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Mlc regulation of *Salmonella* pathogenicity island I gene expression via *hilE* repression

Sangyong Lim[2], Jiae Yun[1], Hyunjin Yoon[1], Chehwee Park[1], Boowon Kim[1], Byeonghwa Jeon[1], Dongho Kim[2] and Sangryeol Ryu[1,*]

[1]Department of Food and Animal Biotechnology, School of Agricultural Biotechnology, and Center for Agricultural Biomaterials, Seoul National University, Seoul 151-921, Republic of Korea and [2]Radiation Application Research Division, Advanced Radiation Technology Institute, Korea Atomic Energy Research Institute, Jeongeup 580-185, Republic of Korea

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ABSTRACT

The global regulator Mlc is a repressor of several genes and operons that are involved in sugar uptake and metabolism. A *Salmonella enterica* serovar Typhimurium *mlc* mutant showed reduced levels of invasion and cytotoxicity compared to the wild-type, and exhibited reduced expression levels of *hilD*, *hilA* and *invF*, which are regulatory genes in the *Salmonella* pathogenicity island 1 (SPI1). However, the effects of Mlc on *hilD* expression and bacterial invasiveness were not seen in the *hilE* mutant, and *hilE* expression was increased in the *mlc* mutant, which suggests that Mlc exerts positive effects on the expression of SPI1 genes by reducing the expression of HilE, which is known to down-regulate the expression of SPI1 genes through direct interaction with HilD. We found that the two known promoters of *hilE* were not modulated by Mlc, and we identified a third promoter, designated P3, which was repressed by Mlc. The gel mobility shift assay and footprinting analysis revealed that Mlc repressed *hilE* in a direct manner by binding to two distinct sites in the *hilE* P3 promoter region. The specific down-regulation of *hilD* observed in the presence of Mlc regulon-inducible sugars, such as glucose and mannose, could not be detected in the *mlc* mutant. Based on these results, we propose that Mlc functions to sense the availability of sugars and is linked to virulence gene regulation by its ability to control *hilE* expression in *Salmonella*.

INTRODUCTION

Salmonella enterica serovar Typhimurium is a facultative intracellular pathogen that initiates disease, which is normally limited to gastroenteritis in humans. However, this bacterium causes systemic disease in mice and has been used as an animal model of typhoid fever. Since *Salmonella* is acquired usually by oral ingestion of contaminated materials, a key step in the infection process is passage across the intestinal epithelium by invasion of M cells in Peyer's patches (1). Many of the genes required for intestinal penetration and invasion of host cells are carried on the 40-kb region at centisome 63, which is called *Salmonella* pathogenicity island 1 (SPI1) (2). SPI1 contains at least 37 genes, which encode various components of the type III secretion systems (T3SSs), its regulators and its secreted effectors (3). The effector proteins mediate actin cytoskeleton rearrangement, in the form of large membrane ruffles, which engulf the bacteria into the host cell (4,5).

The expression of SPI1 is controlled in response to a specific combination of environmental signals, including pH, oxygen tension, medium osmolarity, bile, Mg^{2+} concentration, short-chain fatty acids (SCFAS), and growth stage of the bacteria (3,6). Although many factors and a complex mechanism related to environmental stimuli are involved in the modulation of SPI1 genes, they are thought to converge on the activation of several transcriptional regulators encoded within SPI1, such as HilA, HilC, HilD and InvF (7–9). HilA plays a crucial role in the expression of genes that encode the SPI1-T3SS apparatus, the *prg* and *inv*/*spa* operons, by binding upstream of *prgH* and *invF* (10). The expression of *invF* leads to the induction of several effector genes encoded both within SPI1 (*sic*/*sip* operon) and outside SPI1 (*sigD*/*sopB* and *sopE*), with SicA as a co-factor (11). HilC and HilD, which are members of the AraC/XylS family, have been postulated to act as a derepressor of *hilA* expression by counteracting the action of negative regulatory elements at the *hilA* promoter (12,13). However, it has been shown that HilD provides an essential activating function for *hilA* in the absence of negative regulators (14). HilC and HilD also directly activate the alternative

*To whom correspondence should be addressed. Tel: 82 2 880 4856; Fax: 82 2 873 5095; E-mail: sangryu@snu.ac.kr

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promoter of the *invF* operon, which is independent of HilA (15,16).

Similar to HilC and HilD, RtsA activates the expression of SPI1 genes by binding upstream of the master regulatory gene, *hilA*, to induce its expression (9,17). Recently, Baxter and Jones (18) have shown that HilE is an important *Salmonella*-specific regulator of *hilA* expression. A null mutation in *hilE* causes an increase in *hilA* expression and invasion. Using two-hybrid analysis, it has been shown that HilE interacts with HilD, which suggests that HilE represses *hilA* expression by inhibiting the activity of HilD through a protein–protein interaction (19,20). Several other genes have been identified that exert positive or negative effects on these direct regulators of SPI1 in response to the changes in environmental conditions. These genes include those that encode several two-component regulatory systems (PhoP/Q, PhoBR, OmpR/EnvZ and SirA/BarA), post-transcriptional systems (CsrAB, RNase E, PNPase and Lon), nucleoid proteins (HU, FIS, H-NS and Hha), signaling molecules (ppGpp and Np_nN), and other regulatory proteins (FliZ, FadD and CpxA) (6,7,19,21,22).

The phosphoenolpyruvate: sugar phosphotransferase system (PTS) is the major sugar transport system in many Gram-positive and Gram-negative bacterial species. In the animal model, attenuation of virulence has been noted for *Salmonella* strains that carry mutations in the *pts*, *crr*, *cya* or *crp* genes, which encode the general energy-coupling enzymes of the PTS, enzyme IIA^{Glc} of the PTS, adenylate cyclase and cyclic AMP receptor protein, respectively (23,24). Mlc is a global regulator of carbohydrate metabolism and controls several genes involved in sugar utilization (25–27). Therefore, it seemed possible that Mlc also affects the virulence of *Salmonella*. In the present study, a *Salmonella* Typhimurium *mlc* mutant was constructed to investigate the contribution of Mlc to the virulence phenotype. We have found that Mlc activates SPI1 gene expression by repressing *hilE* expression.

Table 1. The bacterial strains and plasmids used in this study

Strains or plasmids	Genotype	References
***S.* Typhimurium**		
SL1344	Wild type, *rpsL hisG*	16
MGS-7	SL5283, r^-m^+ *galE recD*Δ*mutS* Cam^r	28
BJ2462	SL1344, *hilE*::*cam*, Cam^r	20
SR1304	SL1344, *mlc*::*kan*, Kan^r	This study
SR1305	SR1304, *hilE*::*cam*, Cam^r	This study
E. coli		
SR5055	MC4100 *mlc*::*kan*, Kan^r	Laboratory collection
BL21(DE3)	F^- *ompT hsdS$_B$* with a λ prophage carrying the T7 RNA polymerase	Novagen
Plasmid		
pUC19	Amp^r	Laboratory collection
pJB3	*hilD* expression from *lac* promoter in pZC320	14
pJB5	*hilD*::*lacZ* reporter vector, Amp^r	14
pMAB69	*hilE*::*lacZ* reporter vector, Tet^r	18
pKB	pUC19 containing the *mlc* promoter and structure region	This study
pET-15b	Amp^r, N-terminal His-tag vector	Novagen
pET-Mlc	pET-15b containing the *mlc* structure gene	This study

MATERIALS AND METHODS

Bacterial strains and growth condition

The strains used in this study are listed in Table 1. Bacteria were routinely grown in LB broth (1% bactotryptone, 0.5% bacto yeast extract, 1% NaCl) at 37°C. When necessary, the medium was supplemented with ampicillin (100 μg/ml) or kanamycin (100 μg/ml). Tryptone broth (TB) (1% bactotryptone, 0.8% NaCl) buffered with 0.1 M MOPS (pH 7.0) was used to investigate the effect of various carbohydrate sources on the expression of *hilE* and *hilD*. As needed, the following supplements were added to the TB: 0.2% (w/v) glucose, mannose, arabinose or glycerol. Low oxygen bacterial growth condition (SPI-inducing condition) was used to induce invasion gene transcription (8,14). Briefly, a stationary-phase culture that had been grown overnight with shaking was used as the stock culture. The stock culture was inoculated into fresh LB broth at a 1:100 dilution, and grown in static culture to the late exponential phase for 4 h ($OD_{600} \sim 0.6$). The isogenic *hilE*/*mlc* double-mutant strain SR1305 was obtained through P22 HT-mediated transduction to SR1304 of the mutant allele (*hilE*::cam) from the *hilE* mutant strain BJ2462 (18).

Construction of *S.* Typhimurium *mlc* mutant

The MGS-7 strain (Table 1) was used for the transfer by transduction of chromosomal DNA from *E. coli* to *S.* Typhimurium and for efficient recombination into the genomic DNA of the recipient, as strains that carry the *galE* mutation are particularly suitable as hosts for phages P1 and P22 (28). When *Salmonella galE* mutants are grown in the presence of high concentrations (~1%) of galactose and glucose, the LPS produced is the smooth form (P22-sensitive phenotype), whereas when they are grown in the absence of galactose, the LPS produced is rough (P1-sensitive phenotype). *Escherichia coli* phage P1 transductions were performed to transfer the *mlc*::*kan* region of *E. coli* to MGS-7 (*S.* Typhimurium *galE* mutant), and phage P22 transductions were performed from the *mlc*-mutated MGS-7 to the wild-type *S.* Typhimurium SL1344. Mutation of the *mlc* gene was confirmed by PCR.

Plasmid construction

pKB, which contains the *mlc* promoter and structural region, was constructed by inserting the DNA fragment that spans −473 to +1281 relative to the *mlc* translation start codon into the PstI site of plasmid pUC19. An 1750-bp PCR fragment of the *mlc* region was amplified from the chromosomal DNA using the forward primer 5′-GTCTGACAGAACTGCAGGAAGAACCTTTCG-3′ and the reverse primer 5′-GATATGGCAAGGGC CTGCAGCTTGAGTTAG-3′ (*Pst*I site underlined). The pET-Mlc plasmid used for purification of the *Salmonella* Mlc protein was generated by cloning the DNA segment that spans positions −13 to +1306 relative to the translation start codon into the NdeI and BamHI sites downstream of the His-tag element in plasmid pET-15b (Novagen). The PCR fragment of the *mlc* structural gene was amplified using the forward primer 5′-AAAGGGAG TGACATATGGTTGCTGATAGTC-3′ and the reverse primer 5′-AAATAATACAGTGGATCCAGTCTAAGA TAT-3′, which introduced NdeI and BamHI sites (underlined), respectively. The clone was verified by DNA sequencing.

Invasion and replication assays

The HeLa and RAW264.7 cells were cultured in DMEM and the HEp-2 cells were grown at 37°C under 5% CO_2 in RPMI 1640 medium that was supplemented with 10% fetal bovine serum (FBS), penicillin (50 U/ml) and streptomycin (50 U/ml). Confluent monolayers for infection with bacteria were prepared in 24-well tissue culture plates, and *Salmonella* ($\sim 2 \times 10^6$ CFU/well), which were washed with PBS and suspended in pre-warmed medium, were then added to the cell monolayers ($\sim 2 \times 10^5$ cells/well) at a multiplicity of infection (MOI) of 10. Invasion assays were conducted with bacteria that were grown to exponential phase in static cultures, using previously described protocols (29). Survival of the opsonized *S.* Typhimurium strains in RAW 264.7 cells was determined as previously described (30,31). Briefly, aerated cultures grown to stationary phase were opsonized for 15 min in DMEM that contained 10% FBS and 10% normal mouse serum, and the bacteria were then added to the RAW 264.7 cells.

LDH assay for cytotoxicity

Lactate dehydrogenase (LDH) is a cytoplasmic enzyme, the presence of which in the culture medium reflects the loss of plasma membrane integrity. LDH activity in the culture supernatants was measured using the colorimetric Cytotox 96 kit (Promega). The RAW 264.7 murine macrophage-like cell line was seeded into a 96-well plate at a density of 5×10^4 cells/well and incubated for 24 h at 37°C. Before infection with the bacteria, the medium was replaced with serum-free DMEM medium. The cells were infected with *Salmonella* bacteria, which were grown to exponential phase without shaking, at an MOI of 10. After 1 h of incubation, gentamicin (100 μg/ml) was added, to kill the extracellular bacteria. At 4 h post-infection, the culture supernatants were collected for analysis. Cytotoxicity was quantified colorimetrically with the CytoTox 96 kit and the percentage of cytotoxicity was calculated according to the formula: 100 × [(experimental release − spontaneous release)/(total release − spontaneous release)], in which spontaneous release is the amount of LDH activity in the supernatant of uninfected cells, and total release is the LDH activity in macrophage lysates.

β-Galactosidase assay

β-Galactosidase assays were performed according to the standard method of Miller (32).

Primer extension assay

Total RNA was isolated from the bacteria using the Trizol reagent (Life Technologies). To study SPI1 gene expression, the ^{32}P-labeled primers (50 000 c.p.m.) (Table 2) were co-precipitated with 30 μg of total RNA. Primer extension reactions were performed as described by Lim *et al.* (29).

Real-time PCR analysis

Bacterial cells were grown to exponential phase in LB medium without shaking, and then total RNA was isolated using RNeasy Mini Kit (Qiagen). After DNase treatment (Ambion), cDNA was synthesized from 1 μg of RNA using Omniscript RT kit (Qiagen) and random hexamers (Invitrogen) according to the manufacturer's instruction. Quantification of cDNA was carried out using IQ SYBR Green PCR Supermix (Bio-Rad), and real-time amplification of PCR product was analyzed using iCycler IQ™ (Bio-Rad). The amplification program consisted of one cycle at 95°C for 5 min, followed by 40 cycles at 95°C for 30 s, 52°C for 30 s, and 72°C for 30 s. The relative amount of cDNA was calculated using a standard curve obtained from PCR on serially diluted genomic DNA as templates. mRNA expression levels of target genes were normalized to 16S rRNA expression level. The sequences of the primers used are presented in Table 3.

Purification of the Mlc protein

Cultures of *E. coli* strain BL21(DE3) that carried pET-Mlc were grown at 37°C in LB that was supplemented with ampicillin (100 μg/ml). Mlc under the control of the T7 promoter was induced with 0.01 mM isopropyl-β-D-thiogalactopyranoside (IPTG) in the early log phase of growth. After induction, the cells were allowed to grow for 5 h, and were then harvested by centrifugation. The N-terminal-His6-tagged Mlc protein was purified from cell extracts by Ni^{2+} affinity chromatography, according to the Novagen standard protocol.

Gel mobility shift assay

The 340-bp *hilE* promoter DNA fragment (−581 to −241) was amplified by PCR using the hilE-F2 (5′-GTAGCGTTGGATCGTTTCGTGTTC-3′) and hilE-R2 (5′-TCCACCGAATCGGAATATAGACAATTC-3′) primers. The PCR product was purified from an agarose gel using a gel extraction kit (Qiagen), and labeled with [γ-^{32}P] ATP. The binding buffer for this assay contained

Table 2. Oligonucleotide primers used for primer extension analysis

Gene	Nucleotide sequences (5′ → 3′)	Complementary region[c]
hilA	TAATAATATTGTTATAACTAACTGTGATTA	~−216 to −245
invF$_A$	CATTGTGTCGGCTTTCAGAAAATGACATAT	~−1 to +28
invF$_D$	GGAGTTAATATGAAAAAATTTTATAGCTGT	~−447 to −476
hilC	GGAAATTTGTTCGGCTGTTGAAGGTGATTA	~+45 to +74
hilD	TTTAATTTGCTGCCGGGTATTTGTCAAAAG	~+73 to +102
hilE	CAATGAAAGAACGTTCCATTTTCCAGCCA	~+2 to +30[a]
hilE	ATATCAATATCATTTCTTATTTTTATCCGA	~−188 to −217[b]
prgH	CTGTCAGCAATGGAAACTCACAGCCGTTCA	~+71 to +100
sigD	AGGTTTTTTGTAGGCTTTTAAAAGCCTCCT	~+44 to +73

[a]hilE3 primer used for the analysis of P1 and P2 transcript.
[b]hilE5 primer used for the analysis of P3 transcript.
[c]Numbers of nucleotides were determined relative to translational start site respectively.

Table 3. Primers for real-time PCR analysis

Gene	Primer sequence (5′ to 3′)		Size (bp)
	Forward	Reverse	
hilA	GTCCGGTCGTAGTGGTGTCT	CGGCAGTTCTTCGTAATGGT	182
invF	TGTCGCACCAGTATCAGGAG	AAATAGCGCGAAACTCAGGA	155
hilE	AAAGCCGGATCAAAGGTTTT	CTTTCACCGTTTTCCCGTTA	180
rrs	CGGGGAGGAAGGTGTTGTG	CAGCCCGGGGATTTCACATC	178

20 mM Tris-acetate (pH 8.0), 3 mM magnesium acetate, 200 mM potassium glutamate, 100 μg/ml BSA, 1 mM dithiothreitol and 1% sucrose. For the Mlc–DNA binding interactions, 2 nM of labeled DNA fragments were mixed with the Mlc protein and 100 ng of poly dI-dC (Amersham Pharmacia) as the DNA competitor in 20 μl of buffer. For competition assays, an excess of unlabeled probe was added. The binding mixture was incubated at room temperature for 15 min and analyzed by electrophoresis on a 6% polyacrylamide gel.

DNase I footprinting

A DNA fragment that contained the *hilE* promoter was amplified by PCR using [γ-^{32}P]ATP-labeled hilE-F2 primer and unlabeled hilE-R2 primer. The PCR product was purified from an agarose gel using a gel extraction kit (Qiagen). Mlc–DNA binding was performed in 40 μl of binding buffer under the conditions used in the gel mobility shift assay. DNase I solution (5 μl; 10 ng DNase I per reaction) was added to the binding mixture, which was then placed at room temperature for 1 min. DNase I activity was terminated by the addition of 200 μl of stop solution that contained 0.4 M sodium acetate, 10 mM EDTA and 100 μg/ml yeast tRNA. After phenol extraction and ethanol precipitation, the pellet was dissolved in sequencing dye, and resolved on a 6% polyacrylamide gel that contained 8 M urea.

RESULTS

Mutation of the *mlc* gene reduces *Salmonella* invasiveness

The process of invasion into non-phagocytic epithelial cells, which is known to be mediated by proteins secreted by the SPI1-encoded type III machinery (3), is an important initial step in the pathogenesis of *S.* Typhimurium. We examined the ability of an *mlc* mutant, SR1304, to invade cultured HEp-2 and HeLa epithelial cells. SL1344 was used as the control strain, and the invasiveness of this strain was arbitrarily set at 100%. When the *Salmonella* bacteria were grown in static cultures to the exponential phase (SPI1-inducing condition), the invasiveness of SR1304 for both epithelial cell lines was reduced by about 3-fold, compared to that of SL1344 (Figure 1A and B).

S. Typhimurium induces the apoptosis of infected macrophages. This process is rapid, specific and depends on the T3SS encoded within SPI1 (33,34). To discover whether the *mlc* mutant grown under the SPI1-inducing condition affects the capacity to induce apoptosis in macrophages, the release of the cytoplasmic enzyme LDH was determined in a cytotoxicity assay (33). Figure 1C shows that SL1344 killed 42% of the macrophages, whereas SR1304 killed only 16% of the macrophages 4 h after infection, which demonstrates that the *mlc* gene plays an important role in the expression of genes involved in cytotoxicity. We also tested the effect of the *mlc* mutation on the ability to replicate within the RAW264.7 macrophage cell line. After cultivation to the stationary phase with aeration, the bacteria were opsonized with 10% normal mouse serum and added to the cell monolayer (30). When the number of intracellular bacteria were measured at 2 and 18 h post-infection, the degree of replication was similar for SL1344 and SR1304 (Figure 1D). Collectively, these results suggest that the main target of Mlc action in *Salmonella* virulence is SPI1, which is known to encode various genes that are required for *Salmonella* invasion of host cells.

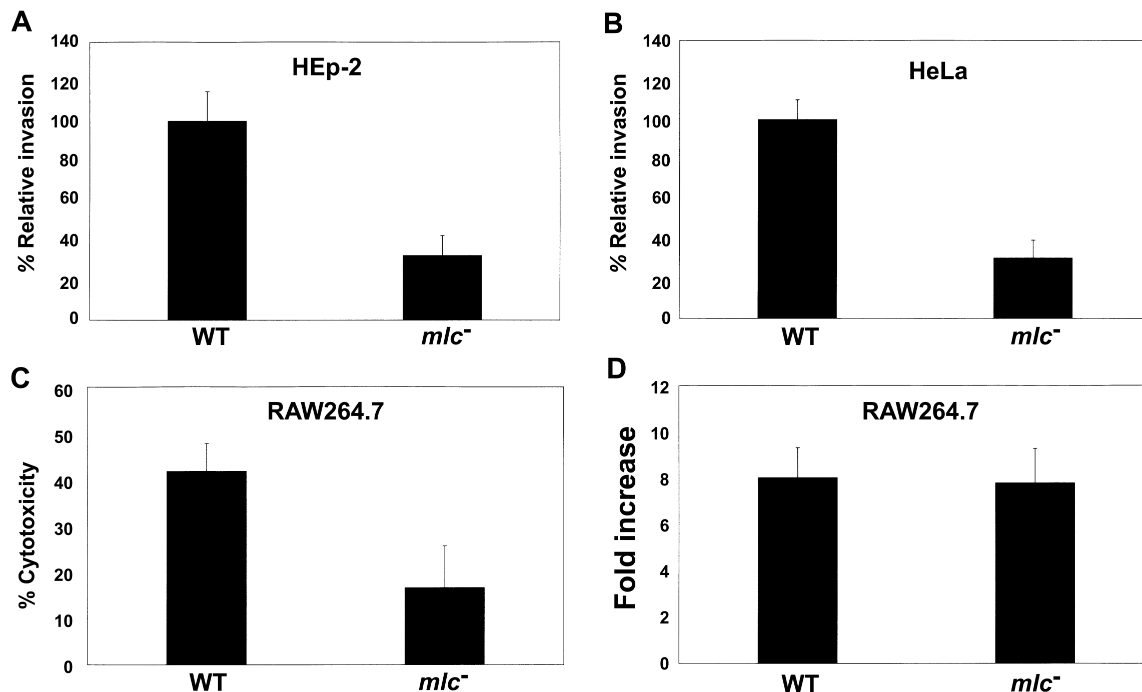

Figure 1. Analysis of the virulence of the *Salmonella mlc* mutant strain. All of the cell infection experiments were carried out with wild-type (SL1344) and *mlc*$^-$ mutant (SR1304) strains. HEp-2 (**A**) and HeLa cells (**B**) were infected with *Salmonella* grown to exponential phase without shaking. Infected cells were lysed 2 h after infection, and dilutions of the suspension were plated onto LB agar medium, to enumerate colony-forming units (CFUs). The data are presented as percentages of the CFU of the wild-type strain. (**C**) For the cytotoxicity assay, RAW 264.7 macrophage cells were infected with *Salmonella* grown to exponential phase without shaking, and then assayed for LDH release. (**D**) The intracellular replication assay was carried out with opsonized bacteria grown to stationary phase under aerobic conditions. After 2 and 18 h of infection, the eukaryotic cells were lysed and the viable intracellular (gentamicin-protected) bacteria were counted. The values shown represent fold-increases, which were calculated as the ratios of intracellular bacteria 2 and 18 h after bacterial entry. These assays were performed at least twice in triplicate, to allow calculation of means and standard deviations.

The expression levels of *hilA*, *invF* and *hilD*, but not of *hilC*, are decreased in the *mlc* mutant

To identify the target gene in SPI1 that is regulated by Mlc, the expression patterns of the SPI1-regulatory genes, which include *hilA*, *invF*, *hilC* and *hilD*, were examined by primer extension analysis of total RNA from SL1344 and SR1304 grown statically to the exponential phase. The transcriptional start sites of these genes have been reported previously (12,16,35,36). SR1304 exhibited a >10-fold reduction in *hilA* and HilA-dependent *invF* ($invF_A$) expression, and about a 3-fold reduction in *hilD* and HilC/D-dependent *invF* ($invF_D$) expression, compared to SL1344. However, *hilC* expression was unchanged (Figure 2A). We also observed reduced expression of the *prgH* and *sigD* genes, which are known to be regulated by HilA and InvF (10,11,29,35), in SR1304 (Figure 2B). The expression levels of *hilA* and *invF* were previously shown to be affected by both HilC and HilD (8,9,16) but since *hilC* levels are unaffected by the *mlc* mutation (Figure 2A) we speculated that Mlc was regulating *hilA* and *invF* expression indirectly probably by modulating the expression of *hilD* or the higher regulator of *hilD*.

Mlc modulates the expression of SPI1 genes by repressing *hilE*

Subsequently, we studied whether *hilE* expression was affected by Mlc. Baxter *et al.* (20) have found that HilE plays an important regulatory role in the expression of the *Salmonella*-invasive phenotype by affecting *hilA* transcription through direct interaction with HilD. To study the effect of Mlc on *hilE* expression, we measured the expression of *hilE-lacZY* on the low-copy plasmid pMAB69, which contains the *hilE* promoter region from −886 to +121, relative to the translation start site. When these cells were grown in static cultures to exponential phase, SL1344-pMAB69 expressed 28.9 ± 3.5 Miller units of β-galactosidase, whereas SR1304-pMAB69 expressed 49.5 ± 5.3 Miller units of β-galactosidase; the level of *hilE* expression in the *mlc* mutant was almost 1.8-fold higher than that in the wild-type strain. The *hilE* mRNA level measured by real-time PCR also revealed that *hilE* expression was increased in SR1304 by about 2-fold (Figure 3A). These results suggest that Mlc can act as a negative regulator of *hilE*.

The role of HilE in the regulation of SPI1 gene expression by Mlc was further studied by comparing the invasive abilities of the *hilE* and *hilE*/*mlc* mutant strains of *Salmonella*. Invasiveness for HEp-2 cells was reduced to 39.1% of the wild-type level by *mlc* mutation, whereas it was increased by 1.57-fold by *hilE* mutation (Figure 3B). However, the *hilE*/*mlc* double mutant showed an almost similar invasion ability to that of the *hilE* mutant (Figure 3B), which suggests that the effect of the *mlc* mutation on the invasive phenotype is mainly dependent

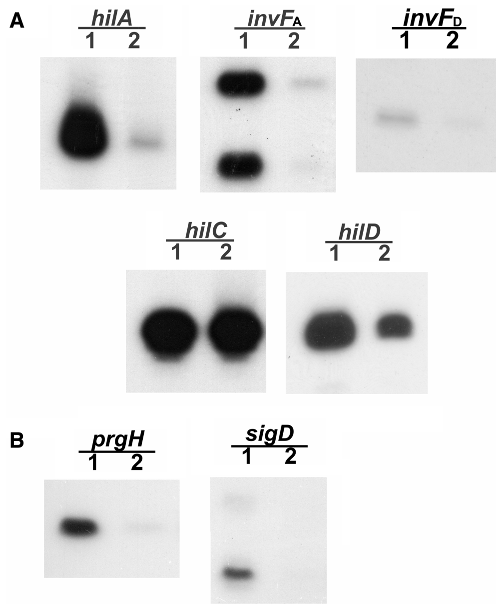

Figure 2. Effects of *mlc* mutation on the expression of SPI1-regulatory genes (**A**) and apparatus/effector genes (**B**) in *S.* Typhimurium, as analyzed by primer extension analysis. Total RNA was prepared from SL1344 (lane 1; wild type) and SR1304 (lane 2; *mlc*$^-$ mutant), which were grown to exponential growth phase in LB medium without shaking. Aliquots of 30 μg of total RNA were co-precipitated and annealed with end-labeled primers. Reactions were performed as described in the Materials and Methods section. The products were resolved on a 6% sequencing gel.

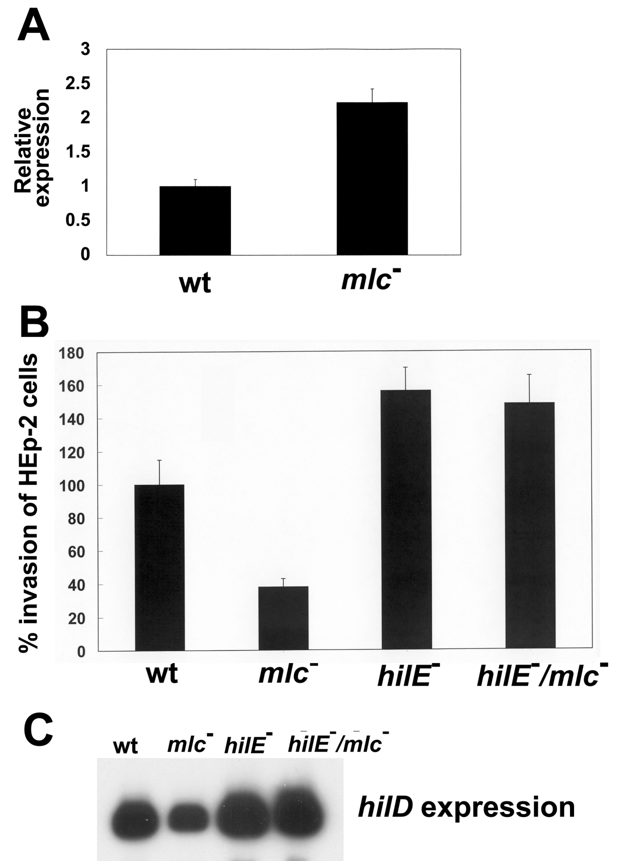

Figure 3. Effects of the *hilE*/*mlc*/double mutation on *Salmonella* invasiveness and *hilD* expression. (**A**) mRNA level of *hilE* gene determined by real-time PCR analysis. SL1344 (wt) and SR1304 (*mlc*$^-$) were grown in LB medium without shaking. Total RNA was isolated from aliquots of cells obtained at the exponential phase. Expression levels of the target genes were normalized to that of 16S rRNA gene. Real-time PCR assay was performed three times in duplicate. (**B**) HEp-2 cells were infected with SL1344 (wild-type), SR1304 (*mlc*$^-$ mutant), BJ2462 (*hilE*$^-$ mutant) and SR1305 (*hilE*$^-$/*mlc*$^-$ double mutant), which were grown to exponential phase without shaking. The CFU of intracellular bacteria were assessed 2 h after infection. The values are presented as percentages of the wild-type invasion, which was set at 100%. This assay was performed at least twice in triplicate, to allow calculation of means and standard deviations. (**C**) Thirty micrograms of total RNA from the indicated strains, extracted at the exponential growth phase in LB medium, were subjected to primer extension analysis.

upon HilE function. The requirement of *hilE* for Mlc function was reflected in the levels of *hilD* transcription. While *hilD* expression was reduced in the *mlc* mutant, it was slightly higher than the wild-type level in both the *hilE* and *hilE*/*mlc* mutant strains (Figure 3C). Additionally, the over-expression of HilD from the plasmid pJB3 containing *hilD* gene under the control of a *lac* promoter caused the induction of *hilA* by ~2-fold and *invF* by ~6-fold irrespective of *hilE* or *mlc* mutation when real-time PCR was used to compare the expression levels (data not shown). Since HilD activates the transcription of *hilA* (14), which in turn can activate HilA-dependent $invF_A$ expression (10), and directly activates HilC/D-dependent $invF_D$ expression (16), these results establish that the *mlc* mutation exerts a negative effect on SPI1 gene expression, mainly by increasing the level of *hilE* expression. The low level of *hilD* expression in the *mlc* mutant can be explained by the fact that the increased level of HilE in the *mlc* mutant can repress the activity of HilD, which is known to act as an activator of its own expression (9,36).

The *hilE* P3 promoter is repressed by Mlc

Baxter and Jones (18) have identified two independent *hilE* promoters, P1 and P2. We performed a primer extension assay of total RNA isolated from SL1344 grown to exponential phase in static culture, using the primer described by Baxter and Jones (18), to discover which promoter is regulated by Mlc. The transcriptional start site of one transcript, designated P1, agreed with the previous report, although the promoter activity was very low under the conditions used in our experiment (Figure 4A). We detected a stronger transcript, designated P2, upstream of P1, although the transcription start site of P2 was mapped 12 nucleotides upstream of that reported by Baxter and Jones (18) (Figures 4A and 5C). This difference could be attributed to differences in the experimental model conditions; in our study, total RNA from a *Salmonella* strain was used in the primer extension assay, whereas Baxter and Jones (18) used total RNA prepared from *E. coli* harboring the *hilE* reporter plasmid pMAB69. However, the activities of the two promoters were not changed by *mlc* mutation. Using a primer extension assay with the hilE5 primer (Table 2), the promoter of *hilE*, which is designated as P3, was newly

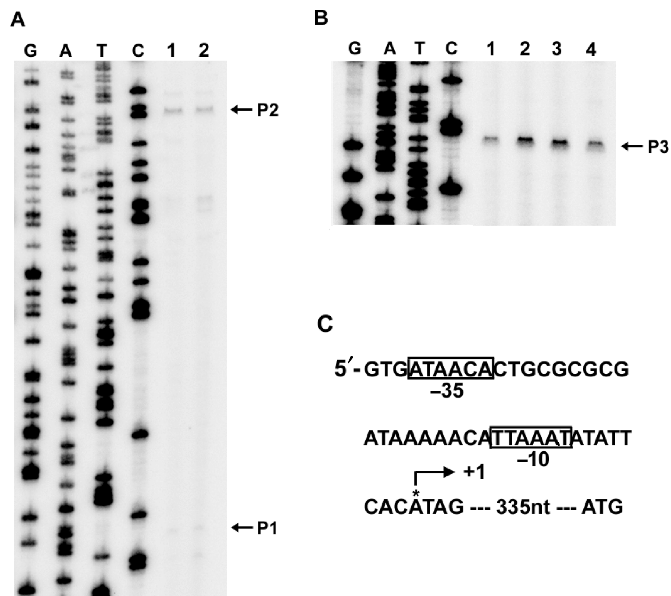

Figure 4. Identification of an Mlc-regulated *hilE* promoter. Primer extension analysis was performed using total RNA from SL1344 (lane 1: wild type), SR1304 (lane 2: *mlc*$^-$ mutant), SR1304 harboring pUC19 (lane 3) and SR1304 harboring pKB (lane 4; Mlc over-expression), all of which were grown to exponential phase in LB medium without shaking. Aliquots of the RNA (30 µg) were subjected to primer extension analysis, and a sequence ladder was generated using the same end-labeled primer that was used for the primer extension analysis. (**A**) The effects of the *mlc* mutation on the P1 and P2 promoters were examined using the hilE3 primer (see Table 2). (**B**) The transcriptional start site of the new promoter (P3), which is modulated by the *mlc* mutation, was identified using the hilE5 primer (see Table 2). (**C**) The start site (+1) is indicated by an arrow, and the promoter elements that resemble the consensus −10 and −35 sites are boxed.

identified; this promoter initiates 335 nucleotides upstream of the translation start site of *hilE* (Figure 4B, lane 1). We observed putative −10 (TTAAAT) and −35 (ATAACA) motifs for *hilE* P3 (Figure 4C) (37). Transcription from the P3 promoter was increased substantially by *mlc* mutation (Figure 4B, lane 2). The specificity of the Mlc effect was verified by complementation, as the plasmid pKB, which expresses *mlc*, restored the transcriptional level of *hilE* in SR1304 almost to that of the wild-type strain (Figure 4B, lane 4).

Mlc directly represses *hilE* by binding to the P3 promoter

A gel mobility shift assay was performed with purified Mlc, to test whether Mlc directly represses the *hilE* P3 promoter. The Mlc-His6 protein was produced in *E. coli* and purified to >90% homogeneity by Ni^{2+} affinity chromatography. The activity of the purified Mlc-His6 protein was verified with the control gel mobility shift experiment employing the *Salmonella ptsG* DNA fragment that contains the known Mlc-binding site (data not shown). When the labeled *hilE* promoter DNA was incubated with 2-fold dilutions of the purified Mlc-His6 protein (100–800 nM) in the presence of the non-specific DNA competitor poly dI-dC, the concentration-dependent formation of protein–DNA complexes was observed (Figure 5A). The addition of cold probe released the labeled probe from the retarded complex (Figure 5A, right panel), which indicates specific binding of Mlc to the *hilE* P3 promoter DNA.

The precise locations of the Mlc-binding sites were determined by DNase I footprinting with His-tagged Mlc. Two Mlc-binding sites, one at position −10 to +12 (Mlc 1) and the other at position −86 to −108 (Mlc 2) with respect to the transcriptional start site of *hilE* P3, were identified (Figure 5B). Inspection of the DNA sequences at these sites showed high-level homology with the known consensus Mlc-binding sequence, which has the conserved TT-9 bp-AA motif and an AT-rich region at positions ±7 to ±11, showing imperfect dyad symmetry (38) (Figure 5C). These results clearly demonstrate that Mlc can regulate directly the *hilE* P3 promoter by binding to the promoter.

Sugars that induce the Mlc regulon repress *hilD* expression by activating *hilE*

Sequestration of Mlc by the unphosphorylated form of glucose permease, enzyme $IICB^{Glc}$, is known to displace Mlc from its DNA-binding sites, thereby allowing transcription of its target genes (25–27). The Mlc regulon can be induced by glucose and, to a lesser extent, by mannose (39). The effects of various carbon sources on the expression of *hilE* and *hilD* were studied with two Mlc regulon-inducing sugars, glucose and mannose, and two Mlc regulon-non-inducing carbohydrates, arabinose and glycerol. SL1344 and SR1304 carrying either pMAB69 (*hilE-lacZY*) or pJB5 (*hilD-lacZY*) were cultivated in TB that was buffered to pH 7.0 with 0.1 M MOPS, to minimize pH effects arising from growth in the presence of the various carbon sources. In the wild-type strain, glucose and mannose increased *hilE* expression 2-fold and *hilE* activation reduced *hilD* expression (Figure 6). The expression levels of *hilE* and *hilD* remained almost unaffected in the presence of glycerol. Interestingly, arabinose also activated *hilE*, albeit to a lesser degree than either glucose or mannose. Nevertheless, this caused a minute increase in *hilD* expression. In the absence of sugars, the *mlc* mutation increased *hilE* expression 1.5-fold, with a concomitant reduction in *hilD* expression. On the other hand, the expression in SR1304 of both *hilE* and *hilD* was increased in the presence of glucose, mannose or arabinose. These results imply that multiple regulatory pathways are involved in the regulation of *hilE* and *hilD* by carbohydrates.

DISCUSSION

Analysis of the *Salmonella mlc* mutant revealed that invasiveness for epithelial cells was impaired by the *mlc* mutation (Figure 1). It is well known that SPI1 is required for the invasion of host cells and induction of macrophage apoptosis (2,3,40). The genetic evidence to date is consistent with a regulatory cascade of transcriptional activation, in which HilD, HilA and InvF act sequentially to activate SPI1 expression. In brief, HilD binds directly to sites upstream of *hilA* and $invF_D$ and acts as an activator (14,16,36). HilA activates the transcription of

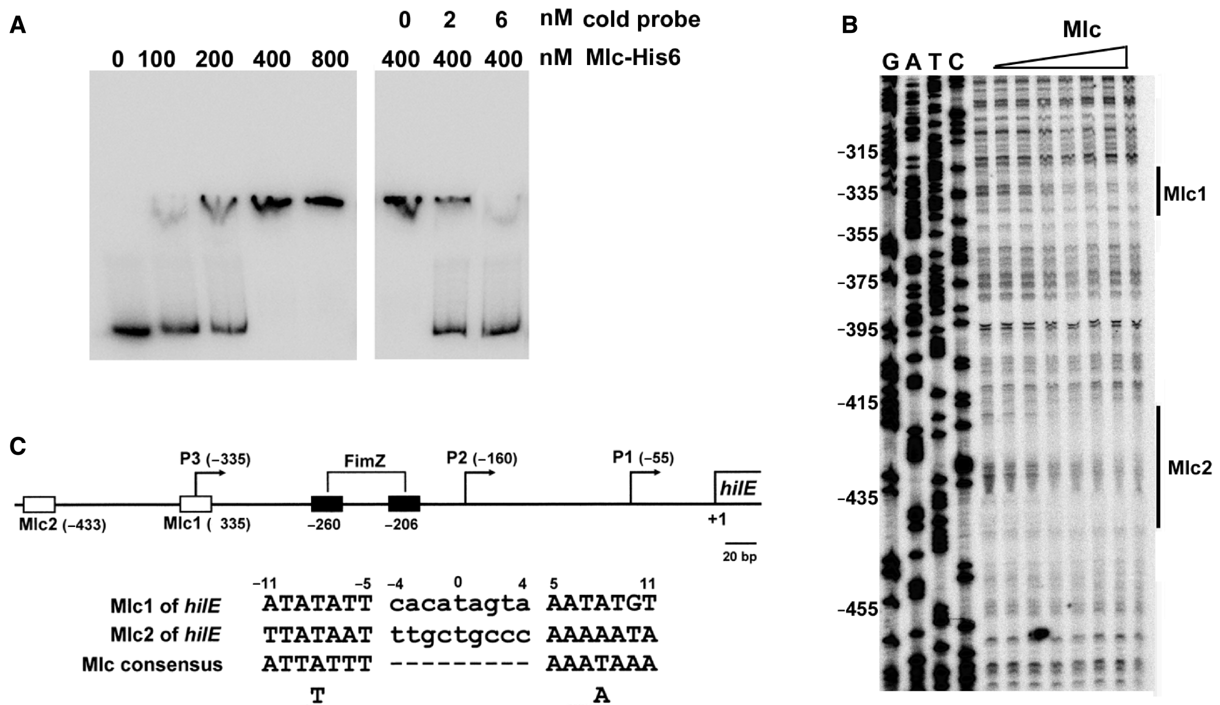

Figure 5. The Mlc protein binds to the *hilE* promoter. (**A**) Gel mobility shift assay of *hilE* promoter DNA with the purified Mlc-His6 protein. Labeled *hilE* promoter DNA (2 nM) was incubated with 100 ng of poly dI-dC DNA competitor and various amounts of Mlc-His6, as indicated at the top of the left panel. Labeled DNA (2 nM) was mixed with 400 nM of Mlc-His6 and various concentrations of unlabeled *hilE* promoter DNA (cold probe), as indicated at the top of the right panel. The DNA–protein complexes were resolved by electrophoresis in a 6% polyacrylamide gel. (**B**) DNase I footprinting analysis of the *hilE* promoter DNA was performed with a probe for the non-coding strand. The *hilE* promoter DNA was incubated with purified Mlc-His6, which was diluted through a 2-fold series of dilutions in 1× binding buffer to the desired concentration (lanes 1–8: 0, 18.75, 37.5, 75, 150, 300, 600 and 1200 nM, respectively). The protected regions of the two Mlc sites are indicated with solid vertical lines and marked as Mlc1 and Mlc2. The numbering on the left is based on the translational start site for *hilE*. (**C**) Schematic representation of the locations of the promoters and putative protein-binding sites in the *hilE* promoter. The numbering is relative to the translational start site for *hilE*. The transcriptional start sites of P1 (−55), P2 (−160) and P3 (−335) are shown with arrows. The binding sites for Mlc and FimZ are shown by open and black boxes, respectively, and the numbers under the boxes indicate the centers of the binding sites. The sequence of each Mlc-binding site in the *hilE* promoter region is numbered relative to the center of the binding site and is indicated along with the consensus sequence for the Mlc-binding site. The locations of the P1 and P2 promoters and FimZ-binding sites have been reported by Baxter and Jones (18). The map is drawn to scale.

$invF_A$ and SPI1-T3SS apparatus genes required for the secretion of effector proteins, whereas the expression of effector proteins is regulated by InvF (10,11). This regulatory cascade implies that the reduced invasive phenotype of *mlc* mutant is the result of *hilD* repression, which affects both *hilA* and *invF* expression (Figures 1 and 2). The 2–3-fold decrease in *hilD* expression caused by the *mlc* mutation is sufficient to account for the nearly 10-fold decrease in *hilA* and $invF_A$ transcription, owing to the nature of the feed-forward regulatory loop, in that moderate effects on HilD production are amplified (9). Although the *Salmonella mlc* mutant was less invasive for epithelial cells than the wild-type strain, the extent of the reduction in invasiveness and cytotoxicity of the *mlc* mutant was not as great as we had expected, considering the observed reduction in SPI1 gene expression (Figure 2). This may be partly due to the expression of *hilC*, a de-repressor of *hilA*, which was not reduced in the *mlc* mutant (Figure 2A). The *hilC* mutation had little effect on SPI1 gene expression or the invasive phenotype, whereas the over-expression of *hilC* suppressed the *hilD* mutation, thereby promoting high levels of *hilA* and *invF* expression, which suggests that HilC can partially compensate for the loss of function of HilD and the invasive phenotype (8,9,15). We have reported previously that there may be an independent *hilC* regulatory pathway that is not applicable to *hilA* or $invF_A$ regulation when bacteria respond to changes in osmolarity (29).

Our data clearly demonstrate that Mlc directly regulates *hilE* expression by binding to the *hilE* P3 promoter (Figure 5). The two Mlc-binding sites in *hilE* P3 identified by the gel mobility shift assay and DNase I footprinting analysis show high-level homology with the known consensus Mlc-binding sequences; additionally, the two Mlc-binding sites are separated by 98 nt, as is the case for other promoters that have two Mlc-binding sites (41). The transcriptional start site of the *hilE* P3 promoter lies 335 nt upstream of the ATG start codon of *hilE* (Figures 4B and 5C). It is unusual that the 5′-untranslated region (UTR) of mRNA is more than 300 nt in bacteria (42). However, this length of 5′-UTR has been observed in other SPI1 regulators. The HilC/D-dependent transcriptional start site of the $invF_D$ promoter is 631 bp upstream of the *invF* open reading frame (16). The UTR of the *hilA* gene is also up to 350 nt in length, and is suggested to be involved in the complex regulation of *hilA* in response

to environmental signals (8,12). It has been demonstrated that the region 190–270-bp upstream of the *hilE* promoter is required for the activation of *hilE* P2 expression by FimYZ, which is a response regulator that is involved in the expression of type 1 fimbriae and motility genes (18, Figure 5C). Therefore, we cannot rule out the possibility that additional *cis*- or *trans*-acting regulatory elements, which have not yet been characterized, are involved in *hilE* expression.

Glucose and mannose induce the Mlc regulon (39) by dephosphorylating $EIICB^{Glc}$, which then sequesters Mlc from its binding sites (27,43). It has been suggested that glucose plays a negative role in the expression of invasion-associated genes in *Salmonella*. The addition of excess glucose to the culture medium results in the reduction of cell association by *S.* Typhimurium (44). The glucose present in DLB (LB broth diluted 1:5) decreased *hilA* expression 2.5-fold, as compared to DLB without glucose, while lactose and arabinose had little effect on *hilA* expression (45). The results presented in this study support the notion that *hilE* expression is activated as a result of Mlc sequestration by unphosphorylated $EIICB^{Glc}$ in the presence of glucose. We observed a 2-fold activation of *hilE* and concomitant repression of *hilD* in the presence of glucose or mannose, which are known inducers of the Mlc regulon, when TB was used (Figure 6). However, the effect of the sugar was not seen when LB was used (data not shown). We speculate that the nutrient-rich LB broth may mask the effect of the sugar on *hilE* expression because the effect of sugar addition on *hilA* expression was more distinct in DLB than LB (45). In addition, *hilE* expression was also increased in the presence of arabinose, which suggests an additional regulatory mechanism mediated by arabinose.

Interestingly, carbohydrate regulation of *hilE* and *hilD* expression in the *mlc* mutant created a different story. All of the carbohydrates tested in this study, with the exception of glycerol, increased *hilD* expression by ~1.1–1.2-fold, even though *hilE* expression in the *mlc* mutant was increased by ~30% in the presence of the sugars tested (Figure 6). These results suggest that the presence of complex regulatory mechanisms for *hilE* is required for optimal regulation of the SPI1 genes, as can be expected from the complex regulatory networks identified in *Salmonella* (46). It has been suggested that the two-component regulatory system PhoR-PhoB leads to increased *hilE* P2 expression and subsequent repression of *hilA* and invasion genes (7,18). The PhoR sensor kinase phosphorylates PhoB when extracellular P_i levels are low, and the phosphorylated PhoB then binds and activates the promoters in the Pho regulon. However, even in the absence of PhoR, many carbon sources are known to activate the Pho regulon via CreC, which is a PhoR homolog (47). Thus, carbon metabolism sensed by PhoB/R may also affect *hilE* expression (22), and this may be one of the reasons for the elevated *hilE* expression in the presence of carbohydrates in the *mlc* mutant (Figure 6A).

Recently, Teplitski *et al.* (48) have reported that the expression of *sirA*, which is a response regulator for BarA (49), is decreased in the presence of 50 mM glucose. The repressive effect of glucose was also observed for the

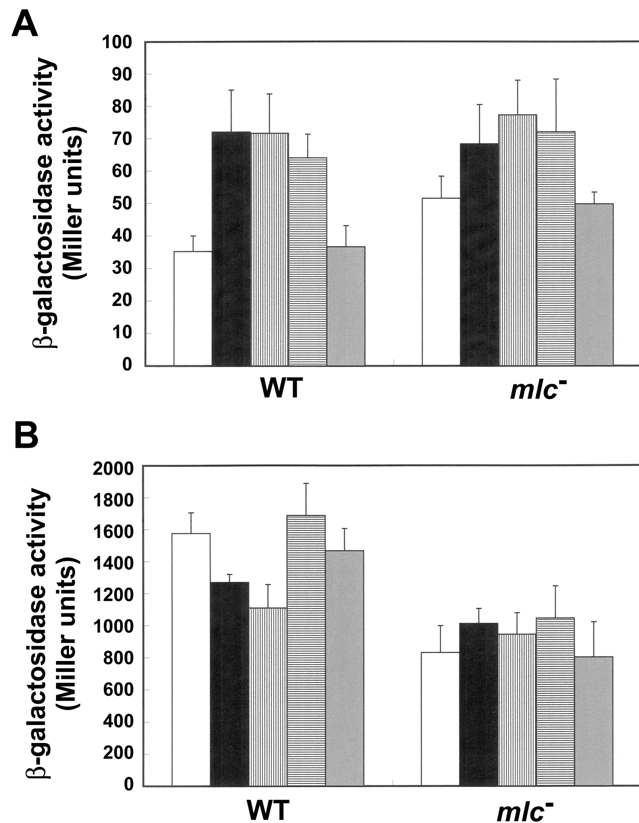

Figure 6. Effect of various carbohydrates on *hilE* and *hilD* expression levels. The β-galactosidase activities were measured for (**A**) the *hilE-lacZY* reporter plasmid pMAB69, and (**B**) the *hilD-lacZY* reporter plasmid pJB5, in both SL1344 (wild-type) and SR1304 (mlc^- mutant). Cultures were grown to exponential phase in MOPS-buffered TB medium in static cultures without carbohydrate (white bars) or with 0.2% glucose (black bars), mannose (vertical hatched bars), arabinose (horizontal hatched bars) or glycerol (gray bars). The β-galactosidase assays were performed at least three times.

downstream members of the SirA regulon, such as *csrB*, *csrC* and *hilA* (48,49). Collectively, these results show that SPI1 is not activated when *Salmonella* is grown in the presence of glucose (Figure 6; 44,45,48). Since the environmental conditions faced by *Salmonella* change constantly during passage through the intestine of the animal host, this bacterium should use multiple signals to modulate the virulence genes needed for survival. It is known that *S.* Typhimurium uses bile or SCFAs including acetate, propionate and butyrate, as environmental signals to modulate its invasion of the gastrointestinal tract (50–52). We propose that *Salmonella* can use the glucose concentration of the mammalian intestinal tract as one of many signals for the regulation of invasion genes. *Salmonella* may use Mlc to sense the availability of sugars, thereby allowing decisions as to when and where to initiate the expression of genes involved in invasion. *S.* Typhimurium typically invades the distal small intestine (ileum) of the mammalian upper gastrointestinal tract (53). Simple sugars, such as glucose, are rarely encountered in the distal ileum, as most are absorbed in the proximal portion of the small intestine (54). The relatively

high glucose concentration in the proximal small intestine may repress SPI1 gene expression through Mlc, perhaps together with PhoR-PhoB and/or SirA, whereas upon transit to the distal ileum, the glucose concentration becomes low enough to activate the invasion genes of SPI1. The effects of various sugars on the differential regulation of SPI1 genes require further investigation to understand their roles in *Salmonella* pathogenesis.

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