YscU/FlhB of *Yersinia pseudotuberculosis* Harbors a C-terminal Type III Secretion Signal

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Background: After auto-proteolysis and dissociation, YscUCC is secreted by the T3SS of *Yersinia pseudotuberculosis*.

Results: YscUCC harbors a specific C-terminal T3S signal and its deletion triggers an increase of YscF secretion without affecting Yops secretion.

Conclusion: C-terminal end of YscU participate in YscF secretion regulation but not in the substrate specificity switch.

Significance: This is the first report of a C-terminal T3S signal.

All type III secretion systems (T3SS) harbor a member of the YscU/FlhB family of proteins that is characterized by an auto-proteolytic process that occurs at a conserved cytoplasmic NPTI motif. We have previously demonstrated that YscU CC, the C-terminal peptide generated by auto-proteolysis of *Yersinia pseudotuberculosis* YscU, is secreted by the T3SS when bacteria are grown in Ca2+-depleted medium at 37 °C. Here, we investigated the secretion of this early T3S-substrate and showed that YscU CC encompasses a specific C-terminal T3S signal within the 15 last residues (U15). U15 promotes C-terminal secretion of reporter proteins like GST and YopE lacking its native secretion signal. Similar to the “classical” N-terminal secretion signal, U15 interacted with the ATPase YscN. Although U15 is critical for YscU CC secretion, deletion of the C-terminal secretion signal of YscU CC did neither affect Yop secretion nor Yop translocation. However, these deletions resulted in increased secretion of YscF, the needle subunit. Thus, these results suggest that YscU via its C-terminal secretion signal is involved in regulation of the YscF secretion.

Many pathogenic and symbiotic Gram-negative bacteria make use of the type III secretion system (T3SS) to export and translocate effector proteins into eukaryotic host cells during infection (1). Human pathogenic *Yersinia* spp. *Y. pestis*, *Y. enterocolitica*, and *Y. pseudotuberculosis* possess a common virulence plasmid that encodes the T3S-components (Ysc proteins) as well as the effector and translocator proteins (Yop proteins) (2). Once translocated into eukaryotic cell, Yop effectors counteract the host immune defense mechanisms such as phagocytosis and apoptosis to promote bacterial survival and spreading (3). These processes are highly regulated, and it has been shown that *Y. pseudotuberculosis* up-regulates Yop expression after the contact with eukaryotic cells has been established (4). This target cell contact can be mimicked *in vitro* by depletion of calcium from the growth medium and a shift of the growing bacteria from 26 °C to 37 °C, resulting in a massive Yop secretion into the culture supernatant (5, 6). The role of calcium in Yop regulation has been an invaluable tool to investigate regulation of *yop* genes as well as Yop secretion in *Yersinia* spp.

The hallmark of the T3SS is the needle complex that is formed by a base multi rings substructure spanning the bacterial envelope and a needle-shaped structure emerging from the bacterial surface (7–9). The base of the needle complex forms a hollow structure through which the T3SS substrates are passing in an unfolded state (10). During assembly, the basal body is first built up by generating a machinery precursor that is secretion competent only for the early substrates that are components involved in the needle and inner rod formation (e.g. YscF, YscI, and YscP). After activation of the T3SS, the secretion of early substrates is modulated in favor of the secretion of translocator and effector proteins, the so called middle and late substrates, respectively (11, 12). This phenomenon is described as the substrate specificity switch that was first identified by Macnab and coworkers in the flagellum (13, 14).

Effector proteins have a secretion signal at their N-terminal region that allows their specific recognition by the machinery (15). It was previously suggested that the secretion signal is guided by the mRNA as an alternative of the protein, but today, the general consensus is that the secretion signal resides within the first 20 residues of the secreted substrates (16, 17). Despite extensive work devoted to the T3S signal characterization, no consensus sequence has yet been identified. However, sequence comparisons showed that N-terminal region of T3S-substrates are unstructured with an elevated solvent accessibility (18–20). Also, an enrichment in some residues like serine has been observed in T3S-substrates N-terminal region (21). These characteristics provide the secretion signal with a high tolerance for introduction of point mutations that barely affect the secretion efficiency (22). These features of the T3S signal were used to develop prediction software to identify T3S-substrates (20, 23). In *Yersinia*, the T3S-ATPase YscN, recognizes the secretion...
signal of the effector proteins when they are in complex with their cognate chaperone (24). It has been show that InvC, the YscN homologue in Salmonella enterica triggers the dissociation of the chaperone/effector complex in an ATP-dependent manner, which subsequently allows effector secretion (25).

Recently, we identified YscU CC as a new early substrate of Y. pseudotuberculosis T3SS (26). YscU is an inner membrane protein with a large cytoplasmic domain denoted YscU C (27). YscU belongs to the FlhB family of proteins found in all T3SSs. This class of proteins is characterized by an auto-proteolytic process that occurs at a conserved NPTH motif within the cytoplasmic domain (28). The cleavage of YscU between Asp263 and Pro264 of the NPTH motif generates a 10-kDa C-terminal peptide, named YscU CC (29–31). After cleavage, YscU CC forms a stable complex with YscU CNP, the N-terminal part of the cytoplasmic domain that is linked to the N-terminal membrane domain of YscU (32). Auto-proteolysis of YscU is essential for proper yop expression and Yop secretion suggesting that YscU is critical for calcium regulation of the Yersinia T3SS (11, 31). In addition, yscU mutants affected in auto proteolysis interfere in the association and secretion of YscU CC is essential for Yop secretion during growth in Ca2+-depleted LB medium (26). Indeed, we showed that depletion of calcium induced intra-molecular dissociation of YscU CC from the remaining part of YscU. Importantly, addition of ~1 mM Ca2+ blocked secretion of both YscU CC and Yops. These results prompted us to suggest that YscU is the actual Ca2+-sensor in Yersinia T3SS (26). We have here studied the secretion of the early substrate YscU CC and showed that YscU CC encompasses a specific C-terminal T3S signal sequence corresponding to the last 15 residues of the protein (U15). C-terminal U15 can promote secretion of reporter proteins like glutathione S-transferase as well as YopE lacking its native T3S signal sequence. In addition, we showed that U15 interacts with the ATPase YscN in an orientation-dependent manner. Moreover, our data highlighted an involvement of the YscU C-terminal end in the regulation of YscF secretion, the needle subunit.

Experimental Procedures

Bacterial Strains, Plasmids, and Growth Conditions—Bacterial strains and plasmids used in this study are listed in supplemental Table S1. Standard molecular biology methods were used to generate the different plasmid constructs used in this study. The PCR primers used in the different cloning strategy are listed in supplemental Table S2. For the complementation assay of YopE secretion, the divergent yera-yopE operon was cloned into pBADmycHis A. The expression of yopE and yera is under control of their native promoter and co-regulated with the T3S machinery. The different yopE variants were cloned similarly. The sequences of all the constructs were systematically checked (Eurofins MWG Operon). The pET-yscN plasmid was generated by GenScript Corporation. Escherichia coli strains were grown in Luria-Bertani broth (LB) or on Luria agar plates at 37 °C. Y. pseudotuberculosis strains were grown at 26 °C in LB or on Luria agar plates. Antibiotics were added to the medium for selection according to the resistance markers carried by the plasmid. The following concentrations were used: kanamycin, 50 μg/ml and carbenicillin, 100 μg/ml.

Yop Secretion Analysis—To induce Yops secretion, Yersinia pseudotuberculosis strains were first grown at 26 °C for 2 h in Ca2+-depleted LB medium (medium containing 5 mM EGTA and 20 mM MgCl2) and shifted at 37 °C for 3 h. Cultures were started at an A600 of 0.1. Samples were treated as described previously (26) and separated on Tris-Tricine polyacrylamid gels. Proteins were either stained with Coomassie R250 or, alternatively, transferred onto a PVDF membrane for immunoblotting. Anti-Yop, anti-YscU, anti-YscP, anti-DnaJ, and anti-YscF antibodies were diluted 1:5,000. Horseradish peroxidase-conjugated anti-rabbit antibody was diluted at 1:10,000 (GE Healthcare). Proteins were detected with a chemiluminescence detection kit (GE Healthcare). Quantification by densitometry was made using Multi Gauge software (Fuji film). The bands to be quantified were selected and quantitated after background subtraction.

HeLa Cell Cytotoxicity Assay—Yersinia cultures were started at an A600 of 0.1 in LB medium containing 1 mM Ca2+. After 1 h of growth at 26 °C, cultures were shifted at 37 °C for 2 h. HeLa cells were infected for 45 min at multiplicity of infection of 10 and 20. The cytotoxicity was assayed as previously described by Rosqvist and coworkers with some modifications (3). For immuno-staining samples were subsequently fixed with 4% PFA and permeabilized with 0.5% Triton X-100 (Sigma Aldrich). Nonspecific binding were prevented by treatment with PBS, 0.1 M glycine, and PBS, 1% bovine serum albumin. Alexa Fluor 488 phalloidin (Life Technologies) and DAPI were used to, respectively, stain cells actin cytoskeleton and nucleic acids. Samples were mounted in mounting medium (Dako) and examined with a fluorescence microscope (Nikon Eclipse C1 plus).

Co-purification Assay—For YscN/YopE interaction analysis, the plasmid encoding His6-YscN was introduced into BL21(DE3) strain. Cells were grown at 37 °C. At A600 = 0.7 protein production was induced by addition of 1 mM IPTG for 2 h. Cells were pelleted 10 min at 6,000 × g. Cell pellets were resuspended in PBS, 1% Triton X-100, and sonicated on ice. Broken cells were centrifuged 20 min at 20,000 × g at 4 °C, and the supernatant was incubated with HIS-Select Nickel Affinity Gel (Sigma Aldrich) for 1 h at 4 °C. Resin was washed, resuspended in PBS, 0.1% Triton X-100, and used in an interaction assay. Yersinia strains bearing plasmids encoding different YopE constructs were grown in conditions that allow induction of the T3SS. Cells were lysed as described above, and supernatants were incubated with gel-bound YscN for interaction. After 2 h of gentle rocking at 4 °C, samples were washed with PBS, 0.1% Triton X-100. Proteins that remained bound to the gel were eluted using SDS-PAGE sample buffer. Sample were separated on SDS-PAGE and transferred onto PVDF membrane for immuno-detection. Similar protocol was used for GST-YscU CC/YscN interaction with some modifications. GST constructs were bound to glutathione-Sepharose resin (GE Healthcare).
C-terminal Secretion Signal of YscU\textsubscript{CC}

Healthcare), and His6-YscN was in solution for the interaction assay.

Results

**GST-YscU\textsubscript{CC} Is Secreted and Interferes with Yops Secretion**—Earlier results have shown that YscU\textsubscript{CC} is secreted by the T3SS (26), and we were therefore interested to localize the minimal T3S signal within YscU\textsubscript{CC}. A classical approach to identify and characterize secretion signals is to generate hybrid proteins with reporter proteins such as GFP, adenylate cyclase, or GST (glutathione S-transferase) and follow the secretion pattern of these hybrids. We therefore generated GST-tagged YscU\textsubscript{CC} variants and introduced the plasmids expressing these constructs into the *Yersinia pseudotuberculosis* strain, YPIII/pIB29 (MEK). To induce the T3SS, bacteria were grown in Ca\textsuperscript{2+}-depleted medium and shifted from 26 °C to 37 °C. YscU\textsubscript{CC}-GST (Ucc-GST), in contrast to GST and GST-YscU\textsubscript{CC} (GST-U\textsubscript{CC}), could not be detected in the cell lysates of both *Yersinia* and *E. coli* BI21 (DE3) strains after induction. This hybrid protein was therefore not further studied. The level of Yops detected in the supernatant of the strain expressing gst-U\textsubscript{CC} was around 70% lower than the level observed for the strain expressing only gst (Fig. 1A, top panel compare lanes 1 and 2). Next we tested whether GST-U\textsubscript{CC} was secreted by the T3SS. For this, anti-GST antibodies were used to detect GST in the supernatants and pellets from strains expressing gst and gst-U\textsubscript{CC}. Two bands were detected in the supernatant of the strain expressing gst-U\textsubscript{CC} (Fig. 1A, middle panel). The high molecular weight band (36 kDa) corresponds to GST-U\textsubscript{CC} while the lower band (25 kDa) corresponds to GST, most likely generated by degradation of the hybrid protein. No band was detected in the supernatant of the strain expressing only gst (Fig. 1A, upper panel). These results show that YscU\textsubscript{CC} fused to the C terminus of GST promotes secretion of the GST-U\textsubscript{CC} hybrid protein. Furthermore, GST-U\textsubscript{CC} was not detected in the supernatant when the bacteria were grown in medium supplemented with Ca\textsuperscript{2+} (data not shown), demonstrating that GST-U\textsubscript{CC} secretion is dependent on the T3SS. Similar amounts of GST and GST-U\textsubscript{CC} were detected in the cell pellets showing that the absence of GST in the supernatant was not linked to expression defect or protein instability (Fig. 1A, lower panel).

YscU\textsubscript{CC} Contains a C-terminal Secretion Signal—The data presented above indicated that unlike Yops, YscU\textsubscript{CC} does not need to be located at the N terminus to promote secretion of GST through the T3SS. In contrast, our results suggested that YscU\textsubscript{CC} harbors a C-terminal T3S signal. To define this putative C-terminal secretion signal, truncated variants of GST-U\textsubscript{CC} were generated by deleting the three and six last residues of YscU\textsubscript{CC} (GST-U\textsubscript{CC}\textsubscript{3} and GST-U\textsubscript{CC}\textsubscript{6}). GST-U\textsubscript{CC}\textsubscript{3} and GST-U\textsubscript{CC}\textsubscript{6} also interfered with Yops secretion but at a lower extent than GST-U\textsubscript{CC} (Fig. 1A, upper panel). Interference in Yop secretion by GST-YscU\textsubscript{CC} has been observed before by Riordan and Schneewind (30). The authors proposed that interaction sites within YscU\textsubscript{CC} mediate GST-YscU\textsubscript{CC} interaction with some components of the machinery (e.g. YscL, YscQ, and YscK), which may interfere in Yop secretion. It is possible that GST-YscU\textsubscript{CC}, GST-U\textsubscript{CC}\textsubscript{3} and GST-U\textsubscript{CC}\textsubscript{6} interfere in Yop secretion in a similar manner. However, YscU is a critical secretion regulator; it is then most plausible that the interference in Yop secretion is due to increased level of YscU\textsubscript{CC} that may hamper the effectors secretion regulation. Nevertheless, neither GST-U\textsubscript{CC}\textsubscript{3} nor GST-U\textsubscript{CC}\textsubscript{6} was detected in the culture supernatant indicating that those two construct are not secreted by the T3SS (Fig. 1A, middle panel). Similar amounts of GST and GST-U\textsubscript{CC} proteins were detected in the cell pellets demonstrating that GST-U\textsubscript{CC}\textsubscript{3} and GST-U\textsubscript{CC}\textsubscript{6} are produced and stable (Fig. 1A, lower panel). Altogether, these data show that the absolute C terminus of YscU\textsubscript{CC} harbors a T3S signal.

![Figure 1: Secretion of GST-YscU\textsubscript{CC}](image-url)
To further explore the C-terminal T3S signal of YscU_{CC}, GST-U_{CC} constructs with large deletions in the N-terminal region of YscU_{CC} were generated. According to the atomic structure of YscU_{CC} residues Leu^{221} to Ile^{236} form a random coil that is followed by an α-helix ranging from Pro^{327} to Arg^{339} (Fig. 2A) (32). The structural organization of the last 15 residues (Trp^{340} to Leu^{354}) remains unknown (Fig. 2A). Based on these structural features three variants were produced: GST-U_{CC34}, GST-U_{CC28}, and GST-U_{CC15} (Fig. 2A). GST-U_{CC34} contains the 34 last residues that form a random coil and the α-helix. In the GST-U_{CC28} variant, the random coil is no longer present. GST-U_{CC15} contains only the last 15 residues of YscU_{CC}. These three variants were introduced in Y. pseudotuberculosis YPIII/pIB29(MEK) strain and probed for secretion. Immuno-detection of GST in the cell pellets showed that these constructs were stable and expressed at equivalent levels (Fig. 1B, lower panel). While GST remained cytoplasmic, GST-U_{CC34}, GST-U_{CC28}, and GST-U_{CC15} were detected in the supernatant showing that those three variants were secreted via the T3SS (Fig. 2B, middle panel). These data show that YscU_{CC} harbors a C-terminal T3S signal and that the last 15 residues are sufficient to promote secretion of GST. After cleavage at the NPTH motif, YscU_{CC} forms a stable complex with YscU_{CN}. The crystal structure of the YscU_{CN}/YscU_{CC} complex showed that YscU_{CC} N-terminal residues are fully or partially buried within the structure while the C-terminal residues are most likely exposed and unstructured (Fig. 2A) (32). These structural features of YscU_{CC} make the N-terminal residues not accessible for interaction with the ATPase YscN in order to be secreted. On the other hand, the C-terminal residues of YscU_{CC} may constitute an alternative to overcome this structural situation.

C-terminal Targeting of YopE to the T3SS—It is known that most of the late T3S substrates interact with specific T3S-chaperones (35). T3S-chaperones bind to N-terminal region of the effectors (residues 25 to 100) to maintain the effectors in a partially unfolded state that facilitate interaction with the T3S-machinery and subsequent secretion. Here we identified and characterized within YscU_{CC}, the first C-terminal T3S signal. It is plausible that YscU_{CC} is an exception to the N-terminal location of the T3S signal. However, it is an opportunity to study the separation of the secretion signal from the chaperone binding site by putting the last 15 residues of YscU_{CC} (U_{15}) at the C terminus of a classical effector lacking its native secretion signal. For this, we used YopE, which is a well characterized substrate of Yersinia T3SS. YopE harbors a "classical" N-terminal secretion signal corresponding to the 15 first residues and the deletion of this sequence blocks YopE secretion (15, 17, 36). To test whether the last 15 residues of YscU_{CC} (U_{15}) can promote secretion of YopE_{ASP} (lacking the 15 first residues) we construct different variants of YopE and introduced the corresponding plasmids into the strain YPIII/pIB29 (MEK). As expected YopE was detected in the supernatant of the strain expressing the wild type YopE while no YopE was secreted by the strain producing YopE_{ASP} (Fig. 3A, lines 1 to 3). These data confirmed that the 15 first residues of YopE are critical for secretion. In the constructs U_{15}-YopE_{ASP} and YopE_{ASP}-U_{15}, the 15 last residues of YscU_{CC} were fused to YopE_{ASP}, N or C termini, respectively. None of these two constructs were secreted into the supernatant at levels that allowed their detection with Coomassie staining (Fig. 3A, lines 4 and 5). However, YopE_{ASP}-U_{15} was detected in the supernatant by immunoblotting while neither YopE_{ASP} nor U_{15}-YopE_{ASP} was detected in the culture supernatant (Fig.
YopE corresponds to the wild type structs were cloned into pBAD-HisA plasmid (Invitrogen) under an arabinose inducible promoter and expressed in trans. The capacity of U_{15} to promote translocation into eukaryote cells was also evaluated. For this, we followed the development of the cytotoxicity phenotype of HeLa cells after infection with strain expressing different variants of YopE. Thirty minutes after infection a full cytotoxicity phenotype was observed with the strain expressing YopE (Fig. 4B) while no cytotoxicity was observed when HeLa cells were infected with strains carrying an empty plasmid or plasmids expressing YopE_{ASP} or YopE_{ASP}-E_{15rev} (Fig. 4, A, C, and D). On the other hand U_{15rev}-YopE_{ASP} provokes cytotoxicity on around 30% of the HeLa cells (Fig. 4E). The cytotoxicity phenotypes of YopE_{ASP}-U_{15} and U_{15rev}-YopE_{ASP} correlate with their secretion efficiency.

YscU_{CC} Interacts with the ATPase YscN—It was recently suggested that the ATPase YscN interacts with the secretion signal of YopR, and that this interaction is critical for YopR secretion by the T3SS (24). Previous studies showed that surface-exposed residues of YscU (U_{15}) were fused to the N and C termini of YopE respectively. The signal peptide of YopE, E_{15} and its reverse sequence E_{15rev} were detected for wt YopE showing that the YopE native secretion signal is more efficient than U_{15rev}. YopE signal peptide contains only one charged residue (Lys') while six charged residues are present in U_{15} (Glu^{342}, Arg^{343}, Glu^{347}, Lys^{348}, His^{350}, and Glu^{352}). As demonstrated by a Kyte and Doolitle plot, the charged residues within U_{15} make this peptide more hydrophilic than YopE signal peptide (Fig. 2B). These hydrodynamic discrepancies between U_{15} and YopE native signal peptide might explain this difference in secretion level. Indeed, charged residues as well as hydrophobic pockets at the interaction surface are parameters that guide protein-protein interaction (38, 39). It is probable that U_{15rev} has a lower affinity for the machinery components that recognize the secretion signal.

We also investigated the ability of the native secretion signal of YopE (denoted E_{15}) to promote secretion of YopE_{ASP} when placed at the C terminus. Surprisingly both YopE_{ASP}-E_{15rev} and YopE_{ASP}-E_{15} were not detected in the culture supernatant (Fig. 3). Unlike U_{15}, E_{15} cannot promote C-terminal secretion and that regardless of the orientation, showing that U_{15} has features that are not shared with classical secretion signal.

The capacity of U_{15} and U_{15rev} to promote translocation into eukaryote cells was also evaluated. For this, we followed the development of the cytotoxicity phenotype of HeLa cells after infection with strain expressing different variants of YopE. Thirty minutes after infection a full cytotoxicity phenotype was observed with the strain expressing YopE (Fig. 4B) while no cytotoxicity was observed when HeLa cells were infected with strains carrying an empty plasmid or plasmids expressing YopE_{ASP} or YopE_{ASP}-E_{15rev} (Fig. 4, A, C, and D). On the other hand U_{15rev}-YopE_{ASP} provokes cytotoxicity on around 30% of the HeLa cells (Fig. 4E). The cytotoxicity phenotypes of YopE_{ASP}-U_{15} and U_{15rev}-YopE_{ASP} correlate with their secretion efficiency.

YscU_{CC} Secretion Signal, Orientation Does Matter—To be secreted via the T3SS, proteins have to interact with component of the T3S-machinery, such as the ATPase YscN (24, 25, 37). It is known that protein-protein interactions depend on the spatial arrangement of the side chains from surface-exposed residues (38). Thus the location (N- or C-terminal) as well as the orientation of the signal peptide might affect the secretion efficiency. To test this hypothesis we assayed the secretion of both U_{15rev}-YopE_{ASP} and YopE_{ASP}-U_{15rev}. U_{15rev} corresponds to the peptide that contains the same residues as U_{15} but in a reverse order. YopE_{ASP}-U_{15rev} was not secreted whereas U_{15rev}-YopE_{ASP} was detected in the supernatant after Coomassie Blue staining (Fig. 3A, lines 6 and 7). Thus, U_{15} promotes secretion when placed at the N terminus but only when the residues are placed in a reverse orientation. These results indicated that the orientation of U_{15} is important for secretion and most likely for interaction with the machinery. The level of U_{15rev}-YopE_{ASP} detected in the supernatant is significantly higher than YopE_{ASP}-U_{15} secreted level but is only about 5% of the amount detected for wt YopE showing that the YopE native secretion signal is more efficient than U_{15rev}. YopE signal peptide contains only one charged residue (Lys') while six charged residues are present in U_{15} (Glu^{342}, Arg^{343}, Glu^{347}, Lys^{348}, His^{350}, and Glu^{352}). As demonstrated by a Kyte and Doolitle plot, the charged residues within U_{15} make this peptide more hydrophilic than YopE signal peptide (Fig. 2B). These hydrodynamic discrepancies between U_{15} and YopE native signal peptide might explain this difference in secretion level. Indeed, charged residues as well as hydrophobic pockets at the interaction surface are parameters that guide protein-protein interaction (38, 39). It is probable that U_{15rev} has a lower affinity for the machinery components that recognize the secretion signal.
residues of some T3S-chaperones in complex with their cargo effector also interact with the ATPase (40–42). The results presented above showed that U₁₅ and U₁₅rev, respectively, placed at the C-terminal or the N-terminal end of YopE₆₅₃ promoted secretion, indicating that both U₁₅ and U₁₅rev interacted with YscN. To test this hypothesis, His₆-YscN bound on a nickel-agarose resin was incubated with different variants of YopE to evaluate the capacity of these proteins to co-purify with YscN. YerA, the cognate chaperone of YopE, was co-expressed in all constructs to avoid degradation YopE and to maintain YopE chaperone binding domain in a partially unfolded state to facilitate interaction in all YscN. YscN was eluted and co-purified with YscN and in a secretion signal-dependent manner (Fig. 5A). As expected, YopE₆₅₃ did not co-purify with YscN. U₁₅rev-YopE₆₅₃, the variant with the highest secretion efficiency after wild type YopE, co-purified with YscN (Fig. 5B). This interaction is strictly dependent on the N-terminal U₁₅rev peptide since YopE₆₅₃ and YopE₆₅₃-U₁₅rev were not co-purified with YscN (Fig. 5B). After YopE, U₁₅rev-YopE₆₅₃ is the variant that co-purified best with YscN (around 60% of YopE amount) correlating with both secretion efficiency and translocation into HeLa cells. Importantly although U₁₅-YopE₆₅₃ was not secreted, this variant interacted with YscN albeit at a lower level compared with U₁₅rev-YopE₆₅₃ (Fig. 5B). Surprisingly, we were unable to detect interaction between YopE₆₅₃-U₁₅ and YscN (Fig. 5B), although YopE₆₅₃-U₁₅ was secreted via the T3SS. However, the YopE₆₅₃-U₁₅ level of secretion was relatively lower than YopE; and it is possible that this low level of secretion is a consequence of a low affinity of YscN for YopE₆₅₃-U₁₅ hybrid protein. Thus, interaction between the secretion signal and the ATPase YscN is critical to allow secretion; however our results suggest that interaction per se may not be sufficient to promote secretion. In line with our findings, we could also show an interaction between YscUₐ₇ and YscN (Fig. 5). A similar result has previously been described for Spa40 and Spa47 that are the homologues of YscU and YscN in Shigella flexneri (43).

Deletion of the C Terminus of YscU Affects YscF Secretion—It has been shown earlier that YscU auto-proteolysis followed by YscUₐ₇ dissociation constitute critical steps for regulation of the substrate specificity switch (14, 29, 30). In addition, we recently showed that YscUₐ₇ is secreted by the T3SS when bacteria are grown in conditions allowing Yop secretion (26). These previous published studies with the data presented above in the present article, suggested that the C-terminal end of YscUₐ₇ would be essential for Yops secretion. Surprisingly, when YscUₐ₇, YscUₐ₆, and YscUₐ₅ which correspond to YscU constructs having C-terminal deletions, were introduced into a ΔyscU strain (YPIII/pIB75), Yop secretion was not affected (Fig. 6A). Similarly, these mutants induced a cytotoxic response after infection of HeLa cells that was indistinguishable from the corresponding yscU wt strain (Fig. 6B). Thus, neither Yop secretion into the culture supernatant nor Yop translocation into HeLa cells was affected by the deletion of the last 9 residues of YscU. The fact that these mutants showed secretion and translocation of Yops at wild-type level strongly suggests that both auto-proteolysis and dissociation of YscUₐ₇ were not affected by the C-terminal deletions. As expected, secretion of YscUₐ₇, however, was impaired by YscU C-terminal deletions (Fig. 7A, second panel from the top) suggesting that YscUₐ₇ secretion is not required to trigger dissociation, but it is likely that YscUₐ₇ secretion is a result of dissociation. Interestingly, while similar amounts of intracellular YscF (needle subunit) were detected for the different strains, we observed an increase of YscF secretion for the C-terminal deletions mutants (Fig. 7A, bottom and third panel from the top). These results suggested that the C-terminal signal sequence of YscU is involved in YscF secretion control. Elongated needles have been reported for ΔyscP mutants and yscU point mutants defective in auto-proteolysis (11, 44, 45). However, in contrast to mutants carrying C-terminal deletion of YscU, Yop secretion is impaired in a yscP mutant as well as in the yscU processing mutants. Altogether, the different phenotypes observed for mutants expressing YscUₐ₇, YscUₐ₆ and YscUₐ₅ constructs suggest that the C-terminal end of YscUₐ₇ is directly involved in the secretion regulation of the
C-terminal Secretion Signal of YscUC

FIGURE 5. The C-terminal signal sequence of YscUC interacts with ATPase YscN. A, His$_6$-YscN bound on a His-Select resin was incubated with lysates containing different variants of YopE, co-expressed with the YopE chaperone YerA. After several washes, proteins associated to the resin were eluted and separated on the gel for an immunoblotting with anti-YopE antibody (upper panel). Level and stability of the proteins in the lysates were analyzed by immunoblotting with anti-YopE antibody (lower panel). As can be seen the wt signal sequence of YopE is essential for interaction with YscN. Both U$_{15}$ and U$_{30}$ interacted with YscN when placed at the N-terminal end of YopE$_{359-418}$, NB: No interaction could be noticed when U$_{15}$ was placed in the C-terminal end of YopE$_{359-418}$. For description of the different constructs used, see Fig. 3 legend. The amount of co-purified wild type YopE was set at 100% for densitometry quantification, and the amount of U$_{15}$-YopE$_{359-418}$ was evaluated at 62.8%. B and C, GST and GST-YscUC$_{CC}$ constructs immobilized on glutathione-agarose columns were incubated with purified His$_6$-YscN. After several washes, proteins associated to the resin were eluted and separated on the gel for an immuno-detection with anti-YscN antibody. The upper panels show the Coomassie staining of the GST constructs bound onto the glutathione-Sepharose (B) YscN interacts associated with GST-YscUC$_{CC}$ but not with GST alone. The binding affinity of YscN to YscUC$_{CC}$ decrease of about 50% after deletion of the 3 and 6 last residues of YscUC$_{CC}$. YscN barely binds to GST-YscUC$_{326-359}$. C, a similar experiment using large N-terminal deletions within YscUC$_{CC}$ showed that the last 28 residues of YscUC encompass the binding site for YscN.

needle subunit, YscF. Previously, it has been shown in Y. enterocolitica that strains bearing the processing mutants YscU$_{N263A}$ and YscU$_{P264A}$ secrete elevated amount of YscF as a consequence of reduced secretion of YscP (12, 45). Interestingly, the secretion of YscP was not affected in any of the YscU mutants with C-terminal deletions (Fig. 7B).

FIGURE 6. C-terminal deletions of YscU do not affect Yop secretion and Yop translocation into HeLa cells. The ability of wt YscU, YscU$_{3}$, YscU$_{6}$, and YscU$_{9}$ (respectively, YscU with deletion of the last 3, 6, and 9 residues) to support Yop secretion was assayed by introducing plasmids expressing these constructs into the ΔyscU-null mutant YPIII/pIB75 in trans. A, culture supernatants were TCA precipitated, separated on Tris/Tricine gel, and stained with Coomassie R-250. The C-terminal deletion constructs supported Yop secretion at a similar level as wild type YscU. B, HeLa cell cytotoxicity assay. Unlike uninfected cells (a) that showed no morphology changes, strains expressing YscU (b), YscU$_{3}$ (c), and YscU$_{9}$ (d) were fully cytotoxic i.e. YopE was translocated at the same level in all strains.

Discussion

We recently published results showing that YscUC$_{CC}$, the 10-kDa C-terminal polypeptide generated by YscU auto-proteolysis, is secreted via the T3SS of Y. pseudotuberculosis (26).
Furthermore, the physiological conditions that allow YscU<sub>CC</sub> secretion are identical to the conditions required for Yop proteins secretion, i.e. after incubation of the bacteria at 37 °C in Ca<sup>2+</sup>-depleted medium. These results indicated that YscU<sub>CC</sub> putatively harbored a specific T3S signal and we have here addressed this question. First, YscU<sub>CC</sub> (U<sub>CC</sub>) was cloned at the C-terminal end of the GST protein reporter and the resulting hybrid protein, GST-U<sub>CC</sub> was probed for secretion. Remarkably, GST-U<sub>CC</sub> was found to be secreted via the T3SS in a Ca<sup>2+</sup>-dependent manner. These results demonstrated that C-terminal localized U<sub>CC</sub> can promote secretion of reporter a protein like GST via the T3SS. C-terminal targeting of T3S-substrates (natives substrates or hybrid proteins) to the machinery has never been reported. In fact, studies of several T3SS effector and translocator proteins from different species demonstrated that the secretion signal is localized at the N terminus (1, 17, 24). To further investigate YscU<sub>CC</sub> secretion signal, GST-U<sub>CC</sub> truncated variants with deletions at the N or C-terminal regions of YscU<sub>CC</sub> were probed for secretion. The secretion profiles obtained for these different variants showed that YscU secretion signal is localized within the last 15 amino acids of the protein (U<sub>13</sub>). Moreover, when YopE<sub>ASP</sub>, the T3S-substrate lacking its native secretion signal was fused to U<sub>15</sub>, the resulting hybrid protein YopE<sub>ASP</sub>-U<sub>15</sub> was also found to be secreted by T3SS. Thus, in addition to the well-studied T3S-associated “classical” N-terminal secretion signals, we identified a C-terminal secretion signal that mediates secretion of both endogenous and exogenous proteins. The ability of T3SS to export proteins directly from the bacterial cytoplasm into the extracellular medium or into the host cell cytosol has been exploited for heterologous protein production or antigen delivery during vaccination (46, 47). In this context, the newly identified C-terminal T3S signal may have interesting biotechnological applications for production of proteins with N-terminal secretion signal which the unfolded substrates are threaded. The T3S-unfoldase hypothesis is the first step in the unfolding and secretion processes (24, 25). As it has been previously demonstrated for the T3S-substrate YopR (24), we showed here for YopE and YscU<sub>CC</sub> that the secretion signal is required for interaction with the ATPase YscN (Fig. 5). The secretion profiles as well as the results from protein/protein interaction assays demonstrated that YscN interacts with YscU<sub>CC</sub> secretion signal regardless of the localization at the C terminus (GST-U<sub>CC</sub> and YopE<sub>ASP</sub>-U<sub>13</sub>) or at the N terminus (U<sub>15</sub>-YopE<sub>ASP</sub>). However, to promote secretion after interaction with YscN, YscU<sub>CC</sub> secretion signal must be correctly oriented according to its localization. Indeed, the forward secretion signal U<sub>15</sub> promoted C-terminal secretion while the reverse sequence U<sub>15</sub>-rev exclusively promoted N-terminal secretion. Although the levels of YopE<sub>ASP</sub>-U<sub>15</sub> and U<sub>15</sub>-rev-YopE<sub>ASP</sub> in the culture supernatants were considerably lower than the level of wild type YopE, the secretion of these hybrid proteins was specific and dependent of U<sub>15</sub> and U<sub>15</sub>-rev, respectively. Hence, the orientation of the amino acids constituting YscU<sub>CC</sub> secretion signal is essential for secretion, which strongly argues for a polarized threading mechanism guided by the orientation of the T3S signal. In line with this idea, the hybrid protein U<sub>15</sub>-YopE<sub>ASP</sub> was not secreted despite the interaction with the ATPase YscN.

Several studies have demonstrated the involvement of the YscU/FlhB family of protein in the substrate specificity switch (11, 13, 14, 34). YscU is anchored in the inner membrane via four transmembrane segments. The membrane integrated part of the protein is connected to a large cytoplasmic domain via a linker sequence. Accumulated data indicate that the different portions of the protein have distinct functions (27). Recently, we showed that the linker sequence forms an α-helix due to interactions of the positively charged residues with the negatively charged lipids of the cytoplasmic membrane inner leaflet (52). This interaction and subsequently the α-helix formation are critical for YscU function and Yops secretion. Auto-proteolysis at the conserved NPTH motif is also critical for YscU function since mutants defective in auto-proteolysis present altered secretion of YscF and Yops (29–31, 45). Previously we showed that Ca<sup>2+</sup>-depleted conditions trigger dissociation and secretion of YscU<sub>CC</sub> which is critical for the substrate specificity switch (26). We describe here yet a novel phenotype associated to YscU. Similarly to yscP mutants (11, 44), deletion mutants within the YscU C-terminal secretion signal secrete elevated levels of YscF, the needle subunit, which indicates that the secretion signal has a negative influence on YscF secretion. These results are in line with earlier findings showing that the 33 last amino acids of FlhB are redundant for hook-basal body assembly. Interestingly this domain of FlhB has an inhibitory effect on the substrate specificity switch suggesting that the C-terminal region of FlhB is involved in the temporal regulation of the flagellar assembly (33, 34). In fact it cannot be excluded that YscU via its C-terminal secretion signal possesses a negative activity similarly to FlhB, that affects the switch from secretion of early to late substrates. However, unlike yscP mutants, strains with YscU C-terminal deletions were still able to secrete...
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Yop-proteins at wild-type level, suggesting that the C-terminal end of YscU\textsubscript{CC} is rather involved in the needle formation than the substrate specificity switch. Needle length control and substrate specificity switch are two closely related events that implicate both YscU and YscP (11, 29, 31). Indeed, it has been shown that deletion of YscP as well as auto-proteolysis mutants of YscU secrete elevated amount of YscF (44, 45, 53). It has been proposed that these mutants form long needles because of their inability to make the substrate specificity switch and secrete effectors. Thus, needle length control and substrate specificity switch were considered as interdependent processes. We speculate that the processing and the dissociation of YscU\textsubscript{CC} are implicated in the substrate specificity switch while YscU secretion signal together with YscP are involved in needle control. More work is however necessary to investigate the YscU/YscP interaction and how this interaction affects the needle formation.

Author Contributions—F. H. L. and H. W. W. designed the study and wrote the paper together. F. H. L. performed the cloning, the cell infection experiments, and purified the proteins for the pull down assay. F. H. L. and H. W. W. analyzed the results and approved the final version of the manuscript.

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