The 33 carboxyl-terminal residues of Spa40 orchestrate the multi-step assembly process of the type III secretion needle complex in Shigella flexneri

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INTRODUCTION

Shigella flexneri, the causative agent of bacillary dysentery in humans, is able to enter and to disseminate in epithelial cells by using a specialized Mxi-Spa type III secretion apparatus (T3SA) (Parsot, 2009). The T3SA is widespread among Gram-negative bacteria that are pathogenic for animals and plants or are symbionts (Viprey et al., 1998; Dale et al., 2001, 2002) and is devoted to the injection of virulence effectors into target cells. The T3SA is composed of a cytoplasmic region called ‘the bulb’, a basal body consisting of a pair of rings that span the inner and outer membranes, and an extracellular needle protruding outside the bacterium. This apparatus is evolutionarily and structurally related to the export machinery of flagellar systems (Macnab, 1999; Blocker et al., 2001). Nine T3SA constituents are related to components of the basal bodies of bacterial flagella (Allaoui et al., 1994; Aizawa, 2001; Blocker et al., 2003; Macnab, 2004), including the inner-membrane proteins MxiA (FlhA), Spa9 (FlhP), Spa24 (FlhR), Spa29 (FlhQ) and Spa40 (FlhB), and the cytoplasmic proteins Spa47 (Flhi), MxiN (FlhI) and Spa33 (FlhN) (Kane et al., 2002; Allaoui et al., 1992b, 1993b, 1995; Penno et al., 2006; Jouihri et al., 2003; Andrews & Maurelli, 1992; Sasakawa et al., 1993; Schuch & Maurelli, 2001; Morita-Ishihara et al., 2005). Additionally, two cytoplasmic proteins, Spa13 and MxiK, which have no obvious counterpart in the flagellar system, are also implicated in

The type III secretion apparatus (T3SA) is a central virulence factor of many Gram-negative bacteria. Its overall morphology consists of a cytoplasmic region, inner- and outer-membrane sections and an extracellular needle. In Shigella, the length of the needle is regulated by Spa32. To understand better the role of Spa32 we searched for its interacting partners using a two-hybrid screen in yeast. We found that Spa32 interacts with the 33 C-terminal residues (CC*) of Spa40, a member of the conserved FlhB/YscU family. Using a GST pull-down assay we confirmed this interaction and identified additional interactions between Spa40 and the type III secretion components Spa33, Spa47, MxiK, MxiN and MxiA. Inactivation of spa40 abolished protein secretion and led to needleless structures. Genetic and functional analyses were used to investigate the roles of residues L310 and V320, located within the CC* domain of Spa40, in the assembly of the T3SA. Spa40 cleavage, at the conserved NPTH motif, is required for assembly of the T3SA and for its interaction with Spa32, Spa33 and Spa47. In contrast, unprocessed forms of Spa40 interacted only with MxiA, MxiK and MxiN. Our data suggest that the conformation of the cytoplasmic domain of Spa40 defines the multi-step assembly process of the T3SA.
the assembly of the type III secretion system (T3SS) (Jouihri et al., 2003; Penno et al., 2006).

Among the battery of T3SA substrates, three classes can be defined based on their order of transit through the T3SA: first, the needle subunits of the T3SA, MxiH and MxiI; then the translocators IpaB, IpaC and IpaD; and finally effectors such as IpgB1, IpgB2, IpgD and IpaA. The first switch in secretion from needle subunits to translocators is regulated by the Spa32 protein. Indeed, mutations in spa32 (Magdalena et al., 2002; Botteaux et al., 2008), as well as in invl from Salmonella (SP-1) (Kubori et al., 2000) and yscP from Yersinia (Journet et al., 2008), lead to abnormally long needles, suggesting that the system is locked in the export of needle subunits. Likewise, Salmonella mutants deficient in fliK, the spa32 counterpart in the flagellar system, make polyhooks and no filament. Interestingly, extragenic suppressive mutations that restore filament assembly on polyhook structures (polyhook-filament phenotype) have been mapped in fliB, a gene encoding a major component of the export apparatus (Kutsukake et al., 1994; Williams et al., 1996; Fraser et al., 2003). FliB exists in two processed and unprocessed forms (Minamino & Macnab, 2000). There is a highly sensitive cleavage site between amino acids Asn269 and Pro270 in the cytoplasmic domain of FliB which is cleaved via an autocatalytic process involving its own tertiary structure (Ferris et al., 2005). These residues lie on a conserved amino acid sequence NPTH in YscU of Yersinia and in Spa40 of Shigella. The uncleaved form of FliB permits hook assembly but not flagellin secretion (Fraser et al., 2003). The deletion of the entire NPTH motif of YscU leads to an uncleaved form of the protein and abolishes secretion of the needle subunit (YscF) (Björnfot et al., 2009).

As for YscU and FliB, Spa40 is also predicted to contain four transmembrane domains followed by a large cytoplasmic one (Allouei et al., 1994). Deane et al. (2008) reported that the C-terminal domain of Spa40 (Spa40CT) is also cleaved within NPTH into two subdomains, Spa40CN and Spa40CC, which remain tightly associated after cleavage. The crystal structures of the cytoplasmic domains of Spa40 (Deane et al., 2008), EcsU from enteropathogenic Escherichia coli (EPEC), SpaS from Salmonella (Zarivach et al., 2008) and YscU from Yersinia (Wiesand et al., 2009) reveal a tight association of the cleaved fragments and show that the NPTH sequence lies on a loop which, when cleaved, swings away from the catalytic N257 residue, resulting in different surface features in this region. This structural rearrangement suggests a mechanism by which non-cleaved forms of these proteins interfere with the substrate specificity switching of the apparatus (Deane et al., 2008).

While the role of Spa40 in Ipa protein secretion has been genetically proven (Sasakawa et al., 1993), its involvement in the secretion process remains poorly understood. In this study we investigated the molecular role of Spa40 in the assembly/function of the T3SA. What has emerged is a picture of Spa40 as a central element of the export machinery with a critical role in T3SA assembly prior to the specificity switch.

**METHODS**

**Media and growth conditions.** S. flexneri strains were derivatives of the wild-type strain M90T (serotype 5) (Sansonetti et al., 1982; Allouei et al., 1992a). E. coli Top10 strains were transformed with pSU18, pQE30 or pGEX4T1 derivatives; strain DH5α (pIR) was transformed with derivatives of the suicide vector pGP704, and SM10–pIR was used to transfer derivatives of pGP704 to S. flexneri. Bacteria were grown in tryptic casein soy broth at 37 °C. Antibiotics were used at the following concentrations: ampicillin, 100 μg ml⁻¹; kanamycin, 50 μg ml⁻¹; streptomycin, 100 μg ml⁻¹; and chloramphenicol, 25 μg ml⁻¹.

**Construction of the spa40 null strain** For the construction of a non-polar mutant of spa40, the αpha-3 cassette specifically designed for the purpose was used (Menard et al., 1993). A non-polar mutant of spa40 was constructed by replacing the 706 bp SflⅠ–Accl fragment internal to spa40 with the Smal-digested αpha-3 cassette that confers resistance to kanamycin. In pN67, the suicide mutant vector, the spa40 gene was interrupted between codons 62 and 298. The structure of promoters (using primers Spa40-i and Spa40-2) and the resulting strains were designated SM144 or spa40.

**Construction of targeted mutations in the cytoplasmic domain of Spa40.** Plasmid pNL3 was constructed by inserting the 1050 bp EcoRI/HindIII-digested DNA fragment carrying the spa40 gene into the corresponding sites of the low-expression vector pSU18 (Invitrogen). Substitutions of Spa40 residues at positions G224, A286, L310 and V320 were made on plasmid pNL3 (Spa40) or pAB51 (GST-Spa40CT) (Botteaux et al., 2009), according to the procedure of the QuickChange Mutagenesis kit (Stratagene). Each primer used created a restriction site to easily confirm the introduced mutation (see Supplementary Table S1). Deletion of Spa40 residues NPTH at positions 258–261 was done by amplifying the first 771 bp of the spa40 gene using primers Spa40-3 and Spa40-13, and the last 243 bp of the spa40 gene with primers Spa40-14 and Spa40-4. Then, the two PCR fragments were assembled by PCR using primers Spa40-3 and Spa40-4 and cloned into EcoRI/HindIII-digested pSU18. The resulting plasmid encoding Spa40(NPTH) deletion was named pAB94. The same deletion was made on pGEX-Spa40CT, and the resulting plasmid encoding GST-Spa40(NPTH) was named pAB98.

**Construction of plasmids.** Plasmid pAB63, expressing GST-Spa40CN (residues 205–258), was constructed by inserting a 159 bp BamHI–EcoRI fragment obtained by PCR using primers Spa40-15/Spa40-17 into the corresponding sites of pGEX4T1. Plasmid pAB70, expressing GST-Spa40CC (residues 259–342), was obtained by inserting a 416 bp BamHI–Smal PCR DNA fragment obtained using Spa40-18/Spa40-19 primers into the corresponding sites of pGEX4T1. Plasmid pAB71, expressing GST-Spa40CC [glutathione S-transferase (GST) fused in-frame to residues 309–342 of Spa40] was constructed by inserting a 266 bp BamHI–Smal PCR DNA fragment amplified using primers Spa40-20/Spa40-19 into the corresponding sites of pGEX4T1. Plasmid pBAL27 was constructed by inserting a 480 bp PstI–KpnI PCR fragment of the mxiA gene (using primers MxiA2/MxiA6), encoding the last 160 residues of MxiA, into the corresponding sites of pBADHisA. Plasmid pBAL26 was constructed by inserting a BamHI–Xhol PCR fragment of the spa33 gene (using primers Spa33.1/Spa33.2) into the corresponding sites of pBADHisA.
Production of recombinant proteins and GST pull-down assay. 

E. coli strain BL21, harbouring pGEX4T1 or its derivatives (see Table 1) and expressing GST-Spa40_CT or its derivatives, was cultured in L-broth with 100 μg ampicillin ml⁻¹ for 2 h at 37 °C, then IPTG was added to a final concentration of 0.1 mM. After incubation for 3 h at 30 °C, bacteria were harvested and GST fusion proteins were purified as described by the manufacturer of Glutathione Sepharose 4B (Amersham Pharmacia Biotech). Briefly, GST-recombinant proteins bound to Glutathione Sepharose 4B (50 μl) were mixed with cleared extract of E. coli strains expressing His-tagged Spa or Mxi proteins and incubated overnight at 4 °C. Supernatants were removed by centrifugation and beads were washed four times with bead-binding buffer (1 % Triton X-100 in Tris-buffered saline, pH 7.4). After the final wash, elution was performed with 40 μl glutathione, and 10 μl SDS-PAGE sample buffer was added to each sample. Bound proteins were analysed by immunoblotting using anti-His (Sigma) or anti-GST antibodies (GE Healthcare).

Protein analysis. Crude extracts and culture supernatants of S. flexneri strains were prepared as described by Allaoui et al. (1993a) and Botteaux et al. (2009), and analysed by SDS-PAGE and Western blotting using anti-IpaB (Barzu et al., 1993) and anti-IpaC monoclonal antibodies (Phalipon et al., 1992) and anti-IpaD (Menard et al., 1993), anti-MxiI and anti-Spa32 (Magdalena et al., 2002), and anti-IpaA polyclonal antibodies (Tran Van Nhieu et al., 1997).

Purification of needle complex (NC), electron microscopy and image analysis. NCs were purified as described by Sani et al. (2007) and were negatively stained with 2 % uranyl acetate on glow-discharged carbon-coated copper grids. Electron microscopy was performed with a Philips CM120FEG electron microscope equipped with a field emission gun operated at 120 kV. Images were recorded with a Gatan 4000 SP 4K slow-scan charge-coupled device (CCD) camera at ×80 000 magnification at a pixel size (after binning the images) of 3.75 Å at the specimen level, with GRACE software for semi-automated specimen selection and data acquisition (Pilgram et al., 1998). Single-particle analysis, including multi-reference and non-reference procedures, multivariate statistical analysis and classification, was performed as described by Sani et al. (2007).

RESULTS

Spa32 interacts with a domain that encompasses residues 309–342 of Spa40

To identify interaction partners of Spa32, we used the two-hybrid technique in yeast to screen libraries of prey plasmids containing DNA fragments from a 65 kb region of the virulence plasmid encompassing the mxi and spa genes (Page et al., 2001). The bait plasmid was constructed by fusing the spa32 gene in-frame with the coding sequence of the Gal4 DNA-binding domain. For each screen, approximately 10⁶ diploids carrying the his3 gene under the control of a Gal4-regulated promoter were spread on plates lacking His, and the insert carried by the prey plasmid present in each of the 19 clones that were selected as His⁺ was sequenced. Three Spa40 proteins encoded by prey plasmids

Table 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant features and/or method of construction</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td>Strains</td>
<td></td>
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<tr>
<td>M90T-Sm</td>
<td>S. flexneri wild-type parental strain (M90T)</td>
<td>Allaoui et al. (1992b)</td>
</tr>
<tr>
<td>SM144</td>
<td>spa40 mutant</td>
<td>This study</td>
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<td>Plasmids</td>
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<tr>
<td>pSU18</td>
<td>Expression vector</td>
<td>Martinez et al. (1988)</td>
</tr>
<tr>
<td>pUC18K</td>
<td>pUC derivative carrying the aphA-3 cassette</td>
<td>Menard et al. (1993)</td>
</tr>
<tr>
<td>pJN67</td>
<td>pGIP704 suicide vector carrying the inactivated spa40 gene</td>
<td>This study</td>
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<td>pSU18 expressing Spa40</td>
<td>This study</td>
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<td>pAB94</td>
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<td>This study</td>
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<td>pBADHisB expressing His-Spa47</td>
<td>Botteaux et al. (2009)</td>
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<td>pIN51</td>
<td>pQE30 expressing His-MxiN</td>
<td>Jouihri et al. (2003)</td>
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<td>pM18</td>
<td>pQE30 expressing His-Spa32</td>
<td>Magdalena et al. (2002)</td>
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<td>pIN79</td>
<td>pQE30 expressing His-MxiK</td>
<td>Jouihri et al. (2003)</td>
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<td>pBAL26</td>
<td>pBADHisA expressing His-Spa33</td>
<td>This study</td>
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started at residue L279, two at residue E281, one at residue N283, four at residue R290, two at residue E295, one at residue V304 and six at residue K309. The region common to all preys corresponded to residues 309–342 (LYKTHTKY-SFVDFEHLEVLRIWLEQVENTH) of Spa40.

In a previous work, we demonstrated that residues 206–246 of Spa32 interact with GST-Spa40\textsubscript{CT} (GST fused to the C-terminal 137 residues of Spa40), corresponding to the whole predicted cytoplasmic domain (Fig. 1a; Botteaux et al., 2008). To test whether residues 309–342 of Spa40 are sufficient for Spa32 binding we constructed plasmids pAB70 (encoding GST-Spa40\textsubscript{CC}, i.e. GST fused to Spa40 residues 259–342), pAB71 (encoding GST-Spa40\textsubscript{CC*}, i.e. GST fused to Spa40 residues 309–342), and pAB63 (encoding GST-Spa40\textsubscript{CN}, i.e. GST fused to Spa40 residues 205–258) and performed a GST pull-down assay. A soluble extract of an \textit{E. coli} strain producing His-Spa32 was incubated with GST-Spa40 derivatives or GST alone, and bound to Glutathione Sepharose beads, and proteins retained on the beads were eluted with glutathione. As reported by Deane et al. (2008), GST-Spa40\textsubscript{CT} undergoes a cleavage that results in the appearance of two forms of Spa40 that are detected in the eluted fractions with anti-GST antibodies (corresponding to GST-Spa40\textsubscript{CT} and GST-Spa40\textsubscript{CN}) (Fig. 1c). SDS-PAGE analysis of eluted proteins indicated that His-Spa32 interacts with GST-Spa40\textsubscript{CC} and GST-Spa40\textsubscript{CC*}, but not with GST-Spa40\textsubscript{CN} or GST (Fig. 1c). These results confirmed that the last 33 residues of Spa40 are indeed sufficient for Spa32 binding.

![Fig. 1.](image-url) Residues 309–342 of Spa40 are sufficient for Spa32 binding. (a) Schematic topology of the entire Spa40 protein. The four transmembrane (TM) domains have previously been reported for YscU (Allaoui et al., 1994). Linear presentation of GST–Spa40 constructs used in the GST pull-down assay. (b) Sequence alignment (using CLUSTAL w) of Spa40\textsubscript{CT} with YscU of \textit{Y. enterocolitica} and FlhB of \textit{Bacillus subtilis}. Residues in bold type were mutated in this work. The box represents the Spa40 domain identified as interacting with Spa32. (c) Eluted fractions (EF) from GST and GST–Spa40\textsubscript{CT} variants, after incubation with the cleared extract of \textit{E. coli} producing His-Spa32, were analysed by SDS-PAGE and immunoblotting using anti-GST or anti-His antibodies.
The spa40 mutant is defective in secretion and exhibits a needleless structure

To investigate further the role of Spa40, we inactivated the spa40 gene in the S. flexneri wild-type strain M90T (see Methods). To assess the ability of the spa40 mutant to secrete Ipa proteins, whole-cell extracts and proteins secreted by the wild-type and spa40 mutant were analysed by SDS-PAGE and Coomassie blue staining (Fig. 2a) or by immunoblotting with antibodies against the needle component MxiI or Spa32, translocators (IpaB, IpaC and IpaD) and an effector (IpaA) (Fig. 2b). Similar amounts of these proteins were present in whole-cell extracts of the two strains (Fig. 2b), and they were all secreted by the wild-type strain but not by the spa40 mutant. Secretion was restored upon complementation of the spa40 mutant with pNL3, a low-copy plasmid expressing native Spa40 (Fig. 2a, b).

To characterize the T3SA structure of the spa40 mutant, extensively purified NCs were analysed by transmission electron microscopy (TEM). The spa40 mutant produced T3SAs lacking the needle portion (Fig. 2c). In parallel, we also purified NCs and analysed the T3SAs of mxiA and spa47 mutants that also lack the needle structure (Fig. 2c). Average analysis of 2D projections of purified basal body particles revealed that the spa40 mutant exhibited a structure slightly different from that of the mxiA and spa47 mutants. Indeed, a dense region observed at the lower end of the bulge in mxiA and spa47 mutants was missing from the corresponding structure of the spa40 mutant (Fig. 2c, d), which may reflect the position of Spa40 within the NCs.

Mutational and functional analysis of the C-terminal domain of Spa40 (Spa40_CT)

Sequence alignment of members of the Spa40 family, including FlhB of Salmonella typhimurium and YscU of Yersinia enterocolitica, pointed to a number of conserved residues (Fig. 1b). As some of these residues are known to be mutagenic suppressors in other systems (Williams et al., 1996; Edqvist et al., 2003; Kutsukake et al., 1994), we mutated the corresponding codons on plasmid pNL3 to construct Spa40 variants G224F in the CN domain and A286V, L310A and V320K in the CC domain. The last two residues were chosen for their location within the Spa40-interacting domain and for their conservation among Spa40/YscU family proteins (Fig. 1b). Plasmids expressing Spa40 variants were introduced into the spa40 mutant and tested for their ability to restore secretion. Mutations G224F and A286V did not affect Spa40 function, but mutations V320K and L310A abolished secretion of all tested T3SA substrates (Fig. 3a). The fact that the needle component MxiI was not detectable in the supernatant (Fig. 3a) suggested that the V320K and L310A mutations...
affect the assembly of the T3SA. To test this hypothesis, we investigated the presence of the needle structure in these strains by electron microscopy and found that bacteria expressing the L310A or V320K variant lacked the needle part (Fig. 3c).

### Spa40 interacts via its C-terminal domain with MxiA, MxiN, MxiK, Spa47 and Spa33

Since the L310A and V320K mutations affected the export of MxiI, we reasoned that Spa40 may interact with other T3SA components. To test this hypothesis, we performed GST pull-down assays with other Mxi and Spa components, including MxiA, MxiN, Spa47, MxiK and Spa33, whose corresponding mutated strains exhibit a needleless phenotype (Jouihri et al., 2003; this study; our unpublished data). MxiK, MxiN, Spa47 and Spa33 are soluble proteins, while the overall secondary structure of MxiA indicates the presence of seven hydrophobic domains followed by a large cytoplasmic C-terminal domain. To test for interactions between Spa40 and these proteins, we performed a GST pull-down assay as described above. Analysis of the eluted fraction with glutathione indicated that Spa40 interacted with all tested proteins (Fig. 4), but not with His-DNMT3α, which encodes a His-DNA-methyltransferase-3-α used as a control. All interactions were detected in *E. coli*, indicating that Spa40 binding to its partners does not require intermediate T3SA components. To investigate further the binding domain(s) of Spa40, we performed a similar GST pull-down assay with GST-Spa40CN, GST-Spa40CC and GST-Spa40CN-C. The CC* domain, which is sufficient for Spa32 binding, was found to interact with MxiA*, MxiN and Spa33. Spa47 bound to the CN domain, but not to the CC one (Fig. 4). Lastly, MxiK, the ATPase cofactor, bound weakly to Spa40CN, and not at all to Spa40CC and Spa40CC*.

**Fig. 3.** Residues L310 and V320 are required for protein secretion. (a, b) Whole cell extracts (WCE) and proteins of the culture supernatant (Sup.) of M90T (wild-type; WT), spa40 and spa40 harbouring plasmid pNL3 (expressing Spa40) or its derivatives encoding Spa40G224A, Spa40A286, Spa40L310A or Spa40V320A were analysed by SDS-PAGE and Coomassie blue staining or by immunoblotting with antibodies directed against Mxi, Spa32, IpaD, IpaC, IpaB or IpaA. (c) Electron micrographs of negatively stained bacteria: spa40, spa40 complemented with native Spa40, Spa40L310A, Spa40V320A or Spa40DNPTh. Visualization by electron microscopy of NCs associated with bacteria. Arrowheads indicate the positions of needle substructures protruding from the bacterial surface. Bars, 100 nm.
Mutation of residues L310A and V320K affects Spa40 interaction with Spa32, MxiA and MxiN

As the same domain of Spa40 is critical for Spa32, MxiA, MxiN and Spa33 binding, we constructed two derivatives of GST-Spa40CT mutated at residues V320 and L310. Analysis of eluted fractions of GST-Spa40CT\textsubscript{L310A} and GST-Spa40CT\textsubscript{V320K} revealed that these two mutations affect the correct cleavage of Spa40CT (Fig. 5a, b). We next used these two variants in a GST pull-down assay. GST-Spa40CT\textsubscript{L310A} and GST-Spa40CT\textsubscript{V320K} were not able to interact with His-Spa32. GST-Spa40CT\textsubscript{L310A}, the production of which was remarkably reduced compared with GST-Spa40CT, did not interact with MxiN or MxiK, while the interaction of GST-Spa40CT\textsubscript{V320K} with MxiA\textsubscript{C} was drastically reduced (Fig. 5c). In contrast, GST-Spa40CT\textsubscript{L310A} and GST-Spa40CT\textsubscript{V320K}, like native GST-Spa40CT, were still able to interact with both Spa33 and Spa47 (Fig. 5c). The L310A and V320K mutations may introduce a misfolding by altering the side chains of residues that pack in the hydrophobic core of Spa40. However, as certain interactions are still maintained with these mutants, misfolding is unlikely to explain the absence of interaction with some type III secretion (T3S) components.

Deletion of the NPTH motif abolishes Spa40 processing and affects assembly of the T3SA

To understand better the role of the cleavage per se, we constructed an in-frame deletion of the region encoding the NPTH motif on GST-Spa40\textsubscript{CT}. As expected, the GST-Spa40\textsubscript{CT\textsuperscript{ANPTH}} protein was not cleaved (Fig. 6a). Next, we tested the effect of this mutation on protein secretion by complementing the spa40\textsuperscript{mut} mutant with pAB94, a plasmid encoding Spa40\textsuperscript{ANPTH}. The latter did not restore the secretion defect of the spa40\textsuperscript{mut} mutant (Fig. 6b). MxiI, the minor needle component, accumulated in the lysate of the spa40\textsuperscript{wt}/Spa40\textsuperscript{ANPTH} strain compared with the wild-type and spa40\textsuperscript{pNL3} strains (Fig. 6b). TEM analysis confirmed the absence of the needle assembly in spa40\textsuperscript{wt}/Spa40\textsuperscript{ANPTH} (Fig. 3c), indicating that Spa40 cleavage is important for NC assembly.

Spa40\textsuperscript{ANPTH} interacts with MxiA, MxiK and MxiN, but not with Spa32, Spa33 or Spa47

To test whether the cleavage of Spa40 interferes with its ability to associate with identified interacting partners, we performed a GST pull-down assay. GST-Spa40\textsuperscript{CT\textsuperscript{ANPTH}} was no longer able to interact with His-Spa32, His-Spa33 or His-Spa47 (Fig. 6c). In contrast, the unprocessed form was still able to interact with MxiA\textsubscript{C}, MxiK and MxiN (Fig. 6c). The NPTH deletion entirely removes the loop connecting the central \(\beta\)-strands that form the core of the Spa40 fold,
which could consequently generate a misfolded protein. However, the inclusion of GST-Spa40 CT D NPTH in our analysis is supported by the fact that certain interactions with MxiAC*, MxiK and MxiN are maintained. In conclusion, Spa40 processing is likely to reflect a cytoplasmic conformational change that defines its binding specificity.

**DISCUSSION**

The assembly of the T3SA needle is a carefully orchestrated multi-step process that requires the coordination of many proteins located in the cytosol, in membranes and on the bacterial cell surface. In the present study, we investigated the role of Spa40, and report structural and functional evidence that supports the concept that Spa40 processing directs selective molecular interactions with several T3SA components, such as Spa32, Spa33, Spa47, MxiK, MxiN and MxiA (Table 2). To get insight into Spa32 function in the switch, we first searched for its interacting partners and identified a domain encompassing residues 309–342 of Spa40 that is sufficient for Spa32 binding. The spa40 mutant lacks a dense region at the central base of the NC basal body, which may reflect the position of Spa40 in this structure, confirming earlier work that established that Spa40 is a minor component of the central inner-membrane export apparatus (Zenk et al., 2007).

To characterize further the role of the C-terminal domain, we examined the effect of single amino acid residue mutations within Spa40. Mutations of L310 or V320, both of which are conserved among Spa40 homologues, abolished the needle assembly and Spa32 binding. L310 is part of a conserved consensus sequence LARXLY, located near a positively charged patch mapped to helix a3, which lies directly below the NPTH loop region (Deane et al., 2008). This position suggests that it may play a role in the recruitment of T3SA components (Deane et al., 2008). Indeed, we found that Spa40 interacts with Spa33, Spa47, MxiN, MxiK and MxiA C*. These interactions, except those with Spa47 and MxiK, involve the last 33 residues of Spa40, suggesting that some competition may occur during the assembly process. We have previously reported that a *Shigella* strain mutated in *spa47*, *mxiN* or *mxiK* does not assemble the needle substructure because of the instability of MxiH and MxiI in the bacterial cytoplasm, suggesting that the preliminary function of the Spa47 ATPase is to ensure the transit of the needle components. In line with this hypothesis, the FliH–FliI interaction with FlhBC (Zhu et al., 2002) facilitates the initial entry of exported substrates into the gate, with the energy of ATP hydrolysis.

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**Fig. 6.** Deletion of the NPTH motif abolishes Spa40 cleavage, assembly of the T3SA and binding to Spa32, Spa33 and Spa47. (a) Analysis of eluted fractions (EF) of GST-Spa40 CT and GST-Spa40 CT D NPTH by SDS-PAGE and Coomassie blue staining. (b) Proteins present in culture supernatants (Sup.) and whole cell extracts (WCE) of M90T (wild-type; WT), spa40 mutant, spa40/ Spa40 or spa40 D NPTH were analysed by immunoblotting with antibodies directed against Mxi, Spa32, IpaB, IpaC and IpaA. (c) SDS-PAGE and immunoblotting analysis of eluted fractions (EF) from beads coated with GST-Spa40 CT or GST-Spa40 CT D NPTH after incubation with the cleared extract of *E. coli* producing His-Spa32, His-MxiA C*, His-MxiN, His-MxiK, His-Spa47 or His-Spa33. His-DNMT3a, negative control.
Table 2. Summary of interactions identified between Spa40 and T3S components

Equivalent interactions with YscU from Yersinia or with FlhB of Salmonella are also indicated. –, No interaction; +, interaction; +/−, reduced interaction; ND, not determined.

<table>
<thead>
<tr>
<th>T3S component</th>
<th>YscU</th>
<th>T3S component</th>
<th>FlhB</th>
<th>T3S component</th>
<th>Spa40</th>
</tr>
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<tr>
<td>YscP</td>
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<td>+</td>
<td>Spa32</td>
<td>+</td>
</tr>
<tr>
<td>YscQ</td>
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<td>FliN</td>
<td>ND</td>
<td>Spa33</td>
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<td>Spa47</td>
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</tr>
<tr>
<td>YscK</td>
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<td>+</td>
<td>†</td>
<td>MxiK</td>
<td>+</td>
</tr>
<tr>
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<td>FliH</td>
<td>ND</td>
<td>MxiN</td>
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</tr>
<tr>
<td>YscV</td>
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<td>FliA</td>
<td></td>
<td>MxiA_C</td>
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</table>

†MxiK has no counterpart in Yersinia or Salmonella.

Role of Spa40 in assembly of Shigella T3SS

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being used to disassemble and release the FliH–FliI complex from the protein to be exported (Minamino & Namba, 2008). Recently, the large cytoplasmic domain of YscU has been defined as necessary for the recruitment of the YscN ATPase regulatory complex, YscK–YscL–YscQ, in Yersinia (Riordan & Schneewind, 2008). The interaction identified here between the Spa40_CC domain and Spa47 and MxiK may permit the correct positioning or movement of the Spa40_CC domain and its recruitment of needle subunits or other components. The L310A mutation drastically reduced Spa40–MxiN binding, while the V320K mutation reduced the Spa40–MxiA_C interaction, suggesting that the binding domain of Spa40 is part of the last α-helix. Based on these results, we propose that the specific interaction of Spa40 with MxiA_C or MxiN is essential for the early events of needle assembly.

We investigated the role of Spa40 cleavage per se by constructing a Spa40 variant lacking the NPTH motif. Spa40^ANPPTH was not functional and was no longer able to interact with Spa32, Spa33 and Spa47, but could bind MxiA, MxiN and MxiK. The interaction of MxiA_C or MxiN with Spa40^ANPPTH was not seen with Spa40^L310A and Spa40^V320K variants, which were also uncleaved, suggesting that residues L310 and V320 are directly part of the binding site. Therefore, our results strongly suggest that the altered function of the unprocessed form of Spa40 results from loss of binding or recognition of other T3S components. Studies on EscU and SpaS, the Spa40 counterparts of EPEC and Salmonella, highlight the importance of the cleavage in T3S function (Zarivach et al., 2008). Structural and in vivo analysis of specific mutations within EscU illustrate the conformational effects of auto-cleavage in modulating the EscU region involved in interactions with other T3S components at the inner membrane (Zarivach et al., 2008).

One intriguing result that we obtained concerns the intracellular accumulation of the minor needle component MxiI in strain spa40/Spa40^ANPPTH. This suggests that Spa40 processing controls MxiI secretion before the assembly of the NC basal body. In comparison, a recent study has highlighted the critical role of the inner rod protein YscI, the Yersinia MxiI counterpart, in the substrate specificity switch. Indeed, it was reported that the formation of the inner rod, not the needle, is critical for substrate specificity switching, and that YscP and YscU exert their effects on substrate export by controlling YscI secretion (Wood et al., 2008). Thus, as suggested for PrgJ/YscI (Marlovits et al., 2004), MxiI may form a rod-like structure inside the base that is required for needle stability and may function as a platform for the initiation of needle polymerization (Marlovits et al., 2006; Wood et al., 2008).

In conclusion, based on our results and on accumulating data from the literature, we propose a model in which the cleaved form of Spa40 interacts with MxiK and MxiN, leading to the recruitment of the ATPase Spa47 at the T3S basal body structure. In addition, the novel conformation of Spa40_CC, within the MxiK–Spa47–MxiN complex, exposes certain residues that facilitate its interaction with Spa33, which is known to interact with the two inner-membrane T3S components MxiG and MxiJ. This configuration permits the transit of the needle components MxiII and MxiH. Later, once the needles reach 50 nm, Spa32 could compete with MxiN for interaction with the Spa40_CC domain to stop MxiH secretion, leading to the switch to translocator secretion. Consequently, a conformational change in the ATPase Spa47 would control the switch in substrate specificity from needle components to effectors, as has been suggested for the switch from translocators to later effectors (Botteaux et al., 2009).

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