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mxiA of *Shigella flexneri* 2a, Which Facilitates Export of Invasion Plasmid Antigens, Encodes a Homolog of the Low-Calcium-Response Protein, LcrD, of *Yersinia pestis*

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The plasmid-encoded invasion plasmid antigen (Ipa) export accessory locus of *Shigella flexneri* 2a, *mxiA*, was cloned, and the complete DNA sequence of the gene was determined. The *mxiA* open reading frame was found to encode a polypeptide of 74.03 kDa with a pI of 5.02. A hydropathy analysis of the predicted protein revealed a hydrophilic C terminus and an extremely hydrophobic N terminus without a cleavable signal sequence but with several potential membrane-spanning regions. While a homology search did not reveal any significant relatedness of the *mxiA* DNA sequence to any known bacterial gene sequences, the derived amino acid sequence of MxiA was found to be highly homologous (68%) to the sequence of the protein encoded by the low-calcium-response locus, *lcrD*, of *Yersinia pestis*. The *lcrD* gene encodes an inner membrane regulatory protein that has an N-terminal membrane anchor and that is implicated in facilitating the export of *Y. pestis* outer membrane proteins (G. V. Plano, S. S. Barve, and S. C. Straley, J. Bacteriol. 173:7293–7303, 1991). Congo red binding, HeLa cell invasion, and Ipa excretion were restored in two avirulent *mxiA* fusion mutants when they were transformed with a cloned copy of the *mxiA* gene. Furthermore, the expression of the cloned *mxiA* gene was independent of any vector-specified promoter, suggesting that the transcription of *mxiA* is driven by its own promoter in this clone. In contrast, the overexpression of *mxiA* that resulted when it was placed under the control of the *lac* promoter was found to be deleterious in *Escherichia coli*. We conclude that *mxiA* is a homolog of the *Y. pestis lcrD* locus and may function similarly in *S. flexneri*, either by directly affecting the excretion of virulence factors or by regulating the expression of export accessory genes.

The invasive phenotype of *Shigella flexneri* is conferred by several different temperature-regulated genes located on the 220-kb plasmid of fully virulent strains (4, 10, 11, 21). Although two of these genes, *ipaB* and *ipaC*, have been characterized as encoding surface-expressed virulence factors, the proteins possess no cleavable signal sequences (3, 31, 33). Additionally, it has been demonstrated that both of these antigens are excreted at low levels into the extracellular environment in vitro (2). These data suggest that there is a specific mechanism for the secretion of these essential virulence proteins and that it is independent of the *sec* export apparatus classically associated with the secretion of many bacterial proteins (27).

We recently identified two novel temperature-regulated virulence loci, *mxiA* and *mxiB*, that are encoded on the invasion plasmid (2, 16). Mutations in either one of these genes result in the inability of the bacteria to bind the planar dye Congo red and in the loss of invasiveness for HeLa cell monolayers. Additional characteristics of the mutants include the accumulation of IpaB and IpaC in the cytoplasm and inner membrane and the depletion of these antigens from the outer membrane and culture supernatants (2). On the basis of these observations, we concluded that *mxiA* and *mxiB* specify accessory proteins involved in facilitating the export of at least two of the invasion plasmid antigens (IpaB and IpaC), hence, the designation *mxi*, for membrane expression of invasion plasmid antigens.

Western blot (immunoblot) analysis with antiserum to a MxiA-LacZ hybrid protein identified MxiA as a temperature-regulated 76-kDa polypeptide (2). In an effort to further characterize the *mxiA* gene and its product, the *mxiA* coding region was cloned and its DNA sequence was determined, analyzed, and compared with those of other known prokaryotic genes. In this report, we describe high homology between the *mxiA* product and the product of the *Yersinia pestis* gene *lcrD*, which is involved in the regulation of *Y. pestis* virulence in response to calcium levels in the bacterial growth environment (26).

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used and the plasmids constructed in this study are presented in Table 1. Strains were grown in tryptic soy broth (Difco Laboratories, Detroit, Mich.) at 37°C unless otherwise specified.

DNA sequencing and recombinant DNA techniques. The putative *mxiA* coding region was subcloned on two contiguous *Hin*dIII restriction fragments (1.8 and 1.3 kb) into pUC19, and the resulting recombinant plasmids were designated pGPA010 and pGPA013 (Fig. 1). The *Hin*dIII restriction fragments were derived from DNA upstream of the previously reported cloned *mxiB* operon fusion end joint in pAEH006 (2). These restriction fragments were believed to span the *mxiA* open reading frame (ORF) on the basis of the overlap occurring between pAEH006 and the BS260 (*mxiA*) protein fusion end joint clone pGPA001 (Fig. 1). A third construct, pGPA040, contained the 3.8-kb *Xba*I-*Eco*RI restriction fragment, which overlaps the two *Hin*dIII frag-

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Reference or source
Strains		
Shigella flexneri 2a		
2457T	Wild type containing pSf2a140	8
BS103	2457T cured of pSf2a140	22
BS226	2457T pSf2a140 (*mxiB*::*lacZ* 11.5)	16
BS232	2457T pSf2a140 (*mxiA*::*lacZ* 18.15)	16
BS260	2457T pSf2a140 (*mxiA*::*lacZ* 1103)	2
E. coli K-12 DH5α	φ80d Δ(*lacZYA-argF*) *hsdR17*	GIBCO BRL[a]
Plasmids		
pUC19	Cloning vector; Ap^r $lacZ^+$	36
pKS	Cloning vector; Ap^r $lacZ^+$	Stratagene[b]
pHS4011	11-kb *Eco*RI fragment from pHS4108	21
pGPA001	9-kb protein fusion end joint clone from BS260 in pMLB524	2
pAEH006	12-kb operon fusion end joint clone from BS226 in pMLB524	16
pAEH009	18-kb operon fusion end joint clone from BS232 in pMLB524	16
pGPA010	*mxiA'*; 1.8-kb *Hin*dIII fragment from pAEH006 in pUC19	This study
pGPA013	*mxiA'*; 1.3-kb *Hin*dIII fragment from pAEH006 in pUC19	This study
pGPA040	$mxiA^+$; 3.8-kb *Xba*I-*Eco*RI fragment from pAEH006 in pUC19	This study
pGPA042	$mxiA^+$; 11-kb *Eco*RI fragment from pHS4011 in pUC19	This study
pGPA043	$mxiA^+$; pGPA042 insert in *lac* promoter-driven orientation	This study
pGPA045	$mxiA^+$; 3.8-kb *Xba*I-*Eco*RI fragment from pGPA040 in pKS	This study

[a] GIBCO BRL, Life Technologies, Inc., Gaithersburg, Md.
[b] Stratagene Cloning Systems, La Jolla, Calif.

ments (Fig. 1). This insert originated from an 11-kb *Eco*RI fragment of the 37-kb cosmid clone pHS4108 from *S. flexneri* 5 (Fig. 1), which possesses all the virulence loci necessary to confer the invasion phenotype (21).

The *mxiA*::*lacZ* protein fusion end joint contained in pGPA001 was primed with a *lacZ*-specific oligonucleotide to obtain the sequence through the fusion junction and into the *mxiA* coding region. The sequence generated was then used to define the reading frame as well as to obtain a *mxiA*-specific primer to begin sequencing of the native gene.

Recombinant plasmid DNA for sequencing was isolated and purified with a column kit from Qiagen (Chatsworth, Calif.) in accordance with the manufacturer's procedure. DNA sequencing was performed with purified plasmids pGPA001, pGPA010, pGPA013, and pGPA040 as double-stranded templates by use of a Sequenase version 2.1 DNA sequencing kit (U.S. Biochemical Corp., Cleveland, Ohio). The protocol described by the manufacturer was used.

The complete DNA sequence of *mxiA* and its derived amino acid sequence were analyzed with Genetics Computer Group version 6.2 software (7). The isoelectric point was determined by use of the ISOELECTRIC program. Homology searches were performed at both the DNA and the amino acid levels by use of the FASTA and TFASTA programs to scan the GenBank (release 70.0) and EMBL (release 29.0) data bases.

Restriction digestions and DNA ligations were performed by standard techniques (20).

Bacterial transformations for both *Escherichia coli* and *S. flexneri* were performed by previously reported methods (6, 12).

Cloning of *mxiA* and complementation of *mxi* mutations. The 9-kb *Eco*RI fragment from pHS4011, containing the intact *mxiA* coding region, was ligated into cloning vector pUC19 (36) in both orientations relative to the vector-specified *lac* promoter (pGPA042 and pGPA043; Fig. 1). A third construct, pGPA045, consisted of an *Xba*I-*Eco*RI fragment placed into vector pKS in the reverse orientation relative to the *lac* promoter (Fig. 1). These recombinant plasmids were used to transform *mxi* mutants BS226, BS232, and BS260 to test for complementation of the avirulent phenotype by use of the following assays.

(i) Quantitative CRB assay. Bacterial strains to be tested for Congo red binding (CRB) were grown overnight at 37 and 30°C on tryptic soy agar with 0.025% Congo red (Sigma Chemical Co., St. Louis, Mo.). Bacteria (10 to 20 colonies) were scraped from each plate and resuspended in 600 μl of deionized H_2O, and the A_{660} was measured to account for variations in cell density between samples. Acetone was added to the bacterial suspensions to a 40% final concentration, and the dye was extracted over a period of 10 min at room temperature. The bacteria were centrifuged at 5,000 × *g*, and the A_{488} (absorbance maximum of Congo red) of the supernatant containing the extracted dye was measured. An arbitrary level of CRB was calculated by dividing the A_{488} value by the A_{660} value. CRB was expressed as the percentage of dye bound by wild-type *S. flexneri*. The assay was performed in triplicate. The variability between absolute dye binding values from multiple experiments with the wild-type control (2457T) was less than 10%.

(ii) HeLa cell invasion assay. The ability of *S. flexneri* to invade mammalian cells in tissue culture was measured as previously described (2). Bacterial invasion was determined as the number of HeLa cells invaded/number of total HeLa cells counted and standardized as a percentage of wild-type invasion. A minimum of 100 HeLa cells were counted for each infected monolayer.

(iii) Ipa export assays. The amount of cell surface-associated IpaB on the *mxiA* transformants was measured by the anti-Ipa monoclonal antibody suspension-labelling assay as previously described (2).

Excreted IpaB and IpaC were detected by a modified

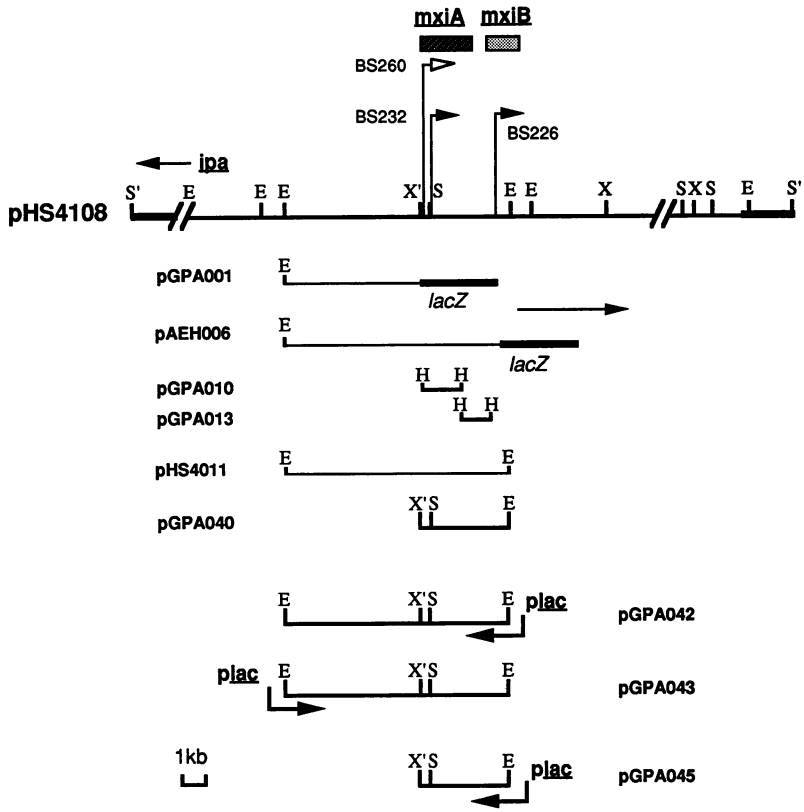

FIG. 1. Cloning and complementation strategy for the *mxiA* locus of *S. flexneri* 2a. Shown at the top is pHS4108, the 37-kb cosmid subclone of the 220-kb invasion plasmid, and the positions of the *mxi* loci relative to the *ipa* operon. Also indicated are the positions of the fusion phage inserts within the *mxiA* locus in mutants BS260 and BS232 and the *mxiB* mutation in BS226. Arrows indicate the direction of transcription. The open arrow represents a protein fusion, and closed arrows indicate operon (transcriptional) fusions. Below pHS4108 are *mxi* fusion end joint clones pGPA001 from BS260 (*mxiA*) and pAEH006 from BS226 (*mxiB*). Plasmids pGPA010 and pGPA013 (subcloned from pAEH006) as well as pGPA040 (subcloned from pHS4011) were used as double-stranded templates to sequence through the *mxiA* open reading frame. The clones used in the complementation experiments consisted of a 11-kb *Eco*RI fragment from pHS4011 ligated into vector pUC19 in both orientations relative to the *lac* promoter (p*lac*) (pGPA042 and pGPA043) and an internal *Xba*I-*Eco*RI fragment placed into vector pKS in the reverse orientation relative to the *lac* promoter (pGPA045). Abbreviations for restriction sites: E, *Eco*RI; H, *Hin*dIII; X', *Xba*I; X, *Xho*I; S, *Sal*I; S', *Sau*3A.

cell-free enzyme-linked immunosorbent assay. Bacteria were washed once with phosphate-buffered saline (PBS), standardized to A_{600}, and exposed to anti-IpaB or anti-IpaC monoclonal antibodies (kindly provided by Ed Oaks) diluted 1:2,500 in 3% casein–PBS. Bacteria were incubated with the primary antibodies for 1 to 2 h at room temperature in uncoated Eppendorf tubes. After three washes with PBS, the monoclonal antibody-labelled bacteria were exposed to a 1:1,000 dilution of a goat anti-mouse immunoglobulin G [F(ab')$_2$]–alkaline phosphatase conjugate (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) for 1 h at room temperature. Just prior to the addition of the alkaline phosphatase substrate (Sigma 104), the bacteria were removed and the tubes were washed once with PBS to remove residual cells. Cell-free antigen-antibody complexes adhering to the tubes were detected by the addition of the enzyme substrate, and the color reaction was stopped after 5 min. The A_{405} of the solution in each tube was measured, and the values were expressed as a percentage of wild-type reactivity. Reactivity from antibody alone adhering to the inside surface of the tubes was always less than 10% of the reactivity of the positive control (2457T). The variabilities between absolute A_{405} values from multiple experiments with 2457T were less than 17% for excreted and cell-bound IpaB and less than 8% for excreted IpaC. All assays were performed in triplicate.

Nucleotide sequence accession number. The DNA sequence of the *mxiA* coding region determined from the 1.8-kb *Hin*dIII fragment from pGPA010 and the 5' end of the 1.3-kb *Hin*dIII fragment from pGPA013 has been submitted to GenBank under accession number M91664.

RESULTS AND DISCUSSION

DNA sequence analysis of the *mxiA* locus. The complete nucleotide sequence of *mxiA* was determined from the *Hin*dIII fragment in pGPA010 and part of the *Hin*dIII fragment in pGPA013. A single large ORF of 1,998 nucleotides was found in-frame with the *lacZ* protein fusion and most likely represented *mxiA* (Fig. 2). The phage insertion in BS260 mapped precisely at nucleotide 387, only 13 codons downstream from the putative start of the *mxiA* ORF (Fig. 1 and 2). A second ORF (ORF2) was found upstream from and in-frame with *mxiA* and continued past the 5' *Hin*dIII restriction site of pGPA010. Mutations in ORF2 also confer the noninvasive and Mxi$^-$ phenotypes, and a detailed analysis of this gene (*mxiC*) will be the subject of another report. A third ORF was also identified in-frame with and at the end

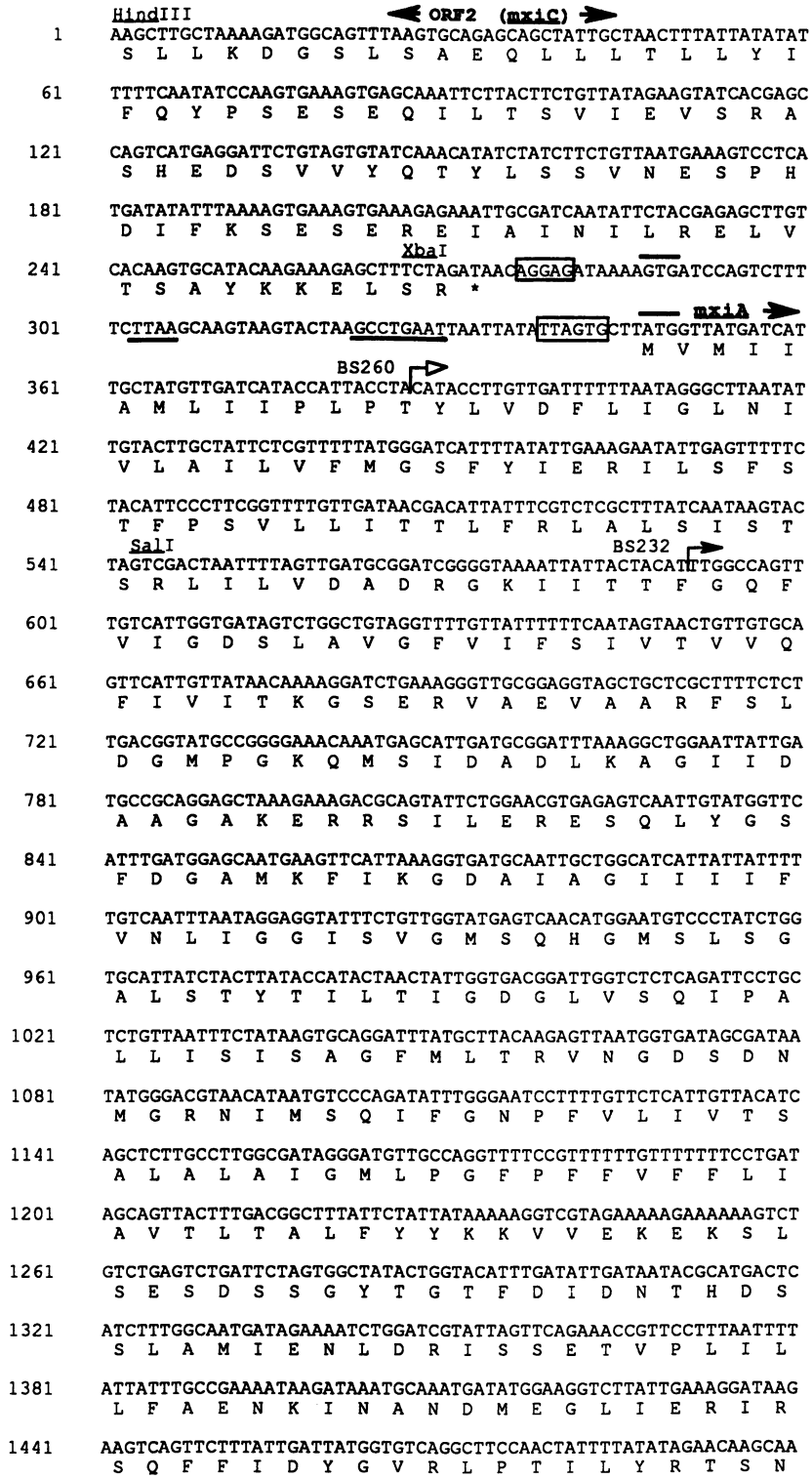

FIG. 2. Nucleotide sequence of *mxiA* and the 3′ coding region of ORF2. The putative Shine-Dalgarno sequence (boxed) and ATG start codon for *mxiA* (overlined) are indicated. An alternate GTG start codon and putative Shine-Dalgarno sequence are also shown. A σ^{28}-like promoter is indicated by lines under the −10 (GCCTGAAT) and −35 (TTAA) sites. One-letter codes for the predicted amino acid sequence are shown for both ORF2 and *mxiA*. Relevant restriction sites are also indicated above the sequence, as are the positions of the λ*plac*Mu fusion phage inserts in *mxiA* mutants BS260 and BS232. The stop codons of ORF2 and *mxiA* are indicated by asterisks below the sequence. The ATG start site of *spa15* (34) is also indicated at the end of the sequence.

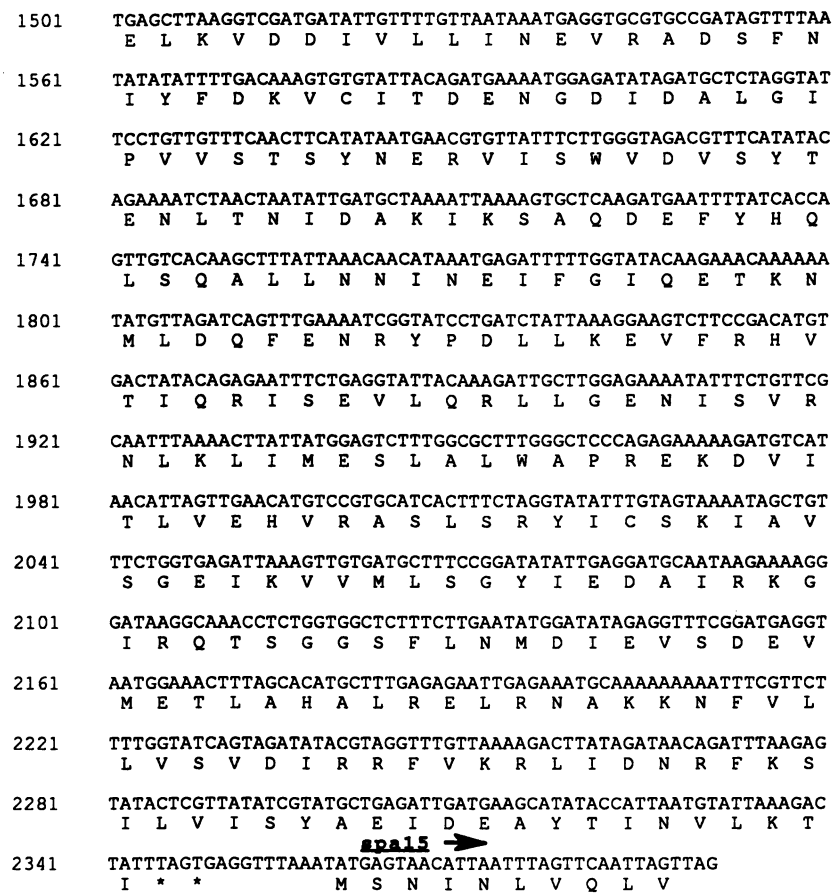

FIG. 2—*Continued.*

of *mxiA*. The 5′ end of this sequence was found to match exactly an uncharacterized ORF, *spa15* (34), which lies between *spa47* and *mxiA*. The *spa47* locus (34) is identical to the Ipa secretion locus *mxiB*, which we have previously described (2, 16).

For determination of the precise location of the λp*lac*Mu fusion phage insert in *mxi* mutant BS232 (16), the cloned fusion end joint from this mutant was used as a template and primed with *lacZ*- and *trpB*-specific oligomers to obtain the sequence through the fusion junction. This sequence was aligned with the sequence of the *mxiA* coding region, and the operon fusion phage insert in BS232 was found to map at nucleotide 590, within the *mxiA* coding region downstream of the *Sal*I restriction site (Fig. 1 and 2). This finding represents a repositioning of the insertion site in BS232, which was originally reported to lie upstream of the *Sal*I site (16). Thus, BS232 can be defined as a *mxiA* transcriptional fusion mutant.

A possible ATG translational start site for the *mxiA* ORF was found at nucleotide 347, and a weak putative ribosome binding site (Shine-Dalgarno sequence; 18) was located upstream (beginning at nucleotide 338) from this designated start site. A second possible start site, utilizing the alternate start codon GTG (nucleotide 287) but possessing a "more favorable" Shine-Dalgarno sequence, was also identified (Fig. 2). Although analysis of further upstream sequences did not reveal a transcriptional start site strongly matching the normal *E. coli* promoter consensus sequence (15), a sequence that resembled the σ^{28} promoter of *E. coli* (13) was identified between 18 and 44 bases upstream of the *mxiA* ATG translational start site. Similarity to the σ^{28} promoter occurred as a 3- of 4-base alignment at the −35 site and a 5- of 8-base alignment at the −10 site, with the number of nucleotides in the spacer region (15 bases) matching the consensus sequence. Transcriptional initiation sites of this type have been found to be involved in the expression of chemotaxis and flagellar genes in *E. coli* as well as *Salmonella typhimurium* (13), although the control of the expression of the σ^{28} protein itself is not clearly understood (14). This observation is significant in light of additional findings discussed below.

The *mxiA* ORF specified a predicted polypeptide of 666 amino acids and with a molecular mass of 74.03 kDa and a pI of 5.02. A hydropathy plot of the predicted *mxiA* gene product revealed that MxiA also possessed an extremely hydrophobic N terminus with six putative transmembrane-spanning regions of 10 to 20 amino acids each (Fig. 3a), possibly representing a membrane anchor. Although it appeared that MxiA did not possess a cleavable signal sequence, a significant amount of the LacZ-MxiA hybrid protein from *mxiA* fusion mutant BS260 was detected in the inner membrane of the bacteria (1). Given the location of the fusion phage insert in BS260 (Fig. 1), this observation suggests that the first 13 amino acids may be the only residues required to target the protein to the inner membrane. Taken together, these findings are consistent with our preliminary analysis of the *mxiA* locus, which suggested that the MxiA polypeptide is a membrane protein (2). The C

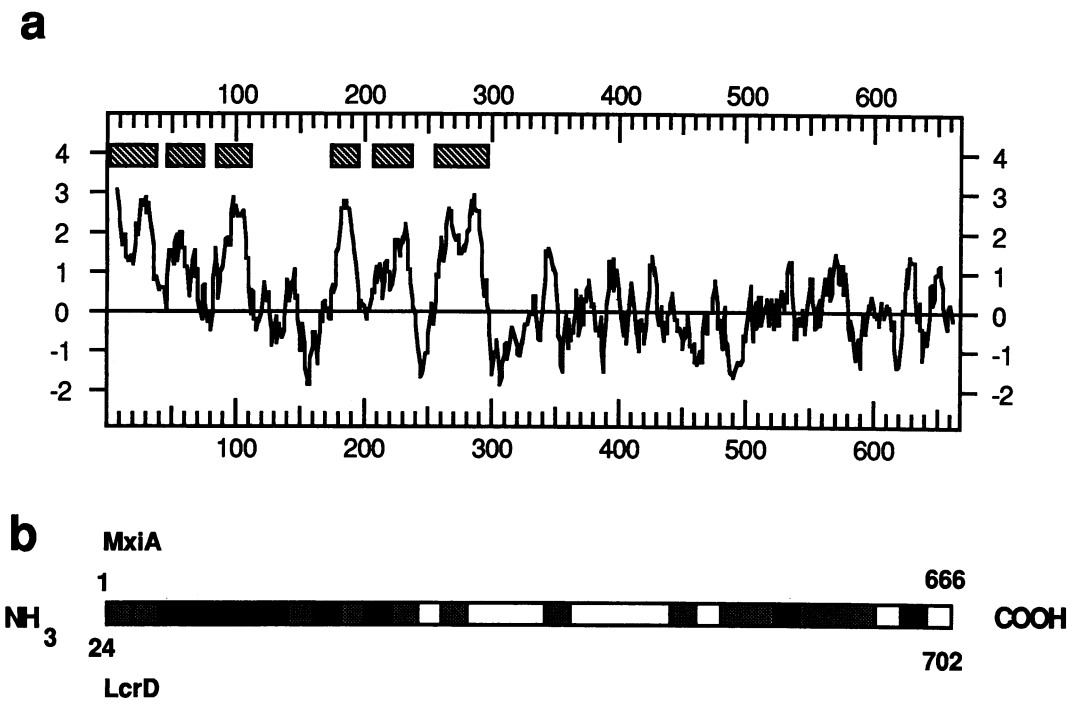

FIG. 3. (a) Hydrophobicity-hydrophilicity plot of MxiA. The analysis was performed with DNA Stryder version 1.1 software for the Macintosh, based on the algorithm of Kyte and Doolittle (19). Positive values represent more hydrophobic residues, and negative values indicate more hydrophilic residues. Hatched bars above N-terminal hydrophobic peaks indicate putative membrane-spanning domains. (b) Regions of amino acid homology between MxiA and LcrD in relationship to the hydropathy plot of MxiA. The amino acid alignment was initially performed by use of the Genetics Computer Group BESTFIT program with an assigned gap weight of 3.0 and a length weight of 0.1. The alignment was then broken down into groups of 20 residues and, on the basis of the identity within each group, assigned one of three shades. These shades were plotted on a horizontal bar representing the entire length of the two proteins. Shades: □, <25% amino acid identity; ▩, 25 to 50% identity; ■, >50% identity. The N-terminal alignment of LcrD begins at residue 24 and ends at residue 702 of 705 total residues.

terminus, from approximately residue 300 to the end of the protein, was essentially hydrophilic, suggesting either a cytoplasmic or a periplasmic location for this part of the protein.

A DNA homology search of the GenBank and EMBL data bases revealed no significant similarities between *mxiA* and any known prokaryotic genes. However, when the amino acid sequence of the predicted *mxiA* polypeptide was compared with those of other known bacterial proteins, greater than 40% identity was found with the predicted translation product of ORF5 of virulence plasmid pYVO3 of *Yersinia enterocolitica* (35). ORF5 was recently shown to be identical to *lcrD* of *Y. pestis*, which encodes a protein involved in the low-calcium response (26). When the deduced amino acid sequence of the *mxiA* polypeptide was compared with that of LcrD, 41% overall identity and 69% similarity were seen (Fig. 4). The highest homology occurred at the amino termini of the two proteins (Fig. 3b and 4). Amino-terminal homology in the first 200 residues represented greater than 85% similarity and 60% identity. High similarity between the amino acid sequences of LcrD and FlbF, a regulatory protein of the aquatic bacterium *Caulobacter crescentus*, has also been reported (26, 28). However, the overall homology between LcrD and MxiA appeared slightly higher than that between LcrD and FlbF, although the N-terminal homology was the same (28). The reason was apparent after a more detailed analysis of the regions of homology between the proteins. When amino acid identity between MxiA and LcrD was analyzed across 20-residue stretches in conjunction with the hydropathy plot, an additional region of moderate to high homology was found in the C-terminal third of the two proteins. This region (Fig. 3b; amino acids 500 to 600) consisted of 100 amino acids separated from the N-terminal hydrophobic region by a 200-residue spacer of low homology (Fig. 3b; amino acids 300 to 500). In contrast, homology between LcrD and FlbF in this region does not appear as high (28). It was also of interest that small regions of lower homology were seen between some of the hydrophobic peaks in the N termini of MxiA and LcrD (Fig. 3b and 4). To our knowledge, this is the first report of significant amino acid homology between *S. flexneri* and *Y. pestis* virulence proteins. In addition to the homology reported with FlbF of *C. crescentus* (28), LcrD and the product of the *invA* gene of *S. typhimurium* are reported to be homologous (26). It is also interesting to note that *flbF* has recently been shown to possess a σ^{28}-like promoter sequence (30). These findings suggest that a family of proteins possessing the same or similar functions may exist among the gram-negative bacteria (32).

One of the functions proposed for LcrD in *Y. pestis* is to export virulence factors, since a mutation in the *lcrD* locus results in the loss of *Y. pestis* outer membrane proteins H and M from the outer membrane and in the accumulation of these proteins within the bacteria (26). In light of an analysis of *lcrD* mutants, however, an alternate function for this gene has been proposed (26). In *Y. pestis*, the *lcrD* locus plays a role in the regulation of gene expression during the low-calcium response (9). Therefore, in addition to displaying a

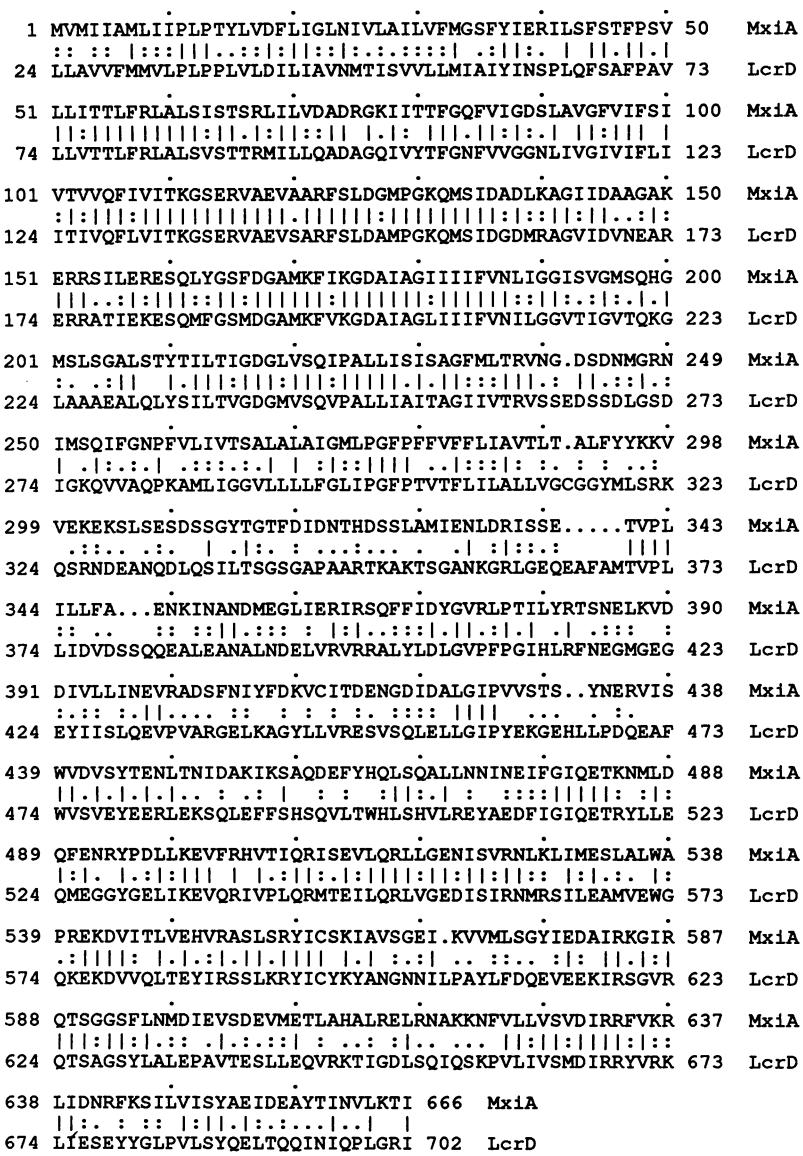

FIG. 4. Amino acid alignment of the predicted proteins encoded by *mxiA* of *S. flexneri* and *lcrD* of *Y. pestis*. Lines between residues indicate identity, colons represent similarity between amino acids, and periods indicate less similarity between amino acids. The amino acid alignment was performed as described in the legend to Fig. 3. The predicted size of LcrD is 705 residues.

secretion-defective phenotype, *lcrD* mutants are also down-regulated for the expression of the V antigen when grown under conditions optimal for the expression of this protein (37°C, no Ca^{2+}). Thus, it appears that LcrD can function as a *trans*-activating factor as well as facilitate the secretion of *Y. pestis* outer membrane proteins. It is highly possible that LcrD actually regulates the expression of the genes directly involved in the export of these proteins (26). In a previous report, we demonstrated that MxiA functions to facilitate the export of IpaB and IpaC (2). Since *mxiA* may function as an *lcrD* homolog in *S. flexneri*, it is also not unrealistic to envision *mxiA* as having a role in the regulation of *S. flexneri* genes similar to the proposed role for *lcrD* in *Y. pestis*. Although we have not specifically identified proteins that are down-regulated as a result of a mutation in the *mxiA* locus, several regulatory loci, both chromosome and plasmid encoded, have been identified in *Shigella* spp. and characterized (5, 17, 24). Until now, temperature and osmolarity have been implicated as environmental factors that modulate gene expression in *Shigella* spp. (23). It is not clear, however, whether cationic (or anionic) effects are also important. Experiments to measure virulence gene responses to these conditions are currently under investigation in our laboratory.

Complementation of the *mxiA* defect in protein and operon fusion mutants of *S. flexneri*. To determine whether the mutant phenotypes in the *S. flexneri* fusion mutants could be complemented by a clone containing the *mxiA* coding region, we transformed pGPA042 (Fig. 1) and pGPA043 (*lac* promoter driven; Fig. 1) into *mxiA* mutants BS260 and BS232. Since the ability of *S. flexneri* to bind the planar dye Congo red correlates with virulence (22) and the *mxiA* mutants were all unable to bind the dye, a quantitative CRB assay was developed and used as an initial screen for complementation

TABLE 2. Complementation of *S. flexneri mxi* mutants with the wild-type *mxiA* locus

Strain	%[a]				
	CRB[b]	HeLa cell invasion[c]	Excretion of[d]: IpaB	Excretion of[d]: IpaC	Surface IpaB[d]
BS103	5	3			
BS260 *mxiA*	14	0	3	11	10
BS260(pGPA042)	64	>100	47	93	58
BS260(pGPA043)	45	>100	76	97	42
BS260(pGPA045)	69	>100	40	97	45
BS232 *mxiA*	13	0	4	17	10
BS232(pGPA042)	56	>100	48	97	53
BS232(pGPA043)	33	>100	57	90	39
BS232(pGPA045)	52	100	37	94	46

[a] Relative to wild-type 2457T values.
[b] Average from three experiments. All values differ significantly from wild-type (2457T) values within 2 standard deviations.
[c] From a single representative experiment.
[d] Average from three experiments. IpaB export values for the *mxiA* transformants were significantly different from those of the positive (2457T) and negative (BS103) controls within 2 standard deviations.

of the transformed *mxiA* mutants. As shown in Table 2, although transformants of both *mxiA* mutants bound significantly higher levels of the dye than did the parental mutants and a plasmidless derivative, BS103, dye binding levels were intermediate in relation to those in the wild type. However, when these transformants were tested in a HeLa cell invasion assay, all were able to enter the monolayers at wild-type levels (Table 2). Moreover, when a smaller, non-*lac*-promoter-driven clone of the *mxiA* coding region, pGPA045 (Fig. 1), was introduced into the *mxiA* mutants, HeLa cell invasion was restored to wild-type levels (Table 2). In contrast to the complementation of the *mxiA* mutants, pGPA042, pGPA043, and pGPA045 transformants of *mxiB* mutant BS226 neither bound Congo red nor were invasive (data not shown).

When the smaller *Xba*I-*Eco*RI fragment from pGPA045 was introduced into vector pBS (similar to pKS but with the polylinker in the reverse orientation) to drive the expression of *mxiA* from the *lac* promoter, the resultant transformants of *E. coli* were extremely slow growing and appeared to readily lyse in liquid culture. Consequently, we were never able to isolate transformants of *S. flexneri mxi* mutants with this DNA. This observation was not surprising in light of our findings suggesting that MxiA is an inner membrane protein. Overexpression of this polypeptide may lead to a general blockage of or interference with an essential export function in the bacteria. In support of this hypothesis, when the amount of excreted Ipa was assessed in the *mxiA* transformants, all strains were found to release IpaB at levels intermediate in relation to those in the wild type, even though these levels were significantly higher than the residual levels excreted by the parental mutants (Table 2). Cell-associated levels of IpaB in these transformants, as measured by the suspension-labelling assay, were also found to be reduced relative to those in the wild type. Therefore, it is possible that because *mxiA* is in a high-copy-number vector in these strains, any increased level of expression of the gene product may interfere with the efficient export of IpaB. In this regard, one phenotypic effect that may be correlated with the decreased excretion of IpaB was observed when the capacity of *mxiA* transformants to produce plaques on HeLa cell monolayers was analyzed. Although the transformants were capable of forming plaques on HeLa cell monolayers, the plaques were generally slower to develop and smaller than those formed by wild-type *S. flexneri* (data not shown). This finding suggests that the decreased excretion of IpaB may lead to a decrease in the efficiency of the organisms in replicating intracellularly and/or spreading from cell to cell.

Our experiments also demonstrated that the ability of the cloned copy of *mxiA* to restore the invasion phenotype to the *mxiA* mutants was independent of its orientation in the vector (Table 2). This finding strongly suggests that *mxiA* is driven by a promoter that lies close to the translational start site, since the cloned copy of the gene in pGPA045, which is fully expressed, is in the reverse orientation relative to the *lac* promoter and contains very little DNA upstream of the *mxiA* ORF. These results are in contrast to those of our previous report suggesting that *mxiA* transcription may be driven by a distal promoter more than 6 kb upstream of the gene (2). Additional evidence supporting the hypothesis that *mxiA* is on a separate transcription unit comes from the finding that the largest *mxiA*-specific message observed in a Northern (RNA) blot analysis of wild-type *S. flexneri* is 2.3 kb (29). If this is the case, then it is unlikely that the GTG codon identified in the *mxiA* ORF represents the true translational start site, since there is not enough DNA upstream of the GTG codon in the smallest complementing *mxiA* clone (pGPA045) to contain a promoter. As indicated above, a σ^{28}-like promoter sequence was, in fact, detected downstream of the GTG codon on the DNA fragment contained in pGPA045 and may serve as the transcriptional start site for *mxiA* as well as genes further downstream.

The *mxiA* locus is the first of at least three plasmid-encoded accessory loci of *S. flexneri* that play a pivotal role in conferring the virulence phenotype on the organism by controlling the export of Ipa polypeptides. We are also investigating the role that *mxiB* plays in this export phenomenon and have not ruled out the possibility that the expression of *mxiB* may be under the regulatory control of *mxiA*. Additionally, ongoing experiments in our laboratory are focusing on the characterization of ORF2, which defines the third *mxi* locus (*mxiC*) essential for virulence. This locus may also function in the multicomponent Ipa export system.

If MxiA does have a regulatory role in modulating export, it may function as a sensor-effector molecule in response to a specific environmental stimulus. In this case, the N-terminal hydrophilic loops of the protein may perform a sensory function, while the C terminus may act as the effector or regulator, with the conserved region representing a common functional domain. Similar structure-function relationships have been proposed for sensor-effector molecules in other bacteria (25). Finally, the pattern of homology that we have found for LcrD and MxiA (a conserved anchor sequence and a more divergent cytoplasmic region with a single domain of high homology) has been demonstrated for other sensor-effector molecules among different bacterial species (25). However, since a functional comparison between MxiA and other sensor or effector molecules cannot as yet be made, other, more complex functional scenarios for this accessory protein are equally as probable.

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