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CadC Has a Global Translational Effect during Acid Adaptation in *Salmonella enterica* Serovar Typhimurium▽

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In *Salmonella enterica* serovar Typhimurium, the membrane-localized CadC is a transcriptional activator of the *cadBA* operon, which contributes to the acid tolerance response. Unlike in *Escherichia coli*, in which transcription of *cadC* is constitutive, in *S. enterica* serovar Typhimurium *cadC* expression is induced by low pH and lysine. Inactivation of *cadC* suppresses the acid-sensitive phenotype of a *cadA* mutation, suggesting the existence of other CadC-dependent genes in addition to the *cadBA* operon. Using a proteomic approach, we identified 8 of the putative CadC-induced proteins and 15 of the putative CadC-repressed proteins. The former include porin proteins OmpC and OmpF. The latter include proteins involved in glycolysis, energy production, and stress tolerance. To better understand the altered levels of OmpC and OmpF, we compared expression of *ompR* in *S. enterica* serovar Typhimurium wild-type and *cadC* mutant strains and determined that CadC exerted a negative influence on *ompR* transcription. Taken together, our findings strongly suggest that CadC may be a global regulator involved in the OmpR regulatory system during acid adaptation.

During the course of infection, *Salmonella enterica* serovar Typhimurium encounters potentially lethal acidic conditions, such as in the stomach or within the macrophage phagolysosome (16). Bacterial survival in acidic host environments requires the adaptive acid tolerance response (ATR), in which exposure to mildly acidic conditions promotes survival in subsequent highly acidic environments (14). Previously, we demonstrated that the *cadBA* operon is involved in the ATR of *S. enterica* serovar Typhimurium (37).

The *cadBA* operon encodes lysine decarboxylase (CadA) and lysine-cadaverine antiporter (CadB); its expression is dependent upon the transcriptional activator CadC, the gene for which is located immediately upstream of the *cadBA* locus (21, 29, 30, 40, 46). Comparative sequence analysis suggests that CadC has three domains: an N-terminal cytoplasmic DNA-binding domain, a central transmembrane domain, and a C-terminal periplasmic domain (46). Although CadC in *S. enterica* serovar Typhimurium and *Escherichia coli* share only 58.4% identity, hydrophobicity plots and sequence alignment indicate similar structures (20). CadC acts as both a signal sensor and transcriptional regulator, responding to the low pH and lysine signal by activating transcription of the *cadBA* operon (21, 29, 30, 40, 46). Following induction, CadA catalyzes decarboxylation of lysine to cadaverine, which is excreted through the lysine-cadaverine antiporter CadB, resulting in neutralization of the external pH (29, 46). However, the role of *S. enterica* serovar Typhimurium CadC in the ATR has not yet been investigated.

Using in vivo expression technology, in which passage through an animal host enables selection of bacterial genes that are induced specifically during infection, *S. enterica* serovar Typhimurium *cadC* was shown to be induced in both the small intestines and spleens of BALB/c mice (19). In addition, microarray analysis demonstrated that expression of *S. enterica* serovar Typhimurium *cadB* was upregulated during macrophage infection (12). The in vivo induction of CadC suggests that it contributes to survival in specific host tissues, enhancing virulence. Although CadC probably plays an important role in the interaction between pathogen and host, little is known about its properties or mode of action.

We aimed to elucidate the function of *S. enterica* serovar Typhimurium CadC in the ATR and discovered that it regulates expression of ≥36 proteins and is related to the OmpR regulatory system. Our findings indicate that CadC may function in adaptation to the host environment by regulating other genes either directly or indirectly.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and growth conditions. The relevant characteristics of the bacterial strains and bacteriophages used in this study are presented in Table 1. Those generated in this study were constructed using suicide vector-mediated gene replacement (11) and phage P22-mediated transductions (8). LB complex medium and Vogel-Bonner E and non-citrate E (NCE) minimal media supplemented with 0.4% glucose were prepared as described previously (25, 45). Lysine decarboxylase broth (0.5% peptone, 0.3% yeast extract, 0.1% dextrose, 0.5% L-lysine, and 0.002% bromcresol purple) was used for the lysine decarboxylase (LDC) assay. The following antibiotics were used: ampicillin (60 μg ml^{-1}), kanamycin (50 μg ml^{-1}), and tetracycline (10 or 20 μg ml^{-1} for minimal or rich media, respectively). L-Lysine was added to a final concentration of 10 mM.

Construction of chromosomal *cadC* and *cadA* knockout strains. The *cadC* and *cadA* knockout mutants were constructed using suicide vector-mediated gene replacement (11). To construct pDMS197 derivatives that contained the sequences up-and downstream of the region to be deleted, PCR amplification was performed using four primers for each strain (Table 2). Recombinant suicide vectors were transferred from *E. coli* χ7213 to *S. enterica* serovar Typhimurium UK1 (wild type) via conjugation. Diaminopimelic acid (13 μg ml^{-1}) was added to media for *E. coli* strain χ7213. *Salmonella* transconjugants containing single-

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TABLE 1. Bacterial strains, bacteriophages, and plasmids used in this study

Strain, phage, or plasmid	Relevant characteristics	Source or reference
S. enterica serovar Typhimurium strains		
SF530 (χ3761)	Wild-type UK1	7
JF4484	UK1 *putPA1303*::Kan^r-*ompR-lacZ*(Op)	1
YK5001	UK1 Δ*cadA*	This study
YK5002	UK1 Δ*cadC*	This study
YK5003	UK1 Δ*cadC putPA1303*::Kan^r-*ompR-lacZ*(Op)	This study
E. coli strains		
DH5α	F$^-$ *endA1 recA1 hsdR17*(r_K^- m_K^+) *supE44 thi-1 gyrA96 relA1* ϕ80d*lacZ*M15 Δ*(lacZYA-argF)U169*	Gibco-BRL
χ7232	pDMS197, (−)DAP[a], Tet^r	11
χ7213	(−)DAP	11
Phages		
P22HT int 105	Used for generalized transduction	41
H5	P22 *c2* mutant	41
Plasmids		
pGEM-T Easy	Multicopy vector for cloning PCR products, Amp^r	Promega
pDMS197	Suicide vector; *oriT oriV sacB*, Tet^r	11
pDMS197-CadC	pDMS197 containing Δ*cadC* gene, Tet^r	This study
pDMS197-CadA	pDMS197 containing Δ*cadA* gene, Tet^r	This study
pMW118	Low-copy-no. cloning vector, Amp^r	Nippon Gene
pMW118-CadC	pMW118 with 1.8-kb DNA fragment containing the promoter region and entire open reading frame of the *cadC* gene, Amp^r	This study

[a] DAP, DL-α,ϵ−diaminopimelic acid.

crossover plasmid insertions were selected on LB agar containing tetracycline (20 μg ml^{-1}). Subsequently, transconjugants were selected on LB agar containing 5% sucrose, using *sacB*-based sucrose sensitivity to identify colonies in which the suicide vector had been lost through a second homologous recombination event (17). Size comparison of PCR products overlapping the deleted region between the wild type and knockout mutants was used to confirm the presence of deletions.

LDC assay. Strains were grown in Moeller lysine decarboxylase broth (Difco) containing the decarboxylase basal medium supplemented with 0.5% L-lysine and bromcresol purple indicator. One or two colonies were inoculated into fresh culture medium. Sterile mineral oil was then added and the culture incubated for 36 h at 37°C. If the dextrose is fermented, the medium turns yellow initially and then purple as the decarboxylase reaction elevates the pH.

RNA preparation and Northern blot analysis. Total RNAs were isolated as described previously (22), with some modifications. In brief, cultured cells were collected and resuspended in lysis solution (20 mM sodium acetate [pH 5.5], 1 mM EDTA, 0.5% sodium dodecyl sulfate [SDS]). The cell lysates were incubated with hot (65°C) 20 mM sodium acetate (pH 4.8)-saturated phenol for 5 min at 65°C. Phenol extraction was performed until no residue was apparent at the interface. RNA was precipitated with 0.1 M KCl and three volumes of ethanol at −20°C, and following centrifugation, the pellet was resuspended in RNase-free water. RNA was separated by denaturing formaldehyde-agarose gel electrophoresis and blotted onto a nylon membrane (Amersham). Northern blot analysis was performed using the DNA probes NcadA and NcadC, which were amplified using oligonucleotide primers NcadA1/NcadA2 and NcadC1/NcadC2, respectively (Table 2) and then labeled randomly using a digoxigenin DNA labeling kit (Roche). Following overnight hybridization at 57°C, blots were washed and signals were visualized using the digoxigenin detection kit (Roche).

Primer extension analysis. Extension analysis was performed using a 20-base oligonucleotide (TScadC [Table 2]) complementary to the *cadC* mRNA. The primer was end labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase (Roche). RNA (5 μg) and labeled primers were incubated at 72°C for 2 min and cooled slowly at room temperature for 1 h in order for the primer to hybridize to the RNA. The primer extension reaction was performed at 37°C for 90 min in a 20-μl reaction mixture comprising 50 mM Tris-Cl (pH 8.5), 8 mM $MgCl_2$, 30 mM KCl, 1 mM dithiothreitol (DTT), 0.4 pmol of ^{32}P-labeled primer, 0.5 mM deoxynucleoside triphosphates, 10 U of RNase inhibitor (Roche), and 10 U of avian myeloblastosis virus reverse transcriptase (Promega). Remaining RNA was eliminated by a 30-min incubation with 40 μg DNase-free RNase (Sigma). Phenol-chloroform extraction was performed on the extension products, followed by ethanol precipitation and resuspension in formamide loading buffer (20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol in formamide). Following denaturation at 90°C for 2 min, products were separated by denaturing polyacrylamide (8%) gel electrophoresis. Gels were dried and visualized using a phosphorimaging analyzer (model BAS-2500; Fuji Photo Film) with Image Reader BAS-2500 software.

ATR assays. ATR assays were performed as reported previously (24), with some modifications. Each test strain was grown overnight in NCE glucose broth and then diluted 1/100 into 3 ml of fresh medium. The diluted cultures were grown with aeration to an optical density at 600 nm of 0.40 (2×10^8 CFU ml^{-1}), after which cultures destined for adaptation were harvested by centrifugation, and the supernatants removed. Cell pellets were resuspended in fresh NCE glucose medium (pH 4.4) containing 10 mM lysine and incubated for 60 min at 37°C. Adapted cultures were harvested, and the cell pellet was resuspended in fresh NCE glucose medium preadjusted to pH 3.0 (acid shock) at 37°C and then incubated for the times indicated. Viability of cells was assayed upon resuspension ($t = 0$) and following acid challenge at 60 and 90 min, by preparing serial dilutions in NCE glucose broth (pH 7.0) and plating onto LB agar. Plates were incubated overnight at 37°C and viable cells enumerated. Percent survival was calculated by dividing the total number of viable cells at each time point by the initial number of viable cells at $t = 0$.

Two-dimensional gel electrophoresis (2-DE) protein analysis. Cells were harvested by centrifugation, washed with 20 mM Tris-Cl buffer (pH 8.0), and resuspended in lysis buffer {0.3 M sucrose, 10 mM EDTA, 20 mM Tris-Cl [pH 8.0], protease inhibitor cocktail [Roche], 0.5 mM DTT, 0.1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate [CHAPS]}, and lysed using 10 ultrasonication treatments of 30 s each. Subsequently, cell debris was removed by centrifugation at 15,000 × g for 20 min at 4°C, and the aqueous supernatant was precipitated with trichloroacetic acid in acetone.

Whole-cell proteins were resuspended in solubilization buffer (7 M urea, 2 M thiourea, 2% CHAPS, 13 mM DTT, protease inhibitor cocktail, 20 mM Tris-Cl [pH 8.0]), and the protein concentration was quantified using the Bio-Rad Bradford protein assay with bovine serum albumin as the standard. Isoelectric focusing (IEF) was performed with the IPGphor system, using commercial 24-cm immobilized pH gradient (IPG) strips with an immobilized pH 4 to 7 gradient (Amersham). Samples (50 μg) were diluted in fresh rehydration buffer (8 M

TABLE 2. Sequences of primers used in this study

Primer	Oligonucleotide sequence, 5′→3′[a]	Use
CadC-F	CCAAGCTTCGGTAGGCTTTATGCAGTT	Complementation
CadC-R	CGGGATCCTTAATCTTCTGCCAGAAAACT	
NcadA1	AAGAGCCTATTCGTGAACTG	Northern blotting
NcadA2	TCATCATCAGATGGGTCAG	
NcadC1	AATAGCCTGTCTTTCGTTTT	Northern blotting
NcadC2	GTCATGGTTCAGCGTTAAAT	
TScadC	CAGCCAGTCTCCAATGCGTA	Primer extension
Hmp1	GAGCTCGCTTTGAATTTGAACCAGTC	Δ*cadC* mutant
Hmp2	GGATCCAAAGAAAGGCATAAAAAGCA	
CadC-Mu1	GGATCCCATTCAGTTTGTCAATCAGC	
CadC-Mu2	TCTAGAGTCATGGTTCAGCGTTAAAT	
CadA-Mu1	GAGCTCTTCCGTTATGGCCTCTTC	Δ*cadA* mutant
CadA-Mu2	GGATCCAGTCTTGTTCCTGGCAAA	
CadA-Mu3	CGGGATCCCGTTCCTGTTCAGCATTGGTAT	
CadA-Mu4	GCTCTAGAGCGTATTCCCAAATTTGAATCG	
OmpC-F	AACTACGGCGTAACCTATGA	RT-PCR
OmpC-R	GAGCAACCACTTCAAAGTTC	
OmpF-F	AGTGGGTTCAATCGATTATG	
OmpF-R	GAATATATTTCGCCAGATCG	
OmpR-F	GTACTGGATTTAATGCTGCC	
OmpR-R	AGACGGTCTGAATATAACGC	
CadCRT-F	GCTGATCGATCTTCTGATGT	
CadCRT-R	GAGTAGGTGTTCAGCAGGTC	
16S RNA-F	AGAGTTTGATCMTGGCTCAG	
16S RNA-R	TACGGYTACCTTGTTACGACTT	

[a] Restriction enzyme recognition sequences are underlined.

urea, 2% CHAPS, 13 mM DTT, 0.5% IPG buffer, and a few grains of bromophenol blue). IEF was performed at 20°C with 50 μA applied per IPG strip. The voltage settings were 200 V for 30 min, a gradient to 500 V for 1 min, and 8,000 V for 1 h, resulting in approximately 80,000 V-h. Following IEF, proteins were separated in 8 to 16% gradient gels, using the Ettan DALT II system. A prestained protein ladder (14.4 to 97 kDa; Amersham) was added to the basic end of the gel. Electrophoresis was performed in running buffer (25 mM Tris base, 192 mM glycine, 0.1% SDS) at 10°C, using a series of current increases (18, 24, and 35 mA for 1, 2, and 5 h, respectively), and was continued until the blue dye reached the end of the gel (at ca. 7 h). Gels were silver stained as described by O'Connell and Stults (36) and scanned using an ImageScanner with ImageMaster Labscan version 3.00 software (Amersham Pharmacia Biotech).

Mass spectrometry and protein identification. Protein spots of interest were excised from the silver-stained 2-D gels. Peptide mass fingerprinting was performed using the Voyager DE-STR matrix-assisted laser desorption ionization mass spectrometer (Applied Biosystems) at the Korea Basic Science Institute. Proteins were identified by searching in ProFound (Genomic Solutions) and MASCOT (Matrix Science) against the NCBInr database.

P22-mediated transductions. *Salmonella* strain YK5003 (Δ*cadC putPA1303*::Kan^r-*ompR-lacZ*) was constructed using phage P22-mediated transduction, as described previously (8). Bacteriophage P22HT int 105 was propagated in a donor strain (JF4484) which was then used to infect the recipient strain (Δ*cadC*). Generalized transduction was used to move *putPA1303*::Kan^r-*ompR-lacZ* between the strains. Transductants were selected on LB agar containing 50 μg ml^{-1} kanamycin. P22 H5 was used to confirm that transductants were phage free and not P22 lysogens (26).

β-Galactosidase assays. β-Galactosidase activity was determined as described by Miller (31). In brief, cells (1 ml) were added to 1 ml Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 2.7 μl ml^{-1} β-mercaptoethanol [pH 7.0]), disrupted with 0.1% (wt/vol) SDS and chloroform, and incubated with 0.4 ml of 4 mg ml^{-1} *o*-nitrophenyl-β-D-galactoside. The reaction mixture was incubated for 2 or 6 min at room temperature, and then the reaction was stopped with 1 M Na_2CO_3. β-Galactosidase activity was expressed in Miller units and calculated using the formula $[1{,}000 \times (A_{420} - 1.75A_{550})]/[\text{time (min)} \times \text{culture volume (ml)} \times A_{600}]$.

RT-PCR assay. For reverse transcription-PCR (RT-PCR), RNA quantity and purity were determined by measuring sample absorbance (A_{260}/A_{280}) in triplicate. Total RNA (1 μg) was used as a starting template; the primers are shown in Table 2. The reaction mixture was denatured (94°C, 4 min), followed by 18 thermal cycles (94°C, 30 s; 54°C, 30 s; and 72°C, 50 s) and a final extension (72°C, 10 min). 16S rRNA was used as a normalization control. Amplified products were separated on a 1.5% agarose gel, stained with ethidium bromide, and visualized. Each reaction was performed at least three times.

RESULTS

CadC activates *cadA* transcription in *S. enterica* serovar Typhimurium. Membrane-bound CadC has been shown to act as a transcriptional activator of the *cadBA* operon in *E. coli*, *Vibrio cholerae*, and *Vibrio vulnificus* (21, 29, 30, 40, 46). However, it is not known whether CadC functions similarly in *S. enterica* serovar Typhimurium. We constructed isogenic *cadC* and *cadA* knockout strains of *S. enterica* serovar Typhimurium. The deleted region in Δ*cadC* encompasses the promoter and nucleotides 1 to 779 of the open reading frame. When *cadC* is expressed from its own promoter in the low-copy plasmid pMW118, it complements the LDC-negative phenotype of the *cadC* knockout strain (Fig. 1A). Low external pH and exogenous lysine are sufficient for induction of *cadA* in the *S. enterica* serovar Typhimurium UK1 wild-type strain (37), and following acid shock (pH 5.8, 10 mM lysine), the time of induction was determined easily. Wild-type serovar Typhimurium strain UK1

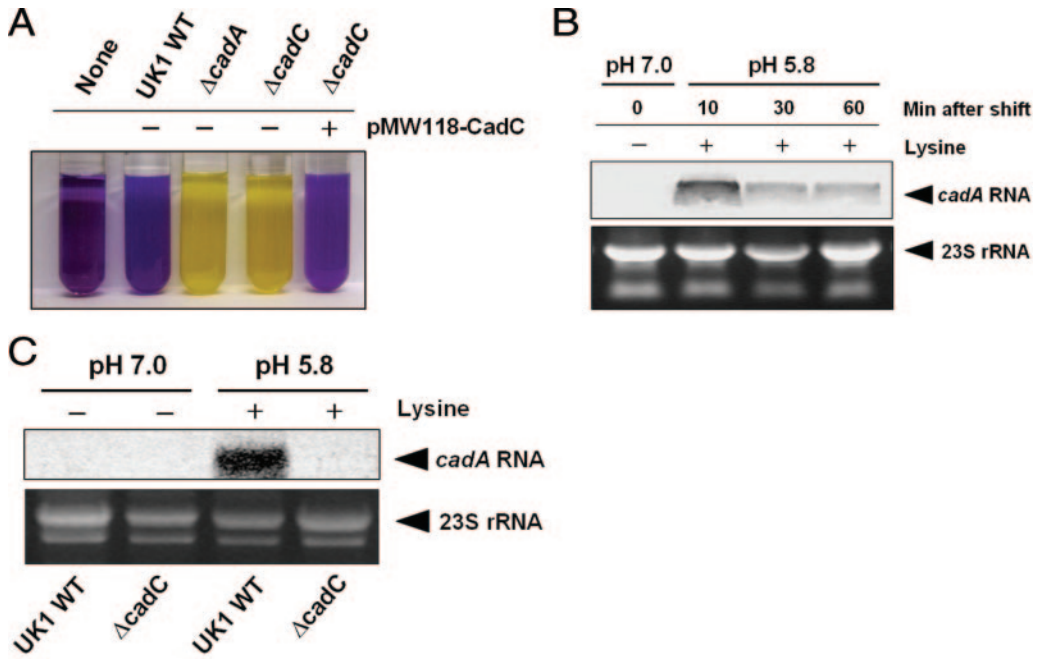

FIG. 1. CadC-dependent transcription of the *cadBA* operon in *S. enterica* serovar Typhimurium. (A) LDC assay indicates that plasmid-borne *cadC* (pMW118-CadC) complements the LDC-negative phenotype of the *cadC* knockout strain. Yellow and purple indicate the absence and presence of lysine decarboxylase, respectively. WT, wild type. (B) Northern blot analysis of *cadA* transcriptional induction following acid shock (pH 5.8, 10 mM lysine) in the UK1 wild-type strain. (C) Northern blot analysis of *cadA* transcription in the UK1 wild-type and Δ*cadC* strains. Cells were grown in E glucose medium at 37°C to mid-log phase (A_{600} = 0.6) and then subjected to acid shock (pH 5.8, 10 mM lysine) for 10 min. 23S rRNA was used as a loading control.

was grown in E glucose medium at 37°C to mid-log phase (A_{600} = 0.6) and then subjected to acid shock (pH 5.8, 10 mM lysine), and total RNA was prepared at various time points. Northern blot analysis indicated that *cadA* mRNA was induced within 10 min of acid shock (Fig. 1B). To determine whether CadC activates transcription of the *cadBA* operon in *S. enterica* serovar Typhimurium, we performed Northern blot analysis on the wild-type UK1 and Δ*cadC* strains following 10 min of acid shock. As expected, there was no induction of *cadA* transcription in the UK1 Δ*cadC* strain (Fig. 1C), indicating that CadC is necessary for expression of the *cadBA* operon.

Transcription of *S. enterica* serovar Typhimurium *cadC* is induced by low pH and lysine. The levels of *E. coli* CadC remain similar in both neutral and acidic media (10, 46), and *cadC* mRNA levels are not greatly affected by pH or lysine (34). However, the *S. enterica* serovar Typhimurium and *E. coli* CadC proteins only share 58.4% identity, well below the median amino acid identity of homologous proteins between these species (90%) (28), including those of CadB (90.2%) and CadA (92.4%). Moreover, there is no significant sequence similarity between the promoters of the *E. coli* and *S. enterica* serovar Typhimurium *cadC* genes.

We examined *cadC* mRNA levels by using Northern blot and primer extension analyses of total RNA isolated from UK1 wild-type cells taken at different intervals following acid shock (pH 5.8, 10 mM lysine). The results indicate that expression of *cadC* mRNA was induced markedly (Fig. 2). This observation was confirmed by primer extension analysis, which indicated that following acid shock the levels of *cadC* mRNA increase rapidly (Fig. 2). Together, these data suggest that the mechanism for activation of the *cadBA* promoter by *Salmonella* CadC may differ from that in *E. coli*.

Mutation in *cadC* suppresses the acid-sensitive phenotype of a *cadA* mutation. Previously, we reported that the LDC system is one of several inducible pH homeostasis mechanisms that contribute to the ATR of *S. enterica* serovar Typhimurium (37). Since CadC is a transcriptional activator of the *cadBA*

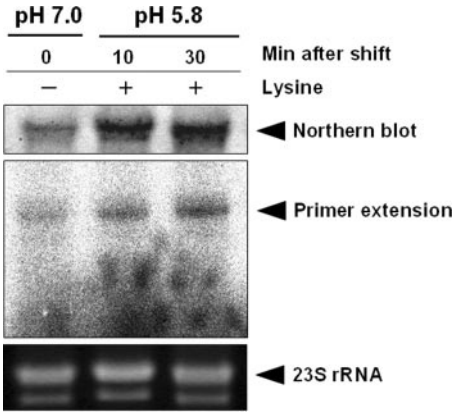

FIG. 2. Induction of *cadC* expression in response to low pH and lysine. Northern blot and primer extension analyses were performed to analyze the expression of *cadC* mRNA under nonstress or acid shock (pH 5.8, 10 mM lysine) conditions. UK1 wild-type cells were grown in E glucose medium at 37°C to mid-log phase (A_{600} = 0.6) and then subjected to acid shock (pH 5.8, 10 mM lysine), and RNA was isolated at the times indicated. 23S rRNA was used as a loading control.

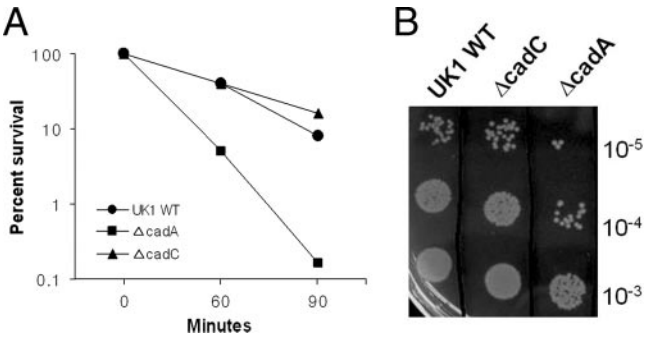

FIG. 3. ATR assays of *S. enterica* serovar Typhimurium UK1 wild-type (WT), Δ*cadA*, and Δ*cadC* strains in NCE glucose medium. Strains were adapted to acid for 1 h in NCE glucose medium (pH 4.4) containing 10 mM lysine prior to acid challenge (pH 3.0) for the times indicated. Cell viability was measured from 10-μl volumes of 10-fold serial dilutions of culture spotted onto LB agar. (A) Percent survival was calculated by dividing the total number of viable cells at each time point by the initial number of viable cells at $t = 0$. (B) Representative cell viability plate, following 60 min of acid shock treatment. The data represent averages from three independent trials.

operon, we hypothesized that the effect of a Δ*cadC* mutation on ATR would be similar to that of a Δ*cadA* mutation. We also reasoned that if CadC regulated additional pathways required for ATR, then the defect exhibited by the UK1 Δ*cadC* strain would be more severe than that of the UK1 Δ*cadA* strain. In order to test this hypothesis, we conducted comparative ATR assays using UK1 wild-type, Δ*cadC*, and Δ*cadA* strains. No differences were observed between the three strains when grown in minimal E glucose medium (data not shown), since the citrate present in the medium contributes to acid shock (pH 4.4) adaptation via internal buffering (37). Thus, ATR assays were performed in NCE glucose medium containing 10 mM lysine. Cells were grown to early log phase ($A_{600} = 0.4$) and then adapted to acid in NCE glucose medium (pH 4.4) for 1 h prior to shifting to pH 3.0. As expected from a previous observation (37), cells with the *cadA* mutation were unable to effect a protective ATR. After 90 min at pH 3.0, survival of the acid-adapted Δ*cadA* strain was approximately 20-fold lower than that of the UK1 wild-type strain. However, survival of the acid-adapted Δ*cadC* strain was virtually identical to or slightly higher than that of the UK1 wild-type strain, indicating that some other acid adaptation was occurring in this mutant (Fig. 3A and B). This observation raised the possibility that CadC may function as a negative regulator of other genes required for the ATR in *S. enterica* serovar Typhimurium. Moreover, serovar Typhimurium CadC shows a high degree of homology to the N-terminal DNA-binding domain of *V. cholerae* ToxR (20), which regulates as many as 17 genes either directly or indirectly (43). These results suggest that during acid adaptation (pH 4.4 for 1 h), CadC may regulate other genes in addition to the *cadBA* operon.

Global differences in protein expression between the wild-type and Δ*cadC* strains of *S. enterica* serovar Typhimurium. To examine the derepression of the ATR exhibited by the Δ*cadC* strain (Fig. 3A and B), comparative 2-DE was conducted on total protein collected from the *S. enterica* serovar Typhimurium UK1 wild-type and Δ*cadC* strains adapted in NCE glucose medium (pH 4.4, 10 mM lysine) for 1 h. We used narrow-pH-range (pH 4 to 7) strips for IEF in order to improve resolution of proteins, since most were found in the 3.5

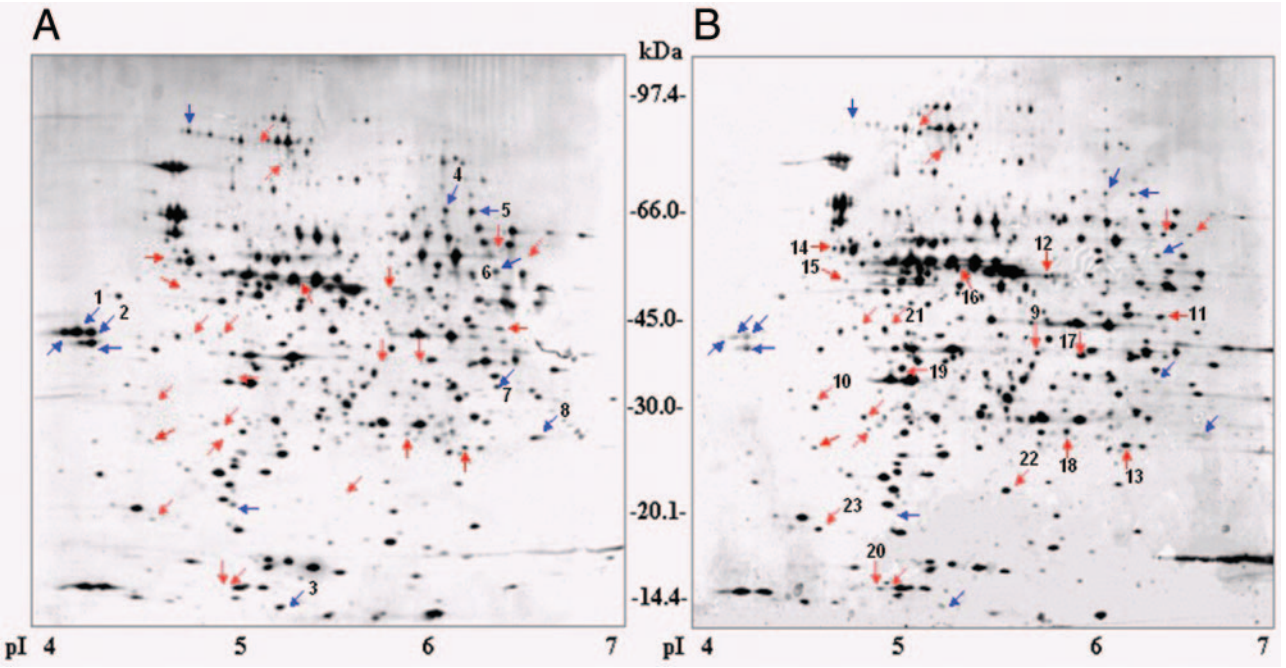

FIG. 4. Comparative 2-D analysis of whole-cell lysates from *S. enterica* serovar Typhimurium UK1 wild-type (A) and UK1 Δ*cadC* (B) strains. Strains were adapted to acid for 1 h in NCE glucose medium (pH 4.4) containing 10 mM lysine. Protein (50 μg) was separated by IEF (pI 4 to 7) and gradient SDS-polyacrylamide gel electrophoresis (8 to 16%). Blue and red arrows indicate putative CadC-induced and -repressed proteins, respectively.

TABLE 3. Identification of putative CadC-regulated proteins by matrix-assisted laser desorption ionization–time-of-flight mass spectrometry

Functional category	Protein[a]	Gene name	Spot no.[b]	NCBI accession no.
Putative CadC-induced proteins				
Cell envelope	Outer membrane porin C	*ompC*	1	2773087
	Outer membrane protein OmpF	*ompF*	2	468741
Pyruvate dissimilation	Putative formate acetyltransferase	*yfiD*	3	16765966
Amino acid biosynthesis	Dihydroxy-acid dehydratase	*ilvD*	4	16767180
	Dihydroxy-acid dehydratase (isoform)	*ilvD*	5	16767180
	D-3-Phosphoglycerate dehydrogenase	*serA*	6	16766363
Regulatory functions	Putative transcriptional regulator	STM4270	7	16767520
Translation	30S ribosomal protein S2	*rpsB*	8	16763606
Putative CadC-repressed proteins				
Glycolysis	6-Phosphofructokinase I	*pfkA*	9	16422627
	6-Phosphofructokinase II	*pfkB*	10	16764677
	Fructose-bisphosphate aldolase	*fbaB*	11	16765470
	Putative NAD-dependent aldehyde hydrogenase	STM4519	12	16767763
Tricarboxylic acid cycle	Succinyl-coenzyme A synthetase alpha subunit	*sucD*	13	16764109
Energy production	ATP synthase subunit beta	*atpD*	14	6625703
Chaperones and heat shock proteins	Trigger factor	*tig*	15	16763828
	Heat shock protein HslU	*hslU*	16	16422658
Amino acid and cofactor biosynthesis	*O*-Acetylserine sulfhydrolase A	*cysK*	17	16765750
	HMP-P kinase[c]	*thiD*	18	1842118
Regulatory functions	Transcriptional activator	*nhaR*	19	16763430
	Transcriptional repressor protein MetJ	*metJ*	20	16767365
Translation	Elongation factor Tu	*tuf*	21	96718
Unknown, hypothetical	Putative periplasmic protein	STM3030	22	16766332
	Hypothetical protein		23	37962772

[a] Proteins were identified using ProFound and MASCOT against the NCBI database.
[b] Protein spot numbers as shown in Fig. 4.
[c] Hydroxy-phosphomethylpyrimidine kinase.

to 7.5 pI range. Comparison of the 2-D proteome maps indicated marked differences in the protein patterns. Thus, it would appear that under the conditions used (pH 4.4 for 1 h), CadC regulates a large number of proteins that may be important for acid adaptation.

In the Δ*cadC* mutant, approximately 12 and 24 proteins were down- and upregulated, respectively (Fig. 4). Most of these proteins were isolated from the 2-D gels and analyzed by peptide mass fingerprinting matrix-assisted laser desorption ionization–time-of-flight mass spectrometry. Eight of the putative CadC-induced proteins and 15 of the putative CadC-repressed proteins were identified using ProFound and MASCOT against the NCBInr database. According to their respective cellular functions, the results were grouped as listed in Table 3. The groups of proteins include cell envelope, pyruvate dissimilation, amino acids biosynthesis, cofactor biosynthesis, regulatory functions, translation, glycolysis, tricarboxylic acid cycle, energy production, chaperone, and heat shock proteins; proteins with unknown function; and hypothetical proteins.

The upregulated proteins in the Δ*cadC* mutant included proteins involved in glycolysis (PfkA, PfkB, FbaB, and STM4519), energy production (AtpD), and stress tolerance (Tig and HslU). To our knowledge, the enhanced glycolytic capacities could contribute to the ATR by lowering the pH through glycolysis during acid adaptation (18). The protein AtpD is a beta subunit of F_1-ATPase, which plays an important role in the ATR (3, 15). Elevated expression of molecular chaperons (Tig and HslU) may also perform essential roles in pH stress. The Tig protein is a ribosome-associated trigger factor that interacts with a wide variety of nascent polypeptides promoting protein folding (23). The downregulated proteins in the Δ*cadC* mutant included outer membrane proteins (OmpC and OmpF). This regulation would be a simple way to provide protection against acids by downregulating expression of both OmpC and OmpF. Moreover, several regulatory proteins were also identified. Taken together, these findings strongly support the notion that CadC is a global regulator in acid adaptation.

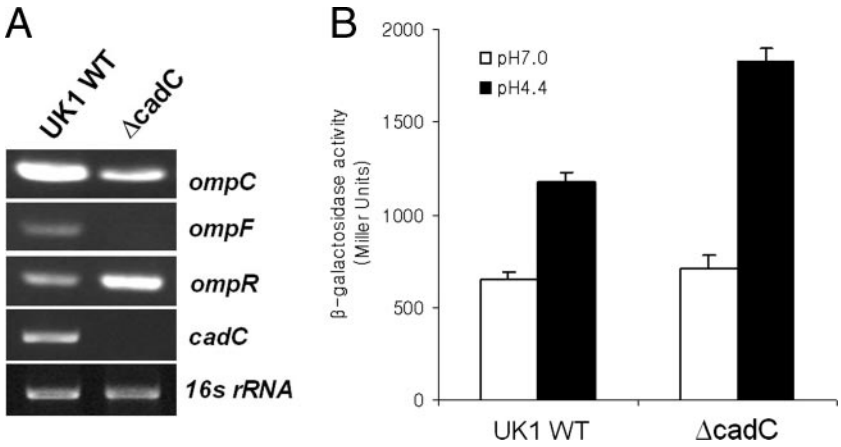

FIG. 5. Effect of *cadC* inactivation on *ompR* transcription. (A) RT-PCR comparison of transcription of the indicated genes. UK1 wild-type (WT) and Δ*cadC* strains were adapted in NCE glucose medium (pH 4.4, 10 mM lysine) for 1 h. 16S rRNA was used as a loading control. (B) The chromosomal *ompR-lacZ* merodiploid strains (*putPA1303*::Kan^r-*ompR-lacZ*) were grown in NCE glucose medium (pH 7.0) to early log phase (optical density at 600 nm of 0.4) and then adapted at pH 4.4 for 1 h. Open and filled bars indicate β-galactosidase activities (Miller units) from unadapted cells and cells adapted at pH 4.4 for 1 h, respectively. Three independent assays were performed for each strain, and standard deviations are indicated by error bars.

Effect of CadC on the regulation of *ompR* during acid adaptation. RT-PCR analysis of the *S. enterica* serovar Typhimurium UK1 wild-type and Δ*cadC* strains confirmed the proteomic data (Table 3), suggesting that CadC positively regulates the transcription of *ompC* and *ompF* during acid adaptation (Fig. 5A). As the serovar Typhimurium *ompC* and *ompF* porin genes are regulated by the products of *ompB*, a two-component regulatory locus encoding OmpR and EnvZ (32, 38), we hypothesized that CadC may regulate the two former genes indirectly via the OmpR regulatory system. We compared *ompR* mRNA levels by using RT-PCR and determined that the presence of CadC has a negative influence on expression (Fig. 5A). Additionally, we compared expression of a chromosomal *ompR-lacZ* fusion in the JF4484 (wild-type) and YK5003 (Δ*cadC* mutant) strains by using β-galactosidase assays. Since OmpR is known to autoinduce its own expression (1), *ompR-lacZ* merodiploid strains (*putPA1303*::Kan^r-*ompR-lacZ*) were used to monitor regulation of *ompR* transcription by CadC. As described previously (1), an *ompR-lacZ* fusion was inserted into the *putPA* operon in the *Salmonella* chromosome. Following adaptation to NCE glucose medium (pH 4.4) for 1 h, the β-galactosidase activity of the Δ*cadC* mutant (YK5003) was approximately 1.6-fold higher than that of the wild-type (JF4484), indicating that *ompR* is transcriptionally downregulated by CadC (Fig. 5B). Therefore, CadC appears to be involved in the OmpR/EnvZ two-component regulatory system.

DISCUSSION

CadC is a global regulator of acid adaptation in *S. enterica* serovar Typhimurium. The major finding in this study is that *S. enterica* serovar Typhimurium CadC plays a role in global regulation during acid adaptation. Previously, only the *cadBA* operon had been identified as CadC dependent (21, 29, 30, 40, 46). However, the data presented in Fig. 4 indicate the presence of additional CadC-regulated genes. Two-dimensional gel electrophoresis analyses showed that *S. enterica* serovar Typhimurium CadC is a global regulator that controls the expression of ≥36 proteins, either positively or negatively. In addition, peptide mass fingerprinting using the NCBInr protein database led to the identification of 23 of the putative CadC-regulated proteins (Table 3). However, it remains unclear whether or not these genes are regulated directly or indirectly by CadC. For example, CadC might regulate other transcriptional regulators, which in turn induce or repress the production of the proteins indicated in Fig. 4.

The enzymes involved in glycolysis were upregulated in the Δ*cadC* mutant. To our knowledge, the rapid dissimilation of glucose results in secretion of relatively greater amounts of acids as by-products of glycolysis. Therefore, we can speculate that during acid adaptation (pH 4.4 for 1 h), the Δ*cadC* mutant may be able to reduce the pH to values lower than those of the wild type and become more resistant to acid killing (pH 3.0). Another possibility is that higher rates of ATP generation through glycolysis enhance the ability of the cells to maintain the internal pH. Moreover, the increased expression of AtpD (F_1-ATPase subunit beta) was also observed in the Δ*cadC* mutant. Under acid stress, there is an apparent need for increased ATPase activity for the organism to protect itself from acidification by pumping protons out of the cells (3, 15). Similar findings have also been reported for the gram-positive *Streptococcus mutans* (2, 18, 39).

The proteomic approach revealed an unanticipated link between CadC and the OmpR-EnvZ regulatory system in *S. enterica* serovar Typhimurium. Although it is clear that CadC acts as a mild repressor of *ompR* transcription, it remains to be determined whether this is a direct or indirect effect. CadC may mediate regulation of *ompR* transcription through protein-protein interactions with OmpR itself. It has been demonstrated recently that EnvZ-dependent phosphorylation of OmpR is required for *ompR* autoinduction and that phosphorylated OmpR (OmpR-P) binds to its own promoter with a greater affinity than the unphosphorylated form (1). Thus, CadC may interact with OmpR-P, hindering its binding to the

ompR promoter region and providing a means of fine-tuning *ompC* and *ompF* expression. The participation of CadC in control of *ompR* expression suggests a role in adaptation to a broad spectrum of environments. Future studies will focus on the mechanism of CadC regulation at the molecular level and the link between *Salmonella* CadC and the OmpR-dependent pathway.

Differential expression of *cadC* between *S. enterica* serovar Typhimurium and *E. coli*. The enteric bacteria *S. enterica* serovar Typhimurium and *E. coli* are closely related, and much of the knowledge obtained about these two organisms is interchangeable (4, 35). For example, both bacteria respond to the low pH and lysine signal by activating transcription of the *cadBA* operon (34). However, the present study indicates that the two species use disparate regulatory strategies to control *cadC* expression. In *S. enterica* serovar Typhimurium, *cadC* transcription is induced by low pH and lysine, whereas its expression is constitutive in *E. coli* (10, 34, 46). Thus, their CadC proteins appear to respond differently to this signal. Dissimilarities in control of CadC expression may be responsible for critical differences in phenotypic traits between these closely related bacteria; such disparities in regulation of homologous genes are known to have phenotypic consequences (48). Although the CadC proteins in *S. enterica* serovar Typhimurium and *E. coli* have similar structures (20), they share only 58.4% identity. Therefore, we can speculate that in serovar Typhimurium, CadC may perform different functions than it does in *E. coli*. Further investigation will be required to identify the molecular mechanisms underlying differential expression of *cadC* in these two organisms.

Effect of a *cadC* mutation on the ATR in *S. enterica* serovar Typhimurium. Previous studies have indicated that in enteroinvasive *E. coli* (EIEC) and *Shigella* strains, silencing of the *cad* locus represents an important pathoadaptive mutation for improving fitness in host tissues (9, 27, 44). Although the *cad* system may be important during transit through the gastrointestinal tract, LDC activity is not found in EIEC or *Shigella* strains (27, 42), and recent findings suggest that inactivation of *cadC* is the main strategy for silencing the *cad* operon (5, 6).

We discovered that mutation of *S. enterica* serovar Typhimurium *cadC* confers a survival advantage under acidic conditions, overcoming the ATR defect caused by lack of expression of *cadA* (Fig. 3). It is possible that pathoadaptive mutation of *cadC* may not be limited to EIEC and *Shigella* strains. A recent report demonstrated an unusually high frequency of LDC-negative *Salmonella enterica* serovar Enteritidis isolates in Japan, all of which had a single-base deletion at the same position in *cadC* (33). The LDC-positive phenotype is a major characteristic of *Salmonella* spp. (13, 47), and the closely related *S. enterica* serovars Typhimurium and Enteritidis cause almost identical diseases. Thus, the lack of *cadC* in *Salmonella* may be considered a pathoadaptive mutation, whose emergence is necessary for enhanced survival within host tissues. However, it has been reported that serovar Typhimurium *cadC* is induced during the course of infection, suggesting that CadC may contribute to survival in certain host tissues, enhancing pathogenicity (12, 19). Therefore, it will be interesting to determine whether or not inactivation of *cadC* is also pathoadaptive in serovar Typhimurium.

In summary, we have shown that *S. enterica* serovar Typhimurium CadC affects global translation and participates in control of *ompR* expression during acid adaptation. Analysis of the other CadC-regulated proteins may provide further insight into the roles played by CadC in pathogen-host interactions. Future studies will be aimed at elucidating the mechanisms underlying regulation by CadC.

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