Temporal Regulation of a Salmonella Typhimurium Virulence Factor by the Transcriptional Regulator YdcR*

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We previously examined Salmonella proteome within infected host cells and found differential expression of many proteins with defined functional roles such as metabolism or virulence. However, the precise roles of other altered proteins in Salmonella pathogenesis are largely unknown. A putative transcriptional regulator, YdcR, was highly induced intracellularly whereas barely expressed in vitro, implicating potential relevance to bacterial infection. To unveil its physiological functions, we exploited quantitative proteomics of intracellular Salmonella and found that genetic ablation of ydcR resulted in severe repression of SrfN, a known virulence factor. Immunoblotting, qRT-PCR, and β-galactosidase assays further demonstrate YdcR-dependent transcription and expression of srfN. Moreover, we found physical interaction of YdcR with the promoter region of srfN, suggesting direct activation of its transcription. Importantly, a Salmonella mutant lacking ydcR was markedly attenuated in a mouse model of infection. Our findings reveal that YdcR temporally regulates the virulence factor SrfN during infection, thus contributing to Salmonella pathogenesis. Our work also highlights the utility of combining quantitative proteomics and bacterial genetics for uncovering the functional roles of transcription factors and likely other uncharacterized proteins as well. Molecular & Cellular Proteomics 16: 10.1074/mcp.M117.068296, 1683–1693, 2017.

Salmonella enterica serovar Typhimurium (S. typhimurium) is one of the leading food-borne bacterial pathogens (1).

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Successful infection is highly dependent on the type III secretion systems (T3SSs) encoded by Salmonella pathogenicity islands 1 and 2 (SPI-1 and SPI-2) (2). T3SS functions as a molecular syringe that is able to translocate bacterial virulence factors (effector proteins) directly into host cytosol. These T3SS effector proteins, upon delivery, can modulate various host cellular processes to promote bacterial infection as well as survival within host cells (3). As an intracellular bacterial pathogen, S. typhimurium has the capability to invade nonphagocytic epithelial cells and proliferate with a membrane-bound compartment known as the Salmonella-containing vacuoles (SCVs) (4).

To understand how bacterial pathogens adapt to their intracellular niches, we previously studied the proteome of intracellular Salmonella within epithelial cells at distinct stages of infection (5, 6). At 6 h post-infection (hpi), Salmonella up-regulates multiple pathways involved in acquisition of metal ions such as iron, suggesting a general shortage of these ions within the host (5). At a later stage of infection (e.g. 18 hpi), intracellular Salmonella undergoes massive metabolic reprogramming and favors glycolysis as well as mixed acid fermentation for its energy requirements, whereas the tricarboxylic acid (TCA) cycle and most respiration pathways are repressed (6). Overall, our quantitative proteomic measurements suggest extensive bacterial adaptations to infected host epithelial cells. In addition, the large-scale proteomic data sets also reveal many other differentially regulated proteins and/or pathways during Salmonella infection. Of interest are transcription factors that were induced during the course of infection. We hypothesize that such regulators are likely to play important roles in bacterial pathogenesis. For instance, one of the MarR family transcription factors, SlyA, was 2-fold upregulated at 18 hpi. SlyA has been shown to contribute to Salmonella...
intracellular survival within macrophages and other aspects of bacterial physiology as well (7, 8, 9). Interestingly, we also identified a putative transcription factor YdcR that was highly induced throughout the infection process, though this protein was barely expressed (i.e. not detected in our proteomic measurements) in bacteria cultured in vitro. YdcR is homologous to the family of a MocR/GabR-type transcriptional regulator that falls into the GntR superfamily, one of the most widespread families of bacterial transcription factors that regulate diverse biological processes (10, 11), but the function of YdcR in Salmonella pathogenesis remains elusive.

Herein we quantitatively studied the protein expression of a Salmonella mutant lacking ydcR (ΔydcR) within host cells in comparison to that of its parental strain, which provides a powerful means to uncover potential YdcR-regulated proteins under physiological conditions. Notably, we identified a Salmonella virulence factor SrfN whose expression within infected host cells is strictly dependent on the presence of YdcR. Furthermore, we found evidence that YdcR directly controls its expression on the transcriptional level by binding to the promoter region of the srfN gene. Importantly, the loss of YdcR resulted in attenuated bacterial virulence in a murine infection model. Our results underscore the power of combining quantitative proteomics and bacterial genetics to study the regulatory roles of diverse prokaryotic transcription factors.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Cell Lines, and Culture Conditions—**The S. typhimurium SL1344 was used in this study, and all Salmonella strains were routinely grown on LB plates with 1.5% agar and 30 μg/ml streptomycin at 37 °C. A single colony picked from the plates was inoculated into LB broth with 30 μg/ml streptomycin, and then the overnight culture was diluted 1:20 into 3 ml of LB broth supplemented with 0.3 mM NaCl (to induce the expression of Salmonella SPI-1 T3SS). The bacteria were harvested for infection assays when they grew to the mid-exponential phase (OD600 ~ 0.9). HeLa cells (a human epithelial cell line) were grown in Dulbecco’s Modified Eagle Medium (DMEM, HyClone, Logan, UT) supplemented with 10% fetal bovine serum (FBS, Gibco, Life Technologies, Carlsbad, CA) under an atmosphere of 5% CO2 at 37 °C.

**Molecular Cloning and Construction of Bacterial Mutants—**The S. typhimurium ydcR deletion mutant (ΔydcR) was constructed using the standard homologous recombination method as previously described (5). The lambda red recombination system (12, 13) was used to construct the ydcR deletion mutant with kanamycin resistance and bacterial strains chromosomally expressing 3×FLAG-tagged YdcR and SrfN. Briefly, the sequences encoding the 3×FLAG epitope were inserted in-frame at the C terminus of the genes of interest before the stop codon. Successful deletion or tagging of the target genes was confirmed by both sequencing and PCR analyses. For construct the complementation construct that harbors a plasmid-borne ydcR gene in the ΔydcR background (ΔydcRΔydcR), the ydcR fragment was amplified, digested, and inserted into a plasmid with an arabinose-inducible promoter and a C-terminal 3×FLAG tag. For β-galactosidase assays, the upstream region (819 bp) of the srfN gene was cloned and inserted into the upstream of lacZ in the single copy plasmid pN387 (14). All primers and strains that were used in this study are listed in the supplemental Table S1.

**Experimental Design and Statistical Rationale—**To uncover potential YdcR-regulated proteins, we performed proteomic analyses of the intracellular ΔydcR mutant at 18 hpi, whereas the wild-type strain was used as a control. Salmonella infection and subsequent isolation from host cells was performed as previously described, with minor modifications (6). Briefly, Salmonella invasion of HeLa cells was performed when cell monolayers reached 70–85% confluence, and the infection was carried out for 45 min in Hank’s balanced salt solution (HBSS) with a multiplicity of infection (MOI) of 30. Subsequently, cell monolayers were treated with 100 μg/ml gentamicin for 1 h to selectively eliminate extracellular S. typhimurium. Cells were then washed with HBSS and fresh DMEM containing 10 μg/ml gentamicin was added. At 18 hpi, cell monolayers were washed extensively with PBS, and lysed in a buffer containing 20 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 0.1% Triton X-100. To recover intracellular bacteria, collected cell lysates were centrifuged at 600 × g for 5 min, and then the supernatant was centrifuged at 4000 × g for 20 min. The bacterial pellets were immediately washed with RIPA buffer to remove residual host proteins. The final pellets were resuspended in the SDS-PAGE sample buffer and heated at 95 °C for 5 min. Then bacterial protein samples were prefractionated by 10% SDS-PAGE, processed into 8 gel bands, and subjected to in-gel trypsin digestion as previously described (15). The resulting peptide samples were reconstituted in HPLC-grade water for LC-MS/MS analyses on a hybrid ion trap-Orbitrap mass spectrometer (LTQ Orbitrap Velos, Thermo Scientific) coupled with nanoflow reversed-phase liquid chromatography (EASY-nLC 1000, 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). Eluted peptides from the capillary column were electrosprayed directly onto the mass spectrometer for MS and MS/MS analyses in a data-dependent acquisition mode. One full MS scan (m/z 350–1500) was acquired and then MS/MS analyses were performed on the 10 most intense ions. Dynamic exclusion was set with repeat duration of 24 s and exclusion duration of 12 s. In total, three independent biological replicates of intracellular Salmonella samples were analyzed in 48 LC-MS/MS experiments.

MaxQuant (http://maxquant.org/), Version 1.5.3.30 was used to generate peak lists from MS raw files, and Andromeda was used to search S. typhimurium LT2 protein database (strain LT2/SGSC1412/ATCC 700720, 11/7/2011, downloaded from UniProt with 5199 sequences) augmented with the reversed sequence of each entry in the database. The precursor mass tolerance was set to 20 ppm, and the fragment mass tolerance for CID MS/MS was set at 0.8 Da. Trypsin was selected as the digestive enzyme with a maximum of two missed cleavages. Oxidation (M) was set as a variable modification. There were no fixed modifications. The minimum ratio counts were set at 2. The false discovery rate (FDR) of peptides and proteins was controlled at <1%. The MaxQuant software was used to calculate the label-free quantitation (LFQ) intensity for each protein. The obtained LFQ intensity values were further processed by using the Perseus software (version 1.5.4.1). Logarithmic values (Log2) of the LFQ intensity for each protein. The obtained LFQ intensity values were further processed by using the Perseus software (version 1.5.4.1). Logarithmic values (Log2) of the LFQ intensity for each protein were used and the missing values were replaced with random numbers from a normal distribution (width = 0.3, shift = 1.8). The p value of each protein was obtained by using the two-tailed Student's t test. Altered proteins with average fold changes > 2 or < 0.5 and p values < 0.05 were considered significant.

**Western Blotting Analyses—**Salmonella strains expressing 3×FLAG-tagged proteins were used to infect HeLa cells in 6-well plates with an MOI of 30. At various time points (indicated) after infection, mammalian cells were lysed and crude fractions of intracellular bacteria were obtained by differential centrifugation. Isolated bacterial pellets were resuspended in the SDS-PAGE sample buffer and run by 10% SDS-
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The assays were started by the addition of 240 μl of 0, 100, 200, 400 in Fig. 6. The reaction mixtures were incubated at room temperature for 30 min and then centrifuged at 14,000 g for 2 min. Bacterial pellets were then resuspended in 1.2 ml of Z buffer (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, and 1 mM MgSO4) plus 50 mM β-mercaptoethanol (freshly added). Then 30 μl of 0.1% SDS were added and mixed upon centrifugation at 1500 g for 3 min. Then the beads were washed three times with a buffer containing 20 mM Tris-HCl (pH 7.5/150 mM NaCl and proteins were incubated with 40 nM DNA fragments in 20 μl of binding buffer (10 mM Tris-HCl (pH 7.5), 100 mM KCl, 1 mM EDTA, 0.1 mM DTT, 5% v/v glycerol, and 10 μg/ml BSA). Molar ratios between DNA and YdcR were set at 1:0, 1:2.5, 1:5, and 1:10 (that is, performed using 0.5 μl of each fragment). The housekeeping 16S rRNA gene was used for normalization and mRNA levels were analyzed using the comparative threshold cycle number (ΔΔCt) method (16).

Expression and Purification of Recombinant Proteins—FLAG-tagged YdcR was expressed in the Salmonella ΔydcR ΔydcR strain with the addition of 0.1% arabinose to induce protein expression. The bacterial cells (from 400 ml culture) were lysed in 30 ml of ice cold PBS buffer via sonication and centrifugation at 5000 × g for 10 min three times. The resulting supernatants were used for YdcR purification by immunoprecipitating with anti-FLAG M2 agarose beads (50% slurry, Sigma). Briefly, the agarose beads were incubated overnight with the clarified lysates at 4°C under rotation, and collected by centrifugation at 1500 × g for 3 min. Then the beads were washed three times with a buffer containing 20 mM Tris-HCl (pH 7.5/150 mM NaCl and proteins were eluted with 150 ng/μl 3×FLAG peptides/20 μl Tris-HCl (pH 7.5)/150 mM NaCl. Finally, the 3×FLAG peptides were removed from the purified proteins by ultrafiltration prior to use.

Electrophoretic Mobility Shift Assays (EMSA)—Putative DNA promoter regions were amplified by PCR, purified with a Gel Extraction Kit (TransGen Biotech) and dissolved in water. Then purified YdcR proteins were incubated with 40 μM DNA fragments in 20 μl of binding buffer (10 μM Tris-HCl (pH 7.5), 100 mM KCl, 1 mM EDTA, 0.1 mM DTT, 5% v/v glycerol, and 10 μg/ml BSA). Molar ratios between DNA fragment and YdcR were set at 1:0, 1:2.5, 1:5, and 1:10 (that is indicated as 0, 100, 200, 400 in Fig. 6B respectively). The reaction mixtures were incubated at room temperature for 30 min and then loaded onto 8% native polyacrylamide gels. Electrophoresis was performed using 0.5×TBE buffer (44.5 mM Tris, 44.5 mM boric acid, and 1 mM EDTA) in ice bath. The gel was washed with Gelstain (TransGen Biotech) and photographed by using a Tanon-1600 Gel Image System (Tanon, Shanghai, China).

β-Galactosidase Assays—Salmonella cells were grown in LB with or without the addition of 0.2% arabinose at 37 °C to an OD600 of ~1.0. Bacterial cells from 1.2 ml of culture were pelleted at 14,000 × g for 2 min. Bacterial pellets were then resuspended in 1.2 ml of Z buffer (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, and 1 mM MgSO4) plus 50 mM β-mercaptoethanol (freshly added). Then 30 μl of chloroform and 15 μl of 0.1% SDS were added and mixed upon vortexing. The assays were started by the addition of 240 μl of 4 mg/ml o-nitrophenyl-β-D-galactopyranoside (ONPG). Upon the observation of a faint yellow color, the reaction was stopped by the addition of 600 μl of 1 M Na2CO3 and the reaction time was noted. Finally, samples were centrifuged at 14,000 × g for 2 min, and the OD420 of the supernatant was recorded. Assay units were calculated as 1000 × OD420/OD600 (total reaction time).

Growth Curve Experiments and HeLa Cell Infection Assays—To assess bacterial growth in LB broth, an overnight culture of either the wild-type or the ΔydcR mutant strain was diluted 1:300 into 3 ml of LB broth. The optical density of bacterial culture was monitored for 13 h. For Salmonella infection assays, HeLa cells in 6-well plates were infected with different strains at an MOI of 10. After 45 min, infected cells were washed with HBSS and incubated in DMEM supplemented with 100 μg/ml gentamicin for 1 h to kill extracellular bacteria. Cells were then lysed and viable intracellular bacteria were enumerated by colony-forming units (CFU) assays to determine the rate of bacterial invasion. Otherwise, cells were washed with HBSS and fresh DMEM containing 10 μg/ml gentamicin was added. At 18 hpi, cells were then lysed and viable intracellular bacteria were enumerated by CFU assays to determine the rate of intracellular bacterial replication.

Mouse Infection Experiments—Animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the Chinese Association for Laboratory Animal Sciences (CALAS) and approved by the ethics committee of Renji Hospital, School of Medicine, Shanghai Jiao Tong University (Shanghai, China). Wild-type S. typhimurium and the ydcR deletion mutant with kanamycin resistance were cultured in LB to the exponential growth phase (OD600 = 0.9) before competitive infection. Twenty female BALB/c mice of 6 weeks old were intracoarally injected with a mixed inoculum (~1 × 108 CFU/mouse) containing an equivalent number of each test strain in 0.1 ml HEPES buffer (pH 8.0) with 0.9% NaCl. Mice were sacrificed at 72 hpi. The spleen, liver, and cecum were harvested and homogenated for the determination of bacterial burdens by plating on LB plates with and without kanamycin (30 μg/ml) simultaneously. The competitive index (CI) was calculated from CFU assays as follows: (mutant/wild-type)output/(mutant/wild-type)input. The competitive infection data were analyzed using the two-tailed Student’s t test.

RESULTS

Profound Induction of YdcR in Intracellular Salmonella During Infection of Host Epithelial Cells—Previously we studied intracellular Salmonella proteome within infected HeLa cells and found extensive reprogramming of bacterial metabolic pathways (5, 6). In addition, our large-scale proteomic datasets also reveal many other proteins with altered expression levels during infection, though most of them cannot be categorized into distinct biological processes. Furthermore, for some differentially regulated proteins very little information is available regarding their molecular functions or potential roles in bacterial pathogenesis. We reasoned that some of these proteins could be of vital interest in understanding the mechanisms underlying Salmonella infection. One of those altered proteins is YdcR, which is annotated as a putative GntR family regulatory protein, and it was profoundly induced at both 6 hpi and 18 hpi (Fig. 1A). In contrast, this protein was barely expressed for in vitro cultured Salmonella. Interestingly, such a pattern of protein expression is reminiscent of that of some SPI-2 encoded virulence factors that are essential for survival and replication of intracellular Salmonella. Indeed, the abundance of two SPI-2 effectors, PipB and PipB2, were both induced in a similar manner as YdcR (Fig. 1A). To further confirm the proteomic changes, we next constructed a Sa-
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YdcR, a putative transcriptional regulator and highly conserved among a number of bacterial species—Salmonella YdcR was initially annotated as a putative GntR family regulatory protein. Further sequence alignment with known regulators of this family reveals that it exhibits similarity to regulators PdxR, TauR, GabR, and DdlR, which belong to the MocR/GabR subfamily (Fig. 2A). In addition to an N-terminal helix-turn-helix (HTH) DNA-binding domain, the members of this subfamily (including YdcR) consist of a very large C-terminal domain that shows sequence similarity to class I aminotransferases. These enzymes often require pyridoxal 5’-phosphate (PLP) as a cofactor for exerting their functions. We found that YdcR also carries the conserved amino acid residues responsible for the interaction with PLP (17) (arrowed in Fig. 2A). In addition, a BLAST search against reference sequences in the NCBI database reveals homologues of YdcR in *Salmonella bongori*. Moreover, YdcR is found to be highly conserved among other bacterial genera including *Citrobacter* spp., *Escherichia* spp., *Klebsiella* spp., and *Shigella* spp. with >85% sequence similarity (some representative YdcR homologues are shown in Fig. 2B), although it has not been characterized in those bacteria. Therefore, it appears that YdcR homologs are at least not confined to nonpathogenic bacteria.

**Comparative Proteomics Reveal Seven Candidates of YdcR-Regulated Proteins**—To shed light on the functional roles of YdcR, if there is any, in *Salmonella* pathogenesis, next we sought to determine the downstream proteins under its regulation in the exact context where its expression was highly induced. To uncover YdcR-regulated proteins, we exploited quantitative proteomics to compare intracellular *Salmonella* protein expression between the wild-type and the isogenic ∆ydcR strains. As *Salmonella* YdcR was most produced at 18 hpi, we isolated bacteria from infected host cells at this point. In total, we identified 1563 bacterial proteins from three biological replicates with at least two unique peptides. Fig. 3A shows the protein-level volcano plot of detected proteins from intracellular *Salmonella* strains. By using peptide intensity-based LFQ, our proteomic datasets reveal fairly high similarity of *Salmonella* proteomes between the wild-type and ∆ydcR strains. In fact, with our significance criteria (fold ratios > 2 and p value < 0.05), only eight bacterial proteins differed in the *Salmonella* strain lacking the ydcR gene (Table I). Among these, four proteins were upregulated (YfgC, PipB, STM2234, and FrdB) and the other four were downregulated (YdcR, SrfN, CysN, and NupC). A complete list of all identified proteins is provided as supplemental Table S2. Notably, the positive control for our quantitative proteomic experiments, YdcR, was the most depressed protein in the entire dataset (Fig. 3A, in the left upper corner). Interestingly, in the volcano plot the only protein near YdcR is SrfN (STM0082), indicating its marked repression in the ∆ydcR strain. Furthermore, like YdcR, SrfN was not detected in the ydcR-deficient strain, though it was abundantly expressed in wild-type *Salmonella* within infected HeLa cells (Table I). Therefore, our proteomic analyses identified SrfN together with other six proteins as candidate proteins under YdcR regulation either in a direct or indirect manner.

**YdcR Transcriptionally Regulates the Expression of Salmonella SrfN**—Next, we sought to investigate if YdcR indeed regulates these altered proteins on the transcriptional level. We measured the mRNA levels of these seven genes (frdB, STM2234, pipB, yfgC, nupC, cysN, and srfN) by qRT-PCR.
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A. HTH DNA binding domain

B. S. enterica

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Fig. 3. YdcR positively regulates the expression of SrfN. A, A volcano plot of intracellular Salmonella proteins detected by LC-MS/MS experiments. The fold changes were calculated by dividing LFQ intensity values from the ΔydcR mutant by those from the wild-type strain at 18 hpi. The logarithmic values of the average fold changes are reported on the x axis. The y axis plots negative logarithmic p values determined from the t test on three biological replicates. Dotted lines denote 2-fold (vertical) and p < 0.05 cutoff (horizontal). B, qRT-PCR analyses of mRNA samples extracted from intracellular Salmonella at 18 hpi (n = 3). Asterisks indicate significant differences (***, p < 0.001).

analyses of extracted RNA samples from intracellular bacteria (Fig. 3B). At 18 hpi, only the transcript level of srfN was markedly altered (6.3-fold lower in the ΔydcR mutant) with p < 0.05. The mRNA level of pipB increased by 2.4-fold, yet this change was not statistically significant (p = 0.08). These results demonstrate that only the transcription of srfN is highly dependent on the presence of a functional ydcR gene.

Furthermore, β-galactosidase assays were used to establish the transcriptional control of the srfN gene by YdcR. To do this, we introduced a plasmid containing a lacZ transcriptional fusion to the downstream region of the srfN promoter and assayed the activities of β-galactosidase in vitro. Like the ydcR deletion mutant, the wild-type Salmonella showed similar β-galactosidase activities (Fig. 4A), suggesting that the wild-type strain failed to increase the level of lacZ gene transcription under this condition. In fact, such observation is consistent with our findings indicating that YdcR is barely expressed under in vitro culturing conditions (Fig. 1). To circumvent this issue and facilitate the examination of YdcR-regulated srfN transcription in vitro, we further introduced a YdcR-expressing plasmid into the ΔydcR strain, whose expression is under the control of arabinose. Using this strain, we detected a significant increase of β-galactosidase activities, indicating YdcR-dependent transcriptional activation of the srfN-lacZ fusion. In line with our hypothesis that YdcR regulates the expression of SrfN at the transcriptional level, we detected the markedly increased β-galactosidase activity only when the inducible ydcR-carrying plasmid and the inducer arabinose were present (Fig. 4B). Taken together, these results indicate that YdcR controls the expression of the srfN gene at the transcriptional level.

YdcR Positively Controls the Expression of Salmonella Virulence Factor SrfN—Salmonella SrfN was first identified by Osborne et al. as an SsrB-regulated virulence factor that contributes to bacterial fitness in a murine model of infection (18). In our previous studies of intracellular Salmonella proteome, we also demonstrated a steady increase of the SrfN expression during infection (data sampled at 1, 6, and 18 hpi) (5, 6). The SrfN induction profile is like that of YdcR, also supporting our hypothesis. To further verify the proteomic observations, next we constructed Salmonella strains chromosomally expressing 3×FLAG-tagged SrfN in the wild-type and ydcR deletion backgrounds. Then these bacterial strains were used to infect host epithelial cells and at 18 hpi intracellular Salmonella was harvested and probed for the expression of SrfN. Consistent with the previous LC-MS/MS measurements, immunoblotting analyses using specific antibodies show abundant SrfN expression in the wild-type bacteria whereas it was barely detected in the ΔydcR strain (Fig. 5A).

Fig. 2. Multiple sequence alignment of YdcR with other transcription factors or its homologs from different bacteria. A, Amino acid sequence alignment shows YdcR is a putative MocR/GabR-type bacterial transcription factor. PdxR_Cg: PdxR of C. glutamicum ATCC 13032 (accession number Q8NS92), TauR_Rc: TauR of R. capsulatus ATCC BAA-309 (accession number D5A4X9), GabR_Bs: GabR of B. subtilis (accession number P94428), Dd1R_Bb, Dd1R of B. brevis (accession number C02DG2), YdcR_St: YdcR of S. typhimurium SL1344 (accession number Q8ZPC8). Arrows indicate those residues that are functional sites involved in PLP binding. B, Amino acid sequence alignment shows YdcR is highly conserved among several bacterial species. YdcR: S. enterica (accession number Q8ZPC8); S. bonngori (accession number S5N885); C. freundii (accession number A0A0D7); E. coli (accession number P77730). The protein sequences were aligned by using the CLUSTALW program (http://www.genome.jp/tools/clustalw/) and the figure was prepared with the BOXSHADE program (http://www.ch.embnet.org/software/BOX_form.html).
Next, we complemented the deletion mutant with a plasmid-encoded copy of ydcR. When the expression of YdcR was induced by the addition of 0.02% arabinose into the cell culture medium, the expression of SrfN was readily detected by immunoblotting analysis. With the addition of more arabinose (0.2%), furthermore, we observed higher levels of both YdcR and SrfN (Fig. 5A). Similar results were obtained with the wild-type bacteria complemented with a YdcR-expressing plasmid. In contrast, the expression level of PipB did not differ significantly for *Salmonella* strains chromosomally expressing 3×/H11003 FLAG-tagged PipB in the wild-type and ydcR mutant backgrounds (Fig. 5B), indicating the specific regulation of SrfN by YdcR. Taken together, these data demonstrate that YdcR positively regulates the expression of *Salmonella* virulence factor SrfN.

**YdcR Directly Binds to the Promoter Region of the srfN Gene**—Like other transcriptional regulators of the GntR family, YdcR also has an N-terminal helix-turn-helix (HTH) DNA-binding domain. Thus, we next explored whether YdcR can directly interact with the promoter region of srfN by conduct-
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Fig. 6. Determination of YdcR-binding sites in the promoter region of srfN by EMSA. A, Various DNA fragments covering different regions upstream of srfN were tested in the EMSA experiments. The numbers represent the positions of the fragments with respect to the translation start site. B, EMSAs were performed by incubating serial concentrations of purified YdcR (ranging from 0 to 400 nm) with the DNA fragments B1, B2, B3, B4, and B5 prior to electrophoretic separation.

YdcR is a MocR/GabR-type transcriptional regulator with a predicted DNA-binding HTH motif. Our proteome data indicate highly induced levels of YdcR in intracellular Salmonella during infection of HeLa cells, though its functions and/or physiological roles in Salmonella infection biology have not been reported thus far. In fact, numerous MocR/GabR-type proteins were found in bacterial genomes. Nevertheless, only four regulator proteins of this family (GabR, PdxR, TauR, and DdIR) have been characterized to some extent (19, 20, 21, 22, 23). For instance, in the presence of PLP and γ-aminobutyrate (GABA), GabR activates the transcription of gabT and gabD, which encode GABA aminotransferase and succinate semialdehyde dehydrogenase, respectively (19, 20). PdxR, on the other hand, activates the transcription of pdxST genes encoding the PLP synthase. In vivo analyses indicate that PLP and PL can lower the transcription of pdxS and pdxT (21). It appears that the four characterized regulators are all involved in regulation of a narrow range of downstream genes (i.e. specific metabolic pathways). In addition, YdcR homologs are found to be present in many bacterial species with high sequence similarity (>85%) such as Citrobacter, Escherichia, Enterobacter, Klebsiella, and Shigella. Clearly, there is still a knowledge gap in understanding the regulatory mechanisms of many other MocR/GabR-type transcription factors and perhaps more importantly the downstream proteins directly under their control.

The goal of our current study is to uncover the functional roles of YdcR in the context of Salmonella infection of host
epithelial cells. We took advantage of quantitative proteomics and examined specifically the *Salmonella* proteome within infected cells in that YdcR was only abundantly expressed during bacterial intracellular replication. Comparative analyses of *Salmonella* protein expression in the wild-type and ydcR-lacking strains permitted us to identify proteins whose expression levels was YdcR-dependent. By using this quantitative approach, we found that expression of *Salmonella* virulence factor SrfN was strictly dependent on YdcR during infection, suggesting a potentially important role of this MocR/GabR-type transcriptional regulator in bacterial pathogenesis.

Next, we provided multiple lines of evidence to support that YdcR, a *Salmonella* transcription factor highly induced within infected host cells, positively regulates the expression of the bacterial virulence factor SrfN. First, we used qRT-PCR to measure the transcript levels of *srfN* and found the regulation of YdcR on the expression of SrfN indeed occurs on the transcriptional level. Further β-galactosidase assays of *Salmonella* strains harboring a lacZ fusion to the promoter region of the *srfN* gene also confirmed that YdcR can mediate transcriptional regulation of *srfN* in bacteria cultured in vitro. Additionally, immunoblotting assays indicated YdcR-mediated expression of SrfN in intracellular *Salmonella* during infection, consistent with our proteomic findings. Lastly, we carried out EMSA experiments by incubating purified YdcR with DNA fragments encompassing various regions upstream of *srfN* and found specific interaction of YdcR with a fragment spanning from position −554 bp to −304 bp, indicating its direct regulatory role in *Salmonella* virulence factor SrfN. Importantly, competitive infection reveals that the loss of YdcR led to reduced bacterial fitness in a murine infection model, thereby suggesting its physiological role in *Salmonella* pathogenesis.

*srfN* was initially discovered by Osborne *et al.* as a gene regulated by SsrB, a master regulator of *Salmonella* SPI-2 T3SS (18, 24). The mRNA level of *srfN* was reduced 8-fold in ΔssrB cells compared with the wild-type bacteria under SPI-2-inducing conditions and SsrB controls *srfN* directly through binding to the *srfN* cis-regulatory element. In line with this finding, we observed concurrent induction of SrfN and some SPI-2 encoded T3SS effectors at 18 hpi (i.e. PipB and PipB2) within infected epithelial cells. Osborne *et al.* also demonstrated that SrfN was a fitness factor and contributed to bacterial proliferation during systemic infection, though direct translocation of SrfN into host cells was not observed (18). Later Yoon *et al.* confirmed the requirement of *srfN* to attain full mouse virulence and demonstrated the translocation of SrfN into macrophage cells by using both cAMP and β-lac-
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Data Availability

The proteomics data reported in this paper have been deposited in the iProx database (URL: http://www.iprox.org/) and are available under the accession number IPX0000899001.

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