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MxiE Regulates Intracellular Expression of Factors Secreted by the *Shigella flexneri* 2a Type III Secretion System

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The *mxi-spa* locus on the virulence plasmid of *Shigella flexneri* encodes components of the type III secretion system. *mxiE*, a gene within this locus, encodes a protein that is homologous to the AraC/XylS family of transcriptional regulators, but currently its role in pathogenesis remains undefined. We characterized the virulence phenotype of a nonpolar *mxiE* mutant and found that this mutant retained the ability to invade mammalian cells in tissue culture and secrete Ipas (type III effectors required for host cell invasion), although it was less efficient than wild-type *Shigella* at cell-to-cell spread. Despite its invasive properties in culture, the *mxiE* mutant was completely avirulent in an animal model. Potential targets for MxiE activation were identified by using promoter-green fluorescent protein fusions, and gene expression was examined under various growth conditions. Six MxiE-regulated genes were discovered: *ospB*, *ospC1*, *ospE2*, *ospF*, *virA*, and $ipaH_{9.8}$. Notably, activation of these genes only occurred within the intracellular environment of the host and not during growth at 37°C in liquid culture. Interestingly, all of the MxiE-regulated proteins previously have been shown to be secreted through the type III secretion system and are putative virulence factors. Our findings suggest that some of these Osp proteins may be involved in postinvasion events related to virulence. Since bacterial pathogens adapt to multiple environments during the course of infecting a host, we propose that *Shigella* evolved a mechanism to take advantage of a unique intracellular cue, which is mediated through MxiE, to express proteins when the organism reaches the eukaryotic cytosol.

Members of the genus *Shigella* are gram-negative, facultative intracellular bacteria that are the causative agents of bacillary dysentery in humans. These organisms invade the colonic mucosa, causing severe inflammation, which eventually leads to mucosal destruction and results in blood and mucus in the stools (30). The key aspect of *Shigella* pathogenesis lies in its ability to invade epithelial cells and spread directly from cell to cell. Epithelial cell invasion occurs by a process similar to phagocytosis and requires major rearrangements of the host cell cytoskeleton (9). Once inside the eukaryotic cell, the bacteria escape from the phagocytic vacuole and are able to multiply in the cytoplasm and spread to adjacent cells via nucleation of host cell actin by the outer membrane protein IcsA (5). The force of the rapid actin polymerization propels the organisms through the cytosol, leading to formation of membrane-bound protrusions, which are then engulfed by neighboring cells. The resulting double membrane-bound vacuole is lysed by bacterial proteins, IpaB and IpaC, allowing the cycle to repeat (51).

The ability of shigellae to invade the host epithelium is dependent upon a 31-kb entry region of the 230-kb virulence plasmid (34). The entry region contains two divergently transcribed loci that encode the genes responsible for invasion of eukaryotic cells, intracellular spread, and production of a type III secretion system. One locus is comprised of the *mxi* and *spa* operons, which encodes ca. 20 different proteins that together make up the type III secretion apparatus. Type III, or contact-dependent, secretion systems are present in a wide variety of gram-negative plant and animal pathogens and function to secrete protein effectors out of the bacterium and inject them into the cytosol of eukaryotic host cells (reviewed in reference 27). Effector molecules perform a wide variety of functions, some of which include translocation of other effectors into the host cell and interaction with host cell cytoskeletal components to facilitate entry of the bacteria. Although the effector proteins are unique to each bacterial species, the proteins comprising the structural apparatus are highly conserved even in distantly related organisms.

Some of the events leading to bacterial uptake, such as cytoskeletal rearrangements, are mediated through secreted or translocated effectors. In *Shigella*, the entry region of the virulence plasmid also contains a cluster of genes encoding the type III secreted effector molecules (the invasion plasmid antigens, IpaA, IpaB, IpaC, and IpaD), which are required for host cell invasion and escape from the phagosome (37). Furthermore, *ipgC*, which encodes a molecular chaperone that associates with IpaB and IpaC in the bacterial cytoplasm, is found immediately upstream of the *ipa* genes in this region. While not secreted, IpgC is required for bacterial entry by stabilizing IpaB and IpaC and preventing their premature association (38).

During a natural infection, secretion of presynthesized effectors is triggered by contact with a eukaryotic cell. However, in the laboratory, type III secretion can be artificially induced in the absence of host cell contact by manipulation of the

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growth medium (e.g., removal of Ca^{2+} for *Yersinia*) or by the addition of specific chemicals, such as Congo red for *Shigella* (3).

Expression of the type III apparatus and its effectors is usually regulated by environmental factors, such as temperature, and often regulated at the level of transcription. In *Shigella*, the AraC-like protein, VirF, is well established as the primary regulator of virulence gene expression. When the bacteria encounter a 37°C environment, VirF activates transcription of both *icsA* and the secondary regulatory gene *virB*, thereby initiating the cascade of virulence gene expression, although exactly how VirF responds to temperature is not yet known (45). This thermal activation is not a result of de novo *virF* transcription since functional VirF protein is present in the bacterial cytosol even at nonpermissive temperatures but rather is due to changes in the amount of supercoiling at the *virB* promoter which allows VirF to bind and activate its transcription (55). VirB, in turn, activates transcription of the *ipa*, *mxi*, and *spa* operons, which encode the type III secretion system that is essential for invasion (54).

A common mechanism of gene regulation in bacteria is via regulatory proteins of the AraC/XylS family. To date, this family contains more than 100 members as identified by sequence homology to the AraC protein of *Escherichia coli* (19). With few exceptions, AraC homologues are transcriptional activators. The defining characteristic of this family of proteins is a C-terminal 99-amino-acid stretch of homology comprising the DNA-binding domain. Importantly, this region contains two helix-turn-helix motifs, at least one of which is responsible for binding to the major groove of DNA. A separate domain (sensor domain) or additional protein acts to bind effector molecules. Members of the AraC/XylS family regulate a broad variety of bacterial functions, which can be divided into three categories: carbon metabolism, stress response, and virulence. The AraC homologues that regulate virulence gene expression have not been shown to bind effectors directly, although they do respond to environmental signals such as temperature, osmolarity, or calcium concentration by an unidentified mechanism (19).

In addition to VirF (mentioned above), the role of several AraC homologues that are involved in bacterial pathogenesis has been the focus of intense research. Many of these proteins function as transcriptional activators of molecules that are secreted through the type III secretion system, their cognate chaperones, and in some cases, type III secretion apparatus structural components as well. The AraC-like InvF protein from *Salmonella enterica* serotype Typhimurium is required for efficient invasion into cultured epithelial cells and activates the expression of genes whose products are secreted through the type III secretion system (12, 28). InvF is encoded by the first gene of the large *inv* operon and acts upon the *sigDE* and *sicA-sipBCDA* operons (12). SigD and the Sip proteins are secreted through the type III apparatus whereas SigE and SicA function as chaperones for these effectors (13, 26, 29). In contrast to other enteric pathogens, transcription of the type III secretion genes in *Salmonella* is not regulated by temperature. These organisms respond to other environmental signals, including oxygen concentration, osmolarity, and pH (4).

VirF, an AraC homologue of *Yersinia enterocolitica*, functions as a transcriptional activator of several plasmid-encoded virulence genes: *yopE*, *yopH*, and the *lcrGVH-yopBD* and *yscB-yscM* operons (31). These Yops are secreted effectors that facilitate formation of channels in the eukaryotic membrane or are translocated into the eukaryotic cell where they are involved in the modulation of cellular signaling (6, 25, 58). LcrG, LcrV, and LcrH each perform multiple functions ultimately related to the secretion and/or targeting of effector proteins (15, 18) and the *yscB-yscM* locus encodes type III structural genes (39). VirF itself is under environmental control, and its synthesis is induced when bacteria encounter a 37°C environment (39). Furthermore, recent evidence suggests that temperature regulation of virulence gene expression in *Yersinia* is modulated through changes in the topology of the virulence plasmid (44).

Clearly, AraC homologues are utilized by several pathogenic bacterial species to regulate expression of type III secretion systems and effectors, which are essential virulence determinants for these organisms. An additional AraC homologue, MxiE, has been described in *Shigella*, although the genes that it may regulate have not yet been identified (2). The purpose of this study was to determine whether MxiE functions as a transcriptional regulator in *Shigella flexneri* and to identify targets of its regulation. Here we describe an additional level of virulence gene regulation in *Shigella* which functions only after host cell invasion and identify six virulence plasmid-encoded genes that are activated by MxiE in the intracellular environment.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are described in Table 1. Bacteria were grown in Luria-Bertani broth at 37°C or tryptic soy broth at 30°C with aeration, unless otherwise stated. Appropriate antibiotics were used at the following concentrations: ampicillin, 100 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; and chloramphenicol, 10 μg ml^{-1}.

The L2 mouse fibroblast cell line was maintained in Dulbecco modified Eagle medium (DMEM; Gibco) containing 10% fetal bovine serum by standard techniques.

Construction of the *mxiE* mutant strain and promoter-*gfp* fusions. Plasmid construction, DNA analysis, and electroporation were performed according to the manufacturer's instructions or previously described standard protocols (46). Oligonucleotide synthesis was performed by using Applied Biosystems automated solid-phase synthesis with standard chemistry at the Uniformed Services University of the Health Sciences (USUHS) Biomedical Instrumentation Center.

The *mxiE*-bearing plasmid was constructed by ligating a 3.5-kb *Eco*RV fragment of pHS4011 (containing *mxiD-J* from pWR100) into pBluescript SK(+). The nonpolar *mxiE* deletion and insertion mutant was constructed by removing the 206-bp *Cla*I-*Hin*dIII fragment of *mxiE*, blunting with Klenow, and ligating an 891-bp *Eco*RI-*Hin*dIII *aphA-3* fragment, which also had been blunted with Klenow, into the site to yield pECK4. pECK4 was linearized with *Not*I and *Xho*I and used to transform BS600 to form BS610. *mxiE*::*aphA*-3 was transduced into 2457T by using P1L4 grown on BS610 to yield BS611.

The promoterless green fluorescent protein (GFP) expression vector, pEBD166, was constructed as follows. The *gfpmut2* allele, a mutant of GFP optimized for fluorescence in flow cytometry applications (10), was amplified from pRRS10. The upstream primer was designed to contain restriction sites for *Kpn*I and *Bgl*II, followed by a strong ribosome-binding site (AGGAGA), located 7 bp upstream of the GFP start codon. The downstream primer contained a *Hin*dIII restriction site. After restriction digestion and agarose gel purification, the GFP-encoding DNA fragment was ligated to *Kpn*I-*Hin*dIII-digested pBAD18, forming pEBD166.

Oligonucleotide primer pairs for the promoter sequences of the *virA*, $ipaH_{9.8}$, *ospB*, *ospC1*, *ospD2*, *ospE2*, *ospF*, *ospG*, and *ipgD* genes were designed based on the *S. flexneri* virulence plasmid sequence and encompassed ca. 500 bp upstream of the start codon of the gene (7). Restriction sites for *Kpn*I and *Bgl*II were incorporated into the upstream and downstream primers, respectively, for cloning into pEBD166. Promoter regions were amplified from 2457T at 94°C for 1

TABLE 1. *Shigella* strains and plasmids used in this study[a]

Strain or plasmid	Relevant genotype and/or phenotype	Source or reference
Strains		
2457T	Wild-type *S. flexneri* serotype 2a	17
BS543	2457T/Δ*icsA*	48
BS600	2457T *galU*::Tn*10* [Δ(*recB ptr recC recD*)::P_{lac}*bet exo cat*]	14
BS610	BS600 *mxiE* (*mxiE*::*aphA-3*)	This work
BS611	2457T *mxiE* (*mxiE*::*aphA-3*)	This work
BS613	BS611/pRRS13 (P_{lac}*mxiE*$^+$)	This work
SCK9	2457T/pRRS13 (P_{lac}*mxiE*$^+$)	This work
SCK45	*mxiE*$^+$/pEBD166	Amp^r transformant of 2457T
SCK78	*mxiE*$^+$/pECK127 (P*virA*::*gfp*)	Amp^r transformant of 2457T
SCK79	*mxiE*::*aphA-3*/pECK127 (P*virA*::*gfp*)	Amp^r transformant of BS611
SCK70	*mxiE*$^+$/pECK116 (P*ipaH*$_{9.8}$::*gfp*)	Amp^r transformant of 2457T
SCK71	*mxiE*::*aphA-3*/pECK116 (P*ipaH*$_{9.8}$::*gfp*)	Amp^r transformant of BS611
SCK39	*mxiE*$^+$/pECK94 (P*ospB*::*gfp*)	Amp^r transformant of 2457T
SCK42	*mxiE*::*aphA-3*/pECK94 (P*ospB*::*gfp*)	Amp^r transformant of BS611
SCK48	*mxiE*$^+$/pECK100 (P*ospC1*::*gfp*)	Amp^r transformant of 2457T
SCK52	*mxiE*::*aphA-3*/pECK100 (P*ospC1*::*gfp*)	Amp^r transformant of BS611
SCK49	*mxiE*$^+$/pECK102 (P*ospD2*::*gfp*)	Amp^r transformant of 2457T
SCK53	*mxiE*::*aphA-3*/pECK102 (P*ospD2*::*gfp*)	Amp^r transformant of BS611
SCK50	*mxiE*$^+$/pECK104 (P*ospE2*::*gfp*)	Amp^r transformant of 2457T
SCK54	*mxiE*::*aphA-3*/pECK104 (P*ospE2*::*gfp*)	Amp^r transformant of BS611
SCK51	*mxiE*$^+$/pECK106 (P*ospF*::*gfp*)	Amp^r transformant of 2457T
SCK55	*mxiE*::*aphA-3*/pECK106 (P*ospF*::*gfp*)	Amp^r transformant of BS611
SCK41	*mxiE*$^+$/pECK98 (P*ospG*::*gfp*)	Amp^r transformant of 2457T
SCK44	*mxiE*::*aphA-3*/pECK98 (P*ospG*::*gfp*)	Amp^r transformant of BS611
SBD150	*mxiE*$^+$/pEBD168 (P*ipgD*::*gfp*)	Amp^r transformant of BS543
SBD165	*mxiE*::*aphA-3*/pEBD168 (P*ipgD*::*gfp*)	Amp^r transformant of BS611
Plasmids		
pBluescript SK(+)	Cloning vector, Amp^r	Stratagene
pUC18K	Vector bearing the *aphA-3* cassette, Amp^r Kan^r	37
pBAD18	Arabinose-inducible P_{BAD} expression vector, pBRori, Amp^r	20
pEBD166	Promoterless *gfpmut2* expression vector, colE1ori, Amp^r	This work
pRRS10	pBluescript KS(+)::*gfpmut2*	This work
pRRS11	3.6-kb *Eco*RV fragment of pWR100, containing *mxiJ-D*, cloned into pBluescript SK(+)	This work
pECK4	891-bp *Eco*RI-*Hin*dIII (Klenow-treated) fragment of pUC18K, bearing *aphA-3*, ligated into pRRS11 at *Cla*I-*Hin*dIII (Klenow treated)	This work
pECK127	*virA*::*gfp* promoter fusion; 560-bp PCR-generated fragment, extending from 502 bp upstream of the start codon to 58 bp downstream of the start codon, ligated with *Kpn*I-*Bgl*II-digested pEBD166	This work
pECK116	*ipaH*$_{9.8}$::*gfp* promoter fusion; 609-bp PCR-generated fragment, extending from 510 bp upstream of the start codon to 99 bp downstream of the start codon, ligated with *Kpn*I-*Bgl*II-digested pEBD166	This work
pECK94	*ospB*::*gfp* promoter fusion; 329-bp PCR-generated fragment, extending from 251 bp upstream of the start codon to 78 bp downstream of the start codon, ligated with *Kpn*I-*Bgl*II-digested pEBD166	This work
pECK100	*ospC1*::*gfp* promoter fusion; 610-bp PCR-generated fragment, extending from 510 bp upstream of the start codon to 100 bp downstream of the start codon, ligated with *Kpn*I-*Bgl*II-digested pEBD166	This work
pECK102	*ospD2*::*gfp* promoter fusion; 637-bp PCR-generated fragment, extending from 509 bp upstream of the start codon to 128 bp downstream of the start codon, ligated with *Kpn*I-*Bgl*II-digested pEBD166	This work
pECK104	*ospE2*::*gfp* promoter fusion; 594-bp PCR-generated fragment, extending from 521 bp upstream of the start codon to 73 bp downstream of the start codon, ligated with *Kpn*I-*Bgl*II-digested pEBD166	This work
pECK106	*ospF*::*gfp* promoter fusion; 574-bp PCR-generated fragment, extending from 499 bp upstream of the start codon to 75 bp downstream of the start codon, ligated with *Kpn*I-*Bgl*II-digested pEBD166	This work
pECK98	*ospG*::*gfp* promoter fusion; 249-bp PCR-generated fragment, extending from 169 bp upstream of the start codon to 80 bp downstream of the start codon, ligated with *Kpn*I-*Bgl*II-digested pEBD166	This work
pEBD168	*ipgD*::*gfp* promoter fusion; 936-bp PCR-generated fragment, extending from 556 bp upstream of the *ipgD* start codon to 380 bp downstream of the start codon, ligated with *Bgl*II-digested pEBD166	This work

[a] Amp^r, ampicillin resistance; Kan^r, kanamycin resistance.

min, 56°C for 1 min, and 72°C for 1 min by using 25 cycles. Each PCR product was digested with *Kpn*I and *Bgl*II and ligated into pEBD166 that had been similarly digested. Plasmids were electrotransformed into *S. flexneri* 2457T and BS611.

Virulence assays. Invasion assays were performed in L2 mouse fibroblasts by using the gentamicin protection modification as previously described (21). Briefly, bacteria at an optical density at 600 nm of 0.72 were washed in DMEM and spun onto subconfluent L2 cell fibroblast monolayers in 35-mm dishes at 6,000 rpm for 10 min. After incubation at 37°C for 30 min, cells were washed with warm phosphate-buffered saline (PBS) three times, and the medium was replaced with warm DMEM containing 50 μg of gentamicin ml^{-1}. After another 30 min incubation at 37°C, the cells were rinsed and treated as described above. Cells were incubated for an additional hour at 37°C and then rinsed three times with warm PBS and treated as described below. To evaluate the formation of protrusions in the eukaryotic cell membrane caused by intracellular movement of the bacteria, infected cells were fixed with a solution of 0.2% glutaraldehyde–2% formaldehyde in PBS for 5 min at 4°C before staining them with Giemsa (47). Plaque assays were performed with confluent L2 fibroblast monolayers as previously described (40). Detection of Ipa synthesis and secretion was as previously described (50). Protein electrophoresis was performed in sodium dodecyl sulfate–10% polyacrylamide gels. Separated proteins were transferred to polyvinylidene difluoride membranes (Schleicher & Schuell, Inc.) and treated with a blocking agent consisting of 5% powdered milk–3% gelatin in Tris-buffered saline. Immunodetection was performed with an anti-IpaB–anti-IpaC–anti-IpaD cocktail and alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G secondary antibody. Blots were visualized by using the chemiluminescent substrate, CDP-Star (Boehringer Mannheim) according to the manufacturer's instructions. Virulence in vivo was assessed with the Serény test as previously described (52).

Flow cytometry. Flow cytometry experiments were performed on an EPICS XL-MCL flow cytometer (Coulter, Miami, Fla.). Regions were set to analyze 10,000 fluorescent, bacterium-sized particles for each sample. The region comprising the nonfluorescent population was set by using the wild-type strain containing the promoterless *gfp* plasmid alone (pEBD166) such that this peak is entirely contained within the first decade (mean fluorescence intensity [MFI] = 0.2 to 0.3). Therefore, nonfluorescent populations are represented by MFIs of ≤0.3. All fluorescence intensity values of >1 represent fluorescent bacteria, and populations with MFIs between 0.3 and 1 are weakly fluorescent. Data were analyzed and compiled by using WinList software (Verity Software House, Topsham, Maine). An induction ratio (IR) was calculated by dividing the MFI of the test bacteria (i.e., bacteria grown in liquid culture at 37°C or grown intracellularly at 37°C) by the MFI of bacteria grown in liquid culture at 30°C as described by Lee et al. (32). Growth at 30°C in liquid culture was chosen as the baseline value since virulence genes are known to be repressed at this temperature (35). Based on control experiments, an IR of ≤1.2 reflects a lack of *gfp* induction under those growth conditions.

Overnight cultures were diluted 1:100 into DMEM containing ampicillin and incubated at either 30 or 37°C with aeration for 2 h. Aliquots were washed with cold PBS and held on ice until analysis. Cells were diluted to allow no more than 1,000 events per s during flow cytometry analysis. To assess promoter activity from bacteria in the intracellular environment, strains were grown to exponential phase in tryptic soy broth containing ampicillin at 37°C and used to infect L2 cells in the gentamicin protection assay as described above with the following modification. After the final incubation, intracellular bacteria were released by the addition of 1 ml of 0.2% Triton X-100 in PBS to each dish and incubation at room temperature for 2 min. Cells were removed by pipetting up and down, and the lysates were held on ice until analysis. Undiluted cell lysates were analyzed by flow cytometry. Samples were run in triplicate, and experiments were repeated at least twice.

RESULTS

Characterization of the virulence phenotype of a *mxiE* mutant. We constructed a nonpolar *mxiE* deletion-insertion mutant such that sections of both the DNA-binding domain and the putative sensor region were removed in order to abolish both activities (Fig. 1A). To this end, the central third of the molecule was excised and replaced with the *aphA-3* kanamycin resistance gene cassette (Fig. 1B). The *mxiE* mutant was able to bind Congo red, an indication that it still has a functional type III secretion system (49). In concordance with this observation, the *mxiE* mutant was able to synthesize and secrete IpaB, IpaC, and IpaD, as well as the wild-type strain (Table 2 and data not shown). Secretion of IpaB and IpaC is essential both for host cell invasion and for efficient cell-to-cell dissemination (51).

Upon initial characterization of the *mxiE* mutant, we noticed that this strain produced smaller plaques than the wild-type strain (Table 2). Moreover, the plaque morphology of the mutant strain was irregular compared to the wild-type plaques (Fig. 2). Although the small plaque size indicated that the *mxiE* mutant was somewhat compromised in its ability to spread from cell to cell, it was able to invade cultured cells and form protrusions just as efficiently as wild-type *Shigella* (Table 2). The defect in the *mxiE* mutant that leads to the reduced plaque size phenotype was also evident in the Serény test, which measures a strain's ability to invade eukaryotic cells and spread from cell to cell within an animal host (52). The *mxiE* mutant was completely avirulent in the Serény test (Table 2). Pathology as severe as that seen with wild-type *Shigella* could be restored by complementation of the mutant with full-length *mxiE* driven from a constitutive promoter. Clearly, in assays that measure postinvasion events, the *mxiE* mutant strain was partially (plaque formation) or fully (Serény test) attenuated. These results led us to examine the influence of MxiE on genes that may be needed postinvasion for a productive disseminated infection in the host.

Analysis of *virA* and *ipaH*$_{9.8}$ expression. Given that MxiE shows homology with members of the AraC/XylS family of transcriptional regulators and that *mxiE* is found within the region encoding the type III secretion system, we strongly suspected that MxiE would play a role in the regulatory cascade of virulence factors. Moreover, MxiE is quite homologous to InvF of *Salmonella*, which is required for expression of genes that are secreted through the type III secretion machinery (12). Since the results above demonstrated that MxiE is essential for *Shigella* virulence and appears to be involved in postinvasion events, we examined the influence of MxiE on the expression of genes that may be involved in postinvasion processes.

VirA and IpaH$_{9.8}$ are two of at least 15 proteins that are secreted through the *Shigella* type III secretion system (16, 42, 57). Demers et al. suggested that transcription of both *virA* and *ipaH*$_{9.8}$ is regulated by the type III secretion machinery, although the transcriptional regulator was not identified (16). To test whether MxiE is the type III system component responsible for *virA* activation, a transcriptional fusion of the *virA* promoter to *gfp*, the gene encoding GFP, was constructed, and expression of this fusion was examined in both the *mxiE* mutant and wild-type strains. The GFP reporter system is at least as sensitive as the β-galactosidase system (33) and has the advantage of allowing us to quantify the amount of GFP expression from individual bacteria by using a flow cytometer. GFP expression from bacteria grown in liquid culture (extracellular) at 30 or 37°C was compared to that of bacteria that were allowed to invade cultured cells and replicate intracellularly. Gene expression was examined after bacterial invasion of eukaryotic cells since transcription of *virA* and *ipaH*$_{9.8}$ was reported to be transiently activated after entry into host cells (16). An IR was calculated by dividing the MFI of bacteria grown at 37°C, either in liquid culture or intracellularly, by the

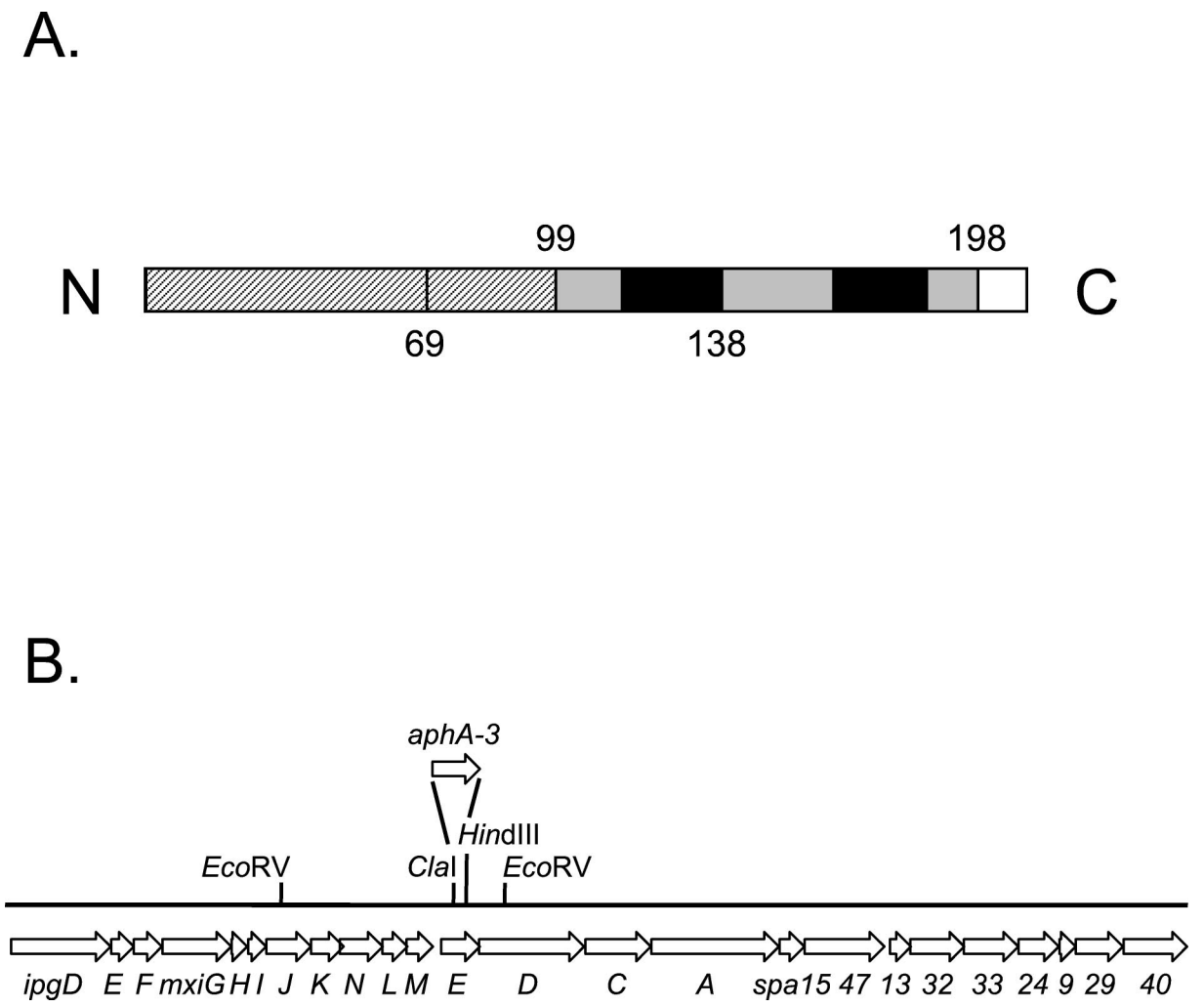

FIG. 1. (A) Predicted domains of MxiE. The AraC domain (residues 99 to 198) is highlighted in gray, and the helix-turn-helix motifs within this region are indicated by black boxes. The striped boxes denote the putative sensor domain. The region between residues 69 to 138 was deleted in construction of the *mxiE* mutant. (B) Genetic organization of the *ipg-mxi-spa* loci adjacent to *mxiE*. The *Eco*RV sites denote the fragment that was cloned to form pRRS11. The 206-bp *Cla*I-*Hin*dIII internal fragment of *mxiE* was replaced with *aphA-3* to form strain BS611 as described in Materials and Methods.

MFI of bacteria grown at 30°C in liquid culture as described in Materials and Methods.

The level of GFP expression from the *virA* promoter in both the wild-type strain and the *mxiE* mutant was the same whether the organisms were grown at 30 or 37°C in liquid culture (Table 3 and Fig. 3). These results indicate that *virA* expression is not temperature dependent since no induction was seen at 37°C in liquid culture compared to 30°C, where virulence genes are repressed (35). Moreover, gene expression was not strictly secretion dependent because activation of type III secretion using Congo red, a dye known to induce type III secretion in the absence of host cell contact (3), also did not influence expression of *virA* (data not shown). In contrast, when bacteria were recovered from the intracellular environment and analyzed, GFP expression from the *virA* promoter was found to be induced in the wild-type strain but remained near baseline levels in the *mxiE* mutant (IRs of 2.9 and 1.3, respectively [Table 3]). Therefore, *virA* was only induced in the intracellular environment and required functional MxiE for full induction.

Bacteria containing the $ipaH_{9.8}$ promoter-*gfp* fusion were very weakly fluorescent when grown extracellularly at either 30 or 37°C (Table 3). There was no difference in reporter expression between the wild-type and *mxiE* mutant strains when they were grown in liquid culture at either temperature. When the bacteria were allowed to invade eukaryotic cells, *gfp* was induced from the $ipaH_{9.8}$ promoter in the wild-type strain (IR of 2.7) (Fig. 3 and Table 3). In contrast, GFP levels were reduced in the *mxiE* mutant under these conditions (IR of 0.6) and, in fact, the MFI of the *mxiE* mutant bacteria recovered from the cytoplasmic environment was even lower than that of the bacteria grown in liquid culture. These results demonstrate that induction of *gfp* from the $ipaH_{9.8}$ promoter was dependent upon the presence of MxiE; the fluorescence intensity decreased nearly fivefold in its absence. Furthermore, as seen with *virA*, no induction from the $ipaH_{9.8}$ promoter could be

TABLE 2. Virulence properties of a *mxiE* mutant strain

Strain	Description	% Invasion[a]	% Plaquing efficiency[b]	Plaque diam (mm)[c]	Serény test result[d]	Ipa synthesis and secretion[e]
2457T	Wild type	0.39	0.47	1.33	+	+
BS611	*mxiE*::*aphA-3*	0.41	0.45	0.87	−	+
BS613	*mxiE*/P_{lac}*mxiE*$^+$	0.59	0.68	0.84	+	ND
SCK9	wt/P_{lac}*mxiE*$^+$	0.50	0.71	1.29	ND[f]	ND

[a] Calculated by dividing the number of bacteria recovered from gentamicin-treated cells, assayed in triplicate, by the number of bacteria present in the inoculum and then multiplying the result by 100.
[b] Calculated by dividing the number of plaques formed on confluent L2 monolayers at 72 h, assayed in triplicate, by the number of bacteria present in the inoculum and then multiplying the result by 100.
[c] Calculated by measuring the diameter of the zone cleared on L2 monolayers. Each value represents the average of 10 plaques measured from each dish, assayed in triplicate in three separate experiments.
[d] Keratoconjunctivitis in the guinea pig eye (52).
[e] Detection of IpaB, IpaC, and IpaD in culture supernatants or cell-associated pellets by immunoblotting after induction of secretion using Congo red as described in Materials and Methods.
[f] ND, not determined.

detected after growth in the presence of Congo red (data not shown).

Analysis of *osp* expression. In addition to *virA* and $ipaH_{9.8}$, we chose to examine expression of a recently described set of virulence plasmid-encoded proteins of unknown function, termed Osps (outer *Shigella* proteins). The Osps were identified through N-terminal sequencing of proteins collected from the culture supernatant of a type III secretion constitutive mutant (7). Six unrelated Osp groups were identified (B to G), some of which have multiple homologous members possibly due to gene duplication. We tested the ability of MxiE to induce expression from the promoters of the *osp* genes under differing growth conditions by using promoter-*gfp* fusions as described above.

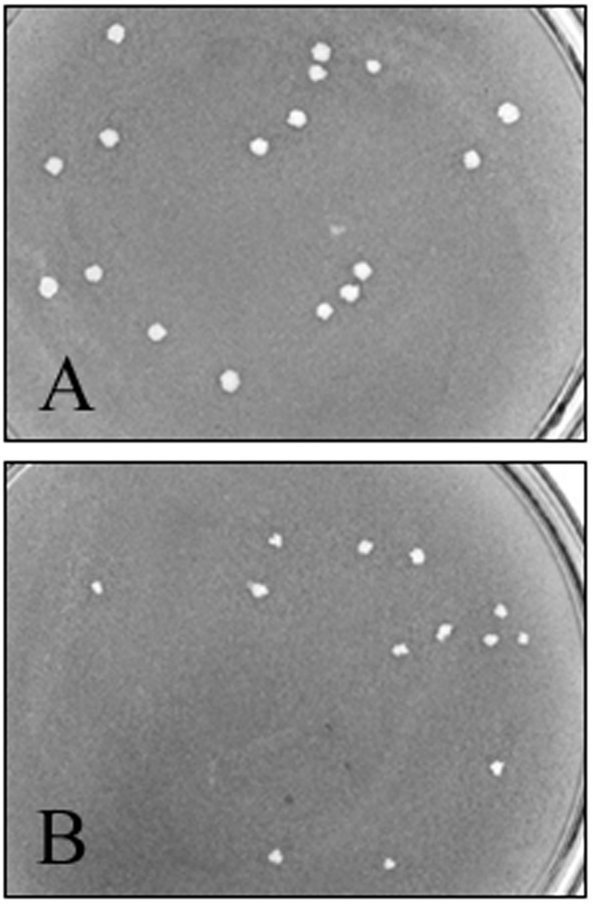

FIG. 2. Effect of the *mxiE* mutation on plaque formation in L2 monolayers. Plaques were visualized by staining with neutral red 72 h postinfection. (A) 2457T, wild-type parent; (B) BS611 (*mxiE*::*aphA-3*). Magnification, ×2.3.

Four distinct *gfp* expression profiles could be identified from the flow cytometry results by using *osp* promoter fusions. In the first expression group (group 1), which includes *ospB* and *ospE2*, the organisms displayed a moderate, but equivalent, level of fluorescence when grown in liquid culture (extracellularly) at either 30 or 37°C (Table 3 and Fig. 3). In contrast, the MFI increased by a factor of 6 to 7 when the bacteria were recovered from the intracellular environment compared to growth in liquid culture. Full, intracellular induction of *gfp* required MxiE. In the absence of MxiE, the MFI of bacteria grown inside eukaryotic cells was reduced compared to growth in liquid culture. In contrast, GFP expression from bacteria grown in liquid culture was not affected by the presence or absence of MxiE. The expression of genes in group 1 was not temperature dependent since no induction was seen at 37°C in liquid culture compared to 30°C, nor was gene expression dependent upon type III secretion because Congo red also did not influence their expression (data not shown). Thus, expression from the group 1 promoters *ospB* and *ospE2* was only upregulated in the intracellular environment and this was dependent on the presence of MxiE (ca. 25-fold induction in the presence of MxiE compared to without MxiE).

The expression profiles of the second group of promoters, which includes *ospC1* and *ospF*, were very similar to that of group 1 (Fig. 3). The main difference between group 2 promoters and those described above is that these promoters were nonfluorescent or only very weakly fluorescent when grown extracellularly but displayed an increase in fluorescence intensity when the bacteria were allowed to invade eukaryotic cells (Table 3). As seen with group 1 promoters, intracellular induction of *gfp* was dependent on the presence of MxiE. Neither growth at 37°C in liquid culture nor the addition of Congo red to the medium was able to induce expression of the group 2 promoters (Table 3 and data not shown). The expression profile of $ipaH_{9.8}$ is consistent with those in group 2.

TABLE 3. MFIs of promoter-GFP fusion constructs under differing growth conditions[a]

Promoter	Presence of MxiE	Avg MFI ± SD[b] at:			Intracellular IR[c]	Fold induction[d]
		30°C (liquid)	37°C (liquid)	37°C (intracellular)		
virA	+	20.00 ± 0.17	18.83 ± 0.06	57.77 ± 3.79	2.9	
	−	11.93 ± 0.15	13.33 ± 0.06	15.40 ± 0.27	1.3	2.2
ospB	+	1.53 ± 0.02	1.82 ± 0.006	10.70 ± 0.46	7.0	
	−	1.52 ± 0.02	1.66 ± 0.006	0.52 ± 0.01	0.3	23.3
ospE2	+	3.27 ± 0.03	3.09 ± 0.02	18.73 ± 0.23	5.7	
	−	3.06 ± 0.12	2.83 ± 0.006	0.73 ± 0.001	0.2	28.5
$ipaH_{9.8}$	+	0.60 ± 0.004	0.61 ± 0.02	1.61 ± 0.03	2.7	
	−	0.61 ± 0.006	0.64 ± 0.002	0.35 ± 0.004	0.6	4.5
ospC1	+	0.25 ± 0.003	0.25 ± 0.001	0.96 ± 0.084	3.8	
	−	0.23 ± 0.001	0.24 ± 0.002	0.23 ± 0.001	1.0	3.8
ospF	+	0.34 ± 0.00	0.40 ± 0.003	1.04 ± 0.014	3.1	
	−	0.35 ± 0.003	0.38 ± 0.004	0.26 ± 0.001	0.7	4.4
ospG	+	24.87 ± 0.06	42.27 ± 0.25	22.37 ± 0.15	0.9	
	−	28.93 ± 0.15	41.27 ± 0.35	22.67 ± 0.50	0.8	1.1
ipgD	+	5.61 ± 0.09	13.63 ± 0.06	20.73 ± 7.28	3.7	
	−	5.14 ± 0.03	12.10 ± 0.10	24.43 ± 4.86	4.8	0.8
ospD2	+	0.28 ± 0.001	0.33 ± 0.005	0.26 ± 0.003	0.9	
	−	0.30 ± 0.007	0.34 ± 0.003	0.26 ± 0.005	0.9	1.0
Vector alone[e]	+	0.24 ± 0.002	0.25 ± 0.001	0.23 ± 0.001	1.0	
	−	0.24 ± 0.01	0.23 ± 0.006	0.22 ± 0.005	0.9	1.1

[a] A graphical depiction of a representative of each sample is shown in Fig. 3.
[b] The MFI was measured by using a flow cytometer and is based on a logarithmic scale. Values represent the means ± the standard deviations of triplicate determinations from one experiment. Experiments were repeated at least twice for each promoter, and trends were the same in each experiment.
[c] The IR was calculated by dividing the MFI of bacteria grown intracellularly by the MFI of bacteria grown in liquid at 30°C, as described in Materials and Methods. An IR between 0.8 and 1.2 indicates no difference in *gfp* induction compared to background (30°C [liquid]).
[d] Fold induction indicates the level of *gfp* induced in the wild type compared to that in the *mxiE* mutant. The fold induction was calculated by dividing the IR of the wild-type strain by the IR of the *mxiE* mutant strain.
[e] Nonfluorescent bacteria are represented by an MFI of ≤0.3.

The third promoter group was temperature regulated, i.e., upregulated at 37°C. Both *ospG* and *ipgD* fall into this group. The amount of GFP detected from these strains nearly doubled when the organisms were grown at 37°C compared to growth at 30°C (Table 3). This induction was the same whether or not the bacteria contained functional MxiE. Furthermore, in the *ospG* reporter strain this induction was confined to growth in liquid culture. GFP levels were similar to baseline levels when bacteria were recovered from the intracellular environment (IRs of 0.9 and 0.8 for the wild type and *mxiE* mutant, respectively). The *ipgD* promoter was used as an internal control of the flow cytometry system. Expression of IpgD, a type III protein, is well characterized and known to be significantly upregulated at 37°C (1). As expected, *gfp* expression from the *ipgD* promoter was temperature dependent, with an IR of 2.4 at 37°C in liquid culture. Moreover, *ipgD* also was induced in the intracellular environment (a 37°C environment), although the presence of MxiE did not affect this induction (Table 3). Similar to the other promoter groups, the addition of Congo red to the culture medium had no effect on GFP expression in these strains (data not shown).

Finally, group four promoters were unaffected by any of the growth conditions tested. This group includes *ospD1* and *ospD2*. The *ospD2* promoter fusion was completely inactive in this system (Fig. 3). Strains containing the *ospD2-gfp* promoter fusion were practically nonfluorescent under all conditions tested, and the MFIs were nearly identical whether tested in the presence or absence of MxiE (Table 3). Similar results were obtained by using an *ospD1-gfp* promoter fusion (data not shown).

DISCUSSION

In this study we analyzed the role of MxiE, a member of the AraC/XylS family of transcriptional regulators and part of the Mxi-Spa type III secretion system. A deletion-insertion mutant of *mxiE* rendered the bacteria avirulent in an animal model (the Serény test). The wild-type phenotype could be restored by complementation with *mxiE* driven from a constitutive promoter, demonstrating that *mxiE* is an essential virulence gene for *Shigella*. The *mxiE* mutant appeared not to be compromised in invasion events in tissue culture and was still able to spread from cell to cell, albeit less efficiently than did the wild-type bacteria. Ipa proteins, important for entry into host cells and translocation of effectors, were still synthesized and secreted as efficiently in a *mxiE* mutant as the wild-type strain.

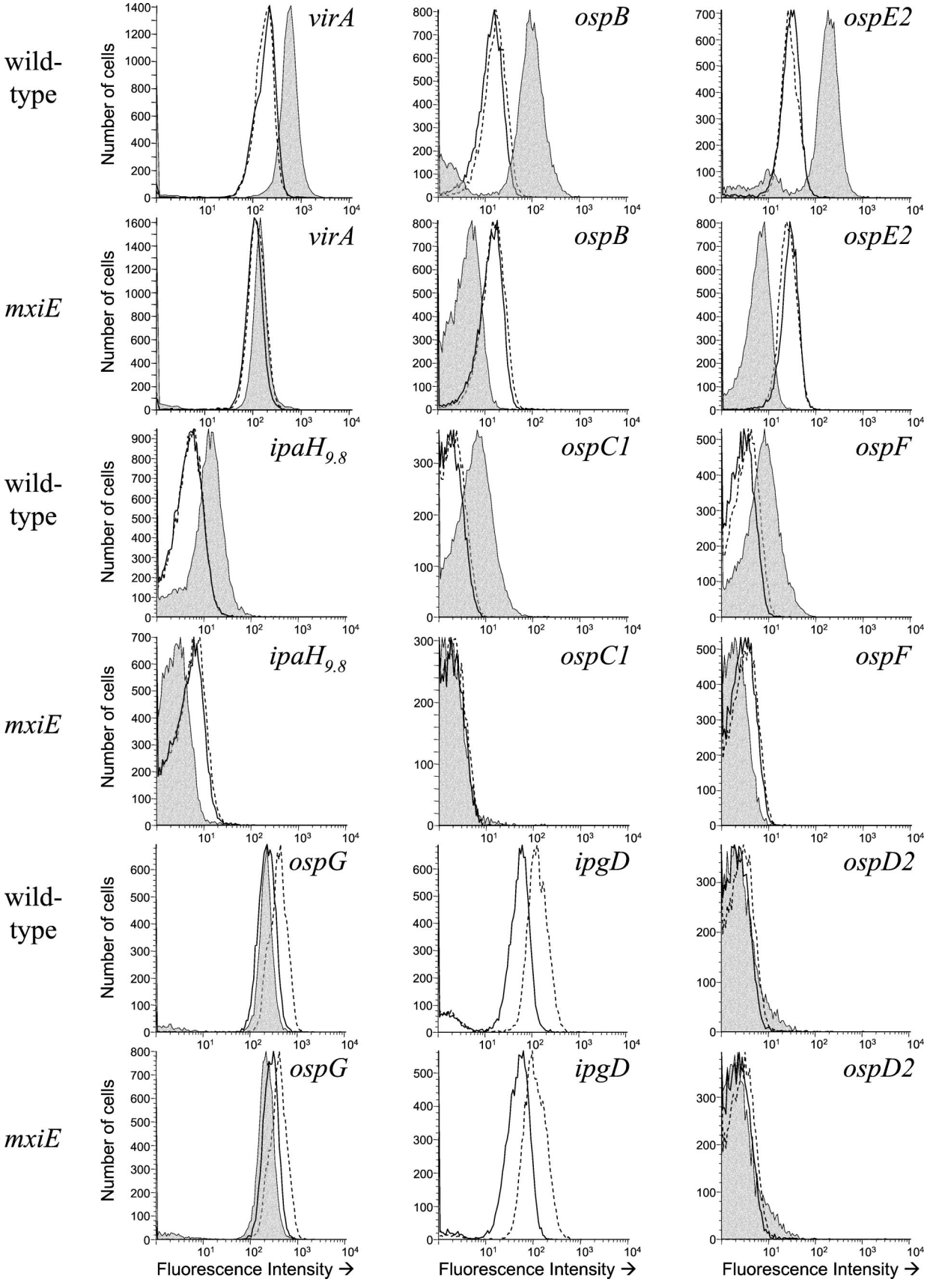

FIG. 3. Flow cytometry analysis of *S. flexneri* 2457T (wild-type) and the *mxiE* mutant (*mxiE*::*aphA-3*) containing promoter-*gfp* fusions. Each promoter examined is indicated above the histogram. The histograms denote GFP expression from extracellular bacteria grown in liquid culture at 30°C (solid black lines), in liquid culture at 37°C (dotted lines), and intracellular bacteria (gray shaded). Histograms depict a representative of each sample. The average MFIs for each sample set are given in Table 3.

Cell-to-cell spread was not affected at least shortly after invasion because the *mxiE* mutant strain was able to form protrusions. These protrusions presumably would be functional in invading neighboring cells since this mutant is also able to form plaques.

The virulence defect of the mutant and the homology to AraC suggested that the role of MxiE might be to regulate expression of factors involved in postinvasion steps of infection. We initially examined the effect of MxiE on the expression of *virA* and $ipaH_{9.8}$. Based on the work of Demers et al., both of these genes were shown to require a member of the type III secretion machinery for expression and were expressed transiently inside eukaryotic cells (16). Furthermore, VirA is not required for invasion, and a *virA* mutant displays a virulence phenotype similar to that seen with the *mxiE* mutant, namely, the *virA* mutant is as invasive as the wild-type strain and the production and secretion of Ipa proteins is not affected (16). In addition, the *virA* mutant forms small plaques and is attenuated in the Serény test. Using a promoterless *gfp* reporter system to analyze potential MxiE-regulated genes by flow cytometry, we demonstrated that MxiE is required to activate transcription from both the *virA* and the $ipaH_{9.8}$ promoters. Notably, these genes were only activated in the intracellular environment and required MxiE for activation. Furthermore, the addition of Congo red to the medium had no effect on expression of these genes. These results are in contrast to a study by Demers et al. (16), who demonstrated the transcription of a *virA-lacZ* fusion in response to Congo red in the growth medium. This discrepancy may be explained by the amount of Congo red used. In our assays, Congo red was added at a concentration of 35 μg/ml, which is already 5 to 10 times higher than the amount needed to induce type III secretion in culture (3). Demers et al. used a 100-μg/ml concentration of Congo red; this concentration is far greater than is necessary to stimulate secretion. The biological effect of such high concentrations of Congo red on secretion needs to be examined more closely.

The lack of MxiE activity during growth in liquid culture is not due to a lack of transcription of *mxi-spa*. *mxiE* is found within a large operon that encodes structural components of the type III secretion system (Fig. 1B). Since gene products found both up- and downstream of *mxiE* are required for host cell invasion and the *mxiE* mutant is still able to invade host cells as well as the wild-type strain, this demonstrates that the *mxi-spa* operon is transcribed in the mutant strain. Furthermore, transcription mapping experiments identified *mxiE* as part of a larger *mxi* transcript (K. A. Lampel, unpublished data). Finally, although there is an intergenic space of 130 bp between *mxiE* and the upstream gene, *mxiM*, no promoter activity could be demonstrated in this region (data not shown). These results imply that transcription of *mxiE* is under the same control as the rest of the *mxi* operon and therefore presumably regulated by VirB. Since *mxi* and *spa* genes are transcribed in response to growth in a 37°C environment, there must be either some sort of posttranscriptional or posttranslational regulation of MxiE activity until it is needed or else there is a cofactor for MxiE that is available only when the bacteria reach the intracellular environment.

In addition to *virA* and $ipaH_{9.8}$, we examined the effect of MxiE on the expression of several Osps. These proteins were identified in the culture supernatant of a constitutive secretion mutant (7). The original grouping of the *osp* gene families was based solely on amino acid homologies, and the different families are not related to each other. Based on results presented here, the *osp* family groupings may need to be reconsidered. One representative of each of the six *osp* gene families was tested and four genes (*ospB*, *ospC1*, *ospE2*, and *ospF*) were found to be activated by MxiE. As we saw with *virA* and $ipaH_{9.8}$, all of the MxiE-regulated *osp* genes were activated only within the intracellular environment and not in liquid culture. Adding Congo red to the liquid medium also had no effect. Two of the *osp* genes, *ospB* and *ospE2*, were very highly activated by MxiE in the intracellular environment, demonstrating an approximately 25-fold increase in fluorescence compared to the *mxiE* mutant. *gfp* induction in these strains was greater than that seen for the remaining MxiE-regulated targets, *ospC1*, *ospF*, *virA*, and $ipaH_{9.8}$, which displayed a two- to fivefold increase in fluorescence compared to the *mxiE* mutant.

The two other *osp* genes tested, *ospD2* and *ospG*, were not regulated by MxiE. The *ospG* reporter was activated by growth at 37°C in liquid culture but not by the intracellular environment. The *ospD2* reporter was not activated under any conditions tested. The fact that no *ospD2* expression was observed in this study is not surprising. The three members of the OspD family show various degrees of homology to ShET-2, a *Shigella* enterotoxin. Enterotoxins induce fluid secretion while the organism is in the intestinal lumen and would not be expected to be needed by the pathogen once inside the intracellular environment. Perhaps other environmental cues (e.g., iron starvation) are required to initiate transcription of the *ospD* genes.

The remarkable finding of the present study is the absolute dependence upon a cytosolic cue for the expression of MxiE-regulated genes. Expression of bacterial virulence factors is tightly regulated during infection of a host to ensure that these proteins are only expressed when and where they are needed. Expression of MxiE-activated genes during initial entry steps may hinder productive infections, whereas expression within the proper environment (inside the host cell) may be essential for later steps of the infection. With this in mind, the MxiE-regulated putative effectors would most likely target eukaryotic proteins or processes. The role of the MxiE-regulated Osps in postinvasion events related to virulence is currently under investigation.

Recently, Toyotome et al. demonstrated that secretion of $IpaH_{9.8}$ occurred later than that of IpaB, IpaC, and IpaD (56). IpaB, IpaC, and IpaD could be detected 30 min after Congo red induction of the type III secretion system, but $IpaH_{9.8}$ was not detected until 2 h postinduction. $IpaH_{9.8}$ is also delayed in its secretion into the cytoplasm but, once secreted, $IpaH_{9.8}$ is targeted to the host cell nucleus (56). IpaH is homologous to YopM of *Yersinia pestis*, which is trafficked to the host cell nucleus via a vesicular pathway, although its activity in the nucleus is not yet known (53). We identified $ipaH_{9.8}$ as a MxiE-regulated gene. The fact that it is delayed in secretion through the type III apparatus is consistent with our hypothesis that MxiE regulates expression of effectors involved in postinvasion events. Moreover, since all of the MxiE-activated genes identified in this study previously have been shown to be secreted by the type III secretion system, MxiE may function as

a central regulator of proteins that are secreted through the type III system after invasion of the host cell.

It is intriguing that of the six MxiE-regulated genes reported here, only *virA* was identified in previous screens for virulence factors. This may be due to the methods used to detect virulence genes. Since MxiE regulation only occurs within the intracellular environment and the *mxiE* mutant retains the ability to invade and form plaques, a screen for genes involved in later steps of infection may require use of an animal model. Induction of genes whose expression is unique to the intracellular environment has been demonstrated in both *Salmonella* and *Shigella* (8, 23, 43). Headley and Payne detected six proteins that were induced in *S. flexneri* when these organisms were recovered from the cytoplasm of cultured cells, although these six proteins were not identified (23). Furthermore, different protein profiles were observed when the organisms were labeled in tissue culture cells compared to liquid medium. Consequently, it is likely that no single method may be sufficient to detect all of the genes that are induced in the intracellular environment. However, by using a variety of approaches, the full complement of *Shigella* genes that are activated intracellularly should be able to be elucidated. The results presented here are an initial step toward that goal.

While this study was under review, Mavris et al. published a study demonstrating that MxiE, in conjunction with IpgC, activates transcription of both *virA* and $ipaH_{9.8}$ (36). These results agree with our findings, but we have, in addition, identified several other MxiE-regulated targets, namely, *ospB*, *ospC1*, *ospE2*, and *ospF*. Moreover, we have characterized the virulence properties of a *mxiE* mutant and shown that *mxiE* is an essential virulence gene in vivo. Importantly, the work presented here extends the observations of Mavris et al. to demonstrate that MxiE activation of its targets occurs only in the intracellular environment. Several possibilities might explain the fact that we did not see MxiE-dependent activation of target promoters in vitro as described by Mavris et al. These researchers demonstrated MxiE-regulated gene activation in the presence of very high concentrations of Congo red, in a constitutive secretion mutant of *Shigella*, or by using *mxiE* on a multicopy plasmid expressed in *E. coli*. Our system employed a wild-type *Shigella* strain in a tissue culture model, which more closely mimics the normal environment encountered by the bacteria during infection. Nevertheless, based on work presented here and that of Mavris et al., it seems likely that the eukaryotic cytoplasm provides a constitutive secretion environment that is the intracellular cue required for MxiE activity in vivo.

The fact that MxiE regulates postinvasion events and that MxiE-regulated targets are, for the most part, previously uncharacterized, is intriguing. Other enteric pathogens, such as *Salmonella* and *Yersinia*, possess two or more distinct type III secretion systems. These systems are differentially regulated and the effectors are involved at different steps of infection. The SPI1-encoded type III secretion system of *Salmonella* is required for bacterial entry into host cells, whereas the SPI2-encoded type III secretion system is involved in intracellular survival and replication (11, 24). In *Yersinia*, the virulence plasmid-encoded type III secretion system (Ysc) is required early in infection for the organism to resist macrophage killing, whereas a newly described type III secretion system (Ysa) found on the *Yersinia* chromosome is believed to play a role late in infection (22). *Shigella* has only one known type III secretion system. The role of this system in invasion is well characterized, although its effectors also are required for some postinvasion steps of infection (41, 51). Perhaps shigellae have evolved unique mechanisms of virulence gene regulation to adapt its single type III secretion system to function in both invasion and postinvasion events, whereas other enteric pathogens evolved separate secretion systems to perform these functions. One of these unique regulation mechanisms is through the intracellular induction of proteins secreted through the type III system in response to MxiE activation.

Virulence gene regulation in *Shigella* is a complex process involving multiple gene products and a variety of environmental signals. Temperature regulation of virulence factors is well characterized in *Shigella*. Here we have identified an essential virulence gene involved in a novel, additional level of regulation of type III secreted proteins in the intracellular environment. Elucidation of the molecular mechanism of regulation by MxiE should yield interesting insights into the evolution of *Shigella* as a facultative intracellular pathogen.

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