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Spa32 Regulates a Switch in Substrate Specificity of the Type III Secreton of *Shigella flexneri* from Needle Components to Ipa Proteins

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Type III secretion systems (TTSS) are essential virulence determinants of many gram-negative bacteria and serve, upon physical contact with target cells, to translocate bacterial proteins directly across eukaryotic cell membranes. The *Shigella* TTSS is encoded by the *mxi*/*spa* loci located on its virulence plasmid. By electron microscopy secretons are visualized as tripartite with an external needle, a transmembrane domain, and a cytoplasmic bulb. In the present study, we generated a *Shigella spa32* mutant and studied its phenotype. The *spa32* gene shows low sequence homology to *Salmonella* TTSS1 *invJ*/*spaN* and to flagellar *fliK*. The *spa32* mutant, like the wild-type strain, secreted the Ipas and IpgD, which are normally secreted via the TTSS, at low levels into the growth medium. However, unlike the wild-type strain, the *spa32* mutant could neither be induced to secrete the Ipas and IpgD instantaneously upon addition of Congo red nor penetrate HeLa cells in vitro. Additionally, the Spa32 protein is secreted in large amounts by the TTSS during exponential growth but not upon Congo red induction. Interestingly, electron microscopy analysis of the *spa32* mutant revealed that the needle of its secretons were up to 10 times longer than those of the wild type. In addition, in the absence of induction, the *spa32* mutant secreted normal levels of MxiI but a large excess of MxiH. Taken together, our data indicate that the *spa32* mutant presents a novel phenotype and that the primary defect of the mutant may be its inability to regulate or control secretion of MxiH.

The gram-negative *Shigella* bacterium is the cause of bacillary dysentery, an invasive disease of the human colonic epithelium (13, 42). The three essential steps for *Shigella* virulence are invasion of epithelial cells, intracellular multiplication, and the spread of the invading bacteria into adjacent cells. The capacity of *Shigella* to enter cells is governed by proteins encoded by a subset of genes within three contiguous operons (*ipa*, *mxi*, and *spa*) in a 30-kb region of the 230-kb pWR100 virulence plasmid (36). The Ipa proteins (invasins) are essential for the invasion of epithelial cells, and their secretion is mediated by the proteins encoded at the *mxi* and *spa* loci whose products constitute a type III secretion apparatus (TTSS) (or secreton) (6, 27).

The major function of TTSSs is to transport proteins from the bacterial cytoplasm into the host cell plasma membrane or cytoplasm upon contact with host cells (5, 11, 12). In *Shigella flexneri*, the *mxi*, the *spa* and the *ipa* operons are expressed at 37°C, but Ipa proteins remain in the bacterial cytoplasm until the secretion machinery is activated by host cell contact or by external, presumably surrogate, signals such as serum or asmall amphipathic Congo red (CR) dye molecule (4, 27, 37). Physical contact between the bacterium and the host cell induces insertion of two Ipas (IpaB and IpaC) into the host membrane to form a 25-Å pore that might be used to translocate the other invasins into target cells (6). The Ipas then catalyze the formation of a localized actin-rich, macropinocytic-like ruffle on the host cell surface which internalizes the bacterium (8, 48). Bacterial internalization initiates a cycle of intra- and intercellular spreading (34).

Th *e Shigella* type III secreton was found by electron microscopy of osmotically shocked and negatively stained cells to be composed of three parts: a cytoplasmic bulb, a transmembrane neck domain, and a 50- to 60-nm-long, extracellular and hollow needle through which secretion of Ipas might occur when bacteria contact epithelial cells (6, 7). This molecular machinery strongly resembles the *Salmonella* SPI1 TTSS1 (17, 20, 21) and flagellar basal bodies. The "needle complex" of *Shigella* is composed at least of MxiD, MxiG, MxiJ, MxiH, and MxiI (7, 47). The major needle component is MxiH, which is essential for the secretion of Ipa invasins (7).

The role of the *Shigella* Spa proteins is poorly understood. Yet, the *spa* region is highly conserved among all TTSS-encoding operons. Sasakawa and coworkers previously reported that *spa32* mutant, which was able to bind CR at 37°C, suggesting a functional secretion apparatus (43, 49). These researchers also reported that cell surface-located Spa32 and contact between bacteria and HeLa cells were required for triggering the release of Ipa proteins from the *Shigella* outer membrane. Very recently, Schuch and Maurelli (45) reported that the *spa33*

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant features and/or method for construction	Source or reference
Strains		
S. flexneri		
M90T-Sm	*S. flexneri* strain is a derivative of the wild-type strain M90T(serotype 5) (41) which is resistant to streptomycin (Sm^r)	1
SF401	*mxiD*-deficient secretion mutant	1
MJ321	*spa32* mutant (pWR100-*spa32*::*aphA-3*), still producing truncated Spa32 protein (the first 91 N-terminal amino acid residues) (Spa32n)	This work
MJ322	MJ321+pMJ7 (native Spa32)	This work
MJ323	MJ321+pMJ8 (His_6-Spa32)	This work
MJ005	pWR100-*mxiD-lacZ*	This work
MJ325	pWR100-*spa32*::*aphA-3-mxiD-lacZ*	This work
E. coli		
DH5α(λ*pir*)	Host for cloning suicide vector pGP704	29
Sm10(λ*pir*)	Host used for conjugation mating with *S. flexneri*	29
XL1-Blue	General host for cloning vectors	Pharmacia
M15	Host for high expression of His_6 recombinant proteins	Qiagen
Top10	Host for high expression of His_6 recombinant proteins	Invitrogen
Plasmids		
pGP704	Suicide vector, with R6K origin, used for the construction of *spa32* mutant	29
pTZ18R	Cloning vector	Pharmacia
pUC19	Cloning vector	New England Biolabs
pMJ1	pUC19 carrying *spa32* and its flanked regions	This work
pMJ2	pMJ1 carrying internal *spa32* in-frame deletion	This work
pMJ3	pMJ2 carrying insertion of the *aphA-3* cassette into *spa32*	This work
pMJ5	pGP704 suicide vector carrying *spa32* inactivated by the *aphA-3* cassette from pMJ2	This work
pMJ7	pTZ18R carrying the *spa32* gene expressed from the p *lac* promoter	This work
pMJ8	pQE30 carrying the entire *spa32* gene; His_6 N-terminal fusion construct	This work
pNJH8	pQE30 carrying the *mxiH* gene; His_6 N-terminal fusion construct	This work
pNJ54	pBAD His *mycA* carrying the *mxiI* gene; His_6 C-terminal fusion construct	This work
pQE30	Expression vector	Qiagen
pBAD His-*mycA*	Expression vector	Invitrogen
pLAC8	pGP704 suicide vector carrying *mxiD-lacZ* fusion	1

gene is required for Ipa secretion and that its product is exported to the surface of the bacteria by the Mxi/Spa TTSS.

We performed here further studies on the *spa32* gene product. We generated a nonpolar *spa32* mutant, localized the Spa32 protein in *Shigella*, and studied the multiple aspects of its complex function.

MATERIALS AND METHODS

Bacterial strains and growth media. *S. flexneri* strains are derivatives of the wild-type strain M90T (serotype 5) (40). The M90T-Sm (Sm^r) and SF401 (*mxiD*) strains have been described previously (1). *Escherichia coli* strains are derivatives of K-12 strain; the M15 strain harboring the pREP4 plasmid (Table 1) was transformed with two pQE30 derivatives (see below), and the Top10 strain (Table 1) was transformed with pBAD derivatives (see below); DH5α(λ*pir*) was transformed with derivatives of the suicide vector pGP704 (29), and SM10-λ*pir* was used to transfer derivatives of pGP704 to *S. flexneri*. Bacteria were grown in Luria-Bertani (LB) medium (Sigma) or tryptic casein soy broth at 37°C (Sigma). Antibiotics were used at the following concentrations: ampicillin, 100 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; and streptomycin, 100 μg ml^{-1}.

Plasmids and strain construction. Strains and plasmids used or constructed in this study are listed in Table 1.

DNA analysis, plasmid construction, and transformation of *E. coli* and *S. flexneri* were performed according to standard methods (39). A 2,475-bp DNA fragment containing the 879-bp *spa32* gene flanked by upstream and downstream regions of 798-bp each was amplified by PCR with *spa32-1* (5′-GCTCGCATG CCTTTTGGAGGATGAT-3′; sense) and *spa32-2* (5′-GGCCGGATCCAAGA ACCATTTACT-3′; antisense) primers and with the pWR100 virulence plasmid DNA as a template. The amplified fragment contains a *Bam*HI site at the 5′ end and a *Sph*I site at the 3′ end. Plasmid pMJ1 was constructed by inserting the *Bam*HI/*Sph*I-digested 2,475-bp PCR fragment (encompassing the *spa32* gene and flanking regions) into the *Bam*HI/*Sph*I-digested pUC19 (Fig. 1B). Plasmid pMJ2 was constructed by an in-frame deletion within the *spa32* gene by removing the 540-bp *Msc*I -*Hin*cII fragment of pMJ1 (from codons 91 to 270 of Spa32) (Fig. 1B). Plasmid pMJ3 was constructed by inserting the 850-bp *Sma*I DNA fragment carrying the *aphA-3* gene (26) into the unique *Sfu*I site of pMJ2 (Fig. 1B). In this construct, the *spa32* gene was interrupted at codon 92, which leads to the production of a truncated Spa32 protein (Spa32n) that contains the first 91 N-terminal amino acid residues, the codons 92 to 271 were deleted, and the last 21 codons were inserted in-frame with the translational start codon located at the 3′ end of the *aphA-3* cassette (26). Plasmid pMJ5 was constructed by inserting the 2,795-bp *Sph*I/KpnI fragment of pMJ3 (encompassing the inactivated *spa32* gene and its flanking regions) into the corresponding sites of the suicide vector pGP704. PMJ5 was then transferred to *S. flexneri* M90T-Sm by conjugal mating, and clones in which the double recombination event had occurred were selected based on their resistance to streptomycin and kanamycin and their sensitivity to ampicillin. The structure of the resulting pWR100::*spa32*, carrying the inactivated *spa32* was confirmed by PCR with the *spa32-1* and *spa32-2* primers, and the corresponding strain was designated MJ321 (and is referred to as the *spa32* mutant).

Plasmid pMJ7, used for complementation experiments, was constructed by inserting the 1,540-bp *Eco*RI DNA fragment from pMJ1 into the corresponding site of pTZ18R (Pharmacia) (Fig. 1B). The introduction of this plasmid into MJ321 gave rise to strain MJ322.

mxiD-lacZ fusions in MJ321 and wild-type strains were constructed by using the pLAC8 (*mxiD-lacZ*) suicide plasmid according to the method of Allaoui et al. (1). Briefly, pLAC8 (*mxiD-lacZ*) was transferred by conjugal mating from *E. coli* SM10-λ*pir* to *S. flexneri* M90T (wild type) and to MJ321 strains, respectively. The appropriate simple integration of the pLAC8 in the virulence plasmid pWR100

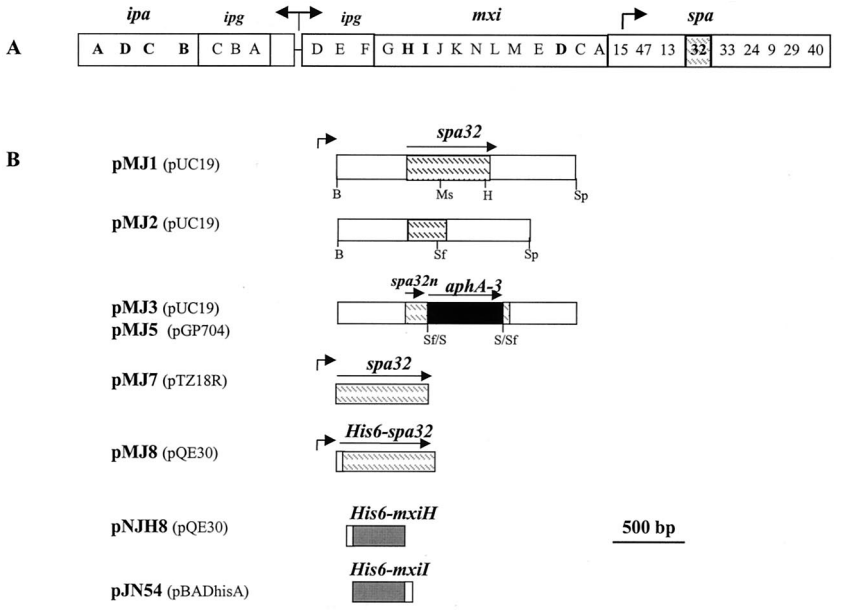

FIG. 1. Plasmids used for the inactivation of the *spa32* gene. (A) Schematic representation of the 30-kb DNA of the pWR100 plasmid required for the entry of *S. flexneri* into eukaryotic cells. Arrows indicate positions and orientation of previously described promoters. Open bars, except for the *spa32* gene (indicated by a dashed box), show the relative positions of the *icsB-ipgABC-ipaBCDA*, *ipgDEF-mxi*, and *spa* loci. (B) Structure of plasmids used to inactivate the *spa32* gene (pMJ2-5), to complement the *spa32* mutant (pMJ7-8), and to produce and purify His-tagged recombinant proteins Spa32 (pMJ8), MxiH (pNJH8), and MxiI (pJN54). The black box represents the nonpolar *aphA-3* gene that confers resistance to kanamycin. The horizontal arrows indicate the extent and the orientation of the corresponding genes, while small arrows indicate positions of the promoters known to control expression of these genes. A small open box represents the His_6 tag motif. Constructs are drawn with the relevant restriction sites: B, *Bam*HI; Ms, *Msc*I; H, *Hin*cII; Sf, *Sfu*I; and Sp, *Sph*I.

(wild type) or pWR100::*spa32* (*spa32* mutated strain) was ascertained by selection of the transconjugants on plates containing streptomycin and ampicillin. The resulting strains were designated MJ005 (M90T-*mxiD-lacZ*) and MJ325 (MJ321-*mxiD-lacZ*).

Construction of recombinant plasmids expressing His_6 hybrid proteins. Plasmid pMJ8, used for the purification of His_6-Spa32 and for complementation experiments, was constructed by inserting the 915-bp *Bam*HI/*Kpn*I PCR DNA fragment, carrying the *spa32* gene, into the corresponding sites of the expression vector pQE30 (Qiagen). The PCR DNA fragment was amplified by using the *spa32-3* sense primer (5′-ACGCGGATCCATGGCATTAGATAATATAAAC C-3′) and the *spa32-4* antisense primer (5′-TGGCGGTACCTCTGTAGTTTT TCGTTAT-3′), which creates a *Bam*HI at the 5′ end and a *Kpn*I site at the 3′ end. Plasmid pNJH8, which encodes His_6-MxiH was constructed as follows. The *mxiH* gene was amplified by using the *mxiHI* sense primer (5′-AGCGGATCC AGTGTTACAGTACCG-3′) and the *mxiH2* antisense primer (5′-CGCGTCG ACTGGATTATCTGAAGT-3′), which creates a *Bam*HI site at one end and a *Hin*dIII site at the other. pNJH8 was constructed by inserting the 263-bp *Bam*HI-*Hin*dIII *mxiH* PCR-digested PCR DNA fragment into the corresponding sites of expression vector pQE-30 (Qiagen) (Fig. 1B). Plasmid pNJ54 encoding MxiI-His_6 was constructed as follows. The *mxiI* gene was amplified by using the *mxiI1* sense primer (5′-CATGCCATGGTTTACATTTATCCAGTC-3′) and *mxiI2*, an antisense primer (5′-CCCAAGCTTAGACTTTAATAAAGTTTC-3′). The amplified DNA contains a *Nco*I site at the 5′ end and a *Hin*dIII site at the 3′ end. Plasmid pNJ54 was obtained by cloning the 305-bp *Nco*I/*Hin*dIII PCR-digested DNA fragment into the corresponding sites of the pBAD His-*mycA* vector.

Preparation of antibodies against Spa32, MxiH, and MxiI. *E. coli* M15 was transformed with pMJ8 (His_6-Spa32) and with pNJH8 (His_6-MxiH), respectively (the two recombinant proteins contain N-terminal His_6 tag fusions). The resulting strains were grown at 37°C to an optical density at 600 nm (OD_{600}) of 0.6 and then induced for 4 h with IPTG (isopropyl-β-D-thiogalactopyranoside) at a 1 mM final concentration. The Top10 strain harboring the pNJ54 plasmid (MxiI-His_6), in which the six histidines were fused to the C terminus of the full-length MxiI protein, was grown at 37°C to an OD_{600} of 0.4 and then induced 4 h with arabinose at a final concentration of 0.2%. In all cases, the His_6-tagged recombinant proteins were found in inclusion bodies and were solubilized in a 0.1 M NaH_2PO_4–10 mM Tris (pH 8) buffer containing 8 M urea. Proteins were further purified according to the Qiagen expression protocol under denaturing conditions. The purified proteins were eluted from the nickel-nitrilotriacetic acid column with a 0.1 M NaH_2PO_4–10 mM Tris (pH 8) buffer containing 6 M urea and imidazole at a final concentration of 200 mM. The eluted protein solutions were further submitted to dialysis in 1 M stepwise reductions in urea concentration until a concentration of 3 M urea was reached. For each His_6-Spa32, His_6-MxiH, or MxiI-His_6, a rabbit was injected three times with 500 μg of the protein sample.

Protein analysis. Proteins were analyzed by sodium dodecyl sulfate (SDS)–12 to 14% polyacrylamide gel electrophoresis (PAGE) (23) and/or Western blot. For some preparations, Tricine gels containing a 16% polyacrylamide concentration were used to separate the MxiH and MxiI proteins. The primary antibodies used in this study were the monoclonal antibodies anti-IpaC and anti-IpaB (1); anti-polyhistidine monoclonal antibodies (Sigma); and anti-IpaD (27), -Spa32, -MxiH, and -MxiI polyclonal antibodies. Goat anti-mouse immunoglobulin G (IgG)-peroxidase conjugate antibodies (Sigma) or donkey anti-rabbit IgG horseradish peroxidase-linked whole antibodies (Amersham Life Sciences) were used as secondary antibodies, and detected proteins were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech).

Preparation of culture supernatants. Two secretion modes are usually used to study Ipa proteins secretion in *Shigella* (37). After their production, the Ipa proteins are stored within the cytoplasm, and only 5% of them are secreted via the TTSS when bacteria are grown to mid log phase. The latter mode of secretion is called uninducible secretion, basal secretion, or leakage. The second mode of secretion is called inducible secretion, a condition during which bacteria are induced by the addition of CR. Under this condition, ca. 90% of the Ipa proteins are instantaneously secreted by the TTSS. This form of secretion is also called full or complete secretion.

Bacteria were grown with aeration on LB medium at 37°C by using a C25 incubator shaker at 175 rpm (New Brunswick Scientific). Proteins were analyzed from 50-ml log-phase cultures (OD_{600} = 0.3). Under inducing conditions, proteins from culture supernatants were prepared from 10-ml portions of these cultures. Bacteria were recovered by centrifugation (10,000 × *g*, 10 min), suspended in 500 μl of phosphate-buffered saline (PBS), and incubated with CR (at a final concentration of 10 μg/μl) for 15 min at 37°C (6, 33). Cells were removed by centrifugation (21,000 × *g*, 20 min), and 250 μl of supernatants was recovered.

Then, 20 μl of these samples, corresponding to 0.8 ml of the original culture, was used for SDS-PAGE and Western blot analysis.

To analyze protein secretion under noninducing conditions, cells were removed from the remaining 40 ml of log-phase cultures (grown at 37°C on LB medium) by centrifugation (10,000 × *g*, 30 min). Portions (30 ml) of culture supernatants were filtered through a 0.2-μm-pore-size syringe filter and needle (18GA2 1.2×50; Becton Dickinson, Drogheda, Ireland) and precipitated by the addition of 40% (wt/vol) ammonium sulfate. The mixtures were incubated overnight at 4°C under mild agitation and then centrifuged (10,000 × *g*, 45 min). The pellets were washed in 800 μl of sterile water and centrifuged at high speed (21,000 × *g*, 45 min). The dried pellets were suspended in 40 μl of PBS. Then, 5-μl portions of these samples, corresponding to 3.5 ml of the original culture, were used for SDS-PAGE and Western blot analysis.

Amino acid sequencing. Proteins were separated by SDS-PAGE, blotted onto polyvinylidene difluoride membrane, and stained with amido black. The bands of interest were applied to a protein sequencer (Eurogentec, Liège, Belgium).

Other tests. Invasion assays, electron microscopy, and β-galactosidase assays were performed as described previously (6, 7, 28, 41).

RESULTS

Spa32 is required for CR-induced secretion of the Ipa invasins. The *spa32* gene is encoded within the 30-kb DNA fragment of the *Shigella* virulence plasmid essential for entry into epithelial cells (Fig. 1A) (25, 44). In a previous work, Watarai et al. (49) constructed an in-frame deletion of *spa32* in *S. flexneri* 2a by using the pCDV442 suicide vector and *sacB* for selection of sucrose-resistant clones. We found that this strain (CS25585) bound CR not only at 37°C but also at 30°C and displayed no secretons at 37°C (not shown). Therefore, we concluded that its CR-binding activity was unrelated to the expression of a functional TTSS. Indeed, the CS25585 strain had been screened for its ability to bind CR on sucrose plates (49), probably forcing the occurrence of an unrelated mutation that otherwise affected the CR-binding ability of this strain. Moreover, we failed, in the present study, to obtain a *spa32* mutant by using a derivative of the pCDV442 that harbored an internal in-frame deletion of *spa32*. Indeed, for all of the clones tested, we found that the virulence plasmid pWR100 was partly deleted after sucrose selection (data not shown).

To characterize the role of *spa32*, we constructed a *S. flexneri* 5a strain, MJ321, in which the *spa32* gene has been inactivated with a nonpolar cassette (Fig. 1B and Materials and Methods). The newly constructed strain was unable to bind CR and was thus white on agar plates at 37°C, suggesting that the *spa32* gene product is required for the secretion of Ipa invasins. To test this, the proteins present in the culture supernatants of M90T (wild-type) and MJ321 strains induced by CR were analyzed by SDS-PAGE and immunoblotting. IpaA (70 kDa), IpaB (62 kDa), IpaC (41 kDa), and IpaD (39 kDa) were secreted by the wild-type strain but not by MJ321 (Fig. 2A). The lack of Ipa secretion was confirmed by using monoclonal antibodies directed against IpaC and IpaB and polyclonal anti-IpaD (Fig. 2B and data not shown). Significant amounts of IpaC were detected in whole-cell extracts of the wild type and the *spa32* mutant, indicating that the lack of secretion was not due to the absence of synthesis or stability of the Ipa proteins (Fig. 2B).

As expected, strain MJ321 was also unable to enter HeLa cells (data not shown). The mutant's inability to secrete Ipa proteins was restored to the wild-type phenotype by complementation of the mutant with expression vectors encoding the Spa32 protein from the p *lac* or the p *tac* promoters (Fig. 1B and Table 1). Indeed, the introduction of pMJ7 or pMJ8, two plasmids encoding the native Spa32 and a His_6-Spa32, respectively, restored the ability of the MJ321 strain to secrete the Ipa proteins and to invade HeLa cells (Fig. 2 and data not shown).

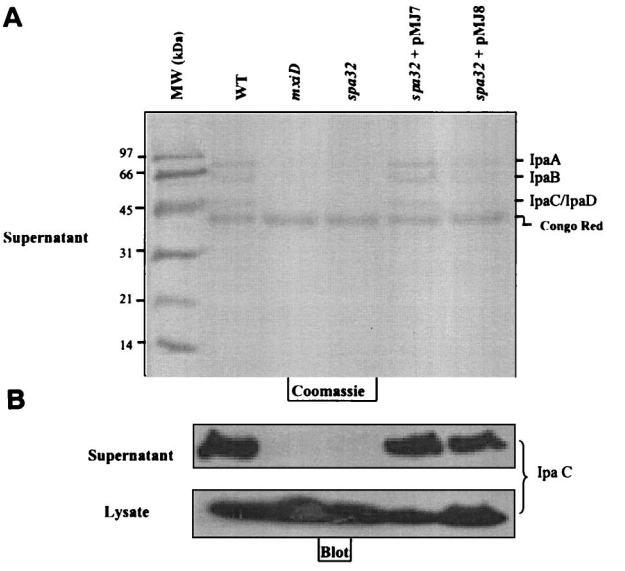

FIG. 2. Spa32 is required for Ipa secretion upon CR induction. Strains used were: M90T (wild type), *mxiD* and MJ321 (*spa32* mutant), MJ322 (MJ321+pMJ7), and MJ323 (MJ321+pMJ8). Cultures of *S. flexneri* strains (grown at 37°C) were harvested by centrifugation, suspended in PBS, and incubated in the presence of CR for 15 min at 37°C to induce Ipa secretion. Equal amounts of the culture supernatants and cell lysates were analyzed by SDS–12% PAGE stained with Coomassie blue (A) or were immunoblotted with a monoclonal antibody specific to the IpaC protein (B).

Spa32 is not required for the initial secretion of Ipa invasins in the absence of CR induction. It was previously reported that low-level secretion of the Ipa proteins via the TTSS in the absence of any induction could be observed in the wild-type strain (37). We therefore investigated whether this process was affected in the MJ321 *spa32* mutant. We precipitated proteins from the culture supernatant of several strains grown until log phase and performed Coomassie blue staining of SDS-PAGE and Western blots. As shown in Fig. 3A and B, we found that IpaB, IpaC, and IpaD (data not shown) were present at wild-type levels in the culture supernatant of the MJ321 strain. As a control, we used the SF401 strain (*mxiD* mutant), which does not secrete any Ipa proteins under similar growth conditions, indicating that the Ipa proteins are released via the Mxi/Spa TTSS (Fig. 3A and B). This unambiguously demonstrates that the *spa32* mutant, despite its apparent inability to bind CR on plates, retained its ability to secrete a small amount of the Ipa proteins prior to CR induction. Therefore, our data provides the first report of a *Shigella* mutant able to secrete a low-level the Ipa invasins in the medium but which has, however, lost its ability to penetrate HeLa cells in vitro.

Electron microscopy analysis of the type III secretons of the *spa32* mutant reveals secretons with ultralong needles. The MJ321 *spa32* mutant's ability to secrete Ipa proteins suggested an at least partially functional TTSS, which we visualized by electron microscopy. Wild-type *Shigella* exhibits secretons with an average needle length of ca. 50 nm (Fig. 4, left panel).

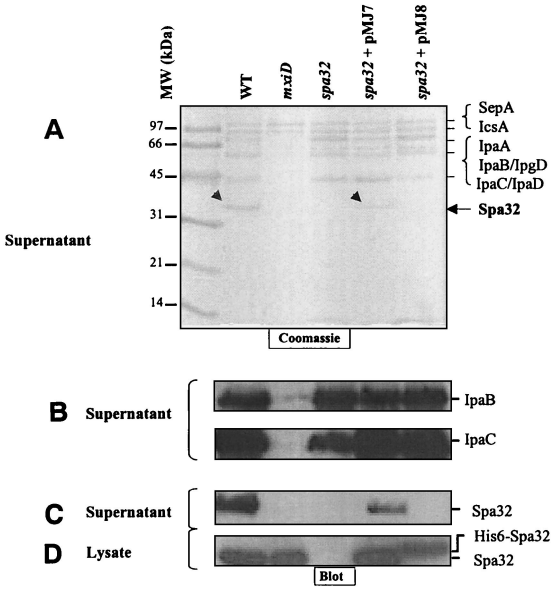

FIG. 3. The *spa32* mutant (MJ321) secretes Ipa proteins even in the absence of CR induction. Culture supernatant proteins were prepared from bacteria grown for 3 h at 37°C until the exponential growth phase was reached (OD_{600} = 0.3). Samples were centrifuged, and equal amounts of precipitated supernatants and cell lysates were analyzed by SDS–12% PAGE and Coomassie blue staining (A) or by immunoblotting with a monoclonal antibody specific for IpaB and IpaC (B) or a polyclonal antibody specific to Spa32 (C and D). The small arrowheads indicate the position of Spa32.

Electron microscopy analysis of the MJ321 strain indicated that the *spa32* mutated strain showed extraordinary long secreton needles up to 400 to 900 nm in length (Fig. 4, middle panel). Complementation of the *spa32* mutant with a plasmid expressing either the native Spa32 (MJ322 stain) (Fig. 4, right panel) or the His_6-Spa32 (MJ323 strain) (data not shown) partly restored the wild-type needle substructures. We found that the two latter strains exhibited intermediate-length needles of up to 50 to 150 nm. Hence, the MJ321 is the first *Shigella* strain that contains the three parts of the secreton (bulb, neck, and an needle) but is no longer able to bind CR on plates, to secrete the Ipa invasins upon CR induction, or to invade HeLa cells in vitro.

MxiH and MxiI are the components of the long needle of the type III secreton of the *spa32* mutant. To analyze the components of the elongated needles observed on the *spa32* mutant (MJ321), we isolated cell surface-associated structures from the MJ321 strain. Log-phase cultures were passed five times each through a 25-gauge needle prior to separation of the culture supernatant and the cell pellet fractions. Supernatant proteins were precipitated and separated by SDS-PAGE on a 16% polyacrylamide gel and then analyzed by Coomassie blue staining and immunoblotting (Fig. 5A). Two other strains—M90T (wild type) and MJ322 (MJ321+pMJ7)—were subjected as controls to the same analysis. As shown in Fig. 5A, the protein patterns show clear differences between strains in the low-molecular-weight range. A thick major band that migrated faster than the 14-kDa marker was detected for MJ321 but is missing in both the wild-type and the *mxiD* mutant strains. However, this protein band was present, although at a lower intensity, in the sample prepared from the MJ322 strain (MJ321+pMJ7), indicating a partial complementation of the *spa32* mutant by pMJ7 (Fig. 5A). When longer 16% acrylamide gels with Tris-Tricine buffers were used, this protein band was revealed to be composed of at least two proteins of ca. 12 and 11 kDa. Upon sequencing the N terminus, the protein from the upper band contained the MALDNI amino acid residues. This sequence corresponds to the N-terminal end of Spa32 and was designated Spa32n (12 kDa); it is produced by the MJ321 strain (see Materials and Methods and Fig. 1B). In contrast, the lower protein band contained a mixture of two profiles of N-terminal amino acid residues: MSVTV and MNYIY (Fig. 5A), which were shown by sequence comparisons with the recently published sequence of the virulence plasmid pWR100 to correspond to the products of the *mxiH* (10 kDa) and *mxiI* (9.3 kDa) genes, respectively (2, 9).

To monitor these proteins more easily, we purified recombinant MxiH, MxiI, and Spa32 proteins tagged with six histidines and generated antibodies against them (see Materials and Methods). The anti-Spa32 polyclonal antibody recognized Spa32n in the supernatant of the *spa32* mutant (data not shown), and similar data were obtained for MxiH and MxiI (Fig. 5B and data not shown). This result indicates that the 91 N-terminal amino acid residues of Spa32 are sufficient to mediate its secretion via the TTSS. The amounts of secreted MxiI in both wild-type and *spa32* mutant strains were similar (data not shown). However, the amount of MxiH detected in the culture supernatant prepared from the MJ321 strain was much higher than in the fractions prepared from for the wild-type strain (Fig. 5B). This difference was not due to the increased MxiH synthesis in the *spa32* mutant strain since approximately equal amounts of MxiH were detected in the whole-cell culture of the wild-type and the *spa32* mutant strains (bacteria and supernatant) from the two strains (Fig. 5B). These results suggest that the elongated needle substructures of MJ321 are due to the increased amount of MxiH secreted by the bacteria.

Spa32 is secreted by the TTSS in the absence of CR but not when secretion is induced. We determined the localization of Spa32 during the bacterial growth cycle. We performed immunoblotting with the anti-Spa32 antibody on bacterial cell fractions prepared under various growth conditions. We found that native Spa32 was associated, in wild-type (M90T) and MJ322 strains, with the supernatant from log-phase cultures but not with the supernatant prepared after addition of CR (Fig. 3C and data not shown). Western blot analysis with antibodies directed against His_6 (results not shown) or against Spa32 indicated that, in contrast to native Spa32, His_6-Spa32 (expressed in MJ323 [MJ321+pMJ8]) was detected in the bacterial lysate fraction but was absent from the supernatant (Fig. 3C and D). In agreement with this result, a protein corresponding to the size of the *spa32* gene product was detectable in a Coomassie blue-stained gel in the culture supernatant of the M90T and the MJ322 (MJ321+pMJ7) strains (Fig. 3A). However, this protein band was missing from the supernatant of the MJ323 (MJ321+pMJ8), thus confirming the lack of His_6-Spa32 protein secretion (Fig. 3A). Moreover, Spa32 was not secreted by the *mxiD* mutant (Fig. 2C), which lacks visible secretons (6), indicating that Spa32 secretion occurs via the TTSS itself. The lack of Spa32 secretion by the *mxiD* mutant was not due to the absence of its synthesis, since Spa32 was

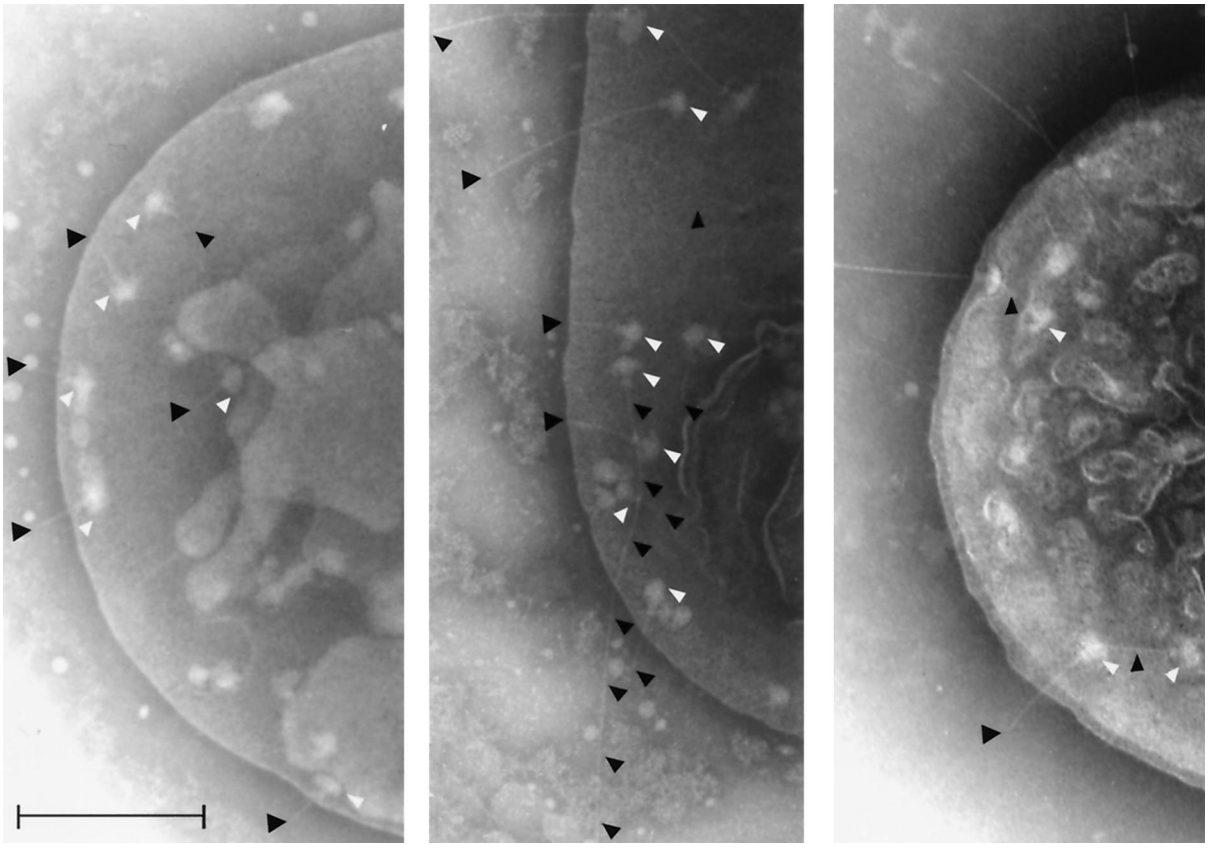

FIG. 4. The *spa32* mutant strain (MJ321) exhibits an elongated needle structure. Electron micrographs of *Shigella* wild-type M90T (left panel), *spa32* mutant (middle panel), and *spa32* mutant complemented with the pMJ7 plasmid encoding the native Spa32 protein (MJ322) (right panel). Osmotically shocked and negatively stained bacteria (grown at 37°C) were visualized by electron microscopy as previously described by Blocker et al. (7). Bar, 200 nm. The white arrowheads indicate the transmembrane region and the cytoplasmic bulb of type III secretons; the needle (if particularly long) or needle tips are outlined or indicated by black arrowheads.

detectable in the cell fraction in amounts equivalent to those seen in the wild-type strain (Fig. 3D). In further support of this, we confirmed by amino acid sequencing and Western blot that Spa32n (the N-terminal 91 amino acids of Spa32) is secreted without any cleavage, as would be expected for a protein secreted by the TTSS (Fig. 5A). In addition, since the His_6 form of Spa32, which is not secreted, partially rescues Ipa secretion upon induction with CR (Fig. 2) and HeLa cell invasion in MJ321, we conclude that Spa32 need not be secreted to be functional. Similarly, we conclude that Spa32 need not be secreted to be functional, regulating the amount of MxiH secreted and thus needle length.

DISCUSSION

In the present study, we generated a *spa32* mutant in *S. flexneri* 5a and showed that, although it can secrete a small amount of Ipa proteins into its growth medium, it is unable to secrete the Ipa proteins upon induction with CR. This mutant is also unable to invade HeLa cells in vitro, suggesting that its "contact-dependent" secretion is impaired. The wild-type phenotype was restored by complementation of the *spa32* mutant with either native Spa32 or a His_6-Spa32 protein. We characterized the *spa32* mutant by electron microscopy of osmotically shocked cells and found that it exhibits a different TTSS (secreton) structure compared to the wild-type strain. Its secretons have transmembrane domains and cytoplasmic bulbs that appear identical to those of the wild-type strain, but the mutant strain has extraordinarily long needles, up to 10 times longer than those found in the wild-type strain. Furthermore, we identified the needle components of this *spa32* mutant by preparing a fraction enriched in surface-associated structures. Three low-molecular-weight proteins were found in this fraction from the exponentially grown *spa32*-deficient strain, whereas they were not detectable in similar fractions from the wild-type strain. By N-terminal amino acid sequencing and Western blot, we showed that the three proteins corresponded to a truncated Spa32 (Spa32n) carrying the first N-terminal amino acid residues of Spa32, MxiH (10 kDa), and MxiI (9.3 kDa). The latter two proteins are required for Ipa secretion and are encoded by adjacent genes of the *mxi* locus (2, 7). These findings are in agreement with the previously proposed composition of the native needle complex of *Shigella*. Indeed, it was recently shown that both MxiH (7, 47) and MxiI are essential components of the *Shigella* TTSS needle (7).

MxiH is conserved in most of the bacteria species that are pathogenic for animals and have a TTSS (7), but MxiH homologues have not been reported in TTSSs of plant pathogens

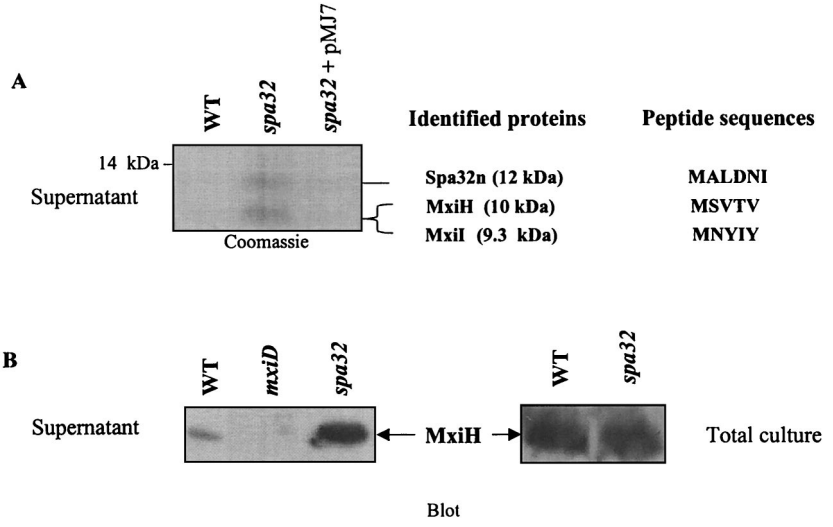

FIG. 5. The higher secreted amount of MxiH in the *spa32* mutant is not due to its overproduction. (A) Wild-type strain (M90T), the *spa32* mutant (MJ321), and its derivative complemented with pMJ7 (encoding the native Spa32) (MJ322) were grown to mid-log phase (OD_{600} = 2). Equal amounts of proteins from the culture supernatants were loaded on 16% Tris-Tricine SDS-PAGE gels, and then stained with Coomassie blue. The positions of the proteins, as well as the sequenced amino acid residues of the N terminus of Spa32n, MxiH, and MxiI are indicated on the right side of each panel. (B) Equal amounts of total culture proteins (supernatant and bacteria; the equivalent of 0.1 OD_{600}) were separated by SDS–16% PAGE and then immunoblotted with the anti-MxiH polyclonal antibody. The strains used were the wild type (M90T), the *spa32* mutant, and the *mxiD* mutant as indicated.

(15). In addition, the only MxiI homologue detected was the highly related *Salmonella* SPI1 TSSS protein PrgJ, which has been shown to be necessary for needle assembly in this organism (17). In *Salmonella*, PrgI is the major component of the needle complex and is secreted by the TTSS itself. Furthermore, PrgI is detected in the supernatant of the *invJ/spaN* mutant, which also displays abnormally long SPI1 TTSS needles (21). Interestingly, Spa32 shares 20% identity over its entire length with InvJ, suggesting a common ability to control the length of the corresponding TTSS needles. Additionally, both proteins share 14% identity with FliK of *Salmonella* (18), which is involved in regulating the hook-filament transition by switching to export of flagellar filament components (30, 32, 35).

One of the remarkable features of the flagellar structure of wild-type *Salmonella* is that the length of the hook, a hollow tubular structure composed of FlgE subunits, is tightly controlled at ca. 55 nm in wild-type *Salmonella* (14). The *Shigella* TTSS needle is of a similar and invariant length (ca. 50 nm [7, 47]). Very recently, Makishima et al. (24) examined hook length and found that it is determined not by molecular rulers (which FliK was initially thought to be) (16, 19) but from the inside of the bacterium by the C-ring proteins (FliG, FliM, and FliN), which appear to monitor the amount of hook protein secreted by the flagellar export apparatus.

Tamano et al. (47) recently reported that increased levels of MxiH in wild-type *Shigella* leads to much longer needles without affecting inducible Ipa protein secretion or invasion of HeLa cells. The elongated needle and the high amount of MxiH secreted in the *spa32* mutated strain grown to mid log phase thus suggested that the deletion of *spa32* might directly upregulate *mxiH* gene transcription or affect the stability of its mRNA or protein product. To test this, we introduced an *mxiD-lacZ* transcriptional fusion in both wild-type and MJ321 strains and found that the the level of β-galactosidase expressed from the *mxi* genes promoter in the *spa32* mutant was 2.5 times higher than that of the wild-type strain (data not shown). This modest increase in *mxiH* gene expression cannot explain the >10-fold increase in the needle length observed in the *spa32* mutant. However, amplification of protein production might also occur at the posttranscriptional or posttranslational level by increased mRNA stability, protein translation, and/or protein stability.

To understand the role of Spa32 in releasing the Ipa proteins in an inducible manner, its cellular location was examined. Spa32 was detected in the supernatant of the wild-type strain growing exponentially (CR-independent secretion). The recent finding that Spa32 is secreted via the TTSS in the Δ*ipa* mutant, which constitutively secretes at least 15 other proteins (9), is supported by our data. In addition, the fact that Spa32 is secreted is in accordance with previous reports on *spa32* homologues, InvJ and YscP, secreted molecules required for the secretion of Sip and Yop virulence proteins of *Salmonella* and *Yersinia*, respectively (10, 38, 46). Finally, the FliK protein has also been shown to be secreted (30), even though its biological action was pinpointed to occur inside the bacterium or as it exits the flagellar export apparatus (19). Interestingly, His_6-tagged Spa32, which is not secreted and is detected in whole-cell protein extracts, restores almost normal needle length, inducible secretion of Ipa proteins, and invasion to the nonpolar *spa32* mutant strain. Of note, FliK and Spa32 might similarly play a crucial role when present inside the bacteria and be secreted concomitantly or after action.

Therefore, this raises the question of why Spa32 is secreted at all? Perhaps, its secretion during type III secreton assembly is an artifact unrelated to its function. However, a body of evidence shows that, upon the completion of the hook assembly, FliK is very efficiently secreted (30). Moreover, any de-

crease in the level of FliK export has been shown to result in polyhook stub-filament structures with an inversely proportional higher number of repeated hooks, each 55 nm in length (19, 30). Thus, we propose that Spa32 might perform two biological functions: the first function, we propose, is exerted inside the bacterium, perhaps at the cytoplasmic face of the C-ring, to mediate the switch in substrate specificity from needle components to Ipa proteins. We propose that the second function, exerted near the entrance of the secreton channel, is to allow inducible Ipa secretion. This second step would be equivalent to the initiation of the hook-filament transition by FliK.

The detailed molecular nature of the biological functions of Spa32 remains to be determined. Yet, there are good reasons to believe that needle unit repeat control in TTSSs operates similarly (but perhaps not identically) to hook unit repeat control in the bacterial flagellum. Indeed, FliK was shown to function together with a membrane protein, FlhB, a component of the flagellar export apparatus, to mediate the switching of export substrate specificity upon completion of the hook assembly (22, 31). FliK was shown to interact with the carboxy-terminal cytoplasmic domain of FlhB. Interestingly, in our laboratory, we have preliminary unpublished data that indicate that the *Shigella* Spa40 protein, which shares 45% sequence identity with FlhB, interacts with Spa32 (3). Since the FlhB/FliK and Spa40/Spa32 protein pairs have counterparts in all bacteria with flagella and/or TTSSs, one can speculate that the mechanisms involved in regulation of hook repeat control and in switching to filament assembly or inducible secretion form common regulatory pathways.

The *spa32* mutant secretes Ipa proteins at a low level but not in an inducible manner. This is the first report of a *Shigella* mutant that has lost its ability to invade HeLa cells, although it can still secrete all of the Ipa invasins. The novel phenotype of the *spa32* mutant regarding Ipa secretion leads us to suggest that the TTSS of *S. flexneri* can exist in at least two secretion modes: (i) a noninducible one that occurs, for instance, in culture media in vitro during type III secreton assembly, and (ii) an inducible one that is activated upon addition of CR or contact with target cells. Furthermore, there are qualitative differences in the levels of secretion of different substrates by the two secretion modes. For example, while the secreton is being assembled, the early P-rod and needle proteins are secreted solely constitutively, and later their secretion is fully turned off, probably because there are no P-rod or needle components left. In a similar manner, Ipa proteins can "leak" at a low level during the later stages of log phase growth when secretons are fully assembled but mostly remain stored inside the bacterium awaiting the signal derived from host cell contact for complete secretion.

The findings of Tamano et al. (47) with a *Shigella* strain that overproduces MxiH and displays highly elongated needles indicate that the length of the TTSS needles is not crucial for correct adherence to and entry of the bacteria into eukaryotic host cells. The question then arises as to why a strain that produces a large amount of MxiH (47) and one deleted for *spa32* (the present study), both of which display highly elongated needles, are differentially responsive to CR on plates and to induction of secretion and also differ in invasion ability. The MxiH-overproducing strain binds CR on plates, is capable of secretion upon CR induction in solution, and is invasive. In contrast, the *spa32* mutant is white on CR plates, is unresponsive to CR induction in solution, and is noninvasive. This may simply reflect the inability of the bulbs in type III secretons of the *spa32* mutant to bind and secrete Ipas efficiently upon addition of CR or host cell contact. Thus, instead of secreting predominantly Ipa proteins at this stage, it can only secrete more MxiH and MxiI (and Ipas at low level). On the other hand, the MxiH-overproducing strain, due to the presence of a wild-type Spa32, is able to switch its substrate specificity despite an elongated needle and is thus ready to respond to CR and host cell contact.

Thus, the primary defect of the *spa32* mutant may be its inability to arrest MxiH export. Spa32 is probably also needed to switch from secretion of needle components to secretion of Ipas. Together with the C-ring components, it might act to monitor the level of MxiH and MxiI secretion and sense when TTSS needles of wild-type length have been assembled. Then, it may transmit a signal to the C-ring to arrest MxiH export and switch the substrate specificity of the export machinery to that of the effectors: Ipas and IpgD, via an interaction with the cytoplasmic domain of Spa40, before it is itself exported. Additional work is required to determine the mechanism by which the TTSS's needle length is controlled, as well as to understand the basis of the inducibility of secretion of the Ipa proteins.

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J.M. and A.H. contributed equally to this study.

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