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Expression of *STM4467*-Encoded Arginine Deiminase Controlled by the *STM4463* Regulator Contributes to *Salmonella enterica* Serovar Typhimurium Virulence

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Arginine deiminase (ADI), carbamate kinase (CK), and ornithine transcarbamoylase (OTC) constitute the ADI system. In addition to metabolic functions, the ADI system has been implicated in the virulence of certain pathogens. The pathogenic intracellular bacterium *Salmonella enterica* serovar Typhimurium possesses the *STM4467*, *STM4466*, and *STM4465* genes, which are predicted to encode ADI, CK, and OTC, respectively. Here we report that the *STM4467* gene encodes an ADI and that ADI activity plays a role in the successful infection of a mammalian host by *S.* Typhimurium. An *STM4467* deletion mutant was defective for replication inside murine macrophages and was attenuated for virulence in mice. We determined that a regulatory protein encoded by the *STM4463* gene functions as an activator for *STM4467* expression. The expression of the ADI pathway genes was enhanced inside macrophages in a process that required STM4463. Lack of STM4463 impaired the ability of *S.* Typhimurium to replicate within macrophages. A mutant defective in *STM4467*-encoded ADI displayed normal production of nitric oxide by macrophages.

Salmonella enterica serovar Typhimurium is a facultatively intracellular bacterium that can cause a diverse spectrum of diseases. In the course of systemic infection of a mammalian host, *S.* Typhimurium can survive within the macrophage phagosome (13). For this purpose, *S.* Typhimurium expresses gene products to avoid killing by microbicides that are produced inside the phagosome (25, 39). Previous studies have also revealed that purine, pyrimidine, and amino acid auxotrophs of *S.* Typhimurium are unable to survive inside macrophages (18, 27). In addition, *S.* Typhimurium appears to activate an alternative metabolic pathway for the utilization of carbon sources during growth inside macrophages (15). These findings demonstrate that bacterial metabolism is a crucial determinant for the successful pathogenesis of *S.* Typhimurium (7, 32).

In general, three enzymes—arginine deiminase (ADI), ornithine transcarbamoylase (OTC), and carbamate kinase (CK)—constitute the ADI system, which catalyzes the conversion of L-arginine into ornithine, ammonia, and carbon dioxide with the formation of ATP (44). This enzymatic pathway is widely distributed in various bacterial species and is known to provide cellular energy, particularly under oxygen-limited conditions (4, 9, 20, 44). In addition to its metabolic function, the ADI system is also employed to protect some bacteria from stressful conditions. In oral streptococci and *Streptococcus pyogenes*, the ADI system helps the bacteria resist acidity by generating ammonia and thus neutralizing an acidic pH (4, 11). Moreover, the ADI system sometimes, though rarely, plays a role in bacterial pathogenesis. ADI is necessary for *S. pyogenes* to invade and survive inside epithelial cells (11). Additionally, a lack of ADI has been found to impair the survival of *Listeria monocytogenes* in the spleen during infection in a mouse model (41).

The *STM4467*, *STM4466*, and *STM4465* genes of *S.* Typhimurium are predicted to encode ADI, CK, and OTC, respectively. The ADI pathway of *S.* Typhimurium appears to be functional, because the expression of the putative ADI cluster increased OTC activity (43). In the present study, we investigated the role of the ADI system in *S.* Typhimurium pathogenesis. We found that an *S.* Typhimurium strain lacking the *STM4467*-encoded ADI was defective in its ability to replicate inside macrophages and was attenuated for virulence in mice. We also revealed that the *STM4463*-encoded regulator contributes to *S.* Typhimurium virulence, at least in part, by upregulating the expression of the ADI gene cluster within macrophages.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. The *S.* Typhimurium strains were derived from strain 14028s. Phage P22-mediated transductions were performed as described previously (28). The bacteria were grown at 37°C in Luria-Bertani (LB) medium. Ampicillin, chloramphenicol, kanamycin, and isopropyl-β-D-thiogalactopyranoside (IPTG) were used at 50 μg/ml, 25 μg/ml, 50 μg/ml and 0.5 mM, respectively.

Construction of strains. *S.* Typhimurium strain CH102, in which the *STM4467* gene is deleted, was constructed using the one-step gene inactivation method (10). The Km^r cassette from plasmid pKD13 (10) was amplified using primers STM67-lamb-F (5′-ACTCCTTCTTTAT

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
S. enterica serovar Typhimurium strains		
14028s	Wild type	18
CH102	Δ*STM4467*	This study
CH110	$P_{STM4467}$::*lacZY* (Km^r)	This study
CH111	$P_{STM4467}$::*lacZY* (Km^r) Δ*STM4463*::Cm^r	This study
CH201	Δ*STM4463*::Cm^r	This study
Plasmids		
pUHE21-2*lacI*^{q}	rep_{pMBI} Ap^r *lacI*^{q}	42
pACYC184	rep_{p15A} Cm^r Tet^r	5
pKD13	$repR_{6K\gamma}$ Ap^r-FRT Km^r-FRT	10
pKD3	$repR_{6K\gamma}$ Ap^r-FRT Cm^r-FRT	10
pKD46	rep_{pSC101}(Ts) Ap^r P_{araBAD} γ β *exo*	10
pCP20	rep_{pSC101}(Ts) Ap^r Cm^r *cI*857 λ P_R*flp*	10
pCE70	$repR_{6K\gamma}$ Ap^r-FRT *lacZY*$^+$	35
p4467	pACYC184-*STM4467*	This study
p4463	pUHE21-2*lacI*^{q}-*STM4463*	This study
pPM4463	pACYC184-*STM4463*	This study

TCTTGTAATTATGTAAAAGGTATAATGTGTAGGCTGGAGCTGC TTCG-3′) and STM67-lamb-R (5′-CGCGACGACCAGTGTGCGTTTG TTTTCCATAACGTCTCCTATTCCGGGGATCCGTCGACC-3′). The resulting PCR product was integrated into the *STM4467* region in strain 14028s, and the Km^r cassette was subsequently removed using plasmid pCP20 (10). In strain CH201, the *STM4463* gene is deleted. For the construction of this strain, the Cm^r cassette of pKD3 (10) was amplified using primers STM63-lamb-F (5′-CGTTGATATCAATAATAAAGATAAGGT GCATTTATGAAGGTGTAGGCTGGAGCTGCTTCG-3′) and STM63-lamb-R (5′-ATTAATGCATGATTTACTCATCGCAAACGGTTCTTAT GAAATATGAATATCCTCCTTAGTTC-3′) and was integrated into the *STM4463* region in strain 14028s. Deletion of the corresponding genes was verified by colony PCR. Strain CH110, which carries a transcriptional *STM4467-lacZ* fusion, was constructed as described previously (14). The *lacZY* genes were introduced into the FLP recombination target (FRT) site in strain CH102 by using plasmid pCE70 (35).

Construction of plasmids. Plasmid p4467 expresses the *STM4467* gene from its own promoter. For the construction of this plasmid, the *STM4467* gene was amplified using PCR with primers STM4467-pACYC-F (5′-TTGTTTTTTGAAGCTTTCTGACCC-3′) and STM4467-pACYC-R (5′-ACGACCAGCATGCGTTTGTTTT-3′) and with chromosomal DNA from strain 14028s as a template. The product was introduced between the HindIII and SphI restriction sites of pACYC184 (5). To construct plasmid p4463, in which the *STM4463* gene is expressed from the *lac* promoter, the *STM4463* gene was amplified using primers STM4463-pUHE-F (5′-AAATGTGATGAATTCCGCCAGTCC-3′) and STM4463-pUHE-R (5′-TGAACCATGGATCCTCCCGGC-3′). The PCR product was introduced between the EcoRI and BamHI restriction sites of pUHE21-2*lacI*^{q} (42). Plasmid pPM4463, which expresses the *STM4463* gene from its own promoter, was also constructed. The *STM4463* gene was amplified using primers STM4463-pACYC-F (5′-GAAAGTCTGAA TTCCGGCCTCTC-3′) and STM4463-pACYC-R (5′-TTTACTCATCGC ATGCGGTTCTTATG-3′). The PCR product was introduced between the HindIII and SphI restriction sites of pACYC184 (5). The sequences of the *STM4467* and *STM4463* coding regions on the recombinant plasmids were verified by nucleotide sequencing.

Determination of ADI activity. The ADI activities of cell extracts were measured using a chemical colorimetric method based on the production of L-citrulline from L-arginine (3). A total of 50 ml of a bacterial culture grown in LB medium was harvested, and the cell pellet was suspended in 3 ml of lysis buffer (10 mM Tris [pH 8.0] containing 0.3 M NaCl) and was disrupted by sonication. After removal of the cellular debris by centrifugation, 0.4 ml of 10 mM L-arginine in 100 mM potassium phosphate buffer (pH 7.2) was added to 1 ml of cell extract. After a 60-min incubation at 37°C, 250 μl of a 1:3 (vol/vol) mixture of 95% sulfuric acid and 85% phosphoric acid and 250 μl of a 3% diacetyl monooxime solution were added, and the mixtures were boiled for 15 min. The development of an orange color was monitored at 490 nm.

β-Galactosidase assay. β-Galactosidase assays were performed in duplicate, and the activity was determined as described previously (36).

Gentamicin protection assay. The gentamicin protection assay was conducted as described previously (7). J774A.1 macrophage cells were grown in Dulbecco modified Eagle medium (DMEM) (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), penicillin (50 U/ml), and streptomycin (50 U/ml). Prior to bacterial infection, a monolayer of 1×10^5 J774A.1 cells was prepared in a 24-well tissue culture plate and was incubated in DMEM–10% FBS without antibiotics at 37°C for 1 h under 5% CO_2. A bacterial culture grown to stationary phase with aeration was applied to the cell monolayer at a multiplicity of infection (MOI) of 10. After 1 h of incubation, the wells were washed three times with prewarmed phosphate-buffered saline (PBS) to remove extracellular bacteria and were then incubated for 1 h with the prewarmed medium supplemented with 100 μg/ml of gentamicin to kill extracellular bacteria. Afterward, the wells were washed three times with PBS, lysed in 1% Triton X-100 for 30 min, and then diluted in PBS. A dilution of the suspension was plated on LB agar medium to enumerate the CFU.

Mouse virulence assay. Bacterial cells grown overnight in LB medium were pelleted, washed, and resuspended in PBS. Eight-week-old C3H/HeN female mice were used to assess the virulence of *S.* Typhimurium strains. Approximately 10^4 bacterial cells in 200 μl of PBS were injected intraperitoneally into groups of mice (5 mice/group), and the survival of the mice was recorded over 3 weeks. To analyze bacterial colonization in organs, the mice were sacrificed at 5 days after infection, and the spleens and livers were removed aseptically. The organs were homogenized in 1 ml of ice-cold PBS and were serially diluted. Bacterial loads were determined by plating the diluents on LB agar plates.

Determination of nitrite concentration. J774A.1 macrophages were infected with bacteria as described above in triplicate. The supernatants were harvested at 18 h after infection. The nitrite concentration was measured using the Griess assay (21). Briefly, 50 μl of culture supernatants was mixed with an equal volume of Griess reagent (Promega). The absorbance was measured after 10 min at 550 nm in an enzyme-linked immunosorbent assay (ELISA) microreader (Sunrise Basic; Tecan). $NaNO_2$ was used to establish the standard nitrite concentration in the supernatants.

RNA isolation and qRT-PCR analysis. For the extraction of RNA from *S.* Typhimurium growing inside J774A.1 macrophages, an infection experiment was conducted as described above except for the increased volume of macrophage cultures (50 ml in a 75-cm^2 T-flask). At 1 h, 6 h, and 18 h after infection, the macrophage monolayers were washed, lysed in 1% Triton X-100, and centrifuged at 1,000 rpm for 5 min to pellet the lysed macrophages. From the supernatant that contained the intracellular bacteria, RNA was extracted using the RNeasy Mini Kit (Qiagen). The RNA samples were then treated with RNase-free DNase (Ambion), and cDNA was synthesized using Omniscript reverse transcription reagents (Qiagen) and random hexamers (Invitrogen). The cDNA was quantified by using the 2× iQ SYBR green Supermix (Bio-Rad), and real-time amplification of the PCR products was performed using the iCycler iQ real-time detection system (Bio-Rad). The calculated threshold cycle (C_T) corresponding to a target gene was normalized to the C_T of the control gene, *rpoD*. The sigma factor gene *rpoD* was chosen as a control because no significant variation in *rpoD* expression was observed inside macrophages (17). The sequences of the primers used in the quantitative reverse transcription-PCR (qRT-PCR) analysis are listed in Table 2.

TABLE 2 Primers used in qRT-PCR analysis

Primer	Target gene	Sequence (5′ to 3′)
STM4467-RT-F	*STM4467*	CTGGCTACTGGATACGCAAA
STM4467-RT-R	*STM4467*	GACGCCGTTATATATCCAGC
STM4466-RT-F	*STM4466*	AACCGCTGGAGGCTGATATT
STM4466-RT-R	*STM4466*	ATGATTCTTCAGCGCCTGTT
STM4465-RT-F	*STM4465*	GGATGCGAAAAGCAAACACT
STM4465-RT-R	*STM4465*	GGACGCGAGCAGTATCTTTC
STM4463-RT-F	*STM4463*	TTGTCAGCGCCTGATTAGTG
STM4463-RT-R	*STM4463*	ACCATTTCGGCTATTGAACG
ssaG-RT-F	*ssaG*	AGTGGATATGCTCTCCCACA
ssaG-RT-R	*ssaG*	AGGCAAATTGCGCTTTAATC
rpoD-RT-F	*rpoD*	GATGAAGATGCGGAAGAAGC
rpoD-RT-R	*rpoD*	GGTAATGGCTTCCGGGTATT

Statistical analysis. Statistical analyses were conducted using the GraphPad Prism program (version 5.0). Survival curves from animal experiments were analyzed by the log rank test, and all other results were analyzed by the unpaired *t* test. Data are presented as means ± standard deviations. A *P* value of <0.05 was considered statistically significant.

Ethics statement. This study was carried out according to the recommended protocol for the care and use of laboratory animals from the Institute of Laboratory Animal Resources at Seoul National University, based on the Korean Animal Protection Law and Korea Food and Drug Administration regulations on laboratory animals. The protocol was approved by the Committee on the Ethics of Animal Experiments of Seoul National University (Institutional Animal Care and Use Committee permit number SNU-120616-1).

RESULTS AND DISCUSSION

The *STM4467* gene encodes ADI in *S.* Typhimurium. In the *S.* Typhimurium genome, the *STM4467*, *STM4466*, and *STM4465* genes are clustered into an operon-like structure (Fig. 1A) and are predicted to encode enzymes of the ADI system: ADI, CK, and OTC, respectively (Fig. 1A). It has been reported that when *Salmonella* is grown in LB medium containing 0.4 M NaCl without agitation, the expression of this gene cluster is induced to promote OTC activity (43). Although this finding suggests that the ADI pathway might be functional in *S.* Typhimurium, the genes responsible for the enzymatic activities of the ADI pathway have remained unknown. Therefore, we compared the ADI activity of wild-type *Salmonella* with that of its isogenic *STM4467* deletion mutant. When the ADI activity assay was conducted using a cell extract prepared from the wild-type strain, an orange color developed (Fig. 1B), which indicated the ADI-catalyzed production of citrulline from arginine (3, 41). However, in the *STM4467* deletion mutant, the enzyme activity was poorly detected (Fig. 1B) and was only ~40% of that present in the wild-type strain (Fig. 1C). Expression of the *STM4467* gene from a plasmid enabled the *STM4467* deletion mutant to produce citrulline at levels even higher than those of the wild-type strain (Fig. 1B and C). Thus, these results indicate that the *STM4467* gene either encodes ADI or is required for full ADI activity.

The *STM4467* gene contributes to *Salmonella* virulence. ADI is necessary for *S. pyogenes* to invade and survive inside epithelial cells (11). Wild-type *L. monocytogenes* survived longer in the spleen during a mouse infection than did a mutant lacking ADI (41). These findings suggested a role for ADI in bacterial pathogenesis.

To explore whether the *STM4467*-encoded ADI contributes to *Salmonella* virulence, we initially compared the replication abilities of the wild-type and *STM4467* deletion strains within murine macrophages. The gentamicin protection assay revealed that the CFU count of the *STM4467* deletion mutant within the macro-

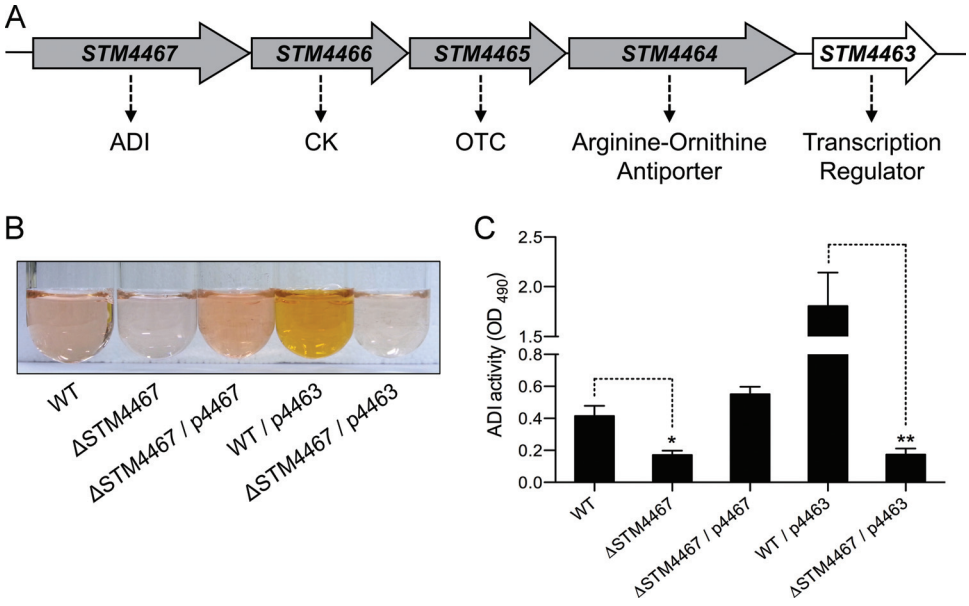

FIG 1 The *STM4467* gene is required for ADI activity. (A) Schematic representation of the ADI pathway gene cluster in *S.* Typhimurium. (B) The ADI activities of the wild-type (WT) strain (14028s), the *STM4467* deletion mutant (CH102), strain CH102 carrying plasmid p4467 (Δ*STM4467*/p4467), the WT strain carrying plasmid p4463, and strain CH102 carrying plasmid p4463 (Δ*STM4467*/p4463) were determined by using cell extracts grown in LB medium. The development of an orange color indicates the ADI-catalyzed production of L-citrulline from L-arginine. (C) Quantification of the ADI activity displayed by the *S.* Typhimurium strains described for panel B. Means and standard deviations from three independent experiments are shown (**, $P < 0.01$). OD_{490}, optical density at 490 nm.

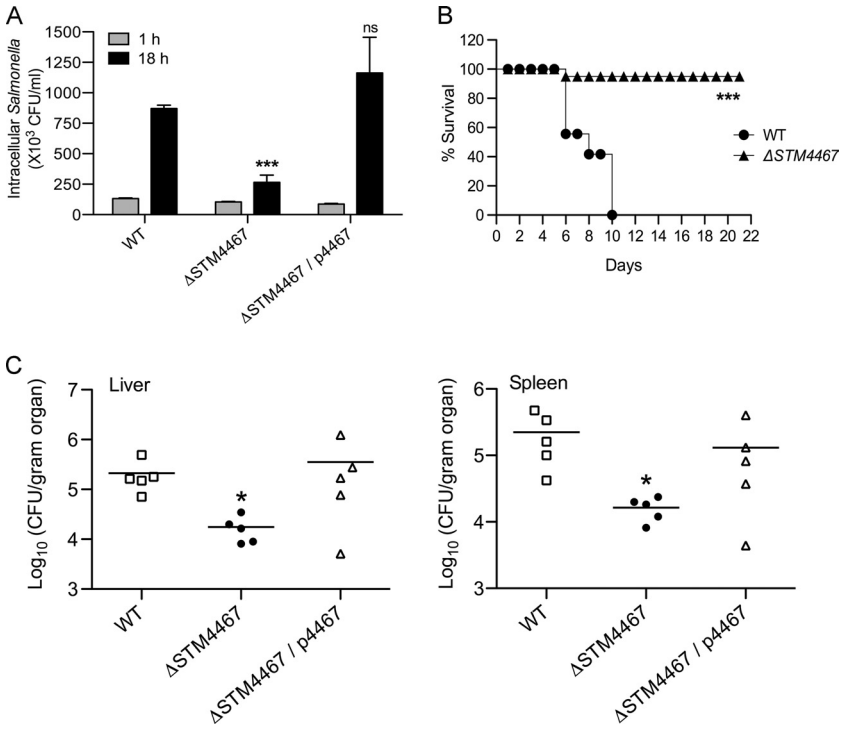

FIG 2 The *STM4467* gene contributes to *S.* Typhimurium virulence. (A) J774A.1 macrophage cells were infected with the wild-type (WT) strain (14028s), the *STM4467* deletion mutant (CH102), or strain CH102 harboring the p4467 plasmid (Δ*STM4467*/p4467). The numbers of intracellular bacteria were determined at 1 and 18 h after infection by using the gentamicin protection assay. Means and standard deviations from at least three independent experiments are shown. Triple asterisks indicate that the numbers of bacteria were significantly different ($P < 0.001$) from those of the WT strain at 18 h postinfection; ns, not significantly different. (B) Groups of C3H/HeN mice (5 mice/group) were injected intraperitoneally with ~10^4 cells of the WT or *STM4467* deletion strain. The survival of the mice was monitored daily for 3 weeks. The results of one of two independent experiments ($P < 0.001$), which yielded similar results, are shown. The results of the other experiment are shown in Fig. S1 in the supplemental material. (C) Groups of C3H/HeN mice (5 mice/group) were infected with the WT strain, the *STM4467* deletion mutant, or strain CH102 harboring plasmid p4467 as described for panel B. At 5 days after infection, the numbers of bacteria in the liver and spleen were determined. An asterisk indicates that the numbers of bacteria were significantly different ($P < 0.05$) from those of the WT strain.

phages at 18 h postinfection was only ~30% of that of the wild type (Fig. 2A). This result was not due to differences in the phagocytosis of the two strains, because the intracellular numbers of the two strains were similar at an earlier time point (i.e., 1 h) after infection (Fig. 2A). The phenotypic defect of the *STM4467* deletion strain was due to the absence of STM4467 function, as evidenced by the fact that expression of the *STM4467* gene from a plasmid enabled the *STM4467* deletion mutant to replicate within the macrophages at a level similar to that of the wild-type strain (Fig. 2A).

To test whether the *STM4467* deletion mutant might be attenuated for virulence in mice, we injected *Salmonella* intraperitoneally into groups of 5 mice. As shown in Fig. 2B, all of the mice inoculated with the wild-type strain died within 10 days, whereas 90% of the mice that received the *STM4467* mutant survived over 3 weeks postinfection. We further verified the virulence phenotype of the *STM4467* deletion mutant by determining the numbers of bacterial cells in organs of mice. In both the liver and the spleen, the numbers of *STM4467* deletion mutant cells were ~10-fold lower than those of wild-type cells at 5 days postinfection (Fig. 2C). This phenotypic difference was due to the absence of STM4467 function, as evidenced by the fact that the *STM4467* deletion strain carrying the STM4467 expression plasmid was able to colonize the liver and spleen as efficiently as the wild-type strain (Fig. 2C). Taken together, these results suggest that in the absence of the *STM4467*-encoded ADI activity, *S.* Typhimurium cannot avoid killing by macrophages and thus is attenuated for virulence.

Expression of the ADI pathway genes is enhanced inside macrophages. The attenuation of the virulence of the *STM4467* mutant suggested that expression of the *STM4467* gene might increase when *Salmonella* cells are inside macrophages. To test this hypothesis, we isolated RNA from wild-type *Salmonella* cells grown within macrophages and determined the transcription levels of genes via qRT-PCR. We found that *STM4467* transcription was enhanced in *Salmonella* cells growing inside macrophages: the *STM4467* mRNA levels increased ~4-fold at 18 h over those at 1 h after bacterial entry into the macrophages (Fig. 3A). In agreement with the hypothesis that the ADI system genes constitute an operon that produces a polycistronic mRNA (2, 22), the transcript levels of the *STM4466* and *STM4465* genes, which putatively encode CK and OTC, respectively (Fig. 1A), were also elevated at 18 h after phagocytosis (Fig. 3A).

In response to environmental cues inside the phagosome, *Salmonella* expresses a series of genes from *Salmonella* pathogenicity island 2 (SPI-2), which mediates its survival within macrophages (7, 24, 29, 31). Therefore, the induction of the SPI-2 gene *ssaG* under our experimental conditions (Fig. 3A) reflects the possibility that upregulation of the ADI pathway could occur in response to environmental cues inside the phagosome. Induction of the ADI pathway was observed only at 18 h after phagocytosis

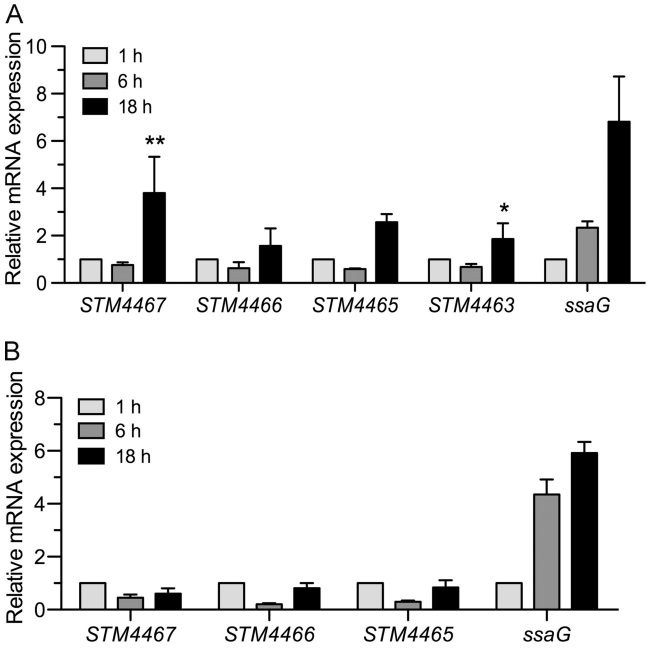

FIG 3 Expression of the ADI system is enhanced inside macrophages in an STM4463-dependent manner. The transcription levels of the *STM4467*, *STM4466*, *STM4465*, *STM4463*, and *ssaG* genes in *S.* Typhimurium growing inside macrophages were determined via qRT-PCR. J774A.1 macrophages were infected with the wild-type strain (14028s) (A) or the *STM4463* deletion strain (CH201) (B), and bacterial RNA was isolated at 1 h, 6 h, and 18 h after infection. To obtain the relative mRNA expression values on the *y* axis, the mRNA levels of each gene were divided by those of the *rpoD* gene, which were further normalized by the transcription levels displayed at 1 h after infection. Means and standard deviations from three independent experiments are shown. Asterisks indicate significant differences (**, $P < 0.01$; *, $P < 0.05$) in mRNA levels between the 1-h and 18-h samples.

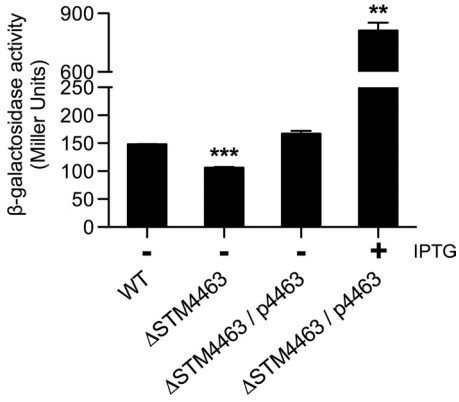

FIG 4 The STM4463 protein promotes transcription of the *STM4467* gene. Transcription levels of the *STM4467* gene were determined using a β-galactosidase assay on *S.* Typhimurium strains carrying an *STM4467-lacZ* fusion. Strain CH110 (wild type [WT]), its isogenic *STM4463* deletion mutant (CH111 Δ*STM4463*), and strain CH111 harboring plasmid p4463 (Δ*STM4463*/p4463) were grown in LB medium with (+) or without (−) 0.5 mM IPTG. Means and standard deviations from three independent experiments are shown. Asterisks indicate that the β-galactosidase levels of bacteria are significantly different (***, $P < 0.001$; **, $P < 0.01$) from those of the wild-type strain.

(Fig. 3A); this timing was slower than that of SPI-2 gene induction, which occurred at 6 h after bacterial entry into macrophages (Fig. 3A) (8). Thus, given that the environment within the *Salmonella*-containing phagosome is dynamically changing, the expression of the ADI system might be important at a stage of infection later than the onset of expression of the SPI-2 genes.

The STM4463 protein promotes the transcription of the *STM4467* gene. As in the structures of other known ADI operons (38, 44), the *STM4463* gene encodes a transcriptional regulator downstream of the ADI gene cluster (Fig. 1A). This gene was formerly known as *rosE*, and the STM4463 protein was identified as a regulator that directly represses the expression of the *std* fimbrial operon (6). However, since homologs of STM4463 have been reported to regulate the expression of the ADI operon in other bacteria (12, 33, 34), we reasoned that the STM4463 protein might act as a regulator of *Salmonella* ADI expression. To examine *STM4467* transcription, we constructed a strain that carried a transcriptional fusion of *lacZ* to the *STM4467* gene. A β-galactosidase assay determined that in the *STM4467-lacZ* strain, the lack of STM4463 reduced *STM4467* expression levels by ~30% (Fig. 4). To further investigate the regulatory role of STM4463, we constructed plasmid p4463, in which expression of the *STM4463* gene is under the control of the *lac* promoter. The β-galactosidase assay revealed that in the *STM4467-lacZ* strain lacking the *STM4463* gene and harboring p4463, *STM4467* transcription was restored to wild-type levels, which further increased ~5-fold when the strain was grown with IPTG to induce STM4463 (Fig. 4). *Salmonella* ADI activity was also enhanced by STM4463 in a process that required STM4467: the overexpression of STM4463 increased ADI activity ~5-fold in the wild-type strain but failed to do so for the *STM4467* deletion mutant (Fig. 1B and C). Cumulatively, these results suggest that the STM4463 protein is a regulator that positively controls the ADI pathway of *S.* Typhimurium.

The STM4463 regulator is necessary for *Salmonella* to express the ADI gene cluster and replicate inside macrophages. Since the STM4463 protein appeared to enhance ADI expression (Fig. 4), we reasoned that the STM4463 regulator might be responsible for intramacrophage induction of the ADI system. To test this possibility, we examined the transcription of the *STM4467*, *STM4466*, and *STM4465* genes within macrophages. qRT-PCR analysis revealed that in contrast to the wild-type strain, the *STM4463* deletion mutant failed to induce the expression of these three genes at 18 h after phagocytosis (Fig. 3B). The failure of the *STM4463* mutant to induce the ADI gene cluster is not due to a general expression defect, because the intraphagosomal induction of the SPI-2 gene *ssaG* was unaffected by the *STM4463* deletion (Fig. 3B).

We then hypothesized that the STM4463 regulator could contribute to *Salmonella* virulence by activating ADI expression within macrophages. Indeed, a lack of STM4463 impaired the ability of *Salmonella* bacteria to replicate inside macrophages. The numbers of intracellular *Salmonella* bacteria with the *STM4463* deletion were ~3-fold lower than those of wild-type bacteria at 18 h after phagocytosis (Fig. 5). This defective phenotype was due to the function of STM4463, as evidenced by the fact that the replication ability of the *STM4463* deletion mutant was recovered by expression of the *STM4463* gene from a plasmid (Fig. 5). The numbers of bacteria of the wild-type strain and the *STM4463* deletion strain inside the macrophages were similar at 1 h after infection, indicating that the STM4463 regulator did not interfere with phagocytosis.

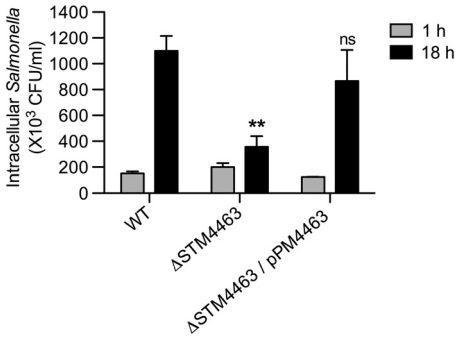

FIG 5 The STM4463 regulator is necessary for *S.* Typhimurium replication inside macrophages. J774A.1 macrophages were infected with the wild-type (WT) strain (14028s), an *STM4463* mutant (CH201), or strain CH201 carrying plasmid pPM4463 (Δ*STM4463*/pPM4463). The numbers of intracellular bacteria were determined at 1 h and 18 h after infection. Means and standard deviations from at least three independent experiments are shown. Asterisks indicate that the numbers of bacteria are significantly different (**, $P < 0.01$) from those of the WT strain at 18 h postinfection; ns, not significantly different.

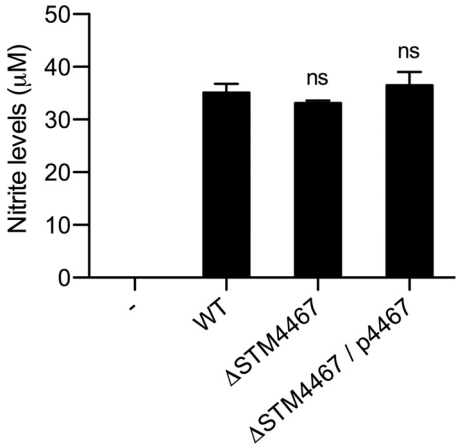

FIG 6 The *STM4467*-encoded ADI has no effect on NO_2^- generation by macrophages. J774A.1 macrophage cells were infected with the wild-type (WT) strain (14028s), an *STM4467* deletion mutant (CH102 Δ*STM4467*), or strain CH102 harboring plasmid p4467 (Δ*STM4467*/p4467). At 18 h after infection, levels of NO_2^- production in the supernatants of macrophages were determined by using the Griess reaction. Note that NO_2^- was nearly absent from macrophages without (−) *S.* Typhimurium infection. Means and standard deviations from three independent experiments are shown. "ns" indicates that the nitrite levels of bacteria are not significantly different from those of the WT strain.

In the wild-type strain, transcription levels of the *STM4463* gene increased ~2-fold at 18 h after phagocytosis (Fig. 3A), concurrently with ADI pathway induction (Fig. 3A). In addition, induction of the STM4463 regulator greatly increased the expression of the *STM4467* gene (Fig. 4). Therefore, although the precise mechanism of control of STM4463 expression within macrophages is presently unclear, we propose that levels of the STM4463 regulator are enhanced by unknown signals present inside the phagosome and, in turn, activate expression of the ADI system.

It is possible that the failure of expression of the ADI gene cluster partially contributes to the defective survival of the *STM4463* mutant within macrophages. The STM4463 regulator repressed the expression of the fimbrial operon *std*, which appeared necessary for full *Salmonella* virulence in mice (6). However, because the effects of *STM4463* and *std* mutations on *Salmonella* virulence were assessed by bacterial colonization of organs of mice that were infected via an oral route (6), it remains unknown whether STM4463 regulation of the *std* operon could affect the survival of *Salmonella* inside macrophages.

***STM4467* does not affect the levels of nitric oxide production inside macrophages.** After phagocytosis, bacterial cells are killed inside the phagosome by the actions of microbicidal products (19, 23). Of these antimicrobials, nitric oxide (NO) is synthesized from arginine by NO synthase (NOS), and the availability of arginine is one of the rate-limiting factors in cellular NO production (1, 16, 37). *S.* Typhimurium appears to have a means to control host arginine metabolism by which it can avoid NO toxicity. A recent study demonstrated that in *Salmonella*-infected macrophages, the upregulation of arginase II quenches arginine and reduces NO production (30). On the basis of these observations, we hypothesized that the enhanced ADI activity within the phagosome might reduce NO levels by consuming arginine, thus helping *Salmonella* to avoid NO-mediated killing by macrophages. To test this hypothesis, we measured NO levels within macrophages with or without *Salmonella* infection. The results indicated that infection by wild-type *Salmonella* dramatically increased the NO levels in macrophages (Fig. 6), in accordance with the observation that NOS activity is inducible upon bacterial infection (26). We found that the levels of NO production stimulated by *Salmonella* bacteria with the STM4467 deletion were comparable to those occurring upon infection with the wild-type strain (Fig. 6). Therefore, this result suggests that the *STM4467*-encoded ADI activity contributes to the intramacrophage survival of *Salmonella* via an alternative mechanism that is unrelated to NO production.

Concluding remarks. In the present study, we established that the ADI pathway contributes to *Salmonella* pathogenesis. The ADI gene cluster was induced inside macrophages in a process that required the STM4463 regulator, the lack of which impaired the ability of *Salmonella* to replicate inside macrophages. To our knowledge, this is the first report to demonstrate the regulation of the ADI genes and their role in a pathogen growing inside host cells. Our findings raise important questions. First, how do levels of the STM4463 regulator increase within macrophages? Since the *STM4463* gene appears to have its own promoter, an unknown *trans*-acting factor might act on the *STM4463* regulatory region in response to environmental cues within the phagosome. Second, how does the ADI pathway contribute to *Salmonella* virulence? We were able to exclude the possibility that the ADI pathway reduced NO toxicity. In *L. monocytogenes* and *S. pyogenes*, ADI enzymes appeared to protect bacteria from acidic stress (4, 11, 43). Since *Salmonella* experiences an acidic pH within the phagosome (13, 40), we tested whether the ADI pathway is also implicated in the survival of *Salmonella* at acidic pHs. However, we found that the *STM4467* deletion did not inhibit the growth of *Salmonella* at pH 5.5 (data not shown). Therefore, as proposed in a recent study (43), the ADI system might provide energy for intracellular *Salmonella* bacteria by producing ATP or might protect it from oxidative stress within the phagosome via production of the polyamine putrescine.

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