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Dam Methylation Controls O-Antigen Chain Length in *Salmonella enterica* Serovar Enteritidis by Regulating the Expression of Wzz Protein▽

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We reported previously that a *Salmonella enterica* serovar Enteritidis *dam* mutant expressing a truncated Dam protein does not agglutinate in the presence of specific antibodies against O9 polysaccharide. Here we investigate the participation of Dam in lipopolysaccharide (LPS) synthesis in *Salmonella*. The LPS O-antigen profiles of a *dam* null mutant (SEΔ*dam*) and the *Salmonella* serovar Enteritidis parental strain were examined by using electrophoresis and silver staining. Compared to the parental strain, SEΔ*dam* produced LPS with shorter O-antigen polysaccharide chains. Since Wzz is responsible for the chain length distribution of the O antigen, we investigated whether Dam methylation is involved in regulating *wzz* expression. Densitometry analysis showed that the amount of Wzz produced by SEΔ*dam* is threefold lower than the amount of Wzz produced by the parental strain. Concomitantly, the activity of the *wzz* promoter in SEΔ*dam* was reduced nearly 50% in logarithmic phase and 25% in stationary phase. These results were further confirmed by reverse transcription-PCR showing that *wzz* gene expression was threefold lower in the *dam* mutant than in the parental strain. Our results demonstrate that *wzz* gene expression is downregulated in a *dam* mutant, indicating that Dam methylation activates expression of this gene. This work indicates that *wzz* is a new target regulated by Dam methylation and demonstrates that DNA methylation not only affects the production of bacterial surface proteins but also the production of surface polysaccharides.

Lipopolysaccharide (LPS) is a key component of the outer membrane of gram-negative bacteria that contributes to the stability and permeability barrier properties of this membrane. LPS is located on the outer leaflet of the outer membrane and consists of three regions: O-antigen polysaccharide, core oligosaccharide, and lipid A (47). The biogenesis of LPS is a complex process involving various steps that occur at the plasma membrane, followed by translocation of LPS molecules to the outer membrane (47, 48, 54, 58). The core oligosaccharide is assembled on preformed lipid A by sequential glycosyl transfer of monosaccharides, while the O antigen is independently assembled on undecaprenyl-phosphate (61). These pathways eventually converge by ligation of the O antigen to the outer core domain of the lipid A-core oligosaccharide acceptor (19, 20, 47, 48, 58, 61, 62). O-antigen assembly occurs by mechanisms referred to as Wzy (polymerase)-dependent and ATP-binding cassette-dependent pathways (for reviews, see references 47 and 58). The *Salmonella* O antigen is assembled by the Wzy-dependent pathway, in which the O-antigen repeating subunits are synthesized at the cytosolic side of the plasma membrane. Each subunit is subsequently translocated across the membrane by an ATP hydrolysis-independent mechanism mediated by the protein Wzx (27, 47, 49, 58). On the periplasmic side of the plasma membrane, translocated subunits polymerize to a certain length, unique to each O antigen, by the concerted actions of Wzy (O-antigen polymerase) and Wzz (O-antigen chain length regulator), and the polysaccharide is ultimately ligated to the lipid A-core oligosaccharide (31, 35, 38).

In *Salmonella* species, O-antigen length contributes to an effective barrier (39) and affects key virulence features, like serum resistance and entry into eukaryotic cells (17, 23, 40–42). Furthermore, O-antigen length can also modulate acquired immunity. Indeed, Phalipon and coworkers demonstrated that in *Shigella flexneri* induction of an O-antigen-specific antibody response depends on the length of the polysaccharide chain (45).

In gammaproteobacteria the DNA adenine methyltransferase (Dam) introduces a methyl group at the N6 position of the adenine of GATC sites in the newly synthesized DNA strand after DNA replication, generating methylated DNA (29, 30, 32, 63). At certain GATC sites, methylation of the newly synthesized strand is hindered by binding of proteins that protect the GATC sites from Dam methylase. The protection against methylation can either cause a temporary delay in methylation or generate GATC sites that are stably hemimethylated or unmethylated (30, 63). Thus, the DNA methyl-

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
S. enterica serovar Enteritidis strains		
#5694	Wild type	F. Collins collection
SEΔ*dam*	Δ*dam*	This study
SEΔ*wzz*::*lacZ*	#5694 Δwzz_{st}::*lacZY*, Km^r	This study
SEΔ*wzz*::*lacZ*Δ*dam*	#5694 Δwzz_{st}::*lacZY* Δ*dam*, Km^r	This study
E. coli K-12 strain DH5α	F$^-$ ϕ80*lacZ*M15 *endA recA hsdR*(r_K^- m_K^-) *supE thi gyrA relA* Δ(*lacZYA-argF*)*U169*	Laboratory stock
Plasmids		
pACYC184	Cm^r Tet^r, cloning vector	52
pCE36	*ahp* FRT *lacZY*$^+$ t_{his} oriR6K	12
pCP20	FLP$^+$ λ*c*I857$^+$ λp_R Rep^{ts} Amp^r Cm^r	7
pIZ833	*E. coli dam* gene, Amp^r	57
pKD3	Template plasmid for mutagenesis, Amp^r Cm^r	7
pKD4	Template plasmid for mutagenesis, Amp^r Km^r	7
pKD46	γ, β, and *exo* from λ phage, *araC*-P_{araB}, Amp^r	7
p*wzz*	*wzz*, Cm^r (pACYC184 backbone)	This study

ation status can affect the interactions between DNA and proteins such as RNA polymerase or transcription factors (63) that regulate (activate or repress) gene expression.

Dam is required for expression of virulence genes in certain bacteria (25), including *Salmonella enterica* (1, 4, 6, 13, 21, 24), but the virulence defects of *dam* mutants are pleiotropic and not completely known. It has been proposed that *dam* mutants could serve as live attenuated vaccines and that the Dam protein itself may provide a potential target for broad antimicrobial activity (21). Recently, *dam* mutants of *S. enterica* serovar Typhimurium have been analyzed as potential live vaccines to prevent salmonellosis in birds and cattle (10, 11). We have reported previously that an *S. enterica* serovar Enteritidis *dam* mutant expressing a truncated Dam protein is attenuated (15). This mutant has limited protective capacity as a live vaccine (55) and is unable to agglutinate in the presence of specific antibodies against O9 polysaccharide (14, 55), suggesting that there is a defective LPS. The aim of the present study was to investigate the participation of Dam methylation in LPS synthesis in *Salmonella* serovar Enteritidis. Compared to the LPS produced by the parental strain, the *dam* null mutant produced LPS with shorter O-antigen polysaccharide chains, indicating that Dam methylation regulates LPS gene expression.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used are listed in Table 1. *Salmonella* serovar Enteritidis #5694 was used to construct all of the mutant strains. Gene deletion was performed as described by Datsenko and Wanner (7). The mutagenic primers used are listed in Table 2. Bacteria were grown in Luria-Bertani (LB) broth (53) supplemented, as required, with antibiotics at the following final concentrations: ampicilin, 100 μg/ml; chloramphenicol, 30 μg/ml; kanamycin, 40 μg/ml; and tetracycline, 20 μg/ml.

Bacterial transformation. *Salmonella* serovar Enteritidis was transformed by electroporation as previously described (9). Briefly, bacteria grown for 24 h were subcultured in 5 ml LB broth for 2 h, harvested into 10% glycerol, and washed twice. Forty-five microliters of bacteria was mixed with 5 to 10 μl plasmid or PCR product and subjected to electroporation in a 0.2-cm electroporation cuvette (Bio-Rad) at a voltage of 2.5 kV, a resistance of 200 Ω, and a capacitance of 25 μF. After this, for recovery, 800 μl of SOC broth was added, and bacteria transformed with a plasmid and bacteria transformed with a PCR product were incubated for 2 and 18 h, respectively. Then 70 μl of bacteria was transferred onto selective plates containing the appropriate antibiotic.

Determination of the DNA methylation status. Genomic DNA was isolated using a Puregene DNA isolation kit (Gentra Systems). Two micrograms of DNA was incubated with MboI, Sau3AI, and DpnI for 4 h at 37°C. The buffer mixtures were the mixtures recommended by the manufacturer (Promega). Restricted DNA was analyzed by electrophoresis on 0.7% (wt/vol) agarose gels along with DNA subjected to the same conditions in the absence of enzyme. After electrophoresis the gels were visualized with UV illumination.

Sensitivity to 2-aminopurine. The sensitivity to the base analogue 2-aminopurine was monitored using LB medium plates containing 100 mg/ml 2-aminopurine (16, 44).

O-antigen agglutination. Individual bacterial colonies were emulsified in 50 μl of saline and checked for autoagglutination. When no agglutination was observed, an equal volume of specific antiserum (Difco) was added. Slides were incubated at room temperature with gentle agitation for 5 min, and agglutination was recorded based on the flocculation of bacteria. Saline and *Salmonella* serovar Enteritidis #5694 were used as negative and positive controls for O9 agglutination, respectively.

Introduction of a *lacZY* transcriptional fusion into the chromosomal *wzz* locus. The *wzz* gene of *Salmonella* serovar Enteritidis strain #5694 was disrupted using the method described by Ellermeier et al. (12). FRT sites generated by excision of kanamycin resistance cassettes were used to integrate plasmid pCE36 (12), generating transcriptional *lacZY* fusions to the *wzz* gene promoter. The resulting strain was designated SEΔ*wzz*::*lacZ*. We also constructed an SEΔ*wzz*::*lacZ* Δ*dam* derivative by using the method of Datsenko and Wanner (7). For complementation experiments, pIZ833 carrying a *dam* gene was introduced into the mutant strains by electroporation.

Molecular cloning of *Salmonella wzz* gene. PCR amplification was performed using Platinum *Pfx* DNA polymerase (Invitrogen). The blunt-ended PCR product was purified using a gel extraction kit (Qiagen) and ligated with T4 DNA ligase (New England BioLabs) into pACYC184, which was digested with EcoRV, blunt ended, and dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics). The ligation mixture was used to transform competent DH5α cells. Plasmids were isolated from Cm^r transformants and screened with restriction endonucleases for inserts that were the appropriate size and correct orientation. The integrity of the insert was confirmed by sequencing (Macrogen Inc.), and the insert was analyzed with Sequencher (Gene Codes Corporation) and Vector NTI software.

LPS analysis. LPS was extracted as described by Marolda et al. (33). Briefly, the optical densities at 600 nm (OD_{600}) of samples (final volume, 100 μl) from an overnight plate culture were adjusted to 2.0. Then the samples were suspended in lysis buffer containing proteinase K as described by Hitchcock and Brown (22), which was followed by hot phenol extraction and subsequent extraction of the aqueous phase with ether. LPS was resolved by electrophoresis in 14% polyacrylamide gels using a Tricine-sodium dodecyl sulfate (SDS) system (26, 56) and was visualized by silver staining. Each well was loaded with the same

TABLE 2. Oligonucleotide primers used in this study[a]

Gene targeted	Primer[b]	Sequence (5′→3′)[c]
Gene deletion		
dam	*dam*::Cm (F)	TTCTCCACAGCCGGAGAAGGTGTAATTAGTTAGTCAG CATGTGTGTAGGCTGGAGCTGCTTC
	dam::Cm (R)	GGCAATCAAATACTGTTTCATCCGCTTCTCCTTGAGA ATTACATATGAATATCCTCCTTAG
Chromosomal *lacZY* fusion		
wzz	*wzz*::Km (F)	TACACTGTCTCCAGCTTCATCCTTTTTTTAGTTAGGGT ATCTAGTGTAGGCTGGAGCTGCTTCG
	wzz::Km (R)	TACCTTTCGAAGCCGACCACCATCCGGCAAAGAAGCT TACATATGAATATCCTCCTTAG
Gene cloning		
wzz	*wzz*-F	GCTTACAAGGCTTTTGGC
	wzz-R	TAGGGTATCTATGACAGTGGAT
Verification of predicted construction		
dam	*rpe*	TACGACAACCTGAACGGTTG
	damX	GCAGCGTGCGGTCAACATG
wzz	*His*	GCGGCCACCGTCAATGATCG
	Ugd	CATTATTTCCAACAGGATGGCGGC
	Kmr	CCATGTTGGAATTTAATCGCGGCC
	*LacZ*revcheck	ACCAGGCAAAGCGCCATTCG
Real-time PCR		
16S rRNA gene	q-16S-F	GCCGCAAGGTTAAAACTCAA
	q-16S-R	AAGGCACCAATCCATCTCTG
wzz	q-wzz-F	CGTCGCTTCGTTCTGTATCA
	q-wzz-R	AGGATGTTACCCAGGACACG

[a] Primers were purchased from Invitrogen Inc. and were designed using the DNA sequence information available for the *Salmonella* serovar Enteritidis strain (*Salmonella* sp. comparative sequencing blast server BLAST Server Database at www.sanger.ac.uk).

[b] F, forward primer; R, reverse primer.

[c] Underlining indicates a sequence homologous to pKD3, pKD4, or pCE36.

LPS concentration, as determined by the keto-deoxyoctulosonic (KDO) assay (43). A densitometry analysis was performed using ImageJ software. The ratio of the relative intensity of the lipid A-core band to the average intensity of the bands corresponding to total O antigen and core + *n* was calculated by quantifying the pixels in a narrow window across the center of each lane. The densitometric analysis was calibrated by determining the ratio of the relative intensity of the lipid A-core region to the average intensity of the O-antigen bands.

β-Galactosidase assays. The expression of the *lacZY* transcriptional fusion was quantified spectrophotometrically as described elsewhere (36, 46). Enzymatic activity, which was expressed in Miller units and normalized for bacterial density (OD_{600}), was calculated using the following equation: $[(A_{420} - 1.75A_{550}) \times 1{,}000]/(\text{reaction time} \times \text{culture volume} \times OD_{600})$, where the reaction time was expressed in minutes and the culture volume was expressed in milliliters. Each sample was analyzed in triplicate for three independent experiments.

Protein analysis. Total membrane fractions were prepared from cells grown in LB medium and harvested at an OD_{600} of 1 as previously described (34). Samples were mixed with 3× protein tracking dye, incubated for 30 min at 45°C, separated on a 14% SDS-polyacrylamide gel electrophoresis (PAGE) gel, and transferred to nitrocellulose membranes. The same membrane was incubated with anti-Wzz affinity-purified polyclonal rabbit antibodies (34) and anti-Flag monoclonal antibodies (Sigma). The reacting bands were detected by fluorescence with an Odyssey infrared imaging system (Li-cor Biosciences) using IRDye800CW affinity-purified anti-rabbit antibodies (Rockland, Pennsylvania) and Alexa Fluor 680 anti-mouse antibodies (Molecular Probes). Densitometry analysis was performed using the Odyssey software and digital images of the membranes, which were incubated simultaneously with both anti-Wzz and anti-Flag antibodies. This resulted in protein bands with two fluorescence colors, and since the anti-Flag antibody cross-reacted with an unknown constitutively expressed membrane protein, we used the density of pixels of this protein band as an internal loading standard for normalization.

RT and real-time PCR. Bacteria were grown at 37°C with agitation to an OD_{600} of 0.6. Cells were lysed, and total RNA was isolated using Trizol reagent (Invitrogen). Contaminating DNA was digested with RNase-free DNase I (Epicentre Biotechnologies), and the purity of all RNA preparations was confirmed by subjecting them to reverse transcription-PCR (RT-PCR) analysis using primers specific for the gene encoding the 16S rRNA (Table 2). After inactivation of DNase, RNA was used as a template for RT. Complementary cDNA was synthesized using random hexamer primers (Invitrogen), deoxynucleoside triphosphates, and Moloney murine leukemia virus reverse transcriptase (Invitrogen). Relative quantitative real-time PCR was performed with an appropriate primer set, cDNAs, and Mezcla Real (Biodynamics) that contained nucleotides, polymerase, reaction buffer, and Green dye, using a Rotor-Gene 6000 real-time PCR machine (Corbett Research). The amplification program consisted of an initial incubation for 3 min at 95°C, followed by 40 cycles of 95°C for 20 s, 60°C for 30 s, and 72°C 20 s. We used primers q-wzz-F and q-wzz-R for *wzz* and primers q-16S-F and q-16S-R for the 16S rRNA gene (Table 2). For the relative gene expression analysis, a comparative cycle threshold method ($\Delta\Delta C_T$) was used (28). The number of copies of each sample transcript was determined with the aid of the software. Briefly, the amplification efficiencies of the genes of interest and the 16S rRNA gene used for normalization were tested. Then each sample was first normalized for the amount of template added by comparison to the 16S rRNA gene (endogenous control). The normalized values were further normalized using the wild-type sample (calibrator treatment). Hence, the results were expressed relative to the value for the calibrator sample, which was 1.

RESULTS AND DISCUSSION

Abnormal O-antigen chain length distribution in a *Salmonella* serovar Enteritidis *dam* deletion mutant. We observed previously that *Salmonella* serovar Enteritidis mutant SD1 expressing a truncated Dam protein (15) does not agglutinate with anti-O9 serum (14). To better understand the role of Dam in LPS expression, we constructed a *dam* deletion mutant of *Salmonella* serovar Enteritidis strain #5694 (SEΔ*dam*) (5).

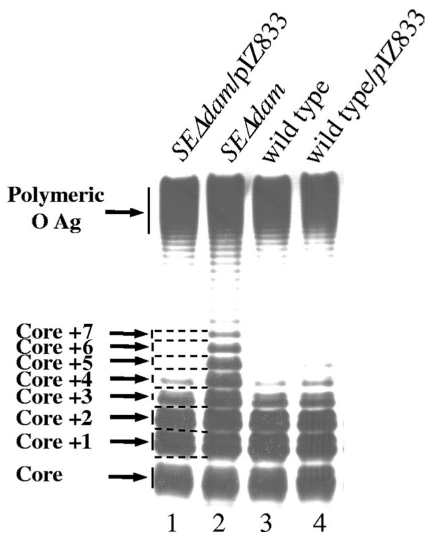


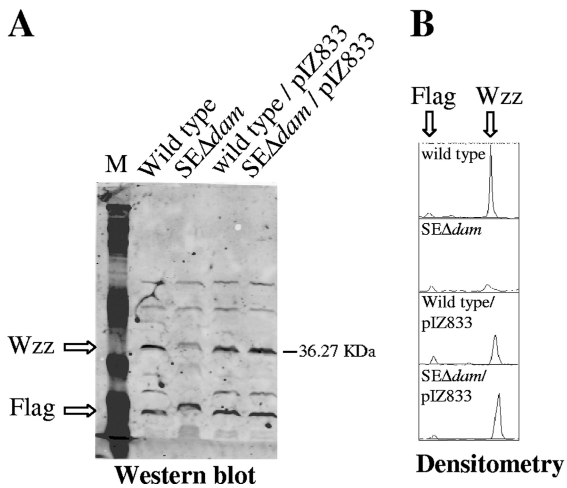
FIG. 1. LPS analysis of *Salmonella* serovar Enteritidis strains. LPS from wild-type strains (wild type and wild type/pIZ833) and *dam* deletion mutants (SEΔ*dam* and SEΔ*dam*/pIZ833) of *Salmonella* serovar Enteritidis were studied. Equal amounts of LPS were loaded in each lane and analyzed by Tricine-SDS-PAGE on a 14% (wt/vol) acrylamide gel, followed by silver staining. The concentration of LPS was determined by measuring the KDO using the purpald assay. Plasmid pIZ833 bears the *dam* gene. The gel shown is representative of nine independent experiments. O Ag, O antigen.

The *dam* null phenotype of SEΔ*dam* was verified by digesting its chromosomal DNA with appropriate restriction enzymes. SEΔ*dam* was susceptible to 2-aminopurine and formed filaments when it was cultured in liquid media, both of which are characteristic phenotypes of *dam* mutants (data not shown). Like the SD1 mutant (14, 55), SEΔ*dam* also showed a reduced level of agglutination with anti-O9 serum, as demonstrated by a 1- to 2-min delay in agglutination compared to the parental strain, which agglutinated almost immediately.

The LPS O-antigen profiles of the SEΔ*dam* and parental strains were examined by electrophoresis and silver staining. A different banding pattern was observed for the LPS O antigen from SEΔ*dam*, which had many more visible bands in the intermediate region of the gel (Fig. 1, lane 2) compared to the banding pattern of the wild-type LPS (Fig. 1, lane 1). This region contains O polymers with lower molecular masses, usually consisting of one to five O-antigen units. On the other hand, the amount of molecules in the polymeric O-antigen region was not substantially different (Fig. 1). Therefore, the observed difference in the SEΔ*dam* LPS pattern is consistent with an increased amount of short polysaccharide chains, suggesting that this mutant has an altered O-antigen polysaccharide chain length distribution. To determine if this defect is associated with the absence of Dam function, we also examined the O-antigen banding pattern of the SEΔ*dam* strain containing pIZ833, which contains a functional *dam* gene. The banding pattern of this strain was similar to the parental banding pattern (Fig. 1, lane 4). Densitometric quantification of LPS gels revealed no significant differences between SEΔ*dam* and the wild-type strain in the amount of total O antigen relative to the lipid A-core region (3.365 ± 0.105 and 3.115 ± 0.165, respectively). This result suggests that the shorter polysaccharide chains observed in the mutant are not synthesized at the expense of longer chains. We concluded that Dam methylation has an effect on the O-antigen polysaccharide chain length in *Salmonella* serovar Enteritidis. This phenomenon was not unique to *Salmonella*, as we also found that a lack of Dam affects the LPS pattern in *Escherichia coli*, increasing the amount of shorter polysaccharides (as seen in SEΔ*dam*) and modifying the size of the banding pattern of the O-antigen region (data not shown). Also, Fälker et al. showed that overproduction of Dam in *Yersinia enterocolitica* results in an increased amount of rough LPS molecules (13). Moreover, these authors suggested that Dam methylation affects the stability of shorter LPS species or influences the addition of the first O-antigen units to a growing chain. Taken together, these findings indicate that Dam methylation plays a role in O-antigen LPS expression in enterobacteria.

Dam participates in the regulation of Wzz synthesis. Since Wzz is responsible for the chain length distribution of the O antigen (37, 47), we investigated whether Dam methylation is involved in regulating *wzz* gene expression. Membrane protein preparations from the parental strain, SEΔ*dam*, and a strain complemented with pIZ833 were examined by Western blotting using anti-Wzz serum, and the results of a representative experiment are shown in Fig. 2A. We also treated the blots simultaneously with the anti-Flag antibody, since this antibody cross-reacts with an unknown constitutively expressed membrane protein. Therefore, the density of the pixels for this protein band serves as an internal loading standard. Normalized densitometry analysis showed that the relative level of the Wzz protein produced by the *dam* mutant was threefold lower (32%) than the level of the Wzz protein produced by the parental strain (Fig. 2B). To investigate *wzz* gene transcription, we determined the galactosidase activities produced by the parental strain and the SEΔ*dam* mutant harboring a *wzz*::*lacZY* transcriptional fusion in the chromosome. β-Galactosidase activity was measured using cells harvested from exponential- and stationary-phase cultures (OD_{600}, 0.4 and 1.0, respectively). As shown in Fig. 3, the transcription of the *wzz* gene in SEΔ*wzz*::*lacZ*Δ*dam* was nearly 50% lower in the logarithmic phase and 25% lower in the stationary phase than the transcription in parental strain SEΔ*wzz*::*lacZ* ($P < 0.01$ and $P < 0.05$, respectively). These results were confirmed by RT-PCR, which revealed that *wzz* gene expression was threefold lower in the *dam* mutant (relative amount of mRNA, 0.313 ± 0.025) than in the parental strain. Next, we investigated whether increased expression of *wzz* in SEΔ*dam* could restore the wild-type O-antigen banding pattern. To do this, strain SEΔ*dam* was transformed with plasmid p*wzz* bearing the *wzz* gene under regulation of the tetracycline resistance gene promoter. As shown in Fig. 4, overexpression of *wzz* in SEΔ*dam* reduced the amount of short polysaccharide chains observed in the *dam* mutant (lanes 1 and 2), resulting in an LPS pattern comparable to that observed for the parental strain (lane 3). Transformation with a plasmid vector (pACYC184) resulted in no changes in the O-antigen LPS pattern of strain SEΔ*dam* (Fig. 4, lane 4).

Collectively, the experiments described above demonstrated that Dam activity modulates *wzz* gene expression. Therefore, it is expected that the *wzz* gene locus contains GATC sequences with the potential to be methylated by Dam. Certainly, an in



Strain	Peak	Area	Proportion Wzz / Flag	Relative production of Wzz (%)
wild type	Wzz	40,80	7.55	100
	Flag	5,40		
SEΔ*dam*	Wzz	12,70	2.43	32
	Flag	5,23		
wild type/ pIZ833	Wzz	34,80	4.79	63
	Flag	7,27		
SEΔ*dam*/ pIZ833	Wzz	53,80	13.45	178
	Flag	4.00		

FIG. 2. Wzz protein in *Salmonella* serovar Enteritidis *dam* mutant. Bacterial strains were cultured in LB medium and harvested at an OD_{600} of 1. Bacterial proteins were extracted, and the Wzz protein was analyzed by Western blotting (A). The intensities of Wzz and FLAG bands were determined by densitometry (B). Plasmid pIZ833 bears the *dam* gene. Prestained SDS-PAGE standards (Bio-Rad) were used as molecular weight markers. The data are representative data for three independent experiments. Lane M contained broad-range prestained SDS-PAGE standards (Bio-Rad).

silico analysis (Fig. 5A) revealed the presence of three GATC motifs in the coding sequence of *wzz* and four GATC sequences upstream of this gene. Dam methylation of any of these GATC clusters could regulate *wzz* expression, although through different mechanisms. On the one hand, it has been proposed that a GATC cluster located in the coding sequence of a given gene affects DNA stability, depending on the methylated state (50). On the other hand, Dam methylation in three GATC motifs (18, 59), two GATC motifs (2), or even a single GATC motif (3, 51) located upstream of a given gene can affect the interaction between regulatory proteins and the DNA binding site. Moreover, Wallecha et al. demonstrated that transcription of the *agn43* gene in *E. coli* is regulated by a GATC motif located downstream of the promoter (60). It remains to be determined whether Dam regulation of the *wzz* gene is also mediated in an indirect manner. For instance, Dam methylation could regulate the expression of other genes whose products are involved in *wzz* transcription. It is known that the PmrA/PmrB and RcsC/YojN/RcsB two-component systems independently promote transcription of the *wzz* gene since a *pmrA rcsB* double mutant of *Salmonella* serovar Typhi-

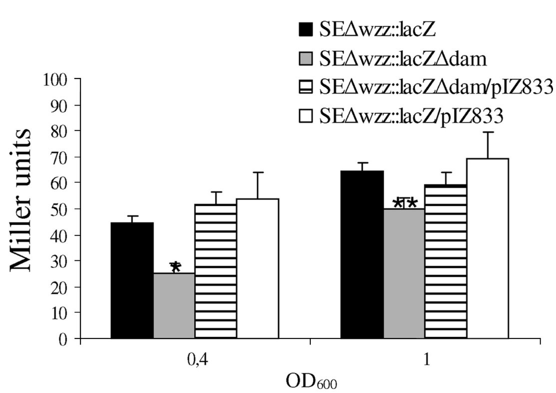


FIG. 3. Activity of *wzz* gene promoter of *Salmonella* serovar Enteritidis strains. The β-galactosidase activities (in Miller units) expressed by strains harboring chromosomal *lacZY* transcriptional fusions to the *wzz* gene in the exponential (OD_{600}, 0.4) and stationary (OD_{600}, 1) phases were determined. The transcriptional activities in SEΔ*wzz*::*lacZ* and in the *dam* deletion mutant SEΔ*wzz*::*lacZ*Δ*dam* of *Salmonella* serovar Enteritidis were investigated. Strains harboring a plasmid bearing the *dam* gene (SEΔ*wzz*::*lacZ*/pIZ833 and SEΔ*wzz*::*lacZ*Δ*dam*/pIZ833) were included as controls. The data are means ± standard deviations of three independent experiments performed in triplicate. *, $P < 0.01$ for a comparison with the wild type; **, $P < 0.05$ for a comparison with the wild type.

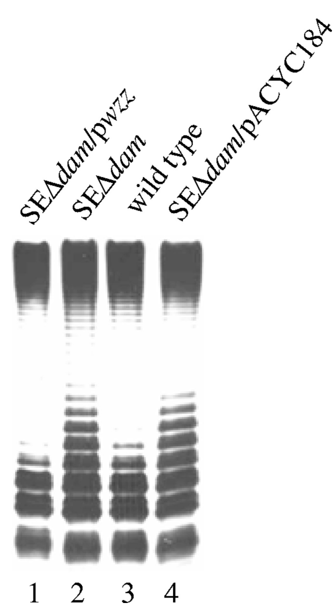


FIG. 4. Production of LPS in *Salmonella* serovar Enteritidis *dam* mutant transformed with the *wzz* gene. Equal amounts of LPS were loaded in the lanes and analyzed by Tricine-SDS-PAGE on a 14% (wt/vol) acrylamide gel, followed by silver staining. The concentration of LPS was determined by measuring KDO using the purpald assay. The data are representative data for seven independent experiments.

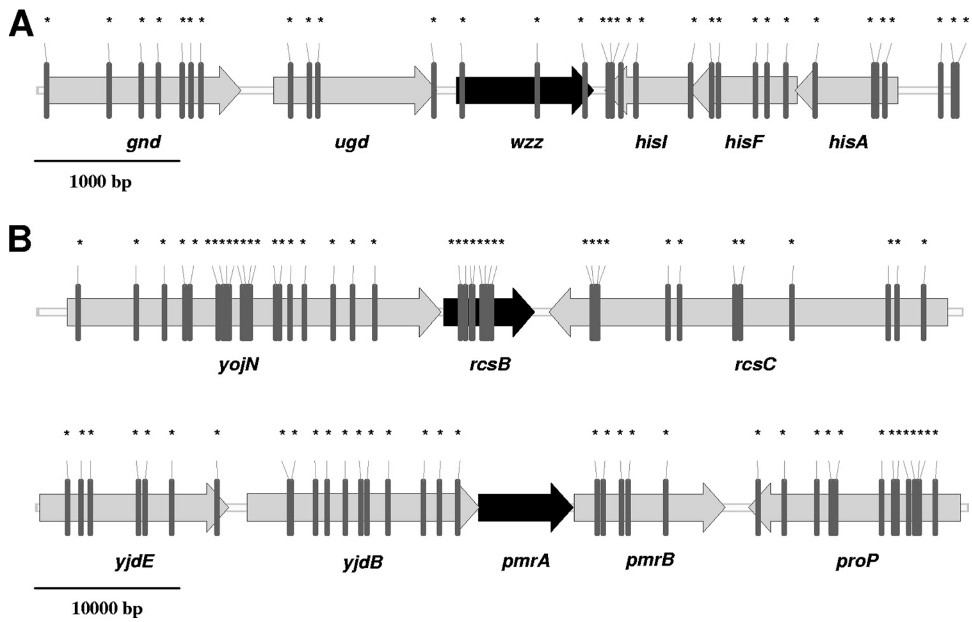


FIG. 5. Distribution of GATC sequences. (A) *wzz* gene region. (B) *pmrA* and *rcsB* gene region. The vertical lines and asterisks indicate the locations of GATC motifs. The diagram is based on the Refseq NC_011294 sequence of *S. enterica* serovar Enteritidis.

murium does not express Wzz (8). In theory, the expression of *pmrA* and *rcsB* could be regulated by Dam methylation since GATC motifs are present upstream of both genes (Fig. 5B). Interestingly, no GATC motifs are present in the *pmrA* sequence, whereas *rcsB* contains eight GATC sequences downstream of the +1 codon in a 224-bp interval, a density threefold higher than the density expected from a random distribution. The regulation of *wzz* in *Salmonella* serovar Enteritidis is highly complex and not completely understood; therefore, further experiments are necessary to investigate whether the expression of *pmrA*, *rcsB*, or a different unidentified factor(s) is regulated by Dam methylation.

In summary, our results unequivocally show that *wzz* gene expression is downregulated in the *dam* mutant of *Salmonella* serovar Enteritidis. The presence of GATC motifs in *wzz*, as well as in the *pmrA* and *rcsB* gene clusters, strongly suggests that Dam methylation is involved in the regulation of LPS synthesis.

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