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Identification of the *cis*-Acting Site Involved in Activation of Promoters Regulated by Activity of the Type III Secretion Apparatus in *Shigella flexneri*

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Bacteria of *Shigella* spp. use a virulence plasmid-encoded type III secretion (TTS) system to invade the colonic epithelium in humans. The activity of the TTS apparatus is tightly regulated in the wild-type strain and is induced upon contact of bacteria with epithelial cells, whereas it is deregulated, i.e., constitutively active, in some mutants. Under conditions of deregulated secretion, approximately 20 proteins are secreted, including VirA, OspB to OspG, and at least three members of the IpaH family, all of which are encoded by the virulence plasmid. Conditions inducing or deregulating the activity of secretion also induce the transcription of *virA* and four *ipaH* genes. The transcription of *virA* and *ipaH9.8* requires both MxiE, a transcriptional activator of the AraC family, and IpgC, the chaperone of IpaB and IpaC, acting as a coactivator. Using reporter plasmids containing *lacZ* transcriptional fusions, we showed that the *ipaH7.8*, *ipa4.5*, *ospC1*, and *ospF* promoters are activated under conditions of deregulated secretion and that both MxiE and IpgC are necessary and sufficient for their activation in both *Shigella flexneri* and *Escherichia coli*. Promoter mapping and deletion analysis of the *ipaH9.8*, *virA*, and *ospC1* promoters identified a 17-bp motif, the MxiE box, which overlaps the −35 region and is essential for the activation of these promoters. The presence of eight MxiE boxes on the virulence plasmid suggests that 11 genes encoding secreted proteins may be regulated by the activity of secretion. We also present evidence that at least one *ipaH* gene that is carried by the chromosome is controlled by MxiE and IpgC.

The type III secretion (TTS) pathway is used by numerous gram-negative pathogenic bacteria to deliver virulence proteins to the membrane or directly to the cytoplasm of host cells, where they interfere with cellular signaling pathways. The TTS system consists of (i) the secretion apparatus that spans the bacterial envelope, (ii) translocators that are inserted into the host cell membrane and effectors that are translocated into the host cell cytoplasm, (iii) specific cytoplasmic chaperones that associate with the translocators and some effectors within the bacterial cytoplasm, and (iv) transcriptional regulators required for the expression of components of the TTS apparatus and/or proteins secreted by this apparatus (21).

The TTS apparatus encoded by the virulence plasmids of *Yersinia* and *Shigella* spp. and the pathogenicity islands SPI-1 and SPI-2 of *Salmonella* spp. is not or is only weakly active during growth of bacteria in broth and is activated upon contact of bacteria with host cells (29, 34, 45). In vitro, increased secretion is obtained following exposure of bacteria to a medium (i) deprived of calcium for *Yersinia* spp. (31, 41), (ii) containing the dye Congo red for *Shigella* spp. (4), (iii) or at a low pH for *Salmonella* spp. (5). In addition, the inactivation of some genes, such as *yopN*, *tyeA*, or *lcrG* in *Yersinia* spp. and *ipaB* and *ipaD* in *Shigella* spp., results in deregulated secretion; i.e., the TTS apparatus of these mutants is active in the absence of inducers (8, 16, 30, 39). Conditions inducing or deregulating the activity of secretion also result in the increased transcription of some genes encoding secreted proteins, such as the *yop* genes in *Yersinia* spp. and the *virA* and *ipaH* genes in *Shigella* spp. (10, 13, 17, 42). In *Yersinia* spp., the mechanism by which the activity of secretion regulates the transcription of *yop* genes involves the secreted protein LcrQ and its cytoplasmic chaperone SycH but is not yet understood (18, 34, 36, 41, 44). In *Shigella flexneri*, the transcription of *virA* and *ipaH* genes requires both MxiE, a transcriptional activator of the AraC family, and its coactivator, IpgC, the chaperone for the proposed IpaB and IpaC translocators (28). Under conditions of nonsecretion, IpaB and IpaC act as anticoactivators by binding to and titrating IpgC, thereby rendering it unavailable to activate MxiE. This mechanism of regulation is reminiscent of that for the flagellin genes in *Salmonella enterica* serovar Typhimurium, in which the secretion of anti-sigma factor FlgM upon completion of the hook-basal body complex is required to liberate sigma factor σ^{28}, which can then transcribe the late genes for flagellin (9, 22).

Bacteria of *Shigella* spp., the causative agents of bacillary dysentery in humans, use a TTS system to invade the colonic epithelium, resulting in tissue destruction and massive inflammation (25). Genes required for the entry of bacteria into epithelial cells are located on a 30-kb region, designated the entry region, of the 210-kb virulence plasmid (27, 38). This region contains genes for the Mxi-Spa TTS apparatus; the secreted IpaA to IpaD, IpgB1, and IpgD proteins; the chaperones IpgC, IpgE, and Spa15; and the transcriptional regulators VirB and MxiE (33). The transcription of genes of the entry region from the divergent *icsB* and *ipgD* promoters is under the control of both VirF and VirB. The expression of VirF, a member of the AraC family that is encoded by the virulence plasmid, is induced at 37°C (14) and activates transcription from the *virB* promoter (23). VirB, a member of the

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ParB family of partitioning proteins, is required for transcription of the *icsB* and *ipgD* promoters (1). Although the TTS apparatus is assembled and effector proteins are synthesized, wild-type *S. flexneri* secretes only a small proportion (~5%) of IpaA to IpaD and IpgD upon growth at 37°C in laboratory medium (3). Secretion is induced by contact with cells (30), addition of the dye Congo red to the growth medium (4), or inactivation of *ipaB* or *ipaD* (29, 32). Under conditions of deregulated secretion, approximately 20 proteins are secreted, including VirA, OspB to OspG, and IpaH (6, 32). The virulence plasmid carries five *ipaH* genes, designated *ipaH1.4*, *ipaH2.5*, *ipaH4.5*, *ipaH7.8*, and *ipaH9.8*, according to the size of the *Hin*dIII fragment that carries each copy (20), and three *ospC*, three *ospD*, and two *ospE* genes (6).

It was previously shown that the transcription of the *virA* gene and four *ipaH* genes is regulated by the activity of the Mxi-Spa apparatus (13). The transcription of these genes is induced upon the entry of bacteria into epithelial cells, during the growth of bacteria in the presence of Congo red, and following the inactivation of *ipaB* or *ipaD*. Under conditions of active or deregulated secretion, activation of the *virA* and *ipaH9.8* promoters is controlled by the MxiE transcriptional activator and the IpgC chaperone acting as a coactivator (28). However, neither the *cis*-acting element(s) involved in the activation of these promoters in response to secretion nor the repertoire of genes whose transcription is controlled by the activity of secretion has been characterized. In the present study, we used reporter plasmids containing the putative promoter regions of a number of genes encoding secreted proteins to investigate (i) which genes are regulated by the activity of secretion, (ii) the *cis*-acting region involved in this regulation, and (iii) whether or not these genes are under the control of MxiE and IpgC. Using promoter mapping and deletion analysis of the *virA*, *ipaH9.8*, and *ospC1* promoters, we identified a motif, designated the MxiE box, which is essential for the activation of regulated promoters. Sequence analysis revealed the presence of eight MxiE boxes on the virulence plasmid, suggesting that 11 genes encoding secreted proteins may be regulated by the activity of secretion. We also present evidence that at least one *ipaH* gene carried by the chromosome is controlled by MxiE and IpgC.

MATERIALS AND METHODS

Bacterial strains and growth media. *S. flexneri* strains are derivatives of wild-type strain M90T (serotype 5) (37); M90T-Sm (Sm^r), BS176 (a virulence plasmid-cured strain), SF622 (*ipaD2*), SF1076 (*ipaB4*), SF1070 (*mxiE ipaB4*) and SF1068 (*ipgC ipaB4*) have been described elsewhere (2, 28, 29). *Escherichia coli* strain DH5α was used for plasmids carrying the *oriT* origin of replication. Bacteria were grown in Luria-Bertani (LB) medium at 37°C. Antibiotics were used at the following concentrations: ampicillin, 100 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; chloramphenicol, 25 μg ml^{-1}; and streptomycin, 100 μg ml^{-1}.

Construction of reporter plasmids. Briefly, a DNA fragment extending from the 3′ end of the nearest insertion sequence (IS) element or coding sequence located upstream from the gene of interest to approximately 100 bp downstream from the translation start site of that gene was amplified by PCR and cloned into the *Bam*HI and *Hin*dIII sites of vector pQF50 (15), upstream from and in the same orientation as a promoterless *lacZ* gene. Coordinates, with respect to the sequence of pWR100 (6), of the 5′ and 3′ ends of the fragment cloned in each plasmid are indicated in Table 1. The construction of *ipaH9.8-lacZ* (pMM10) and *virA-lacZ* (pBD7) reporter plasmids, pMM71 (MxiE), and pKH128 (IpgC) has been described elsewhere (28).

Purification of RNA. RNA preparations were performed essentially as described by Spohn et al. (40). *S. flexneri* strains M90T (wild type) and SF622 (*ipaD*) were grown in 25 ml of LB broth at 37°C to mid-log phase and harvested. Cells were lysed in 3.7 ml of a solution containing 100 mM Tris-HCl (pH 7.5), 2 mM disodium EDTA, and 1% sodium dodecyl sulfate (SDS) and incubated for 5 min at 95°C. Lysates were adjusted to 80 mM KCl and incubated for 10 min on ice. Cellular debris was removed by centrifugation at 8,000 rpm for 10 min in a JA20 rotor (Beckman). To 3.5 ml of supernatant, 4.56 g of CsCl was added, and the RNA was sedimented by centrifugation in an SW65 rotor (Beckman) for 15 to 20 h at 35,000 rpm. The RNA pellet was resuspended in 500 μl of Tris-EDTA (10 mM Tris-HCl [pH 8], 1 mM disodium EDTA), extracted once with an equal volume of phenol-chloroform (1:1), ethanol precipitated, resuspended in 200 μl of Tris-EDTA, reprecipitated, and stored at −20°C.

TABLE 1. Reporter plasmids used in this study

Reporter plasmid[a]	Transcriptional fusion	Cloned fragment		
		Coordinate[b]		Size (bp)
		5′ End	3′ End	
pMM10	*ipaH9.8-lacZ*	173945	174493	547
pMM33	*ipaH9.8-lacZ*	174183	174493	309
pMM34	*ipaH9.8-lacZ*	174203	174493	289
pMM11	*ipaH7.8-lacZ*	63585	64170	584
pMM12	*ipaH4.5-lacZ*	65773	66399	625
pMM13	*ipaH1.4-lacZ*	208426	208995	568
pMM19	*ospC1-lacZ*	78233	78976	742
pMM35	*ospC1-lacZ*	78233	78643	409
pMM37	*ospC1-lacZ*	78233	78616	382
pMM29	*ospD3-lacZ*	76410	76921	510
pMM65	*ospF-lacZ*	12359	12488	128
pMM20	*ospG-lacZ*	176488	176827	338
pBD7	*virA-lacZ*	145165	146008	842
pBD9	*virA-lacZ*	145165	145888	722
pMM30	*icsB-lacZ*	111389	111804	414

[a] The reporter plasmids were constructed with vector pQF50.
[b] Coordinates of the 5′ end and the 3′ end of the cloned fragment are indicated with respect to the sequence of pWR100 (6). In pWR100, the direction of transcription of *ipaH9.8*, *ipaH7.8*, *ipaH4.5*, and *ospG* is clockwise, whereas that of *ipaH1.4*, *ospC1*, *ospD3*, and *virA* is counterclockwise.

Primer extension analyses. Oligonucleotides (10 pmol) were 5′ end labeled in the presence of [γ-^{32}P]ATP (5,000 Ci mmol^{-1}) and T4 polynucleotide kinase (New England Biolabs). Labeled oligonucleotides (0.4 to 1.0 pmol) were then coprecipitated with 15 μg of *S. flexneri* total RNA and resuspended in 5 μl of H_2O–2 μl of each deoxynucleoside triphosphate (2 mM)– 2 μl of 5× reverse transcription buffer (cDNA synthesis kit; Boehringer Mannheim). The reaction mixture was incubated for 1 min at 95°C, 1 μl of reverse transcriptase (20 U ml^{-1}; Boehringer Mannheim) was added, and reverse transcription was carried out at 45°C for 45 min. Samples were then incubated for 10 min at room temperature with 1 μl of RNase A (1 mg ml^{-1}), extracted once with an equal volume of phenol-chloroform (1:1), ethanol precipitated, and resuspended in 5 μl of sequencing loading buffer. After denaturation at 95°C for 2 min, samples were subjected to urea–6% polyacrylamide gel electrophoresis (PAGE) together with the sequencing reaction products obtained by use of the same primers with plasmids containing fragments of the *ospC1*, *virA*, and *ipaH9.8* promoters. Sequencing reactions were performed by the dideoxy chain termination method with [α-^{32}P]dATP (Amersham) and a T7 sequencing kit (Pharmacia).

Enzyme assays. The β-galactosidase activity present in bacteria growing in LB medium and harvested during the exponential phase of growth was assayed by using the substrate *o*-nitrophenyl-β-D-galactopyranoside as described previously (35)

Protein analysis. To prepare whole-cell extracts, bacteria were grown in 3 ml of LB medium. One milliliter of bacteria was centrifuged and resuspended in 500 μl of Laemmli sample buffer (26). Protein samples were boiled for 3 min and analyzed by SDS-PAGE as described previously (26). After electrophoresis, proteins were transferred to a nitrocellulose membrane. Immunoblotting was carried out with a rabbit polyclonal anti-IpaH antibody (28). Horseradish peroxidase-labeled goat anti-rabbit antibodies were used as secondary antibodies and visualized by enhanced chemiluminescence.

TABLE 2. Expression of *lacZ* transcriptional fusions from reporter plasmids in *S. flexneri* strains

Transcriptional fusion	Reporter plasmid[a]	Size of the cloned fragment (bp)	β-Galactosidase activity in derivatives of the following strain[b]:		Ratio[c]
			Wild type	*ipaD*	
ipaH7.8-lacZ	pMM11	584	30	1,700	55
ipaH4.5-lacZ	pMM12	625	150	420	3
ipaH1.4-lacZ	pMM13	568	45	40	1
ospC1-lacZ	pMM19	740	30	440	15
ospF-lacZ	pMM65	128	45	370	8
ospD3-lacZ	pMM29	510	130	220	1.7
ospG-lacZ	pMM20	338	220	250	1
icsB-lacZ	pMM30	414	1,300	760	0.6

[a] The reporter plasmids were constructed with vector pQF50 (Table 1).
[b] β-Galactosidase activities assayed in derivatives of M90T (wild type) and SF622 (*ipaD*) are expressed in Miller units and are the means of at least four independent experiments. Standard deviations (not shown) were within 25% of the reported values.
[c] Activity present in the *ipaD* mutant versus activity present in the wild-type strain.

RESULTS

Use of reporter plasmids to investigate the regulation of genes encoding secreted proteins. To identify the *cis*-acting regions responsible for the regulation of transcription of the *ipaH7.8*, *ipaH4.5*, and *ipaH1.4* genes in response to secretion (13), the putative promoter regions of these genes were amplified by PCR and cloned upstream from the promoterless *lacZ* gene of reporter plasmid pQF50 (Table 1). In each case, the cloned fragment extended from the 3′ end of the upstream IS element or coding sequence to 100 bp downstream from the translation start site of the gene of interest. As a control, we also cloned the *icsB* promoter, which is not regulated by the activity of secretion (13), into pQF50. The resulting reporter plasmids were each introduced into *S. flexneri* strain M90T (wild type), in which the activity of secretion is tightly controlled and promoters regulated by secretion are not active, and strain SF622 (*ipaD*), which displays a phenotype of deregulated secretion and in which promoters regulated by secretion are active (13, 28). β-Galactosidase activity was assayed during growth of the recombinant strains at 37°C (Table 2). As observed for reporter plasmids carrying the *ipaH9.8* and *virA* promoters (28), the reporter plasmid containing a fusion with the *ipaH7.8* putative regulatory region was activated more than 10-fold in the *ipaD* mutant compared to the wild-type strain, indicating that the regulatory region was present and functional on the reporter plasmid. In contrast, reporter plasmids containing fusions with *ipaH4.5* and *ipaH1.4* showed a moderate increase (threefold) and no increase, respectively, in β-galactosidase activity in the *ipaD* mutant. The very low level of β-galactosidase activity expressed from the *ipaH1.4* reporter plasmid suggested that no promoters were present upstream from *ipaH1.4*, i.e., in the 389-bp *ospE1-ipaH1.4* intergenic region. In contrast, the intermediate level of activation of the *ipaH4.5-lacZ* fusion suggested that the promoter and regulatory regions of *ipaH4.5* were indeed present on the reporter plasmid, i.e., in the 429-bp *ipaH7.8-ipaH4.5* intergenic region, and that this promoter was not as tightly regulated as that of the *ipaH7.8* gene. As previously described for an *icsB-lacZ* transcriptional fusion carried by the virulence plasmid (13), the levels of expression of the *icsB-lacZ* fusion carried by the reporter plasmid were similar in the wild-type and *ipaD* strains.

To investigate the potential regulation of representatives of genes encoding proteins that were secreted under conditions of deregulated activity of the TTS apparatus (6), the putative promoter regions of *ospC1*, *ospD3*, *ospF*, and *ospG* were also cloned into pQF50, and the corresponding reporter plasmids were introduced into M90T (wild type) and SF622 (*ipaD*). As shown in Table 2, the levels of expression of both the *ospC1-lacZ* and the *ospF-lacZ* fusions were increased approximately 10-fold in the *ipaD* mutant compared to the wild-type strain, suggesting that the transcription of these two genes was also regulated by the activity of secretion. In contrast, the *ospD3* and *ospG* reporter plasmids expressed similar levels of β-galactosidase activity in both backgrounds, suggesting that the transcription of these two genes was not regulated by the activity of secretion. In addition, similar levels of β-galactosidase activity were obtained in a *S. flexneri* strain cured of the virulence plasmid (data not shown), suggesting that the cloned promoters may not be controlled by regulators encoded by the virulence plasmid.

In conclusion, these and previous results (28) indicated that at least five promoters, including those for *virA*, *ipaH9.8*, *ipaH7.8*, *ospC1*, and *ospF*, and possibly that for *ipaH4.5* were activated under conditions of deregulated secretion.

Primer extension analyses. To determine the transcription start points for representative members of regulated promoters (*ipaH9.8*, *ospC1*, and *virA*), RNA was prepared from both wild-type and *ipaD* strains and used for primer extension analyses. No extension products were observed with RNA isolated from the wild-type strain, a result which was consistent with the lack of transcription of these genes under conditions of nonsecretion (Table 2) (28). The transcription start sites for the three genes were determined from the extension products obtained with the RNA samples from the *ipaD* mutant (Fig. 1). A strong similarity with the consensus sequence for the −10 region (TATAAT) was observed upstream from +1 for each of these promoters (Fig. 2). In contrast, no similarity or a very weak similarity was detected between the −35 regions of these promoters and the consensus −35 sequence (TTGACA).

Identification of a putative regulatory region. A comparison of the *ipaH9.8*, *ospC1*, and *virA* promoter sequences led to the identification of a 10-bp segment (5′-GTATCGTTTT-3′), starting at position −49 and extending to position −40, which was identical in the three promoters (Fig. 2). This 10-bp sequence was also detected upstream from the *ipaH7.8* and *ospF* genes, both of which are also regulated by the activity of secretion (Table 2). Systematic analysis of the sequence of the virulence plasmid indicated that this conserved 10-bp sequence was also present upstream from the *ospE1* and *ospE2* genes, both of which encode proteins secreted by the TTS apparatus (6). A simultaneous comparison of all of these sequences indicated that the conserved region extended over 17 bp, from position −49 to position −33, and that, in each case, it was located 16 or 17 bp upstream from a sequence very similar to TATAAT (Fig. 2). The conservation of this 17-bp sequence, designated the MxiE box (for reasons detailed below), in regulated promoters and its location within these promoters suggested that it may be involved in their regulation. A sequence

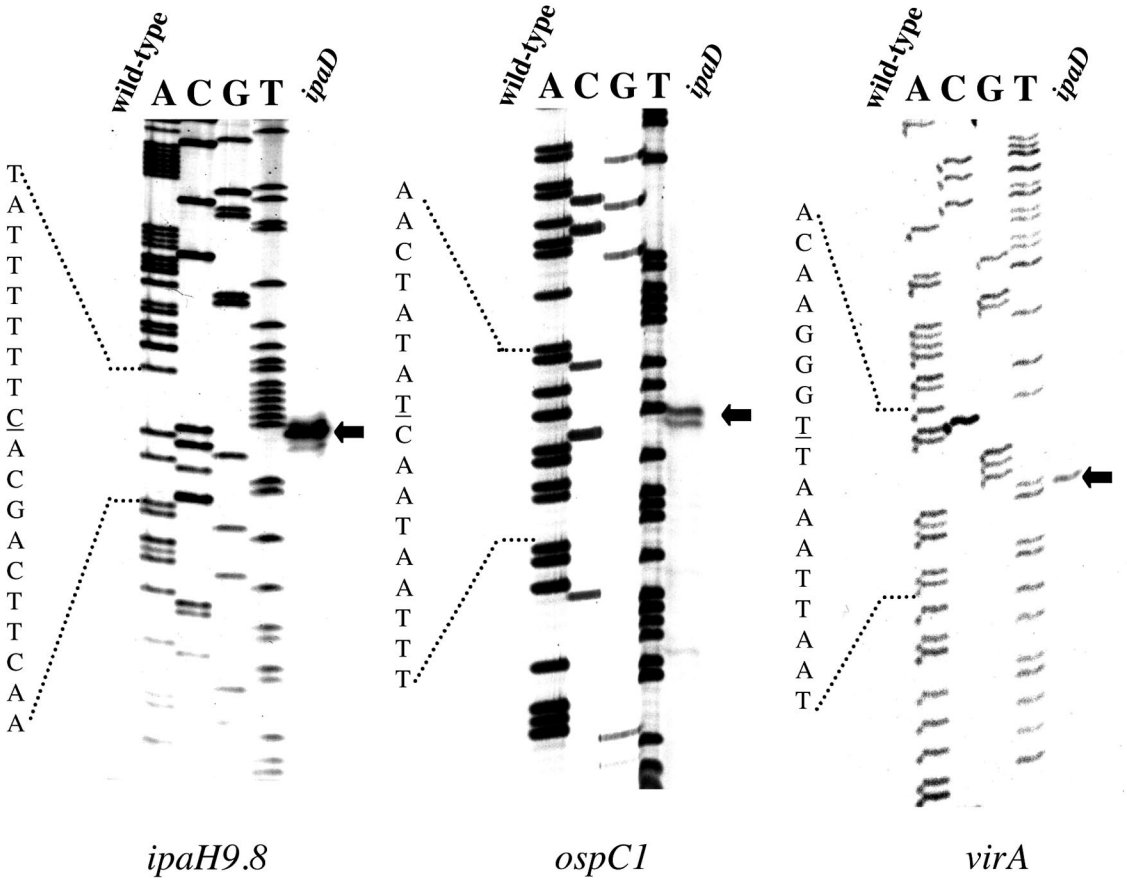

FIG. 1. Mapping of the *ipaH9.8*, *ospC1*, and *virA* promoters. Extension products obtained with RNA isolated from M90T (wild type) and SF622 (*ipaD*) by using primers specific for *ipaH9.8*, *ospC1*, and *virA* were analyzed by PAGE, together with sequencing reaction products obtained by using the same primers. The nucleotide sequence to the left of each panel corresponds to the antisense strand, and the underlined nucleotide corresponds to the major transcription start site. The arrows indicate the major primer extension products.

sharing similarities with the MxiE box was also detected upstream from the *ipaH4.5* gene, the transcription of which was increased threefold in the *ipaD* mutant (Table 2). However, in this latter case, G and T bases, respectively, replaced the conserved T and C bases at positions −48 and −45.

Determination of the role of the MxiE box. To investigate the potential role of the MxiE box in the regulation of the *ipaH9.8* promoter, we constructed plasmids pMM33 and pMM34, in which the cloned regions started at nucleotides −63 and −43, respectively, in relation to the transcription start point and ended at nucleotide +259. In plasmid pMM33, the MxiE box was intact, whereas in plasmid pMM34, it was interrupted at position −43. The two plasmids were introduced into wild-type and *ipaD* strains, and β-galactosidase activity expressed in the recombinant strains was assayed (Table 3). The level of expression of the *ipaH9.8-lacZ* fusion from pMM33, in which the region located upstream from the consensus sequence had been deleted, was still higher in the *ipaD* mutant than in the wild-type strain. In contrast, similar low levels of β-galactosidase were expressed from pMM34, in which half of the MxiE box had been deleted, in both the *ipaD* mutant and the wild-type strain. This result indicated that the promoter carried by pMM34 was no longer regulated by the activity of secretion and suggested further that the MxiE box may represent the binding site for a transcriptional activator.

To confirm the involvement of the MxiE box in the regulation of the *ospC1* and *virA* promoters, we constructed plasmids pMM35 and pMM37, in which the *ospC1* promoter region started at nucleotides −63 and −43, respectively, and plasmid pBD9, in which the *virA* promoter region started at nucleotide −43. Each of these plasmids was introduced into wild-type and *ipaD* strains. As shown in Table 3, deletion of the region located upstream from the MxiE box in the *ospC1* promoter had no effect on the regulation of this promoter. In contrast, deletion of part of the MxiE box abolished the activation of the *ospC1* promoter in the *ipaD* mutant. Likewise, the level of expression of the *virA-lacZ* fusion from plasmid pBD9, in which part of the MxiE box of the *virA* promoter had been removed, was no longer increased in the *ipaD* mutant compared to the wild-type strain. Accordingly, for each of the *ipaH9.8*, *ospC1*, and *virA* promoters, a deletion within the MxiE box led to a loss of the activation of these promoters in the *ipaD* mutant.

Regulation of transcription by MxiE and the coactivator IpgC. It was previously shown that both MxiE, a transcriptional activator of the AraC family, and IpgC, the chaperone of the

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                          -49             -33                    -10        +1
                           .               .                      .          .
ospC1   : atcagagaaa GTATCGTTTTTTTAtAG taaaattcattgctgttcaa TAaAAT tgatat A - 229 - ATG
ipaH9.8: aactgaaaca GTATCGTTTTTTTAcAG ccaattttgtttatccttat TATAAT aaaaaa G -  96 - ATG
virA    : tagtggaaat GTATCGTTTTcTTAaAG agaagaataacattccattt TATtAT gttccc A -  56 - ATG
ospF    : tacatataat GTATCGTTTTaTaAaAG atgataaacaatcaatataa gATAAT atatct A -  20 - ATG
ipaH7.8: tattaaatgt GTATCGTTTTTTTAcAG taatttttaatttgttattc TATAAT aggaat A - 271 - ATG
ospE1   : acccgaaaaa GTATCGTTTTTTTAcAG taaacttcatttagccgac. TATAAT gtaaaa A -  38 - ATG
ospE2   : tacgaaaaaa GTATCGTTTTTTTAcAG taaacttcatttagccgac. TATAAT gtaaaa A -  36 - ATG
ipaH4.5: taaagctcta GgATtGTTTTTTTAaAG actttctcgttttatttgc. atTAAT agacca A -  25 - ATG

MxiE box:           GTATCGTTTTTTTAnAG
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FIG. 2. Comparison of the promoter regions of genes controlled by MxiE and IpgC. Sequences located upstream from the translation start site (ATG) of the various genes were aligned with respect to the transcription start site (+1). The nucleotides corresponding to the transcription start site determined for the *ipaH9.8*, *ospC1*, and *virA* promoters and proposed for the *ospF*, *ipaH7.8*, *ospE1*, *ospE2*, and *ipaH4.5* promoters are indicated in bold and underlined characters, respectively. The number of nucleotides present between the transcription and the translation start sites is indicated. In each promoter region, nucleotides that are identical to those present in the consensus sequence of the MxiE box (last line) and the −10 region (TATAAT) are shown in uppercase bold characters. Coordinates with respect to the transcription start site are indicated at the top.

IpaB and IpaC translocators, are required for the activation of transcription of the *ipaH9.8* and *virA* promoters in response to secretion (28). To investigate whether the *ospC1*, *ipaH7.8*, and *ospF* promoters, which were activated in the *ipaD* mutant, were also under the control of MxiE and IpgC, reporter plasmids containing the *ospC1*, *ospF*, and *ipaH7.8* promoters were introduced into *S. flexneri* strain SF1076 (*ipaB4*) which, like the *ipaD* mutant, displays a phenotype of constitutive secretion, and its derivatives, SF1068 (*ipaB4 ipgC*) and SF1070 (*ipaB4 mxiE*). As expected, each of these plasmids expressed much higher levels of β-galactosidase activity in the *ipaB* mutant than in the wild-type strain (Table 4). In addition, the absence of either MxiE (SF1070) or IpgC (SF1068) reduced the activity of the *ospC1*, *ospF*, and *ipaH7.8* promoters to that observed in the wild-type background. These results indicated that both MxiE and IpgC were required for the transcription of these promoters in an *ipaB* background, i.e., under conditions of deregulated secretion.

TABLE 3. Deletion analyses of *ipaH9.8*, *ospC1*, and *virA* promoters

Transcriptional fusion	Reporter plasmid[a]	Coordinate of 5′ end of the cloned fragment[b]	β-Galactosidase activity in derivatives of the following strain[c]:		Ratio[d]
			Wild type	*ipaD*	
ipaH9.8-lacZ	pMM10	−312	400	4,000	10
	pMM33	−63	480	1,400	3
	pMM34	−43	260	270	1
ospC1-lacZ	pMM19	−398	40	570	15
	pMM35	−63	110	1,100	10
	pMM37	−43	70	80	1
virA-lacZ	pBD7	−163	70	530	8
	pBD9	−43	135	55	0.4

[a] The reporter plasmids were constructed with vector pQF50 (Table 1).
[b] Coordinates are indicated with respect to the transcription start site.
[c] β-Galactosidase activities assayed in derivatives of M90T (wild type) and SF622 (*ipaD*) are expressed in Miller units and are the means of at least four independent experiments. Standard deviations (not shown) were within 25% of the reported values.
[d] Activity present in the *ipaD* mutant versus activity present in the wild-type strain.

Likewise, to determine whether the *ipaH4.5* promoter, which was activated only moderately in the *ipaD* mutant, and the *ospD3* promoter, which was not activated in the *ipaD* mutant, were under the control of MxiE and IpgC, we introduced reporter plasmids carrying these promoters into SF1076, SF1068, and SF1070. As observed in the *ipaD* mutant, the level of expression of the *ipaH4.5-lacZ* fusion was increased threefold in the *ipaB4* mutant compared to the wild-type strain (Table 4). Moreover, in the *ipaB4 mxiE* and *ipaB4 ipgC* mutants, the level of expression of this fusion was decreased to that observed in the wild-type strain. These results confirmed that the *ipaH4.5* promoter was also regulated by the activity of secretion, although to a lesser extent than the *ospC1*, *ospF*, and *ipaH7.8* promoters, and that this regulation was also dependent on MxiE and IpgC. In contrast, the level of expression of the *ospD3-lacZ* fusion was not increased in the *ipaB4* mutant compared to the wild-type strain and was not affected by the inactivation of either *mxiE* or *ipgC*.

Activation of regulated promoters by MxiE and IpgC in *E. coli*. As indicated above, both MxiE and IpgC were required for the activation of the *ospC1*, *ospF*, and *ipaH7.8* promoters and, to a lesser extent, the *ipaH4.5* promoter. To investigate whether MxiE and IpgC were the only virulence plasmid-encoded proteins directly involved in the activation of these promoters, the corresponding reporter plasmids were introduced into *E. coli* strains that expressed MxiE and IpgC from recombinant plasmids (28). Neither MxiE nor IpgC alone increased the levels of expression of the reporter fusions. In contrast, the levels of expression of the *ospC1-lacZ*, *ospF-lacZ*, and *ipaH7.8-lacZ* fusions were increased 4- to 20-fold in the *E. coli* strains producing both MxiE and IpgC (Table 5). These results indicated that MxiE and IpgC were sufficient and necessary for transcription from promoters possessing an MxiE box. The levels of expression of the *ipaH4.5-lacZ* fusion, which was regulated only moderately in *S. flexneri*, and the *ospD3-lacZ* fusion, which was not dependent on MxiE and IpgC in *S. flexneri*, were not increased in the presence of MxiE and IpgC. The lack

TABLE 4. Expression of *lacZ* transcriptional fusions from reporter plasmids in *S. flexneri mxiE* and *ipgC* strains

Transcriptional fusion	Reporter plasmid[a]	β-Galactosidase activity in derivatives of the following strain[b]:			
		Wild type	*ipaB4*	*ipaB4 ipgC*	*ipaB4 mxiE*
ospC1-lacZ	pMM19	30	440	15	20
ipaH7.8-lacZ	pMM11	30	1,000	10	25
ospF-lacZ	pMM65	45	470	50	40
ipaH4.5-lacZ	pMM12	150	500	140	130
ospD3-lacZ	pMM29	130	160	190	140

[a] The reporter plasmids were constructed with vector pQF50 (Table 1).
[b] β-Galactosidase activities assayed in derivatives of M90T (wild type), SF1076 (*ipaB*), SF1068 (*ipaB4 ipgC*), and SF1070 (*ipaB4 mxiE*) are expressed in Miller units and are the means of at least four independent experiments. Standard deviations (not shown) were within 25% of the reported values. Data for the wild-type strain are from Table 2.

TABLE 5. Expression of *lacZ* transcriptional fusions from reporter plasmids in *E. coli* strains containing MxiE- and IpgC-producing plasmids

Transcriptional fusion	Reporter plasmid[a]	β-Galactosidase activity in derivatives of strains expressing[b]:		
		MxiE	IpgC	MxiE + IpgC
ospC1-lacZ	pMM19	5	10	50
ipaH7.8-lacZ	pMM11	190	280	5,500
ospF-lacZ	pMM65	15	40	170
ipaH4.5-lacZ	pMM12	50	90	45
ospD3-lacZ	pMM29	20	60	50

[a] The reporter plasmids were constructed with vector pQF50 (Table 1).
[b] β-Galactosidase activities assayed in derivatives of *E. coli* strains harboring either pMM71 (MxiE), pKH128 (IpgC), or both (MxiE and IpgC) are expressed in Miller units and are the means of at least four independent experiments. Standard deviations (not shown) were within 25% of the reported values.

of activation of transcription from the *ipaH4.5* promoter may be due to its imperfect MxiE box, resulting in weaker recognition by MxiE or in weaker production of either MxiE or IpgC in the *E. coli* background than in the *ipaD* and *ipaB* mutants of *S. flexneri*.

Regulation of expression of chromosomal *ipaH* genes by MxiE and IpgC. Southern hybridization with the constant portion of the *ipaH* genes as a probe indicated that five copies of *ipaH* also exist on the chromosome of wild-type *S. flexneri* strain M90T (7), although it is not known whether any of these genes is complete or expressed. To investigate whether the *ipaH* genes located on the chromosome were regulated in the same manner as those located on the virulence plasmid, we introduced into strain BS176, a virulence plasmid-cured derivative of M90T, plasmids expressing *mxiE* (pMM71) and *ipgC* (pKH128), either individually (MMCP536 and MMCP535) or together (MMCP537). Whole-cell extracts of recombinant strains were analyzed by SDS-PAGE and Western blotting with anti-IpaH serum (Fig. 3). No material cross-reacting with IpaH was detected in extracts of BS176 or its derivatives expressing either MxiE or IpgC alone. In contrast, one protein of approximately 60 kDa, i.e., the same size as IpaH proteins produced from the virulence plasmid, was detected in extracts of the derivative of BS176 expressing both MxiE and IpgC. These results indicated that at least one *ipaH* gene carried by the chromosome was complete and, more importantly, that it was under the control of both MxiE and IpgC.

DISCUSSION

In the present study, we investigated further the repertoire of genes that are regulated by the activity of secretion and the *cis*-acting element involved in the activation of regulated promoters. We present evidence that six and potentially eight promoters carried by the virulence plasmid and at least one promoter carried by the chromosome are regulated by the activity of the TTS apparatus. The mechanism of regulation involves a conserved 17-bp *cis*-acting site overlapping the −35 region of each promoter and two coordinately acting regulators, MxiE and IpgC.

Reporter plasmids carrying the putative promoters and regulatory regions of a number of *ipaH* and *osp* genes were introduced into the wild-type strain, in which the TTS apparatus is not active during growth in broth, and into *ipaD* and *ipaB* mutants, in which the TTS apparatus is constitutively active. The DNA region located upstream from *ipaH7.8*, *ospC1*, and *ospF* contained promoters that were activated approximately 10-fold under conditions of secretion. As previously described for the *ipaH9.8* and *virA* promoters (28), the activation of the *ipaH7.8*, *ospC1*, and *ospF* promoters under conditions of deregulated secretion required both MxiE and IpgC, as these promoters were no longer active in *ipaB4 mxiE* and *ipaB4 ipgC* mutants. Furthermore, each of these promoters was activated in an *E. coli* strain expressing both MxiE and IpgC. The lower β-galactosidase activity expressed from most reporter plasmids in the *E. coli* strain harboring pMM71 (MxiE) and pKH128 (IpgC) than in *S. flexneri ipaD* or *ipaB* mutants might have been due to a level of production of MxiE or IpgC from recombinant plasmids lower than from the virulence plasmid. Primer extension analyses were used to identify the transcription start site of the *ospC1* promoter and those of the *ipaH9.8* and *virA* promoters that are also regulated by the activity of secretion (28). A sequence comparison revealed a conserved motif, designated the MxiE box, overlapping the −35 region of each promoter, and deletion analyses indicated that this conserved

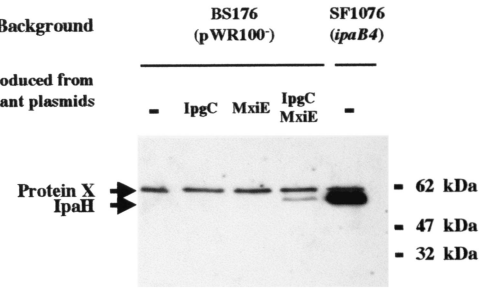

FIG. 3. Production of chromosomally encoded IpaH proteins is activated by MxiE and IpgC. Whole-cell extracts of BS176 (a virulence plasmid-cured derivative of wild-type *S. flexneri* strain M90T), its derivatives harboring plasmid pKH128 (which expresses IpgC), plasmid pMM71 (which expresses MxiE), or both plasmids, and SF1076 (an *ipaB* mutant) were analyzed by SDS-PAGE and probed with rabbit anti-IpaH serum. The positions of IpaH and protein X (a constitutively expressed, chromosomally encoded protein recognized by rabbit anti-IpaH serum) are indicated.

sequence was essential for the activation of these promoters under conditions of secretion. An MxiE box was also detected upstream from the *ipaH7.8* and *ospF* genes, both of which are regulated by the activity of secretion, at an appropriate distance from a putative −10 region and probably represents the regulatory region for each of these genes.

The characteristic features of regulated promoters include (i) a −10 region closely resembling the consensus sequence TATAAT, (ii) no similarities with a canonical −35 region, and (iii) the presence of an MxiE box between positions −49 and −33. The location of the MxiE box is consistent with previous observations indicating that target sequences of most members of the AraC family of transcriptional activators are located adjacent to or overlapping the −35 regions of regulated promoters (19). In *S. enterica* serovar Typhimurium, InvF, which is homologous to MxiE, and SicA, which is homologous to IpgC, are both required for activation of the *sicA*, *sigD*, and *sopE* promoters (12). The target sequence, designated the InvF box, is 11 bp long and is located between positions −51 and −41 with respect to the transcription start point (11, 12, 43). The InvF and MxiE boxes are located at the same positions in regulated promoters and exhibit some limited sequence similarities, including a stretch of six T's between nucleotides −43 and −38.

Using *lacZ* fusions carried by the virulence plasmid, Demers et al. previously showed that the transcription of *ipaH1.4* was also regulated by the activity of secretion (13). However, the region located immediately upstream from *ipaH1.4*, i.e., the 389-bp *ospE1-ipaH1.4* intergenic region, did not appear to contain any promoter, suggesting that the promoter and regulatory region involved in the expression of *ipaH1.4* are located upstream from *ospE1*. Indeed, the region located immediately upstream from *ospE1* has an MxiE box 16 nucleotides upstream from a putative −10 region, suggesting that *ospE1* and *ipaH1.4* belong to the same operon and are transcribed from a regulated promoter located upstream from *ospE1*. The region encompassing the *ospE2* and *ipaH2.5* genes is almost identical to the *ospE1-ipaH1.4* region, except for the presence of IS elements inserted between *ospE2* and *ipaH2.5* (6). The presence of an MxiE box upstream from *ospE2* suggests that the transcription of *ospE2* is also regulated by the activity of secretion. Due to the presence of the IS elements inserted between *ospE2* and *ipaH2.5*, it seems unlikely that *ipaH2.5* is regulated or even transcribed.

The expression of an *ipaH4.5-lacZ* fusion carried by the virulence plasmid was increased 12-fold in an *ipaD* mutant compared to the wild-type strain (13), whereas the expression of the *ipaH4.5-lacZ* fusion carried by the reporter plasmid was increased only 3-fold in *ipaD* and *ipaB* mutants compared to the wild-type strain. As for other regulated fusions, no activation of the reporter plasmid-carried *ipaH4.5-lacZ* fusion was observed in the absence of either MxiE or IpgC, indicating that activation of the promoter upstream from *ipaH4.5*, although less efficient than that of other regulated promoters, was dependent on both MxiE and IpgC. Sequence analysis of the *ipaH4.5* upstream region revealed the presence of a potential MxiE box that, however, differs from the consensus MxiE box by having a G instead of a T at position −48 and a T instead of a C at position −45. In contrast to other promoters that have an MxiE box, the *ipaH4.5* promoter was not activated in the *E. coli* strain expressing both MxiE and IpgC. This result suggests that differences between the MxiE boxes of the *ipaH4.5* promoter and other regulated genes may affect the binding affinity of MxiE which, as discussed above, may be produced in smaller amounts in *E. coli* than in *S. flexneri*. The *ipaH4.5* gene is located 429 bp downstream from *ipaH7.8*, and the greater amplitude of regulation of the virulence plasmid-carried fusion than of the reporter plasmid-carried fusion suggests that, under conditions of secretion, the transcription of *ipaH4.5* occurs mostly through activation of the upstream *ipaH7.8* promoter.

While this study was under review, Kane et al. (24) reported an analysis of the expression of *ospB*, *ospC1*, *ospE2*, *ospF*, *virA*, and *ipaH9.8* with recombinant plasmids and green fluorescent protein as a reporter system. These authors compared the amounts of the reporter protein present in intracellular bacteria following entry into epithelial cells and in extracellular bacteria growing in broth. Increased amounts of the reporter protein were present in intracellular bacteria harboring *virA*, *ipaH9.8*, *ospB*, *ospC1*, *ospE2*, and *ospF* fusions, and this increase was dependent on a functional *mxiE* gene. These authors concluded that these promoters were regulated by MxiE. These results are consistent with the previous demonstrations that the *virA*, *ipaH9.8*, *ipaH7.8*, *ipaH4.5*, and *ipaH1.4* promoters are activated upon entry of bacteria into epithelial cells (13) and that the *virA*, *ipaH9.8*, *ipaH7.8*, *ipaH4.5*, *ospC1*, and *ospF* promoters are controlled by both MxiE and IpgC (28; this study) and with the above hypothesis that the target for the regulation of *ipaH1.4* lies upstream from *ospE1*. No conserved MxiE boxes are present upstream from *ospB*, and the mechanism by which MxiE may control the *ospB* promoter remains to be investigated. Kane et al. (24) proposed that MxiE-regulated genes are activated in the intracellular compartment. However, using kinetic analysis, Demers et al. (13) showed that, although the transcription of *virA* was activated upon entry of bacteria into epithelial cells, it was subsequently repressed during growth of bacteria in the intracellular environment. As shown here and previously (28), the capacity of MxiE to act as a transcriptional activator is dependent on the activity of the TTS apparatus that is sensed by the presence of free IpgC, i.e., not associated with IpaB and IpaC.

It was previously shown that several copies of *ipaH* genes are carried by both the virulence plasmid and the chromosome (20). The 5′ portion of *ipaH* genes carried by the virulence plasmid exhibits a low GC content similar to those of *mxi* and *spa* genes, encoding the components of the TTS apparatus, and the *ipa* operon and *osp* genes, encoding secreted proteins, suggesting that *ipaH* genes were acquired from the same source as other genes of the virulence plasmid-encoded TTS system (6). Thus, it seems likely that chromosomal *ipaH* genes may result from the duplication of plasmid genes. Using recombinant plasmids expressing MxiE and IpgC in an *S. flexneri* strain cured of the virulence plasmid, we showed that the expression of at least one chromosomal *ipaH* gene is also under the control of MxiE and IpgC. The amount of IpaH proteins produced from the chromosome by the derivative of the virulence plasmid-cured strain expressing MxiE and IpgC was much lower than the amount of IpaH proteins produced by an *ipaB* mutant. This result suggests that chromosomally encoded IpaH proteins may represent only a small proportion of the

IpaH proteins that are produced from the virulence plasmid. Alternatively, as in *E. coli*, the low amount of IpaH proteins produced in the virulence plasmid-cured strain containing pMM71 and pKH128 might have been due to a low level of production of MxiE or IpgC from the recombinant plasmids.

In conclusion, we have identified the *cis*-acting regulatory element, or MxiE box, which is involved in the activation of promoters that are regulated by the activity of the Mxi-Spa TTS apparatus. Analysis of the sequence of the entire virulence plasmid indicated that eight MxiE boxes are located upstream from *ipaH9.8*, *ipaH7.8*, *ipaH4.5*, *ospC1*, *ospE1*, *ospE2*, *ospF*, and *virA*. As is the case for *ipaH1.4*, which is located 389 bp downstream from *ospE1* and is proposed to be regulated by the MxiE box located upstream from *ospE1*, the *ospD2* and *ospD3* genes, which are located 431 and 329 bp downstream from *ospF* and *ospC1*, respectively, may also be controlled by MxiE boxes located in front of the upstream genes. This hypothesis suggests that the transcription of 11 genes that are carried by the virulence plasmid and encode proteins secreted by the TTS apparatus is regulated by the activity of secretion. Determination of the complete sequence of the chromosomes of *S. flexneri* will help to determine which chromosomal *ipaH* genes exhibit an MxiE box and whether other chromosomal genes are potentially controlled by MxiE and IpgC.

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