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MarT Activates Expression of the MisL Autotransporter Protein of *Salmonella enterica* Serotype Typhimurium▽

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MisL is a *Salmonella enterica* serotype Typhimurium fibronectin binding protein whose expression is induced during infection of mice. T-POP transposon mutagenesis identified *marT* as a positive regulatory element controlling expression of a *misL*::*lacZYA* transcriptional fusion. Gel shift analysis identified MarT as a transcriptional activator of the *misL* promoter.

The *Salmonella enterica* serotype Typhimurium genome contains an impressive array of genetic determinants devoted to attachment, including some 13 fimbrial operons and three genes encoding adhesins of the autotransporter family, *shdA*, *misL*, and *bigA* (10–12, 15, 23, 34). Only two of the encoded adhesins are well expressed in vitro, including type 1 fimbriae encoded by the *fim* operon (4, 6) and thin curled fimbriae (also known as thin aggregative fimbriae or curli) encoded by the *csg* (*agf*) gene cluster (9, 29, 33), but the expression of both adhesins is also subject to regulation. The lack of in vitro expression of the remaining adhesins has in many cases prevented their identification by methods other than sequence analysis.

The *misL* gene encodes an autotransporter protein of serotype Typhimurium and was identified during sequence analysis of a DNA region termed *Salmonella* pathogenicity island 3 (SPI3) (3). Autotransporter proteins contain an N-terminal signal peptide, an internal passenger domain, and a C-terminal translocator domain. Upon transport across the cytoplasmic membrane and cleavage of the signal peptide, the C-terminal translocator domain forms a β-barrel in the outer membrane through which the passenger domain is exported to the cell surface (27). The MisL passenger domain enables serotype Typhimurium to bind fibronectin to its cell surface, resulting in attachment to fibronectin-coated glass slides and in increased invasiveness for human epithelial cells (5). An intact *misL* gene is required for intestinal colonization in chicks and mice inoculated orally with serotype Typhimurium (5, 25). These data show that MisL is an extracellular matrix adhesin involved in intestinal colonization.

The MisL C-terminal translocator domain can be used to display foreign antigens and elicits specific antibody responses in mice (30, 31, 36). Mice infected with serotype Typhimurium seroconvert to MisL, which provides indirect evidence for in vivo expression of this autotransporter protein (5). Furthermore, expression of *misL* is induced upon serotype Typhimurium infection of macrophages in vitro (7). However, after growth in Luria-Bertani (LB) broth, a *misL-lac* transcriptional fusion does not produce any β-galactosidase activity, suggesting that the *misL* gene may be expressed in response to signals not present in laboratory media (3). Similarly, MisL expression is not detected by Western blotting in serotype Typhimurium grown in LB broth (5). Expression of the MisL protein can be detected with anti-MisL serum in LB broth cultures of serotype Typhimurium strains carrying a cloned *misL* gene whose expression is driven from the *Escherichia coli* arabinose promoter (5). These data suggest that inhibition of MisL expression during growth of serotype Typhimurium in LB broth is mediated by transcriptional control mechanisms. However, the regulatory elements that allow serotype Typhimurium to induce *misL* expression in vivo are not known. The goal of this study was to identify regulatory elements contributing to the control of MisL expression by screening a transposon library for mutants expressing a *misL*::*lacZYA* transcriptional fusion on LB agar plates.

Identification of T-POP insertions that trigger MisL expression in vitro. To identify genes controlling *misL* expression, we constructed a derivative of serotype Typhimurium strain LT2 (21) in which the promoterless *lacZYA* genes were chromosomally inserted behind the stop codon of the intact *misL* gene. To this end, a derivative (pCM2) of the suicide vector pFUSE (2) carrying the 5′ end of the *misL* gene (amplified with the primers 5′-GCTCTAGACTTGCCAACAACTATGCG-3′ and 5′-GCCCGGGCCTGAATCAGAAACTGTATTTC-3′) was transferred from *E. coli* strain S17-1λ*pir* (32) into serotype Typhimurium strain LT2 by conjugation. The resulting strain (MA1) was used to generate a library of random T-POP insertion mutants. To this end, a plasmid encoding the Tn*10* transposase (pNK972) (28) was introduced into serotype Typhimurium *misL*::*lacZYA* (MA1). A bacteriophage P22 derivative (P22 HT105/1 *int*) was grown on a serotype Typhimurium strain carrying the T-POP transposon on an *E. coli* F′ plasmid (TH3923) (28), and the resulting phage lysate was used to deliver the T-POP transposon into MA1(pNK972). Transductants were selected on LB agar plates containing tetracycline

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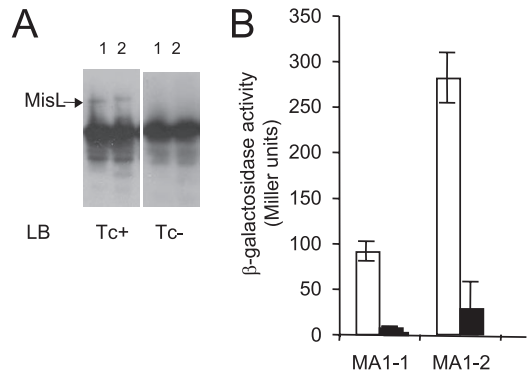

FIG. 1. (A) Analysis by Western blotting of two T-POP mutants (M-1, lane 1; M-2, lane 2) of serotype Typhimurium strain MA1 (LT2 *misL*::*lacZYA*) that exhibited a "blue" colony phenotype on LB–tetracycline–X-Gal agar plates for expression of MisL. Bacteria were grown in LB broth in the presence (Tc+) or the absence (Tc−) of tetracycline. The presence of an approximately 100-kDa protein band (MisL) in the mutants is indicated by an arrow. (B) Quantification of β-galactosidase activity in T-POP mutants (MA1-1 and MA1-2) of serotype Typhimurium strain MA1 (LT2 *misL*::*lacZYA*) grown in LB broth in the presence (open bars) or the absence (closed bars) of tetracycline. The bars represent averages from three independent measurements ± standard deviations.

(20 mg/liter) (the resistance conferred by T-POP) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 60 mg/liter) (to monitor activity of the *misL*::*lacZYA* reporter). Among approximately 40,000 transductants, we detected 21 colonies that were blue or light blue, suggesting expression of the *misL*::*lacZYA* transcriptional fusion. These 21 candidates (M-1 to M-21) were chosen for further analysis.

T-POP contains a tetracycline-dependent promoter that can drive expression of genes that are located adjacent to the transposon insertion. T-POP mutagenesis can thus identify both negative regulatory elements (by insertional inactivation) and positive regulatory elements (by driving their expression from the tetracycline-dependent promoter of T-POP). Expression of MisL was not detected by Western blotting using anti-MisL serum (5) in any of the 21 mutants grown in LB broth. However, after growth in LB broth supplemented with tetracycline, a band of approximately 100 kDa was detected by Western blotting in two of the mutants, M-1 and M-2 (Fig. 1). The size of this band corresponded well to the molecular mass of 98.4 kDa predicted for the mature MisL protein from its amino acid sequence. The fact that MisL expression was detected by Western blotting only after growth of M-1 and M-2 in medium containing tetracycline suggested that T-POP may be inserted upstream of a positive regulatory element in these mutants.

To further characterize the 21 candidates identified by T-POP mutagenesis, we determined the β-galactosidase activities (24) of individual cultures grown in the presence or absence of tetracycline. Expression of β-galactosidase was detected at high levels (between 100 and 300 Miller units) only in M-1 and M-2 grown in the presence of tetracycline (Fig. 1B). The remaining 19 mutants did not express β-galactosidase above background levels, and they were excluded from further analysis. The T-POP insertions in strains M-1 and M-2 were transduced into a serotype Typhimurium *misL*::*lacZYA* strain background, giving rise to strains MA1-1 and MA1-2, respectively. Expression of *misL* in MA1-1 and MA1-2 was tetracycline inducible, as shown by measuring β-galactosidase activity and by detecting MisL expression by Western blotting (data not shown). These results confirmed that the phenotype (i.e., tetracycline-inducible *misL* expression in LB broth) of both mutants was linked to their respective T-POP insertions.

DNA regions flanking the T-POP transposon insertion sites in strains MA1-1 and MA1-2 were cloned by inverse PCR (13) using the primer pair 5′-GCACTTGTCTCCTGTTTACTC C-3′ and 5′-CGCTTTTCCCGAGATCATATG-3′ for amplification. PCR products were cloned into *E. coli* and DH5α (8) using the vector PCR2.1 (Invitrogen), and the respective nucleotide sequences were determined. Sequence comparison using the BLAST algorithm at NCBI (1) revealed that the two insertions were at the same position within the *marT* open reading frame located upstream of nucleotide +7 (relative to the *marT* start codon). The insertion site was adjacent to the sequence 5′-GCTACGC-3′ (+8 to +14), which closely matched the Tn*10* hot-spot consensus sequence (5′-GCTNAGC-3′) (19), thereby providing a possible explanation for the identity of the T-POP insertion sites in strains MA1-1 and MA1-2. Alternatively, strains MA1-1 and MA1-2 may represent siblings, although this possibility appears less likely because delivery of T-POP by phage transduction does not provide bacteria with enough time to replicate prior to being spread on selective agar plates.

MarT activates expression of MisL. The *marT* gene is located on SPI3 and encodes a protein of 285 amino acids, which is most closely related (41% sequence identity) to the transcriptional activator CadC of *E. coli* (35). MarT contains a putative DNA-binding winged-helix-turn-helix domain between amino acids 32 and 180. The N-terminal 31 amino acids of MarT are absent in CadC, but the two proteins share sequence identity over the remaining part of their primary structures. The transposon insertions in strains MA1-1 and MA1-2 generated in each case a truncated *marT* open reading frame that was located downstream of the tetracycline-inducible *tetA* promoter of T-POP. The *marT* open reading frame encodes a second ATG start codon (at nucleotide positions +70 to +72) that may be used to initiate translation of a protein with a truncation of the first 21 amino acids, thus leaving the putative DNA-binding winged-helix-turn-helix domain intact. The tetracycline-inducible expression of *misL* in strains MA1-1 and MA1-2 raised the possibility that the *tetA* promoter might drive the expression of a truncated but functionally active MarT regulatory protein. An alternative possibility was that the *tetA* promoter of T-POP might drive the expression of the downstream *fidL* gene, whose gene product in turn might activate *misL* expression. FidL is a hypothetical inner membrane protein containing no conserved functional domains.

To test the hypothesis that strains MA1-1 and MA1-2 expressed MisL when grown in the presence of tetracycline because the *tetA* promoter of T-POP drove production of a functional MarT protein, we cloned *marT* into an expression vector (pBAD/gIIIA) behind an arabinose-inducible promoter using the primer pair 5′-GGAGCTCGGGGACAATCCATGTCCT GCTACG-3′ and 5′-GCTGCAGTTACCCATGTGTCACCC CGCGG-3′. The resulting plasmid (pCT6) was introduced into serotype Typhimurium strain LT2, and expression of MisL was

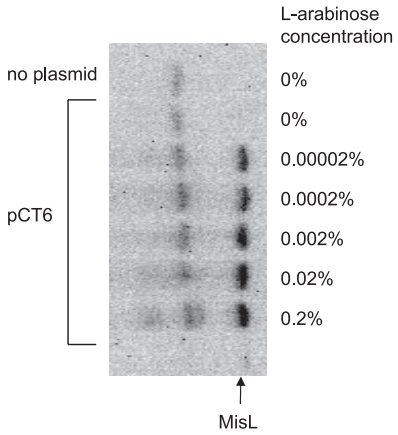

FIG. 2. Detection of MisL expression in serotype Typhimurium strain LT2 by Western blotting. A plasmid in which the arabinose promoter drives the expression of a promoterless *marT* gene (pCT6) was introduced into strain LT2, and the bacteria were grown in the presence of the L-arabinose concentrations indicated on the right. The presence of an approximately 100-kDa protein band corresponding to MisL is indicated by an arrow.

investigated by Western blotting. An approximately 100-kDa protein band was detected by Western blotting with anti-MisL serum only when serotype Typhimurium strain LT2(pCT6) was grown in the presence of arabinose (Fig. 2). The 100-kDa protein was not expressed in serotype Typhimurium strain LT2 lacking pCT6. These data suggested that expression of *marT* in serotype Typhimurium was sufficient to induce expression of the MisL protein.

To determine whether MarT activates transcription of the *misL* gene, plasmid pCT6 was introduced into serotype Typhimurium strain MA1 (*misL*::*lacZYA*). A significant increase in β-galactosidase activity was observed when strain MA1(pCT6) was grown in the presence of 0.02% or 0.2% L-arabinose (Fig. 3). These data supported the idea that expression of *marT*

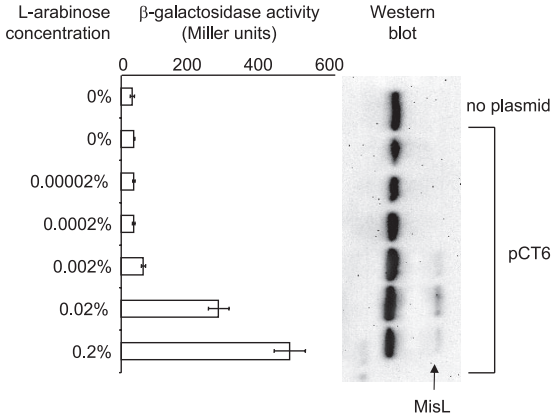

FIG. 3. Expression of *misL* in serotype Typhimurium strain MA1 (LT2 *misL*::*lacZYA*) detected by Western blotting (right) or by measuring β-galactosidase activity (left). Strain MA1 was transformed with a plasmid carrying the promoterless *marT* gene cloned behind the arabinose promoter (pCT6), and the bacteria were grown in the presence of the L-arabinose concentrations indicated on the left. The presence of an approximately 100-kDa protein band in the Western blot, which corresponds to MisL, is indicated by an arrow.

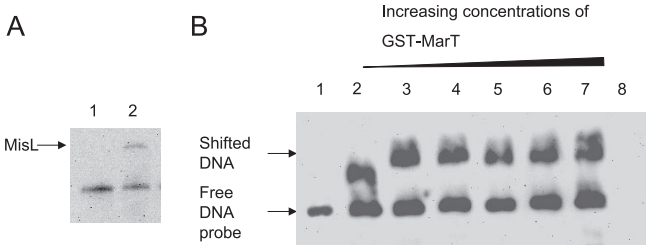

FIG. 4. Induction of MisL expression (A) and binding of the *misL* promoter region (B) by a GST-MarT fusion protein. (A) Western blot of serotype Typhimurium strain LT2 containing plasmid pGEX-4T-2 (lane 1) or a plasmid encoding a GST-MarT fusion protein (pCT17) (lane 2) detected with anti-MisL serum. The presence of an approximately 100-kDa protein band in the Western blot, which corresponds to MisL, is indicated by an arrow. (B) EMSA with a biotin-labeled DNA fragment containing the *misL* promoter region. Lanes 1 to 7 show biotin-labeled DNA in the absence of protein (lane 1) or in the presence of increasing concentrations (1, 2, 4, 6, 7, and 7.5 μg/lane) of purified GST-MarT fusion protein (lanes 2 to 7). Lane 8 contained GST-MarT in the absence of DNA.

from plasmid pCT6 was sufficient to induce transcription of the *misL* gene.

While MisL expression was detected by Western blotting after the growth of serotype Typhimurium LT2(pCT6) in 0.00002% L-arabinose (Fig. 2), induction of *misL* expression in serotype Typhimurium strain MA1(pCT6) required considerably higher concentrations of L-arabinose (Fig. 3). This observation was confirmed by Western blotting, which showed that serotype Typhimurium strain MA1(pCT6) expressed MisL only when grown in medium containing L-arabinose concentrations of 0.002% or above (Fig. 3). These data suggested that the *misL*::*lacZYA* transcriptional fusion reduced expression of the *misL* gene by an unknown mechanism.

Binding of MarT to the *misL* promoter region. To determine whether MarT activates *misL* expression by binding to the *misL* promoter region, we constructed a plasmid (pCT7) encoding a fusion protein between MarT and a C-terminal six-histidine tag (MarT-His) whose expression was under the control of the arabinose promoter by using the primers 5′-GGA GCTCTCCTGCTACGCAGCCACAGCATC-3′ and 5′-GCT GCAGCCCATGTGTCACCCCGCGG-3′ and vector pBAD/gIIIA (Invitrogen). Growth of serotype Typhimurium strain LT2(pCT7) in the presence of L-arabinose resulted only in weak expression of MisL, as suggested by Western blot analysis (data not shown). These data suggested that the MarT-His fusion protein was not fully functional. Attempts to purify MarT-His by affinity chromatography were not successful due to insolubility of the fusion protein. To overcome this problem, the *marT* gene was amplified using the primers 5′ GGGATC CATGTCCTGCTACGCAGCC 3′ and 5′ GGAATTCTTAC CCATGTGTCACCCCGCG 3′, and the resulting PCR product was cloned into vector pGEX4T-2 to give rise to plasmid pCT17. Plasmid pCT17 encoded a fusion protein between glutathione-*S*-transferase (GST) and MarT (GST-MarT), whose expression was under the control of the *lac* promoter. Expression of MisL was detected by Western blotting after growth of serotype Typhimurium strain LT2(pCT17) in the presence of IPTG (isopropyl-β-D-thiogalactopyranoside) (Fig. 4A), suggesting that GST-MarT was functionally active.

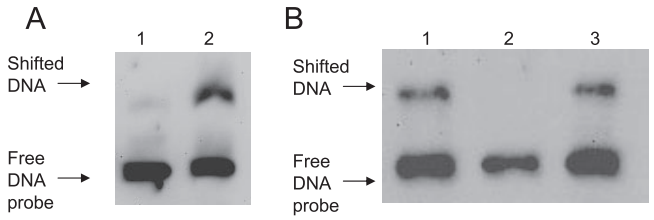

FIG. 5. (A) EMSA for binding of GST or GST-MarT to the *misL* promoter region. Purified GST (Lane 1; 5 μg/lane) or purified GST-MarT fusion protein (lane 2; 5 μg/lane) incubated with a biotin-labeled DNA fragment containing the *misL* promoter region. (B) EMSA for binding of GST-MarT to the *misL* promoter region in the absence of unlabeled DNA (lane 1) or in competition with 10-fold excess of the unlabeled DNA fragment containing the *misL* promoter region (lane 2) or 10-fold excess of an unrelated promoter region (lane 3).

GST-MarT protein (0 to 7.5 μg) affinity chromatography purified (using glutathione-Sepharose [Amersham Pharmacia]) from cell lysates (French press at 12,000 lb/in^2) was tested for its ability to bind the *misL* promoter region using an electrophoretic mobility shift assay (EMSA) (Pierce LightShift Chemiluminescent EMSA kit; Pierce Biotechnology). The reaction mixture was then analyzed by electrophoresis in a nondenaturing 5% acrylamide gel with cold 0.5× Tris-borate-EDTA running buffer. The DNA-protein complexes in the gel were electrophoretically transferred to a positively charged nylon membrane in 0.5× Tris-borate-EDTA at 380 mA for 30 to 60 min and detected using the EMSA kit. Addition of increasing concentrations of GST-MarT to a biotin-labeled PCR product (using the biotinylated primers 5′-AGTAAACG TGGACGAACGCTT-3′ and 5′-TACCGCTATGGCGATGA ATGA-3′) containing the *misL* promoter region resulted in the appearance of a band with higher molecular weight (Fig. 4). Purified GST protein, used as a control, showed no binding activity for the *misL* promoter region (Fig. 5). The specificity of the binding reaction was investigated by competition EMSA. Addition of unlabeled PCR products containing the *misL* promoter to a mixture of GST-MarT and the biotin-labeled *misL* promoter fragment resulted in inhibition of the gel shift (Fig. 5). In contrast, addition of unlabeled PCR products containing an unrelated promoter region (the *vjbR* promoter from *Brucella abortus*) did not inhibit the gel shift of the biotin-labeled *misL* promoter fragment by GST-MarT.

MisL and ShdA are two autotransporter proteins that mediate attachment to fibronectin and contribute to intestinal colonization of serotype Typhimurium in mice (5, 14, 16–18). Expression of these autotransporter proteins is not detected by Western blotting after the growth of serotype Typhimurium under various in vitro growth conditions (5, 17). However, expression of MisL and ShdA is induced in vivo, as demonstrated by seroconversion to MisL during the infection of mice (5) and by a colocalizing immunofluorescence signal detected with anti-ShdA antiserum and anti-O4 antiserum in thin sections of the murine cecum after infection with serotype Typhimurium (17). Our data show that expression of *misL* is controlled by a transcriptional activator, MarT, which is encoded by SPI3.

MarT and its close homologue CadC are members of the family of ToxR-like regulatory proteins. CadC is a transcriptional activator of the *cadBA* operon encoding the lysine decarboxylase CadA and the lysine-cadaverine antiporter CadB of *E. coli*. Under noninducing conditions, expression of *cadBA* is silenced by the histone-like nucleoid structuring (H-NS) protein, which binds the *cadBA* promoter region upstream of the CadC binding site. Under inducing conditions (i.e., acidic external pH and exogenous lysine), CadC binds the *cadBA* promoter region, thereby dissolving the H-NS repressor complex and activating expression of the *cadBA* genes (20). In *S. enterica*, H-NS silences genes with GC contents lower than the genomic average (i.e., genes acquired by lateral gene transfer) by restricting the access of RNA polymerase to DNA (22, 26). SPI3 is a horizontally acquired DNA region with a GC content (47.5%) that is lower than the average GC content (52%) of the serotype Typhimurium genome (3). Chromatin immunoprecipitation indicated the presence of an H-NS binding site in the promoter region of the serotype Typhimurium *misL* gene (22, 26). It is thus possible that H-NS silences *misL* and that binding of MarT to the *misL* promoter region relieves this repression. However, additional experiments are needed to test this prediction.

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