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MxiJ, a Lipoprotein Involved in Secretion of *Shigella* Ipa Invasins, Is Homologous to YscJ, a Secretion Factor of the *Yersinia* Yop Proteins

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Shigella flexneri **causes bacillary dysentery by invading epithelial cells of the colonic mucosa. The invasion process requires the synthesis and secretion of the virulence plasmid-encoded Ipa proteins. Using Tn*phoA* mutagenesis, we have identified two virulence plasmid genes, *mxiJ* and *mxiM*, that encode proteins exported by the general export pathway. Analysis of the MxiJ and MxiM deduced amino acid sequences suggested that *mxiJ* and *mxiM* might encode lipoproteins, which was confirmed by [^{3}H]palmitate labeling of MxiJ:PhoA and MxiM:PhoA fusion proteins. A *mxiJ* mutant was unable to invade HeLa cells, to induce the formation of plaques on confluent monolayers of HeLa cells, and to provoke keratoconjunctivitis in guinea pigs. In addition, secretion of seven polypeptides, including IpaA, IpaB, and IpaC, was abolished in the *mxiJ* mutant. Sequence comparisons indicated that MxiJ and MxiH, which is encoded by a gene located upstream from *mxiJ*, are homologous to the *Yersinia enterocolitica* YscJ and YscF proteins, respectively.**

Shigella spp. are gram-negative microorganisms that cause bacillary dysentery in humans by invading epithelial cells of the colonic mucosa (18). The *Shigella flexneri* invasion process has been extensively studied by using epithelial cell lines (reviewed in references 14 and 38). Following entry by a mechanism similar to phagocytosis (10), *S. flexneri* lyses the membrane of the phagocytic vacuole (40) and moves within the cytoplasm of infected cells (20, 30, 31). Ics (intra-intercellular spread) movement results from the polymerization and reorganization of actin filaments on the bacterial surface and leads to the formation of protrusions by which bacteria pass into adjacent cells (8, 17). Lysis of the cellular membranes of the protrusions releases the bacteria into the cytoplasm of adjacent cells, thus completing the process of intercellular spread (1).

The virulence properties of *S. flexneri* are associated with the presence of a 220-kb plasmid that is found in all invasive isolates (39). The IpaA, IpaB, IpaC, and IpaD polypeptides (invasion plasmid antigens), which are dominant antigens in the humoral immune response during shigellosis (28), are encoded on this plasmid. Subcloning into a cosmid and Tn*5* mutagenesis allowed the definition of a 35-kb fragment of the virulence plasmid that includes the *ipa* genes and is necessary and sufficient for expression of invasive functions (23). Subsequent analysis of this region indicated that the *ipaB*, *ipaC*, *ipaD*, and *ipaA* genes are clustered in an operon and that the IpaB, IpaC, and IpaD proteins are essential for the entry process (6, 7, 41, 42, 45, 46). IpaB is also involved in contact-mediated hemolysis and lysis of the phagocytic vacuole (15).

Recent reports have shown that Ipa proteins are exposed on the bacterial surface and that this localization is dependent on the expression of genes located on the virulence plasmid upstream from the *ipa* locus (2, 3, 16, 47). Transposon insertion in the *mxiA* (membrane expression of invasion plasmid antigens) gene abolishes surface expression and secretion into the culture medium of IpaB and IpaC (3). A Tn*5* insertion in the *spa* (surface presentation of invasion plasmid antigens) locus, located downstream from *mxiA*, also abolishes surface presentation of IpaB and IpaC (47). We have recently characterized the *mxiD* gene, which is located upstream from *mxiA* and encodes an outer membrane protein necessary for secretion of the Ipa proteins (2). In contrast to the N-terminal sequences of the Ipa, Spa, and MxiA proteins, the N-terminal sequence of MxiD exhibits features characteristic of a signal sequence, which suggests that the general export pathway is involved in the localization of the Ipa secretion apparatus.

We describe here the characterization of the *mxiJ* and *mxiM* genes, which are located on large virulence plasmid pWR100 upstream from and in the same orientation as the *mxiD*, *mxiA*, and *spa* genes. By using Tn*phoA* mutagenesis, we obtained MxiJ:PhoA and MxiM:PhoA fusion proteins endowed with high levels of alkaline phosphatase activity, indicating that the PhoA moiety of the hybrid proteins was exported. Analysis of the MxiJ and MxiM deduced amino acid sequences suggested that *mxiJ* and *mxiM* might encode lipoproteins, which was confirmed by [^{3}H]palmitate labeling of MxiJ:PhoA and MxiM:PhoA fusion proteins. After inactivation of *mxiJ* on the virulence plasmid, the mutant strain was not able to invade HeLa cells, to induce the formation of plaques on confluent monolayers of HeLa cells, or to provoke keratoconjunctivitis in guinea pigs (Sereny test). Analysis of concentrated culture supernatants indicated that secretion of seven polypeptides, including IpaA, IpaB, and IpaC, was abolished in the *mxiJ* mutant. Sequence comparisons revealed that MxiJ and MxiH, which is encoded by a gene located upstream from *mxiJ*, are homologous to YscJ and YscF, respectively, which are involved in the secretion of Yop proteins in *Yersinia enterocolitica*.

MATERIALS AND METHODS

Bacterial strains and growth media. *Escherichia coli* DH5α {*endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1* Δ(*lacZYA-*

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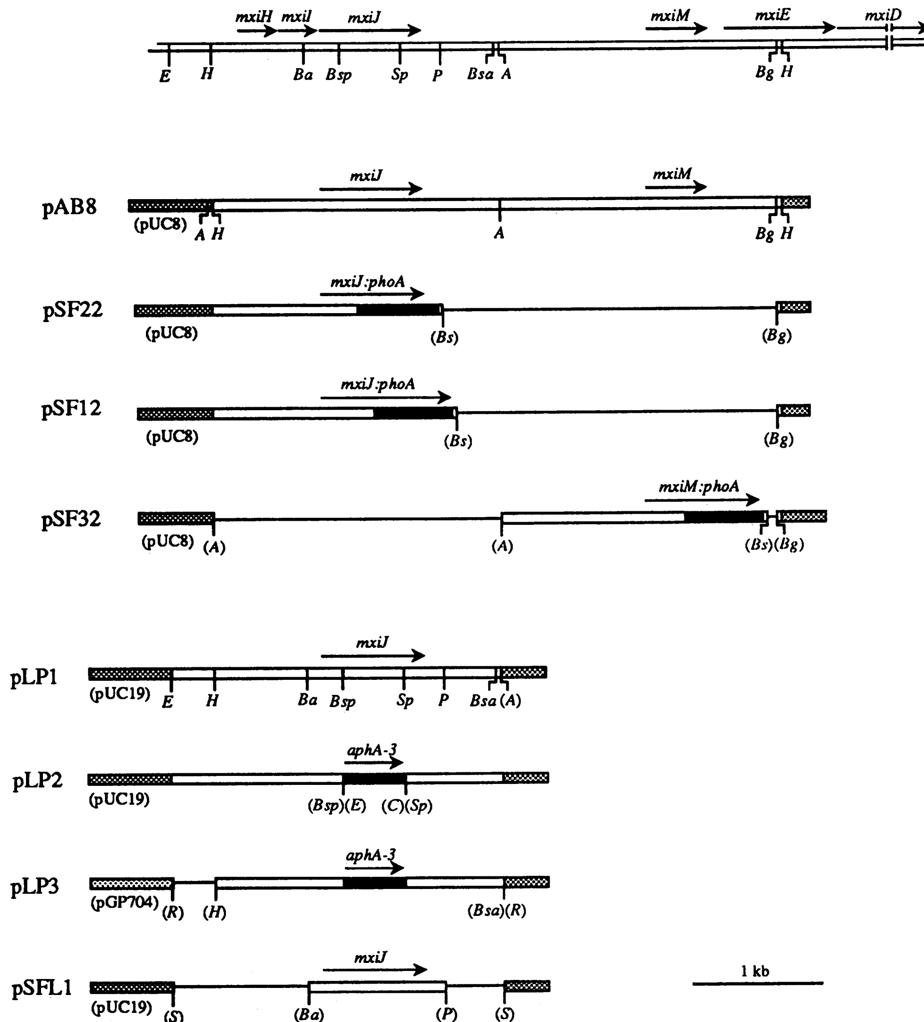

FIG. 1. Structure of plasmids carrying the *mxiH*, *mxiI*, *mxiJ*, and *mxiM* genes. The positions of the *mxiH*, *mxiI*, *mxiJ*, *mxiM*, *mxiE*, and *mxiD* genes are shown at the top. Plasmids are shown in linear form. *S. flexneri* DNA is indicated by open bars, and vector DNA (not shown to scale) is indicated by stippled bars; the vector names are indicated in parentheses. The 850-bp fragment carrying the *aphA-3* gene and the 1,500-bp fragment carrying the *phoA* portion of Tn*phoA* are indicated by solid bars (not shown to scale). The positions of selected restriction sites are shown. Restriction enzyme abbreviations: *A*, *Acc*I; *Ba*, *Bal*I; *Bg*, *Bgl*II; *Bs*, *Bst*EII; *Bsa*, *Bsa*BI; *Bsp*, *Bsp*EI; *C*, *Hinc*II; *E*, *Eco*RI; *H*, *Hin*dIII; *P*, *Pvu*II; *R*, *Eco*RV; *S*, *Sma*I; *Sp*, *Spe*I. Sites in parentheses were filled in during construction. The arrows indicate the positions and extents of the genes.

argF)*U169* F′[ϕ80*dlac*Δ(*lacZ*)*M15*]} (48) was used for plasmid construction, *E. coli* SM10λpir [*thi thr leu tonA lacY supE recA*::RP4-2Tc::Mu (Kn^r) (λ*pir*)] (27) was used to transfer plasmids to *S. flexneri*, and *E. coli* CC118 [*araD139* Δ(*ara leu*)*7697* Δ*lacX74 phoA20 galE galK thi rpsE rpoB argE*(Am) *recA1*] (22) was used for Tn*phoA* mutagenesis. *S. flexneri* M90T-Sm is a spontaneous streptomycin-resistant derivative of strain M90T (1), and strain BS176 is a derivative of strain M90T that has been cured of virulence plasmid pWR100 (39). The bacteria were grown in Luria-Bertani medium, tryptic soy broth, or M9 minimal medium (26). Antibiotics were used at the following concentrations: ampicillin, 50 μg/ml; kanamycin, 30 μg/ml; and streptomycin, 100 μg/ml.

Molecular cloning procedures. DNA analysis and transformation of *E. coli* strains were performed by standard methods (21). Nucleotide sequences were determined by the dideoxy chain termination procedure (37) performed on single-stranded M13 DNA or on alkaline-denatured plasmid DNA. The two DNA strands were sequenced completely. The junction between *mxiJ* (or *mxiM*) and *phoA* was determined by using an oligonucleotide hybridizing to the 5′ part of *phoA*.

Plasmid construction. The plasmids constructed in this study are derivatives of pHS5103 (7); their structures are shown in Fig. 1. Plasmid pAB8 was constructed by inserting a 4-kb *Hin*dIII fragment which was located upstream from *mxiD* in pHS5103 into the *Hin*dIII site of pUC8 (50). In pAB8, the *mxiJ* and *mxiM* genes are located downstream from and oriented in the same direction as the *lac* promoter of the vector. Tn*phoA* mutagenesis was performed as described by Manoil and Beckwith (22) on transformants of *E. coli* CC118 carrying pAB8. Following Tn*phoA* mutagenesis, two *mxiJ*:*phoA* fusions were subcloned by digesting plasmid DNA with *Bst*EII (which cuts 150 bp downstream from the *phoA* coding sequence) and *Bgl*II (which cuts 20 bp up-

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AAGCTTGAGCTCATTAATAGACTGATTTCTGAACATAAAAATATATATGGAGATCAGTATATTGAGTTTTCTGTACTTTTGATAGATGATGATTTTAAAGGTAAATCATATCTTAACAGC  120

                                                                        mxiH M  S  V  T  V  P  N  D  D  W  T  L  S  S  L  S  E  T
AAAGACAGTTATGTAATGTTGAATGATAAACACTGGTTTTTTTAGATAAAAATAAGTGAGGATAAAATGAGTGTTACAGTACCGAATGATGATTGGACATTGAGTTCATTATCTGAAAC  240

 F  D  D  G  T  Q  T  L  Q  G  E  L  T  L  A  L  D  K  L  A  K  N  P  S  N  P  Q  L  L  A  E  Y  Q  S  K  L  S  E  Y  T
TTTTGATGATGGAACTCAAACATTACAAGGTGAACTAACATTGGCACTAGATAAATTAGCTAAAAATCCTTCGAATCCACAGTTGCTGGCTGAATACCAAAGTAAATTATCTGAATATAC  360

 L  Y  R  N  A  Q  S  N  T  V  K  V  I  K  D  V  D  A  A  I  I  Q  N  F  R ***          mxiI M  N  Y  I  Y  P  V  N  Q  V
ATTATATAGGAACGCGCAATCCAATACAGTGAAAGTGATTAAGGATGTTGATGCTGCAATTATTCAAAACTTCAGATAATAGGGAGCATTCATGAATTACATTTATCCAGTCAATCAGGT  480

 D  I  I  K  A  S  D  F  Q  S  Q  E  I  S  S  L  E  D  V  V  S  A  K  Y  S  D  I  K  M  D  T  D  I  Q  V  S  Q  I  M  E
TGATATTATCAAAGCCAGTGATTTTCAATCTCAAGAGATATCAAGTCTGGAAGACGTCGTGTCGGCTAAATATAGTGATATTAAGATGGATACAGATATTCAAGTATCACAAATAATGGA  600

 M  V  S  N  P  E  S  L  N  P  E  S  L  A  K  L  Q  T  T  L  S  N  Y  S  I  G  V  S  L  A  G  T  L  A  R  K  T  V  S  A
GATGGTAAGCAATCCAGAATCATTAAACCCAGAATCTTTGGCCAAGTTACAGACGACGCTCTCAAATTATTCAATAGGAGTATCATTAGCTGGCACGTTAGCAAGAAAAACAGTTTCGGC  720

 V  E  T  L  L  K  S ***mxiJ M  I  R  Y  K  G  F  I  L  F  L  L  L  M  L  I  G↓C  E  Q  R  E  E  L  I  S  N  L  S  Q
TGTTGAAACTTTATTAAAGTCTTAATTTATATGATTAGGTATAAAGGTTTTATTTTATTCTTGTTGCTGATGTTGATTGGATGTGAGCAACGTGAAGAGTTAATTTCTAATTTATCTCAA  840

 R  Q  A  N  E  I  I  S  V  L  E  R  H  N  I  T  A  R  K  V  D  G  G  K  Q  G  I  S  V  Q  V  E  K  G  T  F  A  S  A  V
AGACAGGCAAATGAAATAATATCTGTGCTAGAACGCCATAATATTACTGCTAGAAAAGTTGATGGAGGTAAACAGGGGATCTCGGTACAAGTCGAAAAGGGGACATTTGCATCGGCAGTT  960

 D  L  M  R  M  Y  D  L  P  N  P  E  R  V  D  I  S  Q  M  F  P  T  D  S  L  V  S  S  P  R  A  E  K  A  R  L  Y  S  A  I
GATTTGATGCGCATGTACGATTTGCCAAATCCGGAGAGAGTTGATATCTCACAAATGTTTCCTACAGATTCATTAGTGTCTTCTCCAAGAGCTGAAAAGGCCCGTTTATATAGTGCTATT 1080

 E  Q  R  L  E  Q  S  L  V  S  I  G  G  V  I  S  A  K  I  H  V  S  Y  D  L  E  E  K  N  I  S  S  K  P  M  H  I  S  V  I
GAGCAACGGCTGGAACAGTCTTTAGTTTCTATTGGTGGTGTTATTTCGGCAAAAATACATGTTAGCTATGATCTTGAAGAAAAAAATATATCTTCAAAACCGATGCATATATCAGTAATC 1200

 A  I  Y  D  S  P  K  E  S  E  L  L  V  S  N  I  K  R  F  L  K  N  T  F  S  D  V  K  Y  E  N  I  S  V  I  L  T  P  K  E
GCTATATATGACTCACCGAAAGAGTCTGAACTATTAGTTAGTAATATTAAGCGATTTTTGAAAAACACCTTTTCTGATGTTAAGTATGAAAATATATCTGTCATATTAACTCCGAAAGAA 1320

 E  Y  V  Y  T  N  V  Q  P  V  K  E  V  K  S  E  F  L  T  N  E  V  I  Y  L  F  L  G  M  A  V  L  V  V  I  L  L  V  W  A
GAATATGTTTATACAAATGTACAACCTGTTAAGGAAGTTAAATCGGAATTTTTAACAAATGAAGTAATATATTTATTTCTCGGGATGGCTGTACTAGTTGTCATTCTTTTGGTATGGGCA 1440

                                mxiK M  I  R  M  D  G  I  Y  K  K  Y  L  S  I  I  F  D  P  A  F  Y  I  N  R  N  R  L  N  L
 F  K  T  G  W  F  K  R  N  K  I ***
TTCAAAACAGGGTGGTTCAAGAGAAACAAAATATGATAAGAATGGATGGAATTTATAAAAAATATCTTTCAATAATTTTTGATCCAGCGTTCTATATAAATAGAAATCGGTTGAATTTGC 1560

P  S  E  L  L  E  N  G  V  I  R  S  E  I  N  N  L  I  I  N  K  Y  D  L  N  C  D  I  E  P  L  S  G  V  T  A  M  F  V  A
CTTCTGAACTGTTAGAAAATGGCGTAATCAGAAGTGAGATTAATAATCTCATAATTAATAAATATGATCTAAATTGCGATATTGAACCTTTAAGCGGGGTAACCGCTATGTTTGTTGCCA 1680

N  W  N  L  L  P  A
ACTGGAATTTACTTCCAGCTG 1700
```

FIG. 2. Nucleotide sequences of the *mxiH*, *mxiI*, and *mxiJ* genes. The nucleotide sequence of the *Hin*dIII-*Pvu*II fragment into which the Tn*phoA* insertions in *mxiJ* were localized is shown, along with the deduced amino acid sequences of MxiH, MxiI, MxiJ, and the N-terminal portion of MxiK. The asterisks indicate the positions of the *mxiH*, *mxiI*, and *mxiJ* stop codons. The arrow indicates the proposed processing site in MxiJ.

stream from the distal *Hin*dIII site of pAB8), filling in, and religating, thereby giving rise to pSF12 and pSF22, respectively (Fig. 1). A *mxiM:phoA* fusion was subcloned similarly in two steps by digesting first with *Acc*I and then with *Bst*EII and *Bgl*II to give rise to pSF32 (Fig. 1).

Plasmid pSFL1 (Fig. 1), which was used to complement the *mxiJ* mutant, was constructed by inserting the 1,056-bp *Bal*I-*Pvu*II fragment of pAB8 (from bp 642 to bp 1698 in Fig. 2) into the *Sma*I site of pUC19 (50). In pSFL1, expression of *mxiJ* is under the control of the *lac* promoter of the vector.

Alkaline phosphatase assay. Alkaline phosphatase activity was assayed by using the substrate *p*-nitrophenylphosphate as described previously (22). Alkaline phosphatase specific activity is expressed in Miller units (i.e., milliunits of optical density at 420 nm per minute per unit of optical density at 600 nm).

SDS-polyacrylamide gel electrophoresis and immunoblotting. Electrophoresis in 10% polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS) was performed as described by Laemmli (19). After electrophoresis, proteins were either stained with Coomassie brilliant blue or transferred to a nitrocellulose membrane (44). Immunoblotting procedures were carried out by using a mixture of monoclonal antibodies raised against *S. flexneri* IpaB and IpaC proteins (5, 34).

In vivo labeling with [^{3}H]palmitate. Bacteria were grown at 37°C in M9 medium supplemented with 0.2% glucose, 0.1 mM $CaCl_2$, 1 mM $MgSO_4$, 10 μg of nicotinic acid per ml, and 10 μg of Casamino Acids per ml and were labeled with [^{3}H]palmitate (25 μCi/nmol/ml; Amersham, Internatinal plc, Amersham, United Kingdom) for 4 h as described previously (49).

Virulence assays. HeLa cells were infected as described previously (40). Virulence properties of the strains were also evaluated by using the plaque assay (29) and the Sereny test (43).

Nucleotide sequence accession numbers. The nucleotide sequences reported in this paper have been deposited in GenBank under accession numbers M98390 (for *mxiH*, *mxiI*, and *mxiJ*) and M98391 (for *mxiM*).

RESULTS

Construction of *mxi:phoA* fusions. In a previous study, we identified the *mxiD* gene, which encodes an outer membrane protein involved in secretion of Ipa proteins (2). In contrast to the other *S. flexneri* virulence proteins identified so far, including the Ipa and Spa proteins, the N-terminal sequence of MxiD exhibited features characteristic of a signal sequence, thereby suggesting that its export may rely on the general export pathway. To investigate whether genes located upstream from *mxiD* might also encode proteins exported by the general export pathway, we used Tn*phoA* mutagenesis, which allows the identification of genes that

```
                                    mxiM M  I  R  H  G  S  N  K  L  K  I  F  I  L  S  I  L  L  L  T  L  S  G↓C  A  L  K  S  S
TTAATTAGTGTCTTTGAAGCAGGGAGAGAGGCAGATGATTCGACATGGTAGTAATAAGTTGAAAATATTTATTTTAAGTATATTGCTATTAACACTGAGTGGGTGTGCTTTAAAGTCATC 120

 S  N  S  E  K  E  W  H  I  V  P  V  S  K  D  Y  F  S  I  P  N  D  L  L  W  S  F  N  T  T  N  K  S  I  N  V  Y  S  K  C
ATCTAATTCTGAAAAAGAATGGCATATTGTTCCTGTAAGTAAGGATTATTTTTCTATTCCAAATGATTTATTATGGTCGTTTAATACAACCAATAAAAGTATAAATGTTTACTCTAAATG 240

 I  S  G  K  A  V  Y  S  F  N  A  G  K  F  M  G  N  F  N  V  K  E  V  D  G  C  F  M  D  A  Q  K  I  A  I  D  K  L  F  S
TATTAGTGGTAAGGCGGTTTATAGTTTTAATGCAGGTAAATTCATGGGCAACTTTAATGTTAAGGAAGTAGATGGGTGCTTCATGGATGCACAAAAGATAGCTATAGATAAACTATTTTC 360

 M  L  K  D  G  V  V  L  K  G  N  K  I  N  D  T  I  L  I  E  K  D  G  E  V  K  L  K  L  I  R  G  I ***
TATGCTGAAAGACGGGGTTGTTTTAAAAGGTAATAAGATAAATGATACCATCCTTATAGAGAAGGATGGGGAAGTTAAATTAAAATTAATTCGAGGGATATAATTGTATTGTGAGTAAAT 480

                                                                                                             mxiE M  E  G
ATAAAGGTCTAAATACAAGTAATATGTTTTACATTTACTCTAGTGGACATGAACCAGTTAACGTTGAGCTTGTAAAAGATAAAGAACGTAACATAATTGAGCTGGCTCCAGCATGGAAGG 600
```

FIG. 3. Nucleotide sequence of the *mxiM* gene. The nucleotide sequence of the 600-bp fragment into which the Tn*phoA* insertion in *mxiM* was localized is shown, along with the deduced amino acid sequences of MxiM and the N-terminal part of MxiE. The asterisks indicate the position of the *mxiM* stop codon. The arrow indicates the proposed processing site in MxiM.

encode membrane or secreted proteins, since hybrid PhoA proteins display alkaline phosphatase activity only if the PhoA portion is transported through the cytoplasmic membrane (22).

Plasmid pAB8 (Fig. 1) was mutagenized in *E. coli* by using transposon Tn*phoA*, and restriction analysis of plasmids carried by clones that exhibited a blue color on plates containing 5-bromo-4-chloro-3-indolyl phosphate, the chromogenic substrate for alkaline phosphatase, allowed the identification of three Tn*phoA* insertions. Two insertions were found very close to each other at one end of the pAB8 insertion, and the third was located at the other end of the pAB8 insertion, which suggested that these transposon insertions might affect two different genes. This was confirmed by subsequent analysis (see below), and these genes were designated *mxiJ* and *mxiM*.

To avoid further transposition of Tn*phoA*, derivatives of each plasmid were constructed by deleting the transposon sequence located downstream from *phoA* (see Materials and Methods). The alkaline phosphatase activities expressed by plasmids pSF12 (*mxiJ:phoA*), pSF22 (*mxiJ:phoA*), and pSF32 (*mxiM:phoA*) were assayed in *S. flexneri* M90T; high levels of alkaline phosphatase activity (from 1,200 to 1,500 U) were expressed by each of the three plasmids, confirming that the PhoA portion of the hybrid proteins was exported.

Identification of the *mxiH*, *mxiI*, and *mxiJ* genes. The sequence of the 1,700-bp *Hin*dIII-*Pvu*II fragment, within which the two Tn*phoA* insertions in *mxiJ* were localized, is shown in Fig. 2. The *mxiJ* open reading frame (ORF), which extends from bp 751 to bp 1476, was identified by sequencing the junctions between *mxiJ* and *phoA* in plasmids pSF12 and pSF22. These junctions occurred after codon 19 and codon 111 of *mxiJ* in plasmids pSF22 and pSF12, respectively. The *mxiJ* gene is predicted to encode a 242-amino-acid residue polypeptide with a calculated M_r of 27,477. The N-terminal extremity of the *mxiJ* gene product has a stretch of hydrophobic and nonpolar residues that probably represents the signal sequence involved in the periplasmic or membrane localization of the MxiJ:PhoA hybrid proteins.

Analysis of the nucleotide sequence located upstream from *mxiJ* revealed the presence of two ORFs, the first from bp 188 to bp 439 and the second from bp 452 to bp 745; these ORFs were designated *mxiH* and *mxiI*, respectively. *mxiH* is predicted to encode an 83-amino-acid residue polypeptide (M_r, 9,255), and *mxiI* is predicted to encode a 97-amino-acid residue polypeptide (M_r, 10,621). Although the *mxiH* and *mxiI* ORFs are rather small, their proposed start codons are preceded by potential ribosome binding sites (5′-GAGGA-3′ for *mxiH*, 5′-GGGAG-3′ for *mxiI*), which suggests that these ORFs encode proteins. Downstream from *mxiJ*, we detected the beginning of another ORF, designated *mxiK* (Fig. 2). There are 12 bp between the *mxiH* stop codon and the *mxiI* start codon, 5 bp between the *mxiI* stop codon and the *mxiJ* start codon, and a 4-bp overlap between the end of *mxiJ* and the beginning of *mxiK*. This genetic organization suggests that *mxiH*, *mxiI*, *mxiJ*, and *mxiK* belong to the same transcription unit.

Identification of the *mxiM* gene. The sequence of the 600-bp fragment, into which the Tn*phoA* insertion in *mxiM* was localized, is shown in Fig. 3. The *mxiM* ORF, which extends from bp 35 to bp 463, was identified by sequencing the junction between *mxiM* and *phoA* in plasmid pSF32. This junction occurred after codon 79 of *mxiM*. The *mxiM* gene is predicted to encode a 142-amino-acid residue polypeptide with a calculated M_r of 15,834. As in the case of MxiJ, the N-terminal sequence of MxiM exhibits a stretch of hydrophobic residues that is probably recognized as a signal sequence by the general export pathway. There are 120 nucleotides between the *mxiM* stop codon and the start codon of the downstream gene *mxiE* (2).

MxiJ and MxiM are lipoproteins. The motif Leu-Xaa-Gly-Cys (Xaa means any amino acid residue) which is characteristic of the processing site of lipoproteins (49), was detected at the ends of the putative signal sequences of MxiJ and MxiM, suggesting that *mxiJ* and *mxiM* encoded lipoproteins. To characterize lipoproteins encoded by virulence plasmid pWR100, *S. flexneri* M90T-Sm (wild type) and BS176 (cured of pWR100) were labeled in vivo with [^{3}H]palmitate, and whole-cell extracts were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiographed. As shown in Fig. 4, numerous molecular species, ranging in molecular mass from 10 to 30 kDa, were labeled in both strains. Except for a band corresponding to a 27-kDa protein that appeared to be more strongly labeled in M90T-Sm than in BS176, no clear difference was detected between the patterns of the two strains. This difficulty in identifying virulence plasmid-encoded lipoproteins might be due to the comigration of these lipoproteins with lipoproteins encoded by the chromosome.

To demonstrate that *mxiJ* and *mxiM* encoded lipoproteins, we took advantage of the *mxiJ:phoA* and *mxiM:phoA* fusions, assuming that insertion of Tn*phoA* into a gene encoding a lipoprotein would give rise to a fusion protein with a molecular mass of at least 48 kDa (corresponding to the molecular mass of the PhoA portion of the hybrid) that should still be labeled with [^{3}H]palmitate. The patterns of proteins labeled with radioactive palmitate in BS176 transformants carrying pUC8, pAB8 (*mxiJ mxiM*), pSF12 (*mxiJ:phoA*), pSF22 (*mxiJ:phoA*), and pSF32 (*mxiM:phoA*) are shown in Fig. 4. Proteins with molecular masses of 50, 64, and 59 kDa were detected in strains carrying pSF22, pSF12,

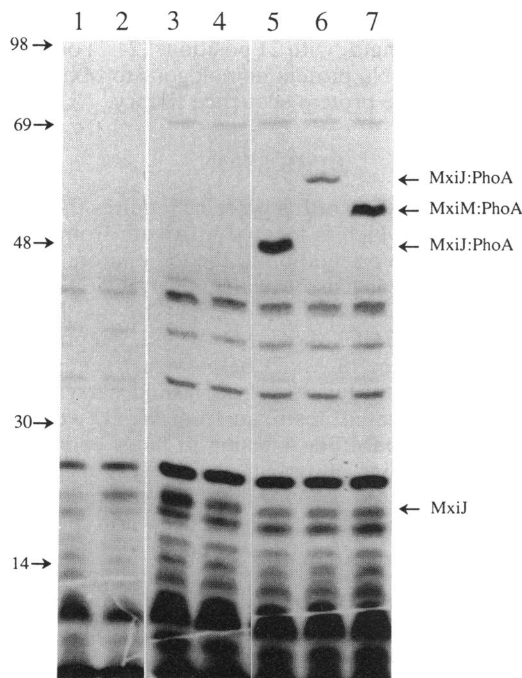

FIG. 4. [^{3}H]palmitate labeling of the MxiJ:PhoA and MxiM:PhoA fusion proteins. Whole-cell extracts of *S. flexneri* grown in M9 medium and labeled with [^{3}H]palmitate for 4 h were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiographed. Lane 1, strain BS176; lane 2, strain M90T-Sm; lane 3, strain BS176 (pAB8); lane 4, strain BS176(pUC8); lane 5, strain BS176(pSF22) (*mxiJ:phoA*); lane 6, strain BS176(pSF12) (*mxiJ:phoA*); lane 7, strain BS176(pSF33) (*mxiM:phoA*). The positions and sizes (in kilodaltons) of protein standards are indicated on the left; the positions of the MxiJ:PhoA and MxiM:PhoA hybrid proteins and of the proposed *mxiJ* gene product are indicated on the right.

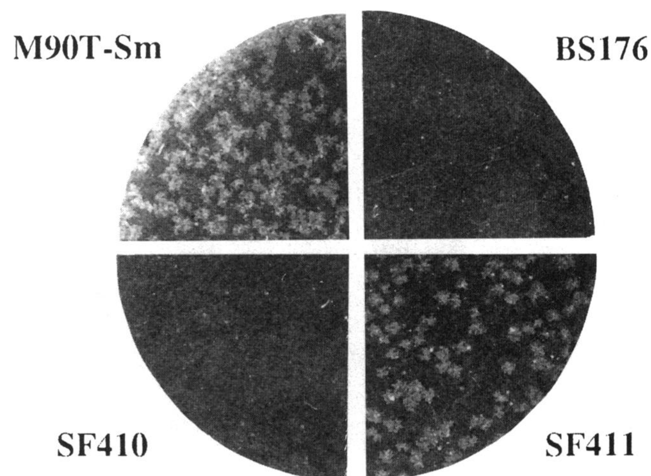

FIG. 5. Formation of plaques on confluent monolayers of HeLa cells infected with *S. flexneri* M90T-Sm (wild type), BS176 (cured of pWR100), SF410 (*mxiJ1*), or SF411 (strain SF410 carrying pSFL1).

and pSF32, respectively, and were not present in strains carrying pUC8 or pAB8. The sizes of these proteins are consistent with the sizes expected for the PhoA fusion proteins, indicating that the MxiJ:PhoA and MxiM:PhoA hybrids were indeed labeled with [^{3}H]palmitate. Moreover, in the extract of BS176 harboring pAB8, the intensity of a band corresponding to a 27-kDa protein was enhanced relative to the intensity of the band in the same strain harboring pUC8. This suggested that a 27-kDa lipoprotein was encoded by pAB8, most probably by *mxiJ*, whose product is predicted to be a 27-kDa polypeptide.

Construction and characterization of a *mxiJ* mutant. To investigate the role of *mxiJ*, we inactivated the *mxiJ* gene on the large virulence plasmid. First, plasmid pLP1 (Fig. 1) was constructed by cloning the 2,150-bp *Eco*RI-*Acc*I fragment of pHS5103 (7) into the *Eco*RI and *Acc*I sites of pUC19 after filling in of the *Acc*I sites. Then, plasmid pLP2 was constructed by replacing the 420-bp *Bsp*EI-*Spe*I fragment of pLP1 (from bp 995 to bp 1413 in Fig. 2) by an 850-bp *Eco*RI-*Hin*cII cassette carrying the *aphA-3* gene, which confers resistance to kanamycin (24). The 2,550-bp *Hin*dIII-*Bsa*BI fragment of pLP2 was then cloned into the *Eco*RV site of pGP704, a derivative of suicide vector pJM703.1 that confers resistance to ampicillin (27), to give rise to pLP3 (Fig. 1). Finally, plasmid pLP3 was transferred to *S. flexneri* M90T-Sm by conjugal mating, and transconjugants were selected on plates that contained streptomycin and kanamycin. Clones in which a double recombinational event had exchanged the wild-type *mxiJ* gene for the mutated copy carried by pLP3 were identified by their sensitivity to ampicillin, and the structure of the resultant large plasmid carrying the inactivated *mxiJ* gene was confirmed by Southern analysis. This mutation in *mxiJ* was designated *mxiJ1*, and the corresponding strain was designated strain SF410.

The virulence properties of the wild-type strain were completely abolished in the *mxiJ1* mutant, since this strain was unable to provoke keratoconjunctivitis in guinea pigs and to invade and to form plaques on confluent monolayers of HeLa cells (Fig. 5). In addition, the *mxiJ1* mutant was not able to bind the dye Congo red. To ensure that these defects were due to the *mxiJ1* mutation, plasmid pSFL1 (Fig. 1), which carries a wild-type copy of *mxiJ* (from bp 642 to bp 1697 in Fig. 2), was used to transform strain SF410 (*mxiJ1*). The transformants had the ability to bind Congo red, to invade HeLa cells, to induce the formation of plaques on confluent monolayers of HeLa cells (Fig. 5), and to provoke keratoconjunctivitis in guinea pigs. These results confirmed that the phenotypes observed with the *mxiJ1* mutant were due solely to the inactivation of *mxiJ*.

MxiJ is involved in secretion of the Ipa proteins. The phenotypes of the *mxiJ1* mutant, especially the lack of Congo red binding, were similar to those of a *mxiD* mutant (2). Since a *mxiD* mutant is unable to secrete the IpaA, IpaB, and IpaC proteins, we analyzed the protein contents of the culture supernatants of strains M90T-Sm (wild type), SF410 (*mxiJ1*), and SF411 (strain SF410 with pSFL1). As shown in Fig. 6, molecular species with molecular masses of 110, 95, 70, 62, 60, 58, 41, 39, and 35 kDa were detected in the culture supernatant of the wild-type strain. The 70-, 62-, 60-, 58-, 41-, 39-, and 35-kDa proteins were absent in the culture supernatant of the *mxiJ* mutant (Fig. 6), but were present in the culture supernatant of the *mxiJ1* strain harboring plasmid pSFL1 (data not shown). The diffuse bands at 56 and 26 kDa correspond to partially hydrolyzed proteins present in the tryptic soy broth medium used to grow bacteria. Immunoblot analysis in which a mixture of monoclonal antibodies directed against IpaB and IpaC was used confirmed that IpaB (62 kDa) and IpaC (41 kDa) were not secreted in the *mxiJ1* mutant (Fig. 6). Similar amounts of IpaB and IpaC were detected in crude extracts of the wild-type and *mxiJ1*

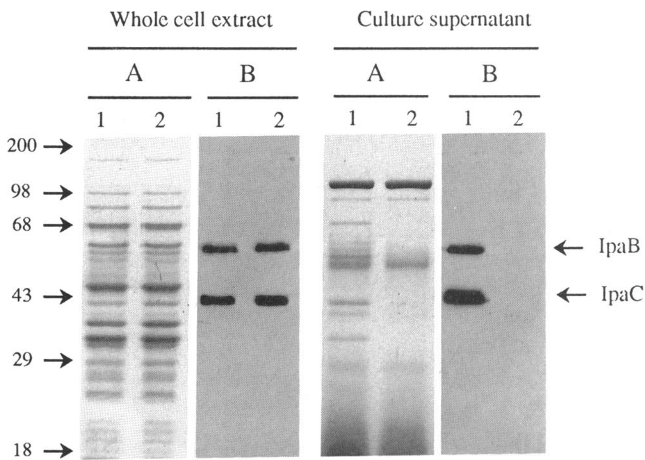

FIG. 6. Expression of the Ipa proteins in the *mxiJ* mutant. Whole-cell extracts and culture supernatants of *S. flexneri* M90T-Sm (wild type) (lanes 1) and SF410 (*mxiJ1*) (lanes 2) were separated by SDS-polyacrylamide gel electrophoresis and either stained with Coomassie brilliant blue (A) or transferred onto nitrocellulose and reacted with a mixture of monoclonal antibodies directed against IpaB and IpaC (B). The positions and sizes (in kilodaltons) of the protein standards and the positions of IpaB and IpaC are indicated by arrows.

strains (Fig. 6), indicating that export rather than synthesis of these antigens was affected by the *mxiJ* mutation. The 70-kDa protein, whose secretion was abolished in the *mxiJ* mutant, has been identified previously as IpaA (2), and the 95-kDa protein, which is secreted in the *mxiJ* mutant, corresponds to a mature form of IcsA (13). Thus, it appears that processing and secretion of IcsA are independent of MxiJ.

MxiJ is homologous to YscJ. The sequences of MxiH, MxiI, MxiJ, and MxiM were compared with the protein sequences translated from the GenBank library (release 71) by using the FASTA computer program (33). This comparison revealed extensive similarity between the sequence of MxiJ and the sequence of YscJ (YlpB) of *Y. enterocolitica* (25). As shown in Fig. 7, the MxiJ and YscJ sequences are homologous over their entire length, with 65 positions (26%) occupied by identical residues.

We also detected a statistically significant similarity between the sequences of MxiH and YscF of *Y. enterocolitica* (25). As shown in Fig. 8, the two sequences are homologous over their entire length, with 21 positions (24%) occupied by identical residues. No protein homologous to MxiI or MxiM was detected in the protein sequence library.

DISCUSSION

Export and secretion of Ipa proteins requires the products of a large locus, which is located upstream from and transcribed in opposite orientation to the *ipa* operon. The distal part of this locus consists of *mxiE*, *mxiD*, *mxiC*, *mxiA*, and five *spa* genes (2–4, 47). In contrast to the N-terminal sequences of the MxiE, MxiC, MxiA, and Spa proteins, the N-terminal sequence of MxiD has a signal sequence. Following Tn*phoA* mutagenesis of a plasmid carrying a 4-kb DNA fragment located upstream from *mxiD*, we obtained MxiJ:PhoA and MxiM:PhoA fusion proteins endowed with high levels of alkaline phosphatase activity, suggesting that the PhoA portion of the hybrids was exported. This was confirmed by a sequence analysis of the wild-type *mxiJ* and *mxiM* genes; the N-terminal sequences of the *mxiJ* and *mxiM* gene products contain basic amino acid residues followed by a stretch of noncharged and hydrophobic residues, which are features characteristic of a signal sequence (reviewed in reference 36).

The presence of the motif Leu-Xaa-Gly-Cys at the end of the putative signal sequences of MxiJ and MxiM suggested that MxiJ and MxiM might be lipoproteins. Prolipoproteins are subjected to a cascade of modifications, including cleavage of the signal sequence by signal peptidase II, which gives rise to mature lipoproteins that have an N-terminal Cys residue modified by two fatty acyl groups through ester linkages and by a third fatty acyl group via an amide linkage (49). Such modifications can be detected after growth in the presence of radioactive palmitate. Direct detection by [^{3}H]palmitate labeling of lipoproteins encoded by the *S. flexneri* virulence plasmid was complicated by the presence of numerous lipoproteins encoded by the chromosome. This difficulty was overcome by taking advantage of the MxiJ:PhoA and MxiM:PhoA fusions; in the fusion-containing strains, proteins, whose sizes corresponded to the sizes of PhoA hybrid proteins, were labeled after growth in the presence of radioactive palmitate, demonstrating that MxiJ and MxiM encoded lipoproteins. A similar strategy has been used to identify *Vibrio cholerae* lipoproteins whose expression is under the control of ToxR (32). Interestingly, only the first two residues of MxiJ are present in the mature MxiJ:

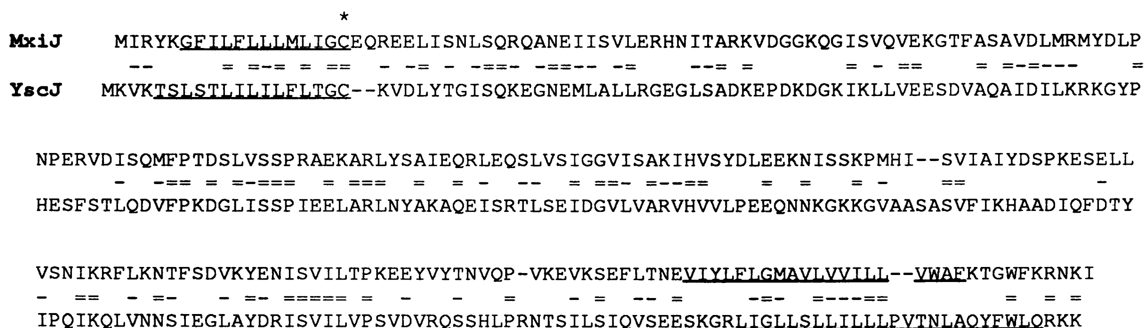

FIG. 7. Sequence comparison of MxiJ and YscJ. The complete sequence of the *S. flexneri* MxiJ protein (this study) is aligned with the sequence of the *Y. enterocolitica* YscJ protein (25). The positions of identical residues (equals signs) and functionally equivalent residues (dashes) are indicated between the sequences. The N- and C-terminal hydrophobic regions of the two proteins are underlined. The asterisk indicates the Cys residue that is proposed to be modified in MxiJ and YscJ.

```
MxiH          MSVTVPNDDWTLSSLSETFDDGTQTLQGELTLALDKLAKNPSNPQLLAEYQSKLSEYTLYRNAQSNTVKVIKDVDAAIIQNFR
                     =  ==   =  -  =           -   -  =   = == ===- =  -   --  =  =  =- -==-  -=-=  =
YscF      MSNFSGFTKGNDIADLDAVAQTLKKPADDANKAVNDSIAALKDTPDNPALLADLQHSINKWSVIYNISSTIVRSMKDLMQGILQKFP
```

FIG. 8. Sequence comparison of MxiH and YscF. The complete sequence of the *S. flexneri* MxiH protein (this study) is aligned with the sequence of the *Y. enterocolitica* YscF protein (25). The positions of identical residues (equals signs) and functionally equivalent residues (dashes) are indicated between the sequences.

PhoA fusion encoded by pSF22, which makes it an extremely short hybrid lipoprotein.

To investigate the role of *mxiJ*, the *mxiJ* gene carried by virulence plasmid pWR100 was inactivated by allelic replacement with a gene mutagenized in vitro. Mutant strain SF410 (*mxiJ1*) was not able to bind the dye Congo red, to invade HeLa cells, and to provoke keratoconjunctivitis in guinea pigs. Expression of *mxiJ* from a plasmid was sufficient to complement the *mxiJ1* mutant for all of these phenotypes, confirming that the virulence defects of the mutant were due solely to the inactivation of *mxiJ* and indicating that *mxiJ* is involved in the invasive phenotype of *S. flexneri*.

Analysis of concentrated culture supernatants by SDS-polyacrylamide gel electrophoresis and Coomassie blue staining indicated that wild-type *S. flexneri* secretes nine polypeptides into the growth medium. This observation extends previous reports indicating that IpaB and IpaC were secreted (3, 16, 47). Seven of these polypeptides, including IpaA, IpaB, and IpaC, are not secreted in the *mxiJ* mutant. In the case of IpaB and IpaC, the absence of secretion is not due to a lack of synthesis, since these antigens are present in whole-cell extracts of the mutant. These results demonstrate that MxiJ is required for the secretion of the Ipa proteins. In addition, we have detected 110- and 95-kDa proteins, whose secretion was not altered in the *mxiJ* mutant. Whereas the 110-kDa protein has not been characterized yet, the 95-kDa protein has been identified as a mature form of IcsA (13). Venkatesan et al. (47) have also noted that a Tn*5* insertion in the *spa* locus did not affect the localization of IcsA (VirG) in the outer membrane.

Sequence comparisons revealed that MxiJ is homologous to YscJ of *Y. enterocolitica*. The *yscJ* gene is the 10th gene of the *virC* operon, which consists of 13 ORFs. In addition to *yscJ*, at least two other genes in this operon, *yscD* and *yscL*, are required for export and secretion of the Yop proteins (25). The similarity detected between MxiJ and YscJ encompasses the entire length of these proteins, which strongly suggests that they have similar localizations and functions. YscJ corresponds to the previously identified YlpB lipoprotein, which has been localized in the outer membrane (9). Localization of the *Klebsiella pneumoniae* lipoprotein pullulanase and of an *E. coli* lipoprotein in the outer membrane is dependent on the nature of the second amino acid residue of the mature protein (i.e., the residue that follows the lipid-modified N-terminal cysteine residue). Replacement of the serine residue by an aspartic acid residue, but not by a glutamic acid residue, directs hybrid lipo-β-lactamase and pullulanase to the inner membrane (11, 35). YlpB contains a lysine residue in position +2 of the mature protein, whereas the second residue of the MxiJ mature protein is a glutamic acid residue. Structural determinants, in addition to the N-terminal sorting sequence, influence the outer membrane localization of other lipoproteins (12). Interestingly, each of the C-terminal sequences of MxiJ and YscJ has a long stretch of hydrophobic residues (underlined in Fig. 7) that is likely to be involved in the localization of these proteins. Having such structural features (i.e., a lipid-modified N-terminal extremity and a hydrophobic C-terminal domain), the MxiJ and YscJ proteins might be anchored in the membrane by both ends. The C-terminal hydrophobic domain of both MxiJ and YscJ is followed by basic residues; therefore, this domain might serve as a stop transfer signal, anchoring the C-terminal end of each of these proteins in the inner membrane, while the N-terminal part would be inserted into the outer membrane by the lipid moiety. This is one of several models that could be consistent with the presence of two membrane domains; additional studies will be required to determine the topology of these proteins.

We have also detected an important sequence similarity between the product of *mxiH*, which is located upstream from *mxiJ*, and the product of *yscF*, which is the sixth gene of the *virC* operon in *Y. enterocolitica* (25). Although the functions of *mxiH* and *yscF* have not been investigated yet, the positions of these genes in loci encoding secretory proteins suggest that they might be important for secretion of the Ipa proteins in the genus *Shigella* and the Yop proteins in the genus *Yersinia*. Inactivation of *mxiH*, as well as *mxiI* and *mxiM*, would be required to confirm that these genes are involved in secretion of the Ipa proteins. We have found previously that MxiD is homologous to YscC, the product of the third gene in the *virC* operon (2). Thus, it appears that three genes in the *Yersinia virC* operon (*yscF*, *yscJ*, and *yscC*) have counterparts in the *Shigella mxi* locus (*mxiH*, *mxiJ*, and *mxiD*). This suggests a common origin for these regions of the virulence plasmids of *Shigella* and *Yersinia* species. In addition, MxiA is homologous to the *Yersinia pseudotuberculosis* LcrD protein (4). In conclusion, not only are the *Shigella* Ipa and *Yersinia* Yop proteins secreted, but their secretion apparatuses have at least four components in common, some of which (MxiD and YscC, MxiJ and YscJ) have an N-terminal signal sequence indicative of the involvement of the general export pathway in their secretion.

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