analyses of *pduJ* and *pduT* mRNA levels in the WT and Δ*fnr* strains grown under anaerobic conditions. Asterisks indicate significant differences (*, $p < 0.05$; **, $p < 0.01$). (**d**) The sequence of the *pdu* promoter region corresponding to 250 bp upstream of the start codon. The putative FNR-binding site is underlined. Putative – 10 and – 35 elements are circled. (**e**) EMSA of FNR with the promoter region (P*pdu*). The arrow denotes free DNA and the asterisk indicates DNA-protein complexes. NC, negative controls in which a DNA fragment with random sequence was utilized

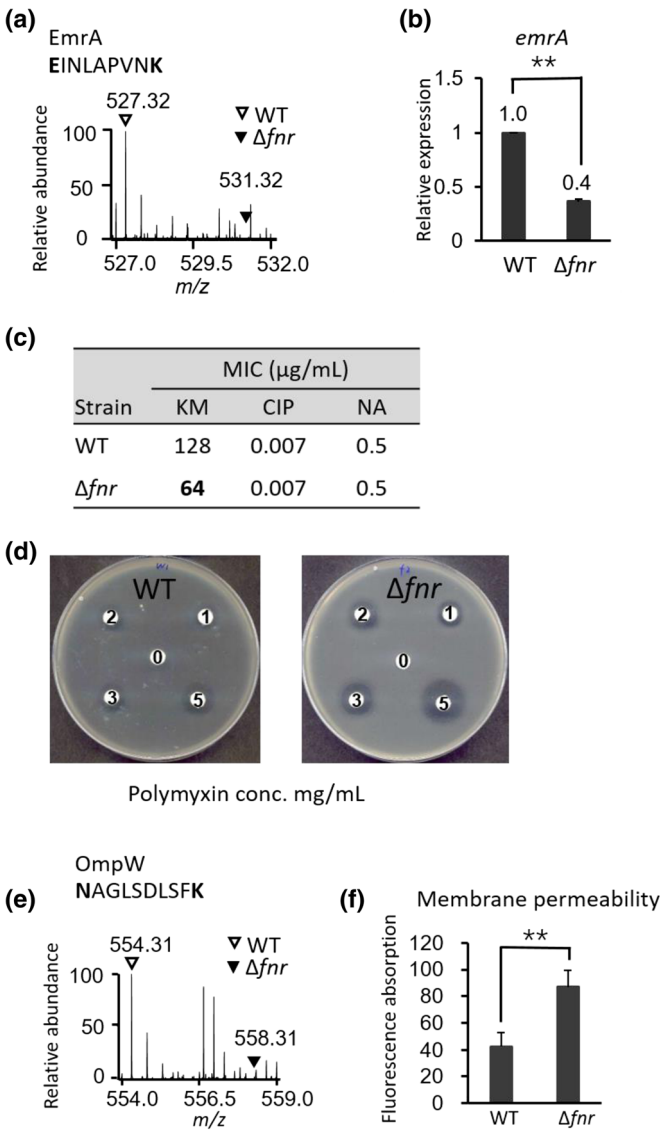
directly binds to the *pdu* promoter and represses its transcription under anaerobic conditions.

S. Typhimurium Lacking fnr Showed Increased Susceptibility to Some Antibiotics as well as Damage to the Outer Membrane

Our data showed differential regulation of quite a few membrane proteins in the Δ*fnr* cells relative to the WT. For instance, the multidrug resistance protein EmrA is markedly downregulated (Figure 4a). Consistent with its protein expression, RT-qPCR analyses showed decreased mRNA levels of *emrA* (2.5 folds less) in the Δ*fnr* mutant as well (Fig. 4b). EmrA is a component of the tripartite efflux system (EmrAB–TolC), which is involved in the export of antibiotics, such as fluoroquinolones across both the inner and outer membranes of *Salmonella* [33, 34]. We reasoned that downregulation of EmrA may render *S.* Typhimurium Δ*fnr* cells more susceptible

to antibiotics treatment. To test this hypothesis, we measured the MIC (minimum inhibitory concentration) of various antibiotics to the WT and Δ*fnr S.* Typhimurium SL1344 cells. It was shown that the Δ*fnr* cells were more susceptible to kanamycin but not to fluoroquinolones as reported previously [34] (Figure 4c). Further disk diffusion tests revealed that Δ*fnr* mutants also displayed increased susceptibility to polymyxin under anaerobic conditions (Figure 4d). Taken together, these data suggest that *Salmonella* strains lacking *fnr* showed increased susceptibility at least to some antibiotics.

Furthermore, an outer membrane protein OmpW was downregulated in the Δ*fnr* strain (Figure 4e). It is known that the outer membrane of Gram-negative bacteria serves as a protective barrier that restricts the permeability of both hydrophilic and hydrophobic compounds. In particular, OmpW is proposed to transport small hydrophobic molecules across the outer membrane [35] and its expression is shown to be activated by FNR in *E. coli* [36]. Therefore, we assayed outer membrane permeability of the WT and Δ*fnr S.* Typhimurium SL1344 cells by utilizing a fluorescent probe 1-N-phenylnaphthylamine (NPN). We found that under anaerobic conditions, the Δ*fnr* cells displayed increased membrane permeability towards this small molecule probe (Figure 4f). Taken together, these data suggest that FNR modulates the expression of certain



Strain	MIC (μg/mL) KM	CIP	NA
WT	128	0.007	0.5
Δ*fnr*	**64**	0.007	0.5

Figure 4. *Salmonella* lacking *fnr* showed increased antibiotics susceptibility and membrane permeability. (**a**) Representative mass spectra of dimethyl labeled peptides from EmrA. Peptides in the WT and Δ*fnr* samples with light and heavy labels are indicated by open and filled triangles, respectively. (**b**) RT-qPCR analyses of *emrA* mRNA levels in the WT and Δ*fnr* strains grown under anaerobic conditions. Asterisks indicate significant differences (**, $p < 0.01$). (**c**) MIC (minimal inhibitory concentration) assays of WT and Δ*fnr* strains to different antibiotics. KM, kanamycin; CIP, ciprofloxacin; NA, nalidixic acid. (**d**) Disk diffusion test assays of sensitivity of WT and Δ*fnr* strains to polymyxin. The numbers labeled in the plate represent the concentration of polymyxin. (**e**) Representative mass spectra of dimethyl labeled peptides from OmpW. (**f**) Membrane permeability assays of WT and Δ*fnr* cells. Asterisks indicate significant differences (**, $p < 0.01$)

membrane proteins, which leads to increased susceptibility to kanamycin and polymyxin and increased membrane permeability in *S.* Typhimurium.

Impaired Bacterial Survival of S. Typhimurium Lacking fnr in a C. elegans Infection Model

Our data demonstrated that FNR regulates a wide spectrum of bacterial proteins in various cellular pathways. Next, we examined the impact of this global regulator on *S.* Typhimurium fitness in a *C. elegans* infection model. We fed *C. elegans* with equal doses of *S.* Typhimurium *fnr* mutants and its parental WT bacteria (Figure 5a). At 24 h post-infection, we recovered bacteria from the intestine of *C. elegans* and enumerated the number of viable bacteria. The data revealed substantially decreased bacterial survival of the *fnr* mutant in the *C. elegans* intestine (Figure 5b), confirming the important role FNR plays in the adaptation of *S.* Typhimurium to the host environment.

Discussion

There are several reports on the regulation of specific genes by FNR in *Salmonella* [24, 27, 37]. For instance, previously, Fink et al. examined the transcriptome of *S.* Typhimurium ATCC 14028s wild type (WT) and its isogenic Δ*fnr* mutant under anaerobic conditions [24]. Our current study is the first proteomic investigation on the regulatory role of FNR in *S.* Typhimurium SL1344 during anaerobiosis. Here, we demonstrate that FNR not only regulates metabolic enzymes which facilitate the adaptation to anaerobic conditions but also controls the expression of virulence factors. *S.* Typhimurium pathogenesis is highly dependent on two distinct type III secretion systems (T3SSs) that are encoded on *Salmonella* pathogenicity island 1 (SPI-1) and SPI-2. The T3SS is a needle-like apparatus that mediates the delivery of virulence factors (called effectors) directly into host cells. Effector proteins translocated by *S.* Typhimurium SPI-1 T3SS enable bacterial invasion, whereas the SPI-2 T3SS transports effector proteins that are important for intracellular survival and replication [38–40].

Several previous studies have implied the role of FNR in regulating SPI-1 in *S.* Typhimurium. Fink et al. reported that FNR activates SPI-1 genes in anaerobic conditions [24]. In contrast, Van et al. subsequently found FNR as a negative regulator of SPI-1 gene expression in aerobic conditions [41]. Later, FNR was suggested to repress SPI-1 at the level of HilA under in vitro SPI-1-inducing conditions [42]. In addition, Contreras et al. found increased invasion of an *fnr* mutant strain during *S.* Typhi infection of intestinal epithelial cells when grown in anaerobic conditions [43]. These studies suggest that FNR may exert a delicate regulatory effect on SPI-1 in *Salmonella*, which needs to be further clarified in different conditions and different stages of infection. In our study, we found that *S.* Typhimurium SPI-1 T3SS was significantly repressed by FNR under anaerobic conditions (Figure 2a–b). RT-qPCR analysis revealed that the repression occurs at the transcriptional level (Figure 2c). This finding is in contrast to the previous transcriptomic study reported by Fink et al [24]. Considering the same culturing conditions, we speculate that the different strains (i.e., 14028s versus SL1344) may account for this discrepancy. Interestingly, our previous studies revealed that another anaerobic global transcription factor ArcA also regulates the expression of SPI-1 genes but in an opposite manner, i.e., activating the expression of SPI-1. Notably, FNR has been suggested to regulate ArcA as well [44]. We consider that such regulation patterns, to some extent, function as a feed-forward regulation which ensures the bacteria to turn on/off the SPI-1 system timely during infection.

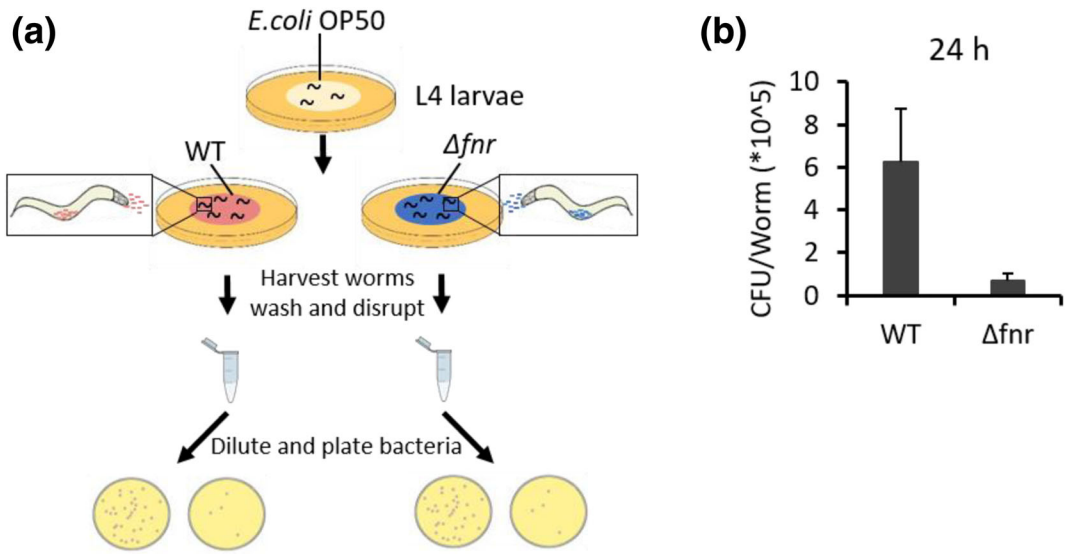


Figure 5. *Salmonella* lacking *fnr* exhibited decreased bacterial survival in the *C. elegans* intestine. (**a**) A schematic diagram of the survival assay (WT *S.* Typhimurium vs isogenic Δ*fnr* strains) in the *C. elegans* intestine. (**b**) Adaptation assays of WT and Δ*fnr* strains upon infection of *C. elegans* intestine at 24 h

Propanediol is a major product of the fermentation of rhamnose and fucose, two common sugars present in intestinal epithelial cells [45]. Accordingly, propanediol degradation is thought to provide a selective advantage to *S.* Typhimurium in anaerobic environments such as the mammalian gastrointestinal tract [31]. Previous studies have shown that the deletion of *Salmonella pdu* genes causes a defect in systemic survival [46, 47]. The 19-kb *pdu* operon encodes 21 genes specifically required for propanediol utilization by *S.* Typhimurium [48, 49]. It is reported that PocR is a positive regulator of *pdu* operon and is subject to global control by ArcA and Crp [48]. A subsequent study [24] and our previous results [16] have shown that the *pdu* operon was activated by ArcA during anaerobic growth. Herein, we found several proteins encoded in the *pdu* operon were significantly repressed by FNR (Figure 3a–b). Further, RT-qPCR analysis and EMSA experiments confirmed that FNR is a negative regulator of the *pdu* operon: FNR directly binds to the *pdu* promoter and represses its transcription under anaerobic conditions (Figure 3c–e). However, the expression of PocR was not changed in the Δ*fnr* cells, indicating that the control of the *pdu* operon by FNR is not through the regulation of PocR. Taken together, we report that FNR and ArcA co-regulate the *pdu* operon in *Salmonella* under anaerobic conditions. These coordinated regulations may be necessary to fine-tune the propanediol utilization during the anaerobiosis of *Salmonella*.

In our proteomic data, the *Salmonella* multidrug resistance protein EmrA showed significantly lower expression levels in the Δ*fnr* cells compared to wide-type, indicating the activation of EmrA by FNR (Figure 4a). EmrA is a component of tripartite multidrug efflux pump system (EmrAB–TolC) which can recognize and export antibiotics and biocides [50]. Thus, we speculate that FNR may participate in regulating antibiotic resistance genes in *S.* Typhimurium. To verify our speculation, we performed MIC and disk diffusion test assays (Figure 4c–d). These results show that *S.* Typhimurium lacking *fnr* displayed increased susceptibility to kanamycin and polymyxin. The key to understanding how bacteria utilize multidrug efflux pumps to increase antibiotic resistance lies in the regulation of pump expression. The multidrug efflux pumps are expressed under precise and elaborate transcriptional control. For example, the expression of *macAB*, an operon encoded for another efflux pump in *S.* Typhimurium, is directly controlled by the two-component signal transduction system PhoPQ [51]. PhoQ is required for *Salmonella* to detect environmental signals and activates PhoP. The activated PhoP then binds to the upstream of the *macAB* operon and subsequently induces the MacAB system, resulting in excretion of toxic compounds into the surrounding environment such as the intestine [50]. However, the regulation of *emrAB* has not been reported previously. Here, we uncovered that FNR activates the expression of *emrA* at the transcriptional level (Figure 4b). It would be worthwhile to determine whether FNR is the direct regulator of *emrAB* and how FNR regulates their function in the future.

Finally, we found that deletion of *fnr* results in perturbation of membrane permeability of *S.* Typhimurium cells (Figure 4f) and decreased bacterial survival in the *C. elegans* host (Figure 5b). Taken together, these findings highlight that FNR participates in the regulation of bacterial metabolism, physiology, and virulence in *S.* Typhimurium.

Conclusions

In this work, we presented the first proteomic investigation on the regulatory role of FNR in *S.* Typhimurium SL1344 under anaerobic conditions. We found FNR represses *Salmonella* virulence genes during anaerobiosis. Notably, we found FNR as a negative regulator of the *pdu* operon by directly binding to the *pdu* promoter and repressing its transcription under anaerobic conditions. Furthermore, *S.* Typhimurium SL1344 lacking *fnr* showed increased susceptibility to some antibiotics and potential damage to the outer membrane. Finally, we demonstrated substantially decreased bacterial survival of *S.* Typhimurium SL1344 lacking *fnr* in the intestine of *C. elegans*.

Acknowledgements

We thank the members of the Liu and Yan laboratories for the critical reading of this manuscript. This work was financially supported by grants from the National Natural Science Foundation of China (21475005 and 21622501), Clinical Medicine Plus X-Young Scholars Project of Peking University, and the Thousand Young Talents Program to XL, and the Hong Kong University Research Council General Research Fund (17127918) and Shenzhen City Knowledge Innovation Plan (JCYJ20160530174441706) to AY.

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