The Needle Component of the Type III Secreton of *Shigella* Regulates the Activity of the Secretion Apparatus*[s]

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Gram-negative bacteria commonly interact with eukaryotic host cells by using type III secretion systems (TTSSs or secretons). TTSSs serve to transfer bacterial proteins into host cells. Two transloca- tors, IpaB and IpaC, are first inserted with the aid of IpaD by *Shigella* into the host cell membrane. Then at least two supplementary effec- tors of cell invasion, IpaA and IpgD, are transferred into the host cytoplasm. How TTSSs are induced to secrete is unknown, but their activation appears to require direct contact of the external distal tip of the apparatus with the host cell. The extracellular domain of the TTSS is a hollow needle protruding 60 nm beyond the bacterial surface. The monomeric unit of the *Shigella flexneri* needle, MxiH, forms a superhelical assembly. To probe the role of the needle in the activation of the TTSS for secretion, we examined the structure- function relationship of MxiH by mutagenesis. Most point muta- tions led to normal needle assembly, but some led to polymerization or possible length control defects. In other mutants, secretion was constitutively turned "on." In a further set, it was "constitutively on" but experimentally "uninducible." Finally, upon induction of secre- tion, some mutants released only the translocators and not the effectors. Most types of mutants were defective in interactions with host cells. Together, these data indicate that the needle directly con- trols the activity of the TTSS and suggest that it may be used to "sense" host cells.

Shigella flexneri causes bacillary dysentery in humans, a disease char- acterized by invasion of, massive inflammation in, and destruction of the colonic mucosa. The genes required for *S. flexneri* invasion are clus- tered on a 31-kb fragment of a large virulence plasmid (1). Within this region, the *mxi/spa* operons encode a type III secretion system (TTSS[5] or secreton), and the *ipa/ipg* operons encode five effector proteins abundantly secreted early in invasion, IpaA to -D and IpgD.

Type III secretons are essential determinants of the interaction of many Gram-negative bacteria with animal or plant hosts, and they translocate bacterial proteins into eukaryotic host cells to manipulate them during infection. TTS apparatuses are encoded by ~25 genes (2), nearly all being essential for function. These devices perform regulated post-translational and co-translational protein translocation across three biological membranes (two from the bacterium and one from the host cell). In *Shigella*, IpaB to -D are essential for invasion and give rise to the TTSS translocon (3, 4) through which at least two other proteins, IpaA and IpgD, are thought to be translocated.

Sequence similarities exist between components of TTSSs and those of the prokaryotic flagellar assembly machinery (5). TTSSs and flagella share all but host cell contact-mediated TTSS induction and the ability to translocate proteins into eukaryotic cells. 10 TTSS proteins are sim- ilar in sequence and/or membrane topology to the cytoplasmic or inner membrane proteins of flagellar hook-basal bodies (6, 7). Others show no significant sequence similarity; however, they show functional conser- vation because, when absent, they lead to similar phenotypic defects in assembly or function of the apparatuses (8).

A part of the TTS machinery, the "needle complex" (NC), resembles hook-basal bodies (9, 10). NCs comprise a 10×60-nm external needle inserted within a 30-nm diameter cylinder traversing both bacterial membranes and the peptidoglycan. NCs are traversed by a 2–3-nm channel (11), which also exists within the entire bacterial flagellum (12– 14). Flagellin and TTSS effectors may transit partially unfolded through this channel, since they are only secreted at the distal tip of their TTS machineries (15–18). The major needle components have been identi- fied. These are small global proteins, such as MxiH (9, 19). A minor component (11, 20), which shares some sequence similarity to MxiH, lies in the periplasmic rod of the secreton (21).

We previously determined the structure of the *Shigella* TTSS needle by x-ray fiber diffraction and electron microscopy at 16 Å resolution and found that its architecture is similar (~5.6 subunits/turn, 24-Å helical pitch, 2–3-nm central channel) to that of the flagellar rod, hook, and filament (10, 22, 23). However, its protein monomer, MxiH, is up to 5 times smaller and displays no primary sequence conservation with any flagellar axial component. Others have shown that a specialized exten- sion of the enteropathogenic *Escherichia coli* TTSS is similarly assem- bled (24). This suggests that these helical parameters represent central structural constraints in the assembly and function of flagella and TTSSs.

A recently understood example of functional analogies in TTSS and flagellar assembly is TTSS-secreted Spa32 (25–29), which works in a manner similar to flagellar FliK (30–33) to regulate the transition from needle/hook to effector/flagellar filament component secretion. The absence of these proteins leads to unusually long needles and the inabil- ity to activate secretion of effectors (26–28, 34), which is reminiscent of the "polyhook" phenotype and the inability to assemble a filament in *fliK*⁻ bacteria (30–33). The similar phenotypic defects of *spa32*⁻ and

fliK⁻ mutants establishes a strong regulatory analogy between needle and hook assembly checkpoints.

The flagellar hook-to-filament transition begins when the hook reaches the right length and signals that it has done so, with a change in the specificity of substrate export (30–33). It immediately continues with the up-regulation of the expression and secretion of the proteins forming the flagellar filament. In TTSSs, the needle also reaches a fixed length and then signals to change the export substrate specificity (25–29). But then, unlike flagella, nothing further appears to happen until the TTSS "detects" host cell contact and secretion of intracellularly stored effectors is activated. Polar translocation of effectors upon contact-mediated activation appears autonomous to each secreton (35). The distance to the host membrane is crucial (4), and the needle must protrude above lipopolysaccharide or bacterial adhesins for efficient activation (36, 37). Hence, the needle is likely to transmit the contact signal from its distal tip, but how this might occur is unknown.

We proposed that the activation signal of the TTSS was transmitted mechanically via shifts in the helical architecture of the needle (8). Flagellar filaments switch their helical architecture to adapt to changes in the direction of motor rotation by subtle rearrangements of the N and C termini of the filament subunit flagellin (38, 39). The helical structure of the needle and an *in silico* analysis of the secondary structure of needle protein MxiH led us to hypothesize an analogy with the N- and C-terminal domains of flagellin. We show here that site-directed mutants, specifically constructed to alter these regions, lead to secretons with 1) altered needle length and/or 2) altered secretion activities/host cell interaction abilities.

EXPERIMENTAL PROCEDURES

Materials—The *S. flexneri mxiH⁻* mutant strain SH116 and the anti-MxiH and anti-Spa32 rabbit antisera were gifts from A. Allaoui (11, 27). C. Sasakawa donated the pKT001 plasmid for *mxiH* overexpression (19). The *S. flexneri* wild type strain M90T and specific anti-Ipa protein rabbit antisera were gifts from P. Sansonetti's team. The anti-MxiG mouse monoclonal antibody was made with the aid of F. Ebel, using purified NCs as an immunogen (40).

Generation of mxiH Mutants for Expression in S. flexneri mxiH⁻—The *mxiH* gene was cloned into pWPsf4 to give pRK*mxiH*, which was electroporated into *S. flexneri mxiH⁻* SH116 (11, 41). The expression plasmid pWPsf4 is a pUC18 derivative that has an NdeI site inserted, so translation starts at the Met encoded by the NdeI site. The PCR primers used in this study are listed in supplemental Table S1. Using pRK*mxiH* as a template, C-terminal deletion mutants were made by inverse PCR using a vector-specific primer to the region 3′ of *mxiH* and a primer containing a GAGAGA sequence, a BamHI site, and the 18 nucleotides 5′ of the deletion. The PCR products were digested with the appropriate restriction enzyme, intramolecularly ligated, and again introduced into *S. flexneri* SH116. Point mutations were created using inverse PCR with primers containing GAGAGA, a restriction site, and 18 bases of *mxiH* past the point mutation. PSNP mutations were made by inverse PCR using primers with GAGAGA, an MfeI restriction site, and 18 bases beyond the PSNP sites. The PSNP residues were mutated with the sequence TG*X* AT*X* CG*X* AG*X*, allowing for a limited number of amino acids to replace the wild type sequence. Ampicillin selection ensured the presence of the recombinant plasmid, whereas kanamycin resistance and/or Congo red (CR) binding was used to ensure that the transformants still possessed the *Shigella* virulence plasmid. For inducible overexpression of the *mxiH* mutants in SH116, the mutant genes were subcloned into pKT001 (19), in which the NdeI site has been removed and the EcoRI-HindIII polylinker has been replaced with that of pWPsf (42) to generate pRK2*mxiH*.

Assays of Ipa Protein Secretion, Contact-mediated Hemolysis Activity, and Bacterial Entry into Cultured Epithelial Cells—*S. flexneri* strains were grown as previously described (4). In most of TABLE ONE, overnight secretion of the Ipa proteins and CR-induced protein secretion were determined as described (43, 44). In Figs. 3, 5, and 6 and some of TABLE ONE, the secretion assays were simplified as follows. For overnight secretion, 30 μl of bacterial supernatant, obtained after centrifugation at 2200 × *g* for 10 min at 4 °C of the total bacterial culture, was separated by SDS-PAGE and silver-stained using the SilverXpress kit (Invitrogen). For CR induction, bacteria collected during exponential growth were resuspended at $A_{600} = 5$ in phosphate-buffered saline (PBS, pH 7.4) with 210 μg/ml CR, incubated for 15 min at 37 °C, and centrifuged at 15,000 × *g* for 10 min at 4 °C. Finally, 30 μl of this bacterial supernatant was separated by SDS-PAGE and silver-stained as above. Contact-mediated hemolysis was measured as described by Blocker *et al.* (4). *S. flexneri* invasion of Henle 407 cells was monitored using a gentamycin protection assay as described by Picking *et al.* (41).

Analysis of Secreton Abundance—For analysis of NCs within the bacterial surface, samples were prepared as in Ref. 11 with the following modifications. In an appropriately sized microcentrifuge tube, 2 ml of cells grown (as above) to midexponential phase were collected by centrifugation, washed once in PBS, and resuspended in 50 μl of PBS. Glass beads were added as described by Blocker *et al.* (11), and the samples were vortexed for 10 s five times to create holes in the surface of the cells. 950 μl of PBS was added to each tube, which was centrifuged at 6500 rpm in a Hettich Micro 20 centrifuge for 3 min to remove unopened cells and beads. 800 μl of supernatant was collected and centrifuged again at 13,000 rpm for 5 min to pellet cell ghosts. The supernatant was removed gently, and the pellet was resuspended in 25 μl of water.

Needle Complex Preparation—NCs were prepared as described by Blocker *et al.* (11), but omitting the final gel filtration step and with the following modifications. 200 ml of bacterial culture grown at 37 °C to A_{600} between 1 and 2, from an overnight culture diluted 1:50 in the morning, were harvested and washed once in PBS. The bacteria were resuspended in 5 ml of cold 0.5 M sucrose, 100 mM Tris, pH 8. EDTA, pH 8, and lysozyme were added to 1 mM and 1 mg/ml, respectively. The bacteria were incubated at 37 °C for 30 min, and spheroplasting was checked microscopically and prolonged (for maximally another 30 min) until over 70% of bacteria were spheroplasted. Protease inhibitors (Complete Mini EDTA-free; Roche Applied Science) were added as well as 1 ml of 10% fresh Triton X-100. MgSO₄ was added to 10 mM as well as a few crystals of DNase. The preparation was left to clear and lose its viscosity at room temperature for a few minutes. Cell debris was removed by centrifugation at 45,000 × *g* for 20 min at 4 °C. The supernatant was collected, staying well away from the soft pellet, and centrifuged again at 100,000 × *g* for 1 h at 4 °C to pellet the NCs. NCs were washed by thorough resuspension in 5 ml of 10 mM Tris, pH 8, 1% Triton X-100, 150 mM KCl, 0.3% sarcosyl, and 5 mM EDTA. More debris was removed by centrifugation at 45,000 × *g* for 20 min at 4 °C. NCs were finally collected by centrifugation again at 100,000 × *g* for 1 h at 4 °C and resuspended in 50 μl of 50 mM Tris, pH 8, 5 mM EDTA, 0.1% Triton X-100.

Electron Microscopy—For analysis of bacterial ghosts, an 8-μl drop of 1% phosphotungstic acid, pH 7, was deposited on a 300-mesh, Formvar- and carbon-coated copper grid into which 2 μl of ghosts were mixed by pipetting. After 1 min, the excess liquid was removed with filter paper, and the grid was air-dried. The samples were observed in a Zeiss Omega 912 electron microscope at ×20,000 magnification. Digital pictures

TABLE ONE

General characterization of MxiH mutants

Mutations (S2A, V3A, N7A, D8A, D9A, T11A, S13A, S14A, L15A, S16A, E17A, and V74A) that did not result in drastic alteration of function (overnight secretion and Congo red induction profiles that were normal and greater than 50% of wild type activity in hemolysis and invasion assays) are not reported in the table.

	Colony color	MxiH in bacterium	MxiH in o/n growth medium	Ipas in bacterium	Ipa/Ipg secretion o/n	CR induction	% Hemolysis	% Invasion
mxiH							N/A	N/A
mxiH/pWPsf4							N/A	N/A
mxiH⁻/⁺							100±10	100±6
T4A							70±25	33±9
V5A							61±5	46±24
P6A							101±7	39±6
W10A							11±13	0.4±1
L12A							28±11	1±2
SSNA							102±5	39±6
PSNT							103±6	32±7
TTDP							104±3	41±4
PANT							101±4	50±5
P44A							101±1	100±6
Q51A							97±8	53±5
L54A							100±6	36±14
Y57A							0±2	1±3
T67A							82±5	39±16
V68A							103±5	41±18
K69A							10±4	4±9
V70A							72±11	95±6
I71A							27±6	19±7
K72A							6±5	14±6
D73A							8±7	19±5
D75A							16±2	78±20
I78A							34±18	24±19
I79A							33±11	21±13
Q80A							5±4	31±23
N81A							103±2	80±45
F82A							105±9	32±49
R83A							5±2	5±9
CΔ3							0±1	N/A
CΔ4							0±2	N/A
CΔ5							0±2	N/A

Colony colour on CR plate
- Very red colony
- Red colony
- Pink colony
- White colony

MxiH expression/secretion
- Much more MxiH than in WT
- More MxiH than in WT
- Equal MxiH as in WT
- Less MxiH than in WT
- No MxiH protein

Ipa storage and o/n secretion
- Much more Ipa than WT (late proteins seen)
- More Ipa than WT (late proteins seen)
- Equal signal to WT
- Somewhat less than WT
- No Ipa/Ipg protein secretion
- Altered patterns of IpaA and/or IpgD storage

CR induction
- More than WT
- Same as WT
- As WT but no IpaA or IpgD (blots)
- All Ipa/Ipg proteins reduced
- No Ipa/Ipg protein secretion

TABLE TWO

Needle complex abundance and needle length on MxiH mutants with gross phenotypic alterations

Mutants altered in any aspect of secretion or grossly defective host cell interaction phenotypes (<30% in both hemolysis and invasion assays) that could not be trivially explained (*e.g.* by instability of the mutant MxiH in the bacterium) were assayed for secreton assembly by the criteria listed below.

	Secreton base abundance[a] on bacteria relative to *mxiH*⁻/⁺	% NCs with needles (n)	Average needle length[b] (% of *mxiH*⁻/⁺, for those NCs with needles)
mxiH⁻/⁺	+	100 (69)	100±23
W10A	+	100 (12)	65±20**[c]
PANT	+	100 (21)	95±10
P44A	+	100 (22)	90±15*
Q51A	+	100 (21)	90±21
Y57A	+	38 (12)	68±24*
K69A	+	75 (8)	88±18
V70A	+	100 (22)	100±23
I71A	+	100 (10)	73±15**
K72A	+	92 (13)	67±12**
D73A	+	100 (24)	83±18**
D75A	+	100 (16)	78±26*
I78A	+	100 (7)	96±19
I79A	+	100 (24)	88±17
Q80A	+	100 (13)	79±18*
N81A	+	100 (24)	79±18**
R83A	+	88 (17)	69±16**
CΔ5	+	0 (18)	N/A

[a] A plus sign corresponds to a value contained within the 0.5–1.5 interval when the ratio of secretons observed for each mutant to that seen in the *mxiH*⁻/⁺ strain was calculated.
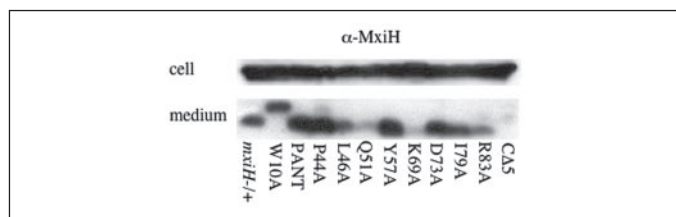
[b] The errors presented are sample S.D. values.

[c] The asterisk indicates the confidence interval of statistical significance in a *t* test relative to *mxiH*⁻/⁺.

were recorded with a ProScan 2K CCD camera using SIS software. Secretons along and on the surface of 10 individual bacterial outlines, always selected as halves of previously dividing bacteria, were counted by hand to yield the estimates in TABLE TWO. Only estimates were made, because lack of visualization of secretons in this type of analysis cannot be taken as an indicator of their absence. Indeed, secreton visualization in this manner is highly dependent on how each bacterial ghost or even ghost area has been depleted of its cytoplasm and inner membrane. In addition, some needles may be too short to see, and needleless secretons are often difficult to identify with certainty.

For analysis of NC preparations, grids were prepared similarly as above. 5 μl of fresh NC sample were deposited on the grid surface. After 1 min, the grid surface was quickly washed three times in water and stained with 5 μl of 2% phosphotungstic acid, pH 7, before the excess liquid was removed, and the grid was left to air-dry. The samples were observed, and pictures were collected as above. The length of NC needles was measured by hand on paper copies of the photographs, from the distal needle tip to the upper, external part of the outer membrane ring. Since the amount of MxiH within cells is known to affect needle length (19) and the pWsf4 *mxiH* expression level did not exactly match that of wild type *mxiH* expression, the lengths are expressed relative to the length of needles within *mxiH*⁻/⁺, which we verified was on average within 10% of that of wild type (not shown), although their length distribution was broader. As a secondary check of the estimate of the number of NCs per bacterium performed above, the number of purified NCs per surface area of the EM grids was assessed but did not vary significantly between any of the mutants analyzed.

Functional Analysis of Shigella TTSS Needle Protein MxiH

FIGURE 1. *A*, domain predictions for the MxiH protein family (using PSI-BLAST, ClustalW, and PREDICTPROTEIN). The protein/species names on the *left* refer to sequences from the following species and with the following data base accession numbers, from top to bottom: *Salmonella typhimurium* U21676, *Chromobacterium violaceum* AE016918, *E. coli* O157:H7 BAB37141, *Sodalis glossinidius* AA566834, *Burkholderia pseudomallei* Q63K18, *Aeromonas hydrophila* AY528667, *Aeromonas veronii* AY289195, *Photorhabdus luminescens* BX571871, *Pseudomonas aeruginosa* G83430, *Yersinia enterocolitica* F40361, *Citrobacter rodentium* AF311901, *E. coli* O157:H7 H91197, and *S. flexneri* Q06079. Analogies with flagellin are shown with *colors* identical to those used in the diagrams *below*. Conserved amino acids are in *boldface type* and *colored* to match their respective domain. *B*, *left*, *top*, *ribbon diagram* of the Cα backbone of the filament model. End-on view from the distal end. *Left*, *below*, side view. 11 subunits are displayed. *Right*, *ribbon diagram* of the Cα backbone of flagellin. Chain colors are as follows: residues 1–44 (*blue*), 44–179 (*cyan*), 179–406 (*green*), 406–454 (*yellow*); 454–494 (*red*). Panel *B* of this figure is adapted from Ref. 45.

RESULTS

In silico analysis of the secondary structure of the TTSS needle protein family (Fig. 1) led us to predict four α-helices, which we denominated N-D0, N-D1a, N-D1b, and C-D0, in reference to the flagellin structure (39, 45), and a β-hairpin around which many conserved amino acids cluster. To test the validity of these predictions, we generated the different types of MxiH mutants listed below. First, since the termini are critical for flagellin polymerization, short deletions were made at the MxiH C terminus to test whether these would prevent polymerization of the protein but not its secretion. Second, all conserved amino acids and the first and last 17 amino acids of MxiH, thought to represent domains N-D0 and C-D0, respectively, were individually replaced with alanines. Finally, random replacements were made within the MxiH PSNP loop, connecting the putative ND1a and ND1b regions (Fig. 1). These three types of sites were chosen, because point mutations in the putative equivalents of these regions in flagellin lock the flagellar filament in different supercoiled forms (46). The mutated genes were transferred into a nonpolar *mxiH* knock-out strain, and the resulting strains were assayed for various phenotypes.

Class I MxiH Mutants Have Varying Degrees of Polymerization Defects—A total of 45 mutants were tested for (i) the ability of the colonies to bind CR on a plate, which is a preliminary indicator of func-tional type III secreton assembly or inducibility (11, 27), (ii) expression/secretion of mutant MxiH, and (iii) the ability to assemble a normal number of NC bases, each with a needle. The majority of point mutations, especially within the N-terminal region of MxiH, had no measurable effect on any of the above phenotypes (Fig. 2 and TABLES ONE and TWO).

Only a minority of the mutants showed severe needle assembly defects. These were detected initially by the white color of the colonies on CR plates, indicating lack of full complementation of the *mxiH⁻* mutant (11). This was confirmed by direct observation of the absence of needles using electron microscopy (EM; TABLE TWO) when the mutant protein was expressed at wild type levels (Fig. 2 and TABLE ONE). Absolute polymerization defects resulted entirely from deletions at the C terminus of MxiH and support the notion that this region is involved in polymerization, as it is in all of the axial proteins of the flagellar filament. Since these mutants do not assemble needles, this explains their inability to secrete, hemolyze, or invade (11).

All other mutants with defects in secretion or grossly decreased hemolysis or invasion had their NC bases examined for abundance and presence of a needle (TABLE TWO). NC abundance was estimated by EM observation of negatively stained bacteria emptied of their cytoplasmic contents (4), but in no mutant was a significant alteration of the

FIGURE 2. **Analysis of MxiH stability and secretion in several key MxiH mutants.** Immunoblot of MxiH expressed within bacterial cells (*cell*) and secreted into the medium (*medium*; 3-fold the amount of overnight growth medium was used relative the amount of bacterial pellet loaded). Anomalous migration and possible proteolytic sensitivity of W10A and CΔ5 are consistent with improper/incomplete folding.
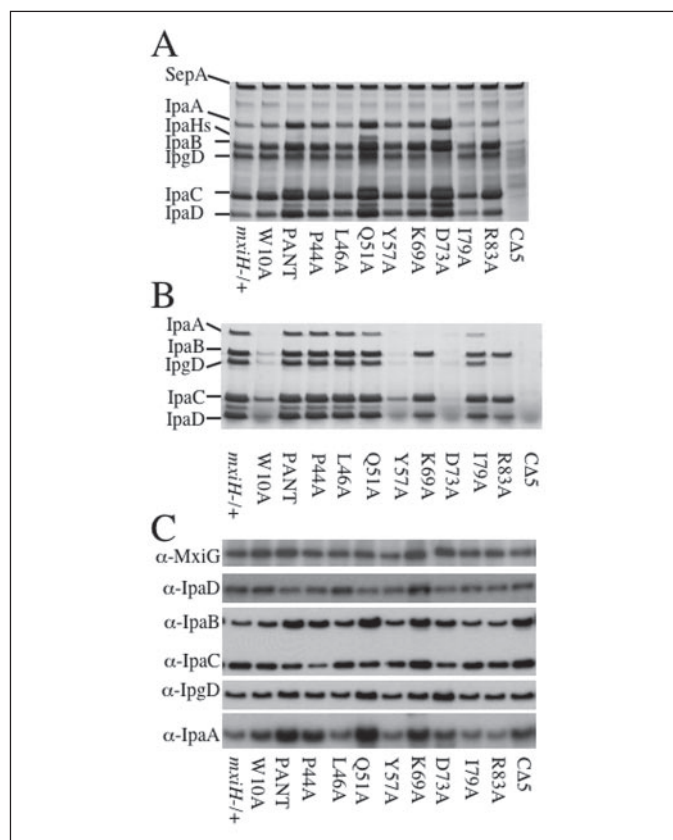

FIGURE 3. **Secretion analysis of key MxiH mutants.** *A*, SDS-polyacrylamide gel, silver-stained, showing complete profiles of proteins secreted in the overnight broth of the MxiH mutants. *B*, SDS-polyacrylamide gel, silver-stained, showing secretion of Ipa/Ipg proteins following induction of bacteria from exponentially growing cultures with CR. *C*, bacterial cell (in stationary phase) pellets from each mutant were separated by SDS-PAGE. Ipas/Ipgs and MxiG (TTSS inner membrane component used as loading marker) were detected by immunoblotting. In all three *panels*, all experimental samples were initially normalized for bacterial density. No growth defect was observed for any of the mutants constructed.

number of NC bases per bacterium noted. This is in agreement with the similar level of MxiG, a TTSS inner membrane protein, which is expressed by all mutants examined (Fig. 3*C*).

However, some mutants (*e.g.* Y57A) were found to have needle assembly defects, as assessed by EM examination of their purified NCs (TABLE TWO). We considered the percentage of NC bases with needles to be a reflection of the ability of the mutant needle protein to polymerize, since in the wild type control, 100% of NC bases make needles. If the number of NC bases with needles was above 75%, we assumed that MxiH polymerization was not substantially defective. By this criterion, the Y57A mutant showed the most severe needle polymerization defect.

The length of needles is normally tightly regulated at 60 nm ± 10%. This was also assessed by comparison with complementation of *mxiH⁻* with wild type *mxiH* (TABLE TWO). In this strain, the length of needles was less accurately controlled (~60 nm ± 20%), but the level of MxiH expressed was similar to wild type (data not shown). Hence, we considered needle lengths below 70% of that in the NCs of *mxiH⁻/⁺* to result from severely abnormal polymerization. All NCs in the W10A mutant had needles, but these were very short (on average 65% of the length of those in *mxiH⁻/⁺*). W10A is also one of two mutant proteins (the other being CΔ5) that are SDS-PAGE-retarded and/or more prone to degradation when secreted into the culture medium as compared with when they are located intracellularly (Fig. 2).

Class II Mutants May Have Needle Length Regulation Defects—Other mutants did not show strong needle polymerization defects by the parameters defined above, yet they were substantially defective in various aspects of secretion, hemolysis, and/or invasion. These mutants (K69A, I71A, K72A, D73A, D75A, I78A, I79A, Q80A, and R83A) all carried point mutations within the C-terminal region of the protein. Most alanine substitution mutants in this region (except V70A and I78A) were found to have needles between 67 and 88% of wild type length (TABLE TWO). These differences were statistically significant for all but the K69A and I79A mutants. This region of the MxiH protein is therefore important in maximally efficient needle polymerization and/or, given the similar average needle length reached in most of these mutants, in precise needle length control.

Class III MxiH Mutants Display Constitutive Secretion—All MxiH mutants were also assessed for the ability to secrete low levels of effector proteins during overnight growth and for increased secretion of effector proteins following induction with an artificial activator, the small amphipathic dye CR (TABLE ONE and Fig. 3). Several of the random mutations within the PSNP loop and alanine substitutions at positions Pro[44] or Gln[51] led to normal NC numbers per bacterium with needles of nearly normal lengths (TABLE TWO). These mutants, however, secreted higher levels of effector proteins prior to induction with CR as compared with *mxiH⁻/⁺* while remaining responsive to CR (Fig. 3, *A* and *B*, respectively). We termed these constitutively "on" mutants.

To determine whether constitutively secreting mutants were still able to respond to CR, the "on" mutants were briefly exposed or not to CR. Fig. 5*B* shows that detection of secretion in this assay is entirely dependent on CR addition, even in the constitutively "on" mutants. This demonstrates that up-regulated constitutive secretion occurs at a much slower rate than CR-induced secretion.

A Class IV Mutant Displays Constitutive Secretion but Is Uninducible by Congo Red—Another mutant, substitution D73A, also led to a constitutively "on" phenotype. However, this mutant was not inducible with CR (Fig. 3, *A*–*C*, and TABLE ONE). We therefore termed this mutant "uninducible." This finding indicates that the ability to secrete Ipa/Ipg proteins is distinct from the ability to respond to induction signals.

Class V Mutants Secrete Only the IpaB to -D Translocators upon Induction with Congo Red—Other MxiH mutants, which also showed mild needle polymerization defects, were altered in their ability to be induced to secrete the complete set of Ipa/Ipg proteins (Fig. 3*B*), despite intracellularly storing and secreting each protein into the overnight culture medium to at least wild type levels (Fig. 3, *A* and *C*). These mutants were K69A, K72A (not shown, except in TABLE ONE), and R83A. We called these "effector" mutants, since they secreted the translocon components normally but not the other effector proteins. These mutants suggest that induced TTSS secretion may be staged at least in part by signals received through the needle itself, since individual MxiH mutations appear to interfere at a specific point in the secretion process. That so many different secretion phenotypes can result from mutations
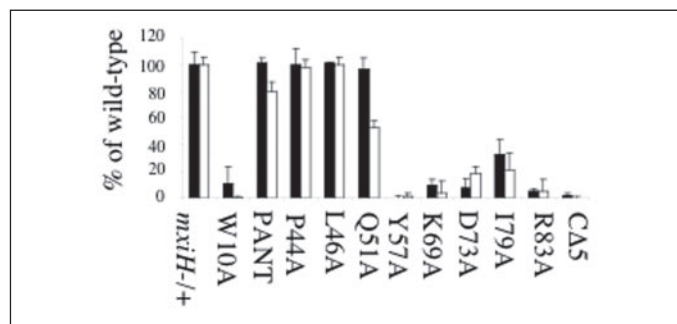
FIGURE 4. **Analysis of the ability of key MxiH mutants to interact with host cells.** The *bar diagram* represents the ability of the MxiH mutants to hemolyze (*black bars*) and invade epithelial cells (*white bars*). Results were normalized to *mxiH*$^{-/+}$, which was set at 100%, and resulted from at least two independent experiments performed in triplicate.

FIGURE 5. **Secretion analysis of the P44A/Q51A MxiH mutants.** *A*, overnight secretion; *B*, CR-induced secretion. SDS-PAGE-separated samples were silver-stained. All experimental samples were initially normalized for bacterial density. No growth defect was observed for any of the mutants constructed.

within a single small protein clearly points at a key role for the needle in regulating TTSS activity.

The Secretion Defects of Some MxiH Mutants Lead to Defects in Their Ability to Perform Contact-mediated Hemolysis and Host Cell Invasion— All MxiH mutants were tested for their ability to perform contact-mediated hemolysis of sheep red blood cells, a more physiological test of the ability of the apparatus to be induced, and for their ability to invade epithelial cells, as normally occurs during shigellosis. Of those with largely normal needle assembly properties, the constitutively "on" mutants displayed host-cell interaction phenotypes similar to *mxiH*$^{-/+}$. However, the D73A "uninducible" mutant, albeit being a constitutive secreter as well, was largely incapable of hemolysis or invasion (Fig. 4). This suggests that 1) CR inducibility and host cell sensing are related abilities and 2) secretion of Ipa/Ipg proteins in a deregulated manner is incapable of leading to proper TTSS translocon assembly/function (even when host cell contact is forced, as it is in both the hemolysis and invasion assays). Therefore, the needle is an essential component of the signaling system that allows detection of host cell contact by the TTSS.

The effector mutants (K69A, K72A, and R83A), which do not secrete IpaA and IpgD, performed very poorly in hemolysis and invasion. However, an *ipaA*$^-$/*ipgD*$^-$ mutant can perform hemolysis normally (4) and invade at low levels (47). Hence, we propose that the nearly complete inability of the effector mutants to perform hemolysis and invasion is due to the defects in needle polymerization that these mutants also show (TABLE TWO).

The importance of needle length in effective TTSS function has recently been demonstrated (37). I71A, which is wild type-like for all secretion parameters but unable to interact with host cells (TABLE ONE) and has needles of only 70% of wild type length supports this notion. When compared with N81A (TABLE ONE), which has needles of 80% of wild type length and normal secretion and host cell interaction phenotypes, this suggests that the minimal fully functional needle length is 80% of that of wild type. However, a series of other mutants, with amino acid substitutions clustered together within MxiH (D75A, I78A, I79A, and Q80A; see TABLE ONE), have very defective host cell interaction phenotypes but normal secretion and needles above 80% of the length of those in *mxiH*$^{-/+}$. This suggests that other parameter(s), which we are currently unable to measure, are also important for efficient interactions with host cells.

A Double Mutant of Two Constitutively "on" mxiH Alleles Is Uninducible—Two point mutations, localized very close to each other within the protein, were identified that led to a "constitutively on" (and inducible) phenotype. To determine whether they led to an identical effect on the protein function, we asked whether their effects were addi-
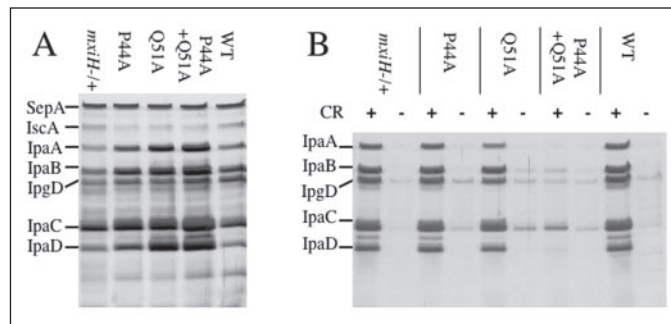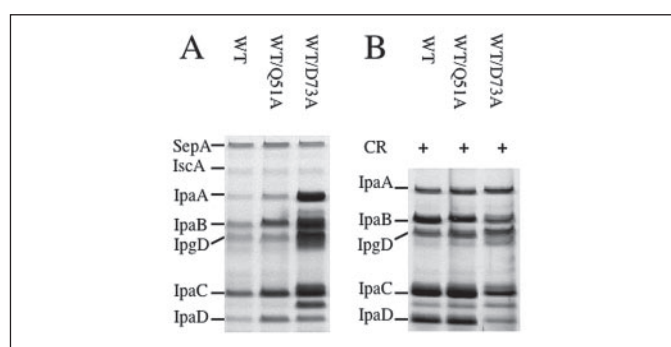
tive. The double mutant (P44A/Q51A) had equivalent numbers of NCs on its surface and assembled needles almost normally (not shown). It secreted Ipas/Ipgs into the overnight culture medium to a level slightly higher than the stronger of the two single constitutively secreting mutants (Fig. 5A; particularly obvious for IpaA to -C secretion). However, it could not be induced to secrete with CR (Fig. 5B) and was also severely handicapped in its ability to cause hemolysis and to invade epithelial cells (not shown). It was therefore classified as another "uninducible" (Class IV) mutant. These data suggest that the two mutations affect the protein in different ways.

Interestingly, although D73A, the other "uninducible" mutant, was white on CR plates (like *spa32*$^-$, which also cannot be induced by CR but can secrete normal levels of Ipas/Ipgs into the overnight culture medium through its superlong needles), the double mutant was red on CR plates, indicating that D73A and P44A/Q51A do not display fully identical phenotypes. When examined, Spa32 secretion into the overnight culture medium was found to occur normally for all mutants in Fig. 3 as well as K72A and the double mutant (not shown). Thus, the "uninducibility" of D73A and P44A/Q51A is not due to a block in Spa32 release upon needle termination (27).

"On" and "Uninducible" MxiH Mutants Act Dominantly in a Dose-dependent Manner in Wild Type Shigella—To understand whether key MxiH mutants could affect the function of wild type MxiH, we wanted to know whether the constitutively "on" and "uninducible" mutations were dominant in a wild type background. As shown in Fig. 6, co-expression of the "uninducible" D73A (or P44A/Q51A; not shown) and wild type MxiH proteins within cells surprisingly generates merely "constitutively on" secretion phenotypes. However, co-expression of Q51A (or P44A; not shown) and wild type proteins leads to either wild

FIGURE 6. **Analysis of the dominance of "on" and "uninducible" mutants in wild type strains.** *A*, overnight secretion; *B*, CR-induced secretion. SDS-PAGE-separated samples were silver-stained. All experimental samples were initially normalized for bacterial density. No growth defect was observed for any of the mutants constructed.

type (Fig. 6) or "constitutively on" secretion phenotypes as the expression level of the mutant protein increases (see "Experimental Procedures"; not shown). Furthermore, none of these mutants was more than mildly attenuated for invasion of HeLa cells. This was expected, since these mutants at most showed constitutive secretion (*i.e.* they remained inducible with Congo red), and constitutive secretion alone does not cause more than a ~50% decrease in hemolysis or invasion (TABLE ONE and Fig. 4). Hence, all mutants analyzed are only partially dominant in a wild type background.

DISCUSSION

In summary, we find that mutations in MxiH affect 1) the ability of normal MxiH protein to function when co-expressed with them in the same cell, 2) needle polymerization (Class I) and perhaps length control (Class II), 3) storage and secretion of individual Ipa/Ipg proteins (Class III), and 4) the inducibility of the secreton (Classes IV and V) and its ability to sense host cells (Class IV).

Since the quaternary structures of needles and of the axial flagellar proteins are nearly identical (23), our data support the relevance of our *in silico* prediction of MxiH secondary structure. Mutations based on the predicted structure have the effects on function expected by analogy with flagellin and the position of mutations known to alter its own quaternary packing.

First, short deletions in the C terminus prevent needle polymerization. Analysis of the secondary structure of CΔ5 MxiH demonstrates that this C-terminal mutant protein is monomeric. Circular dichroism measurements indicate that it contains 56% α-helices, 18% β-sheets, 14% turns, and 15% random coils.[6] This agrees with recent structural and functional analysis performed on external appendages of TTSSs from other species (48, 49). For all of these proteins, free and intact termini are key for polymerization.

Second, individual mutations in the putative CD0, the PSNP loop, and tiny β-hairpin domains of MxiH lead to altered activity of the secretion apparatus. These are the very regions that we predicted would be analogous to the regions known to be involved in switching between helical forms within the flagellin molecule (39, 45, 46). This clearly indicates the importance of this entire region, representing the C-terminal half of the protein and where most of the amino acids conserved throughout the MxiH family are located, in the biological functions of the TTSS needle.

Third, dominance analysis suggests that the ratio of wild type to mutant protein affects the activation state of hybrid needles. It is not within the scope of this work to determine whether wild type and point mutant MxiH proteins are able to co-polymerize within the same needles. However, it seems unlikely that all three mutants tested should specifically exclude wild type MxiH, and the phenotypes observed suggest that this is not the case. Therefore, the partial dominance of the Class III and IV mutations may indicate that the ratio of mutant to wild type protein affects the structure of all MxiH molecules within hybrid needles, as predicted to occur during helical switching in the flagellar filament (50).

The W10A and Y57A mutants had normal intracellular protein stabilities but significant defects in needle polymerization (Class I). Biophysical analysis of C-terminally deleted versions of Y57A demonstrates that this protein has a less stable secondary structure,[6] but that is not the case for W10A. Both of these proteins also largely share secondary structure content with CΔ5. This is surprising, given the drastic nature of each amino acid substitution performed. In addition, for reasons

unclear at present, W10A and CΔ5 mutant MxiHs are SDS-PAGE-retarded and/or more prone to degradation when secreted into the bacterial medium than when they are stored intracellularly. MxiH may be stabilized in the bacterial cytosol by an unknown chaperone, as are the needles and extracellular extension-forming proteins of TTSSs from other bacterial species (48, 51–53). Finally, Q51A, K69A, and CΔ5 are less abundant extracellularly than other mutant proteins shown in Fig. 3. We cannot exclude a secretion defect for K69A and CΔ5, the needles of which are either defective or absent. However, we think this unlikely, since these mutations lie within the C terminus of MxiH, and the secretion signal of all virulence TTSS substrate proteins is at the N terminus. In addition, Q51A makes nearly wild type needles. Therefore, the reason for this discrepancy is also unclear.

Other mutants (Class V) had mild defects in needle polymerization (K69A, K72A, and R83A). These mutants were also unable to secrete the IpaA and IpgD effector proteins upon CR addition. The phenotypic interpretation of mutants where not all NCs carry full-length needles is problematic. Assembling NCs secrete low levels of Ipas, as assembling flagellar hooks secrete low levels of filament proteins (27, 54). However, it is unknown at which point needles become secretion-competent in either type of secretion assay. Nevertheless, when compared with all other C-terminal point mutants that demonstrate mild defects in needle polymerization and/or length control (TABLE TWO), only K69A, K72A, and R83A show defects in secreton inducibility (TABLE ONE). This suggests that their inducibility defects are not linked to their needle polymerization and/or length control abnormalities.

Recent work has indicated that secretion of translocators and effectors may be staged hierarchically in time by the action of a family of cytoplasmic TTSS proteins showing weak sequence homologies to each other (55–62). Deletion of the genes encoding these proteins, reduces translocator secretion while effector secretion is increased, probably at a post-transcriptional level. Our "effector" mutants secrete the translocators normally but not the effectors destined to be translocated. We therefore propose that this novel family of "gatekeeper" proteins responds directly or indirectly to external signals received from the needle.

Quantitative differences are seen in the abundance of certain Ipa/Ipg proteins released into the medium by some "on" and "effector" mutants relative to *mxiH*^{−/+} (Fig. 3*A*). TTSS proteins can be secreted via both co- and post-translational pathways (63–65). Therefore, these differences might be due to differential effects of the mutations on these two pathways. The levels of IpaA stored and secreted (Fig. 3, *A–C*) in several of the mutants are particularly anomalous in being differentially altered relative to those of the other Ipa/Ipg proteins. We are investigating whether this is due to the differential importance of co- and post-translation secretion in the two secretion assays for IpaA (66), relative to the other effectors of invasion.

The constitutively "on" and "uninducible" mutants (Classes III and IV) display nearly normal needle polymerization but strongly altered secretion or/and cell interaction phenotypes. Their partial gain-of-function phenotypes make them the most interesting mutants found. The identification of mutants "uninducible" by CR and their only partial dominance in wild type *Shigella* also hints that this compound might work on the needle itself or an element distal to it in the sensing pathway. We failed to obtain any evidence of specific binding of CR to needles (not shown), but since this inducer is nonphysiological, it may not bind with strong affinity to its target(s).

Our mutations were designed based on a putative structural analogy of MxiH with flagellin, because it was the only axial protein of the flagellum for which an atomic structure was partially known. Neverthe-

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[6] R. Kenjale, W. L. Picking, and W. D. Picking, manuscript in preparation.

less, we have postulated a morphological and functional analogy between the needle and the flagellar hook. The hook shows helical assembly parameters similar to the flagellar filament and stepwise supercoiling (22). Furthermore, the structure of D0 and the interactions between D0 domains are shared by all flagellar axial proteins, including the hook protein, FlgE, because these motifs are the means by which the flagellum is built. However, the atomic structure of the D2 and part of the D1 regions of FlgE (67) indicate that its D1 domains (to which we proposed that our putative ND1a and -b and β-hairpin regions were analogous) contain primarily β-sheets, whereas those of flagellin (and probably MxiH) are largely α-helical. The hook has developed a specific mechanism, utilizing the β-sheets within its D1 and D2 regions, to allow it to function as a flexible "universal joint" (67). A search for mutations affecting this gradual form of supercoiling led to FlgE alleles with mutations sitting in the tip of the D2 domain, at the hinge between the D1 and D2 domains (and interestingly these mutations yield hooks ~80% of wild type length, similar to our Class II mutants) and within the lower D1 and D0 domains of FlgE.[7] The structure of the latter FlgE region is unsolved, but it is the most likely equivalent of the tiny MxiH. It may be that these FlgE mutants will also be affected in the previously noted stepwise helical transition-mediated polymorphism. Yet, any second, completion-coupled switch mechanism may be different for FlgE and the needle protein family.

The data presented here demonstrate that the TTSS needle can act as a secretion regulator and is involved in sensing host cells. Similar results were very recently reported by Plano and co-workers (68) for the *Yersinia* TTSS needle, suggesting that the mechanism of transmission of the host cell detection signal may be conserved at least among the TTSSs of bacterial pathogens of animals.

The mutants described here were made based on a proposed structural analogy to flagellin. This protein can exist in different superhelical states. We have further tested our predictions by investigating whether the mutations described here lead to alterations in the helical packing of MxiH needles (69), which we hope to ascribe in time to changes in the atomic structure of MxiH.

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SUPPLEMENTARY DATA FOR:
THE NEEDLE COMPONENT OF THE TYPE III SECRETON OF *SHIGELLA*
REGULATES THE ACTIVITY OF THE SECRETION APPARATUS

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<u>Table S1</u>: List of primers used to construct MxiH mutants in this work.

Plasmid pWPsf4	Primers	Sequence 5' to 3'
pRK*mxiH*	MxiH1	GAGAGAGAGCATATGAGTGTTACAGTACCGAA
	MxiH2	GAGAGAGAGGGATCCTTATCTGAAGTTTTGAATAATTG
pRK*mxiH*$_Δ$*3*	MxiH23	GAGAGAGAGGGATCCTTATTGAATAATTGCAGCATCAAC
	Rev2	GAGAGAGAGGATCCTCTAGAGTCGACCTGCAG
pRK*mxiH*$_Δ$*4*	MxiH24	GAGAGAGAGGGATCCTTAAATAATTGCAGCATCAACATC
	Rev2	GAGAGAGAGGATCCTCTAGAGTCGACCTGCAG
pRK*mxiH*$_Δ$*5*	MxiH25	GAGAGAGAGGGATCCTTAAATTGCAGCATCAACATCC
	Rev2	GAGAGAGAGGATCCTCTAGAGTCGACCTGCAG
pRK*mxiH*$_Δ$*6*	MxiH4	GAGAGAGAGGGATCCTTATGCAGCATCAACATCCTTAA
	Rev2	GAGAGAGAGGATCCTCTAGAGTCGACCTGCAG
pRK*mxiH*$_Δ$*12*	MxiH5	GAGAGAGAGGGATCCTTAAATCACTTTCACTGTATTGGA
	Rev2	GAGAGAGAGGATCCTCTAGAGTCGACCTGCAG
pRK*mxiH S2A*	MxiH26	GAGAGAGAGCATATGGCAGTTACAGTACCGAATGATG
	START	GAGAGACATATGTGTTTCCTGTTTCCTGTGT
pRKmxiH *V3A*	MxiH27	GAGAGAGAGCATATGAGTGCAACAGTACCGAATGATGATT
	START	GAGAGACATATGTGTTTCCTGTTTCCTGTGT
pRK*mxiH T4A*	MxiH28	GAGAGAGAGCATATGAGTGTTGCAGTACCGAATGATGATTGGA
	START	GAGAGACATATGTGTTTCCTGTTTCCTGTGT
pRK*mxiH V5A*	MxiH29	GAGAGAGAGCATATGAGTGTTACAGCACCGAATGATGATTGGACA
	START	GAGAGACATATGTGTTTCCTGTTTCCTGTGT
pRK*mxiH P6A*	MxiH30	GAGAGAGAGCATATGAGTGTTACAGTAGCAAATGATGATTGGACATTGAG
	START	GAGAGACATATGTGTTTCCTGTTTCCTGTGT
pRK*mxiH N7*A	MxiH62	GAGAGAGAGGCTCAGCGTCCAATCATCTGCCGGTACTGTAACACTCAT
	MxiH31	GAGAGAGAGGCTGAGCTCATTATCTGAAACTTTTGATG
pRK*mxiH D8A*	MxiH63	GAGAGAGAGGCTCAGCGTCCAATCTGCATTCGGTACTGTAACACT
	MxiH31	GAGAGAGAGGCTGAGCTCATTATCTGAAACTTTTGATG
pRK*mxiH D9A*	MxiH64	GAGAGAGAGGCTCAGCGTCCATGCATCATTCGGTACTGTAAC
	MxiH31	GAGAGAGAGGCTGAGCTCATTATCTGAAACTTTTGATG
pRK*mxiH W10A*	MxiH65	GAGAGAGAGGCTCAGCGTTGCATCATCATTCGGTACTGTAAC
	MxiH31	GAGAGAGAGGCTGAGCTCATTATCTGAAACTTTTGATG
pRK*mxiH T11A*	MxiH66	GAGAGAGAGGCTCAGCGCCCAATCATCATTCGGTACTGTAAC
	MxiH31	GAGAGAGAGGCTGAGCTCATTATCTGAAACTTTTGATG
pRK*mxiH L12A*	MxiH42	GAGAGAGAGGCTCAGCGAACTCGCTGTCCAATCATCATTCGG
	MxiH41	GAGAGAGAGGCTGAGCGAAACTTTTGATGATGGAAC
pRK*mxiH S13A*	MxiH43	GAGAGAGAGGCTCAGCGAAGCCAATGTCCAATCATCATTC
	MxiH41	GAGAGAGAGGCTGAGCGAAACTTTTGATGATGGAAC
pRK*mxiH S14A*	MxiH38	GAGAGAGAGGCTGAGCGCATTATCTGAAACTTTTGATG
	MxiH37	GAGAGAGAGGCTCAGCGTCCAATCATCATTCGGT
pRK*mxiH L15A*	MxiH6	GAGAGAGAGGCTAGCGAACTTTTGATGATGGAACT
	MxiH7	GAGAGAGAGGCTAGCTGAACTCAATGTCCAATCAT

pRK*mxiH S16A*	MxiH39	GAGAGAGAGGCTGAGCTCATTAGCTGAAACTTTTGATGATGG	
	MxiH37	GAGAGAGAGGCTCAGCGTCCAATCATCATTCGGT	
pRK*mxiH E17A*	MxiH40	GAGAGAGAGGCTGAGCTCATTATCTGCAACTTTTGATGATGGAACTC	
	MxiH37	GAGAGAGAGGCTCAGCGTCCAATCATCATTCGGT	
pRK*mxiH P44A*	MxiH8	GAGAGAGAGTTCGAATGCACAGTTGCTGGCTGAATAC	
	MxiH10	GAGAGAGAGTTCGAAGGATTTTTAGCTAATT	
pRK*mxiH L46A*	MxiH9	GAGAGAGAGTTCGAATCCACAGGCGCTGGCTGAATACCAAA	
	MxiH10	GAGAGAGAGTTCGAAGGATTTTTAGCTAATT	
pRK*mxiH Q51A*	MxiH11	GAGAGAGAGGCTAGCAAATTATCTGAATATACATTATATA	
	MxiH12	GAGAGAGAGGCTAGCGTATTCAGCCAGCAACTG	
pRK*mxiH L54A*	MxiH13	GAGAGAGAGGCTAGCGAATATACATTATATAGGAACG	
	MxiH14	GAGAGAGAGGCTAGCTTTACTTTGGTATTCAGCCA	
pRK*mxiH Y57A*	MxiH15	GAGAGAGAGGAATGCGCAATCCAATACAGTGA	
	MxiH20	GAGAGAGAGCGCATTCCTATATAATGTAGCTTCAGATAATTTACTTTGGTA	
pRK*mxiH T67A*	MxiH45	GAGAGAGAGGAATGCGCAATCCAATGCAGTGAAAGTGATTAAGGA	
	MxiH44	GAGAGAGAGCGCATTCCTATATAATGTATATTCAG	
pRK*mxiH V68A*	MxiH46	GAGAGAGAGGAATGCGCAATCCAATACAGCGAAAGTGATTAAGGATGTTG	
	MxiH44	GAGAGAGAGCGCATTCCTATATAATGTATATTCAG	
pRK*mxiH K69A*	MxiH17	GAGAGAGAGCCAATACAGTGGCAGTGATTAAGGATGTTGATG	
	MxiH18	GAGAGAGAGCCACTGTATTGGATTGCGCG	
pRK*mxiH V70A*	MxiH47	GAGAGAGAGGAATGCGCAATCCAATACAGTGAAAGCGATTAAGGATGTTGATGCT	
	MxiH44	GAGAGAGAGCGCATTCCTATATAATGTATATTCAG	
pRK*mxiH I71A*	MxiH53	GAGAGAGAGGCTAGCATCAACATCCTTAGCCACTTTCACTGTATTGGAT	Gives I71A,A77S
	MxiH67	GAGAGAGAGGCTAGCATTATTCAAAACTTCAGATAAGGA	
	FixI71	GAGAGAGGATCCTTATCTGAAGTTTTGAATAATAGCAGCATCAACATCCTTAGCC	
	Rev2	GAGAGAGAGGATCCTCTAGAGTCGACCTGCAG	
pRK*mxiH K72A*	MxiH52	GAGAGAGAGGCTAGCATCAACATCAGCAATCACTTTCACTGTATTGG	Gives K72A,A77S
	MxiH67	GAGAGAGAGGCTAGCATTATTCAAAACTTCAGATAAGGA	
	FixK72	GAGAGAGGATCCTTATCTGAAGTTTTGAATAATAGCAGCATCAACATCAGCAATC	
	Rev2	GAGAGAGAGGATCCTCTAGAGTCGACCTGCAG	
pRK*mxiH D73A*	MxiH51	GAGAGAGAGGCTAGCATCAACAGCCTTAATCACTTTCACTGT	Gives D73A,A77S
	MxiH67	GAGAGAGAGGCTAGCATTATTCAAAACTTCAGATAAGGA	
	FixD73	GAGAGAGGATCCTTATCTGAAGTTTTGAATAATAGCAGCATCAACAGCCTTAATC	
	Rev2	GAGAGAGAGGATCCTCTAGAGTCGACCTGCAG	
pRK*mxiH V74A*	MxiH50	GAGAGAGAGGCTAGCATCAGCATCCTTAATCACTTTCACT	Gives V74A,A77S
	MxiH67	GAGAGAGAGGCTAGCATTATTCAAAACTTCAGATAAGGA	
	MxiHV74a	GAGAGAGGATCCTTATCTAAAGTTTTGAATAATTGCAGCATCAGCATCCTTAATC	
	Rev2	GAGAGAGAGGATCCTCTAGAGTCGACCTGCAG	
pRK*mxiH D75A*	MxiHD75a	GAGAGAGCGGCCGCAATTATTCAAAACTTCAGA	
	MxiHD75bnew	GAGAGAGCGGCCGCAACATCCTTAATCACTTTCAC	
pRK*mxiH I78A*	MxiH19	GAGAGAGAGGGATCCTTATCTGAAGTTTTGAATAGCTGCAGCATCAACATCCTT	
	Rev2	GAGAGAGAGGATCCTCTAGAGTCGACCTGCAG	

pRKmxiH I79A	MxiH68	GAGAGAGAGGGATCCTTATCTGAAGTTTTGAGCAATTGCAGCATCAACATCC
	Rev2	GAGAGAGAGGATCCTCTAGAGTCGACCTGCAG
pRKmxiH Q80A	MxiH69	GAGAGAGAGGGATCCTTATCTGAAGTTTGCAATAATTGCAGCATCAACAT
	Rev2	GAGAGAGAGGATCCTCTAGAGTCGACCTGCAG
pRKmxiH N81A	MxiH57	GAGAGAGAGGGATCCTTATCTGAATGCTTGAATAATTGCAGCATCC
	Rev2	GAGAGAGAGGATCCTCTAGAGTCGACCTGCAG
pRKmxiH F82A	MxiH58	GAGAGAGAGGGATCCTTATCTTGCGTTTTGAATAATTGCAGCAT
	Rev2	GAGAGAGAGGATCCTCTAGAGTCGACCTGCAG
pRKmxiH R83A	MxiH59	GAGAGAGAGGGATCCTTATGCGAAGTTTTGAATAATTGCAG
	Rev2	GAGAGAGAGGATCCTCTAGAGTCGACCTGCAG
"*PSNP*" loop	MxiH21	GAGAGAGAGCAATTGCTGGCTGAATACCAAA
	MxiH22	GAGAGAGAGCAATTGTGXATXCGXAGXATTTTTAGCTAATTTATCTAGTG

The Needle Component of the Type III Secreton of *Shigella* Regulates the Activity of the Secretion Apparatus

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