Secretion signal recognition by YscN, the *Yersinia* type III secretion ATPase

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Yersinia **type III machines secrete protein substrates across the bacterial envelope. Secretion signals of some substrates have been identified; however, the mechanisms whereby these signals interact with type III machines are not known. Here we show that fusion of YopR, an early secretion substrate, to the N terminus of dihydrofolate reductase (DHFR) or other tightly folded proteins generates impassable hybrids that cannot travel the type III pathway. YopR hybrids capture YscN, the ATPase that provides energy for type III transport reactions, in the bacterial cytoplasm. Eleven N-terminal residues function as the YopR secretion signal, which is required for both binding to YscN and blocking the type III pathway. When expressed during type III machine assembly, YopR-DHFR blocks all secretion. Delayed expression of YopR-DHFR, when yersiniae have already engaged the type III pathway, blocks secretion of early (YscP) but not of late (effector Yops) substrates. These observations support a model whereby type III machines are programmed to secrete a sequence of proteins that can be disrupted when an impassable early substrate interacts with the YscN ATPase and blocks the transport of late substrates.**

impassable substrates | YopR

Substrates for protein translocation across membranes are chosen by mechanisms that require binding of specific signal peptides to machinery components (1, 2). Fusion of signal peptide-bearing substrates to impassable molecules, proteins that fold rapidly and cannot be accommodated by translocation machines, jam the secretion pathway for other substrates (3–6). The underlying principle of this phenomenon is that the binding of signal peptides to translocases represents an irrevocable, default step in the initiation of proteins into translocation pathways (7). Unlike protein translocases that move their substrates 7–12 nm across a single membrane (8), type III secretion machines provide for protein transport across the bacterial double membrane envelope and host-cell plasma membrane (9, 10). The physical dimensions of type III secretion machines, also called needle complexes, are expansive: 50–100 nm in length with a luminal diameter of 2 nm (10–12).

One can view type III machines as secretion devices that promote self-assembly of a protein conduit between bacteria and host cells (13). Once completed, the virulence plasmid-encoded type III machinery of pathogenic *Yersinia* spp. (*Y. enterocolitica*, *Y. pseudotuberculosis*, and *Y. pestis*) exploits the conduit for the delivery of effectors (YopE, YopH, YopM, YopN, YopO, YopP, YopQ, and YopT) into host cells, providing for bacterial escape from phagocytic killing (14). This hypothesis implies that type III machines are preprogrammed for transport of a defined sequence of substrates that begins with needle-forming machine components (YscF, YscP, and YscX) (15–17), followed by translocators that complete the type III conduit into host cells (LcrV, YopB, and YopD) (18, 19) and finally by effector Yops (14). Several predictions can be derived from this model, and two are most relevant for this study. First, mutations that eliminate early secretion substrates are predicted to prevent downstream secretion events, as the type III conduit cannot be completed. This finding has indeed been observed as mutations in genes encoding early substrates (YscF, YscP, and YscX) abolish type III secretion (17, 20, 21), whereas mutations in genes specifying middle substrates (LcrV, YopB, and YopD) prevent type III injection of effector Yops into immune cells (18, 22, 23). Second, substrate fusions to impassable molecules should generate hybrids that cannot travel the type III pathway, thereby imposing a blockade for other substrates that are initiated later in the preprogrammed sequence of transport reactions.

Results

YopR-Dihydrofolate Reductase (DHFR) Imposes a *Yersinia* Type III Secretion Blockade. Previous work used rapidly folding, thermodynamically stable proteins [β-galactosidase (LacZ), ubiquitin (Ub), DHFR, or GST] to generate impassable hybrids that cannot travel across secretion pores, features that also apply to impassable hybrids of *Yersinia* type III machines (6, 24–27). Unlike canonical pathways that engage signal peptide-bearing hybrids and jam translocases, YopE-DHFR is rejected from the *Yersinia* type III pathway without blocking the secretion of other substrates (28). We wondered whether impassable hybrids generated from other *Yersinia* substrates also are rejected by the type III pathway.

Y. enterocolitica W22703 cultures were induced for type III secretion by chelation of calcium ions and a temperature shift from 26°C to 37°C. Expression of plasmid-borne reporter genes was induced with isopropyl β-D-thiogalactoside (IPTG) or left uninduced. Similar to YopE hybrids, YopH and YopQ fusions to DHFR generated impassable hybrids that were not secreted by *Y. enterocolitica* W22703 (see *Supporting Materials and Methods* and Fig. 6, which are published as supporting information on the PNAS web site). Expression of *yopH-dhfr*, but not of *yopQ-dhfr*, reduced *yop* virulon expression (see abundance of YopE, YopD, and YscP; Fig. 6), as has been observed for *yopE-dhfr*. Class II mutations [e.g., Δ(*yscM1*, *yscM2*)] disrupted posttranscriptional regulation of the *yop* virulon (29) and restored Yop expression and type III secretion in yersiniae expressing *yopH-dhfr* (Fig. 6). Thus, impassable hybrids generated with three effectors, YopE, YopH or YopQ, are rejected from the type III pathway and fail to block secretion of other substrates.

The *yopR* gene, encoding a type III substrate that is secreted into extracellular media but not injected into host cells (20, 30), was fused to *dhfr*, and *yopR-dhfr* was expressed under control of the IPTG-inducible *tac* promoter in plasmid pJS111. *Y. enterocolitica* W22703 (pJS111) cultures were induced for type III secretion via chelation of calcium ions and temperature shift from 26°C to 37°C. Cultures were centrifuged, and proteins in extracellular media (S, supernatant) or bacterial sediments (P, pellet) were detected by Coomassie-stained SDS/PAGE or

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Abbreviations: DHFR, dihydrofolate reductase; IPTG, isopropyl β-D-thiogalactoside; TTSS, type III secretion system; NPT, neomycin phosphotransferase.

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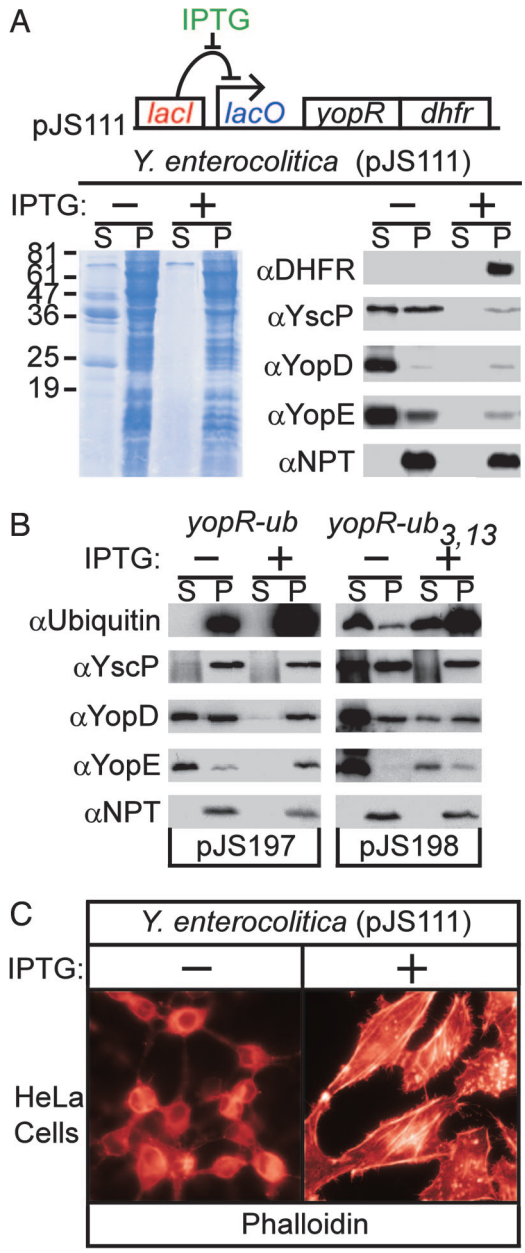

Fig. 1. YopR-DHFR blocks the *Yersinia* type III pathway. (*A*) *Y. enterocolitica* W22703 expressing *yopR-dhfr* (pJS111) under control of the *tac* promoter was induced for type III secretion in the presence or absence of IPTG, inducer of the impassable YopR-DHFR hybrid. After centrifugation of culture aliquots, proteins in the supernatant (S) and the bacterial pellet (P) were separated by SDS/PAGE and stained with Coomassie or transferred to PVDF membrane and immunoblotted with antibodies (α) specific for DHFR, YscP, YopD, YopE, and NPT. (*B*) *Y. enterocolitica* W22703 expressing *yopR-ub* (pJS197) or *yopR-ub*$_{3,13}$ (pJS198 carrying mutations that destabilize the ubiquitin fold) were induced for type III secretion in the presence or absence of IPTG and analyzed as described above. (*C*) HeLa cell infection with *Y. enterocolitica* (pJS111) in the presence or absence of IPTG. F-actin was stained with Texas red-conjugated phalloidin to reveal cell rounding and actin rearrangement as a measure for type III effector injection.

immunoblotting. YopR-DHFR completely blocked secretion of all substrates (YscP, YopD, and YopE) (Fig. 1*A*).

To examine whether *yopR-dhfr* altered expression of type III substrates or machine components in yersiniae, the abundance of YopD, YopE, and YscP was determined in bacterial cultures that had been grown in the presence or absence of IPTG. IPTG induction of *yopR-dhfr* reduced the expression of *yopD* (15% ± 2%) and *yopE* (13% ± 4%) as compared with bacterial cultures without IPTG induction (Fig. 6). Reduced expression of *yopD* and *yopE*, but not the blockade of the *Yersinia* type III secretion pathway, was in part relieved in yersiniae harboring class II regulatory mutations [e.g., Δ(*yscM1*, *yscM2*): *yopD* (36% ± 15%) and *yopE* (48% ± 10%)] (Fig. 6). IPTG induction of *yopR-dhfr* reduced expression of *yscP* by 2-fold (data not shown). Thus, even though YopR-DHFR can cause 10- or 2-fold reduction in the expression of type III substrates or machine components (depending on the strains examined), these changes in gene expression are unlikely to account for the observed blockade in type III secretion.

To test whether tight folding of impassable YopR hybrids is responsible for this blockade, *yopR* was fused to ubiquitin, either wild-type *ub* or *ub*$_{3,13}$, carrying mutations that destabilize the folded structure of its product (24). IPTG-induced expression of *yopR-ub*, but not of *yopR-ub*$_{3,13}$, blocked secretion, indicating that the type III secretion system (TTSS) is indeed blocked by the tightly folded structure of these hybrid proteins (Fig. 1*B*).

YopR-DHFR Blocks *Yersinia* Type III Injection of Effector Yops. To test whether YopR-DHFR blocks *Yersinia* transport of effectors into host cells, HeLa cells were infected with *Y. enterocolitica* W22703 (pJS111). Once inside host cells, effector Yops trigger depolymerization of the actin cytoskeleton and cell rounding, which can be revealed by phalloidin staining and fluorescence microscopy (31). Upon infection with *Y. enterocolitica* W22703 (pJS111), HeLa cells displayed rounding and actin filament rearrangements only in the absence, but not in the presence, of IPTG, indicating that YopR-DHFR blocks TTSS transport of effector Yops (Fig. 1*C* and Fig. 7, which is published as supporting information on the PNAS web site).

YopR Secretion Signal. YopR sequences were examined for their ability to promote secretion of translationally fused neomycin phosphotransferase (*npt*), a reporter protein that can be secreted via the TTSS (32). Full-length YopR$_{1–163}$-NPT was secreted into the culture medium, similar to wild-type YopR (data not shown). Stepwise truncations of 3′ (C-terminal) coding sequence generated YopR$_{1–100}$, YopR$_{1–75}$, YopR$_{1–50}$, and YopR$_{1–25}$ fusions to NPT, all of which were secreted (data not shown). YopR$_{1–15}$-NPT, YopR$_{1–14}$-NPT, YopR$_{1–13}$-NPT, YopR$_{1–12}$-NPT, and YopR$_{1–11}$-NPT also were secreted; however, removal of another amino acid in YopR$_{1–10}$-NPT or any further truncation, as in YopR$_{1–9}$-NPT or YopR$_{1–8}$-NPT, abolished secretion (Fig. 2*A*). Previous work mapped the secretion signals of effector Yops encoded by *yopE*, *yopQ*, and *yopN* (33–35). Two types of mutations suggest that the secretion signals of *yopE*, *yopQ*, and *yopN* may be decoded, at least in part, by means of features of their mRNA. Frame-shift mutations that alter the ORF (and amino acid sequence) of these secretion signals do not impair their function (32); however, synonymous substitutions that change mRNA, but not amino acid sequence, abolish signal function (36). The *yopR* signal does not seem to display such features, as the product of a frame-shift mutation was not secreted; *yopR*$_{1–15,+1}$-*npt* carries a single nucleotide insertion after the start codon and the appropriate suppressor at the *npt* fusion site (Fig. 2*B*). Further, *yopR*$_{1–15,wobble}$-*npt*, carrying 17 synonymous substitutions in the first 15 codons of *yopR*, generated a fusion protein that was secreted (Fig. 2*B*). Thus, in contrast to the secretion signal of effector Yops, neither frame-shift mutation nor synonymous substitutions point to the involvement of mRNA in *yopR* secretion signaling. These results are consistent with a model whereby the first 11 aa of YopR function as a secretion signal.

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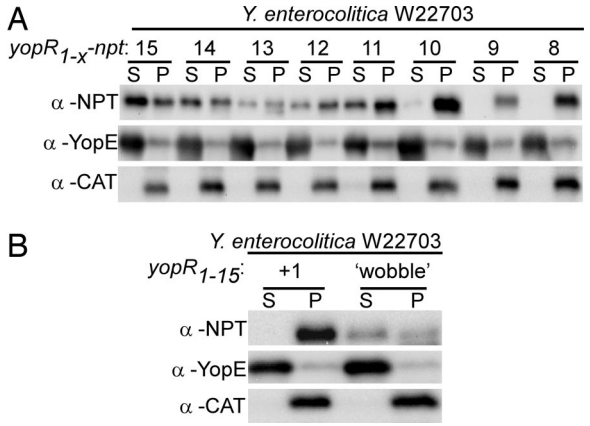
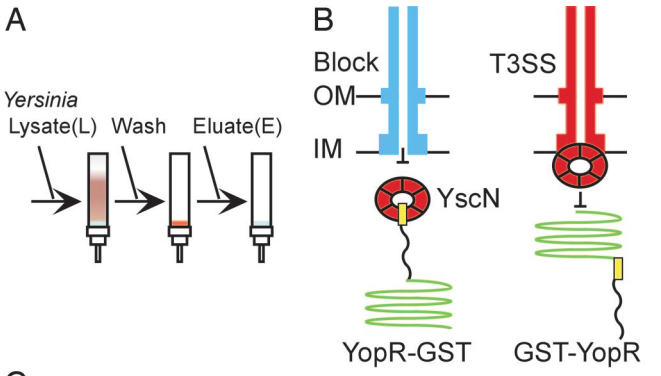


Fig. 2. The YopR secretion signal. *yopR* nucleotide sequences with successive 3′ truncations were fused in-frame to *npt*, and hybrid YopR-NPT was expressed in *Y. enterocolitica* W22703. (*A*) $YopR_{1–15}$-NPT (15), $YopR_{1–14}$-NPT (14), $YopR_{1–13}$-NPT (13), $YopR_{1–12}$-NPT (12), and $YopR_{1–11}$-NPT (11) were secreted, whereas $YopR_{1–10}$-NPT (10), $YopR_{1–9}$-NPT (9), $YopR_{1–8}$-NPT (8) were not. (*B*) $yopR_{1–15,\ [infi]\ +1}$-*npt* harbors a frame-shift mutation (insertion of an adenosine nucleotide immediately after the start codon) and a suppressor mutation at the fusion site with *npt*, which restores the proper reading frame. $yopR_{1–15,\ [infi]\ mut}$-*npt* carries 17 synonymous nucleotide substitutions in *yopR* codons 2–15. After centrifugation, proteins in the supernatant (S) and the bacterial pellet (P) were separated by SDS/PAGE, transferred to PVDF membrane, and immunoblotted with antibodies against YopE, chloramphenicol acetyltransferase (CAT), and NPT.

Impassable YopR Hybrids Bind YscN ATPase. To explore whether the TTSS blockade imposed by YopR hybrids is caused by their physical interaction with specific machine components, plasmids were constructed that express *yopR-gst* (pJS116), *gst-yopR* (pJS154), or $yopR_{\Delta 2–15}$-*gst* (pJS174) under control of the IPTG-inducible *tac* promoter. Plasmids were transformed into *Y. enterocolitica* W22703, and bacterial cultures were analyzed for secretion. As expected, IPTG induced expression of *yopR-gst* blocked secretion, whereas expression of *gst-yopR*, $yopR_{\Delta 2–15}$-*gst*, or *yopE-gst* did not (Fig. 3). *Yersinia* harboring GST hybrids were lysed, membranes were sedimented, and cleared lysates were subjected to affinity chromatography. Load and eluate fractions were analyzed by SDS/PAGE and immunoblotting (Fig. 3*C*). YopR-GST copurified with YscN, the ATPase that generates energy for the TTSS (37, 38), whereas GST-YopR, $YopR_{\Delta 2–15}$-GST, or YopE-GST did not. YopR-GST affinity chromatography captured most of the soluble YscN species in cleared lysates of yersiniae (data not shown). YscN associates with YscL, YscK, and YscQ in the bacterial cytoplasm (39, 40); however, neither these nor any other machine components examined (YscB, YscC, YscD, YscF, YscG, YscI, YscJ, YscK, YscL, YscO, YscP, YscQ, YscR, YscT, YscU, YscX, or YscY) copurified with YopR-GST and YscN (data not shown).

YopR Hybrids Interact with YscN ATPase *in Vitro*. To examine whether purified YscN recognizes the YopR signal peptide *in vitro*, $_{His}$YscN (40), YopR-GST, GST-YopR, and $YopR_{\Delta 2–15}$-GST were purified from recombinant *Escherichia coli* (Fig. 4*A*). By monitoring ATPase cosedimentation with hybrids immobilized on glutathione-Sepharose, we observed $_{His}$YscN binding to YopR-GST, its *in vivo* substrate, but not to GST-YopR or $YopR_{\Delta 2–15}$-GST, hybrids without substrate properties (Fig. 4*B*). Thus, the secretion signal of YopR-GST appears to promote physical association between YopR and YscN, a family member of the type III ATPases that unfold substrates and promotes TTSS transport (38, 41, 42).



C

Yersinia strains	Glutathione-Agarose Chromatography									Block?	
	α -GST			α -YopH			α -YscN			α -YopE	
	L	E	E/L	L	E	E/L	L	E	E/L	S	P
yopR-gst			1.30			0.00			0.35		
gst-yopR			1.59			0.00			0.00		
$yopR_{\Delta 2-15}$-*gst*			1.58			0.00			0.00		
yopE-gst			2.35			0.00			0.00		

Fig. 3. YopR-GST copurifies with YscN, the type III secretion ATPase. *Y. enterocolitica* W22703 expressing *yopR-gst* (pJS116), *gst-yopR* (pJS154), $yopR_{\Delta[infi]2–15}$-*gst* (pJS174), and *yopE-gst* (pJS168) were induced for type III secretion. (*A*) Cells were harvested by centrifugation and lysed via a French pressure cell (L, lysate). The 100,000 × *g* supernatant was subjected to affinity chromatography on glutathione-Sepharose. Columns were washed and eluted with 10 mM glutathione (E, eluate). (*B*) Diagram displays productive (YopR-GST) and nonproductive (GST-YopR) interactions between impassable hybrids [YopR secretion signal (yellow box) and C-terminal domain (black), GST (green)], YscN ATPase (red, hexameric ring), and type III machines [TTSS active (red); TTSS blocked (blue)] that are positioned in the inner membrane (IM) and outer membrane (OM) envelope of yersiniae. (*C*) Samples were separated by SDS/PAGE and immunoblotted with antibodies specific for GST, YscN, and YopH. Block in type III secretion was analyzed by immunoblotting culture supernatants (S) and bacterial pellet (P) fractions for YopE.

Impassable YopR Hybrids Block the Type III Secretion Pathway at an Early Stage. Temperature shift and chelation of calcium are inducing signals that activate *Yersinia* TTSS assembly. Synchronized assembly occurs when yersiniae grown at 26°C are diluted into fresh media at 37°C without calcium, and such newly formed type III machines then are blocked by the expression of *yopR-dhfr* (Fig. 5 *A* and *B*). As *Yersinia* grow and generate new needle complexes, synchrony is lost, and bacterial cultures that were induced (37°C without calcium) for 2 h are expected to harbor both fully assembled TTSS engaged in secretion of middle and late substrates (YopD and YopE) as well as machines undergoing assembly and secretion of early substrates (YscP) (Fig. 5*B*).

What happens when YopR-DHFR is expressed in bacterial cells harboring type III machines at all stages of assembly and function? To address this question, we compared the effect of YopR-DHFR expression in cultures with synchronized type III assembly (simultaneous induction via IPTG and 37°C without calcium) and nonsynchronized secretion (2 h delayed induction). As expected, simultaneous induction of type III assembly and YopR-DHFR blocked secretion of early (YscP), middle (YopD), and late (YopE) substrates. On delayed induction, YopR-DHFR did not block secretion of YopD (middle) and YopE (late substrate); however, YscP secretion was dramatically reduced (Fig. 5 *B* and *C*). Thus, the impassable hybrid YopR-

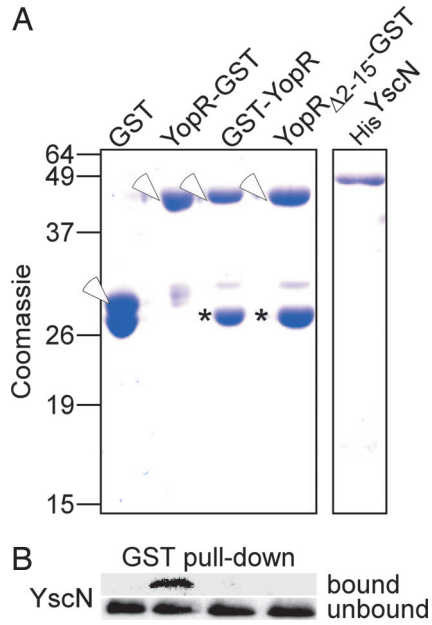


Fig. 4. YscN binds YopR-GST in a secretion signal-dependent manner. (*A*) GST, YopR-GST, YopR$_{\Delta 2-15}$-GST, GST-YopR, and $_{His}$YscN were purified from recombinant *E. coli* strains, and aliquots were separated by Coomassie-stained SDS/PAGE. (*B*) $_{His}$YscN binds YopR-GST immobilized on glutathione-Sepharose, but not GST, YopR$_{\Delta 2-15}$-GST, or GST-YopR. Arrowheads point to purified full-length GST, YopR-GST, GST-YopR, and YopR$_{\Delta 2-15}$-GST; asterisks identify C-terminal GST fragments that appear to arise from proteolysis during purification.

DHFR appears to block the type III machine only at a time when early substrates are being recognized.

Discussion

Type III machines function as conduits for the transport of proteins from bacteria into host cells (43). Assembly of type III machines occurs via deposition of secretion substrates at the distal tip of the growing conduit (needle complexes) (13). In *Yersinia* spp. these include the early substrates YscP and YscF (needle protein) as well as middle substrates LcrV (needle tip protein), YopB, and YopD, which together comprise a functional complex named injectisomes (19). What are the underlying principles that faithfully enable ordered assembly and transport of early, middle, or late substrates? Earlier work characterized *Yersinia* mutants that cannot complete the type III conduit into host cells (18, 22). Mutations that abolished expression of early or middle substrates also blocked the pathway at discrete steps (44). These experiments, however, did not reveal whether ordered recognition and initiation of substrates into the secretion pathway enables type III machine assembly. Alternatively, the three-dimensional fit of proteins that are eventually deposited into quaternary assemblies of the conduit may be the principal mechanism for ordered assembly. In the latter model, type III machines may not be required to establish an order for the secretion of substrates; proteins that "fit" at the end of the conduit can be deposited while a disordered stream of substrates travels along the pathway.

To distinguish between these possibilities, we sought to generate impassable hybrids with the goal of blocking type III secretion. Our goal was simple: if impassable hybrids blocked all secretion, type III machines obviously would not be able to distinguish between substrates. On the other hand, if impassable hybrids blocked secretion of only some (but not all) proteins traveling the pathway, this would provide important evidence for ordered recognition and initiation of substrates.

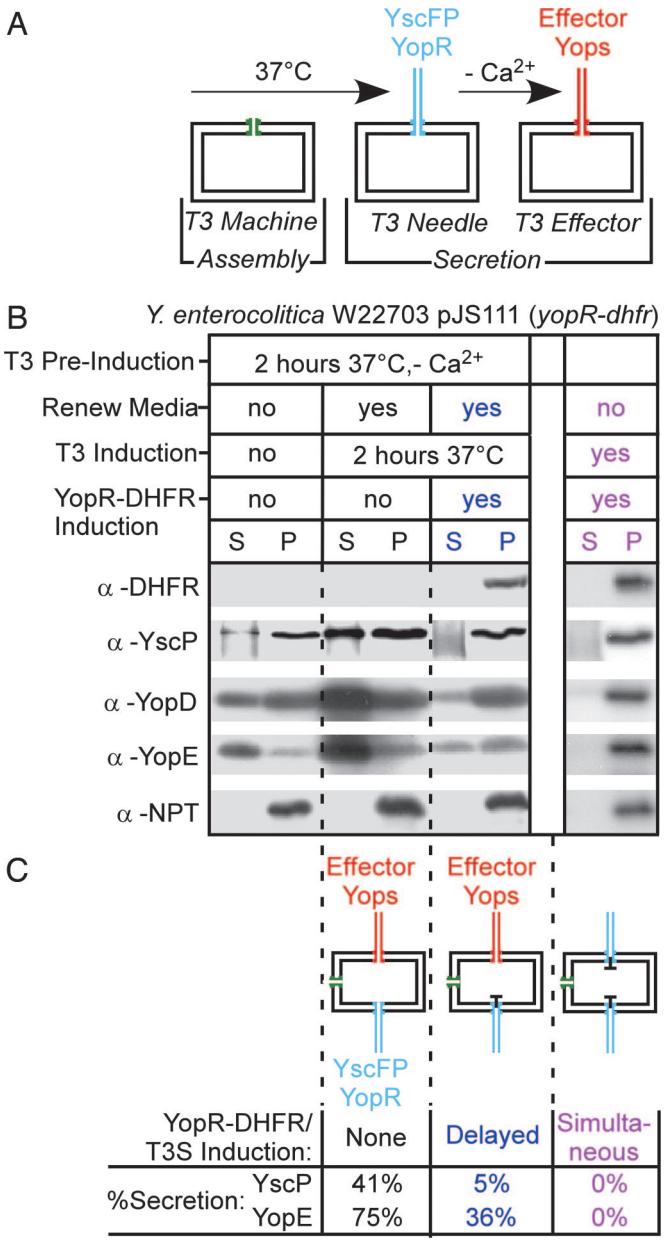


Fig. 5. YopR-DHFR blocks type III secretion of early but not late substrates. (*A*) Diagram displays type III machine assembly (green), early/middle [YscF, YscP, and YopR (blue)], and late [YopE (red)] secretion events. (*B*) *Yersinia* cultures were preinduced for type III secretion without the addition of IPTG (T3 Pre-Induction, 2 h at 37°C, $-Ca^{2+}$). As a control, a culture was simultaneously induced for *yopR-dhfr* expression with IPTG and for type III secretion (no T3 Pre-Induction as in Fig. 1). Two cultures grown without IPTG were aliquoted, centrifuged, suspended in fresh media (Renew Media) with or without IPTG (YopR-DHFR Induction), and incubated for an additional 2 h. As another control, a preinduced culture was incubated for another 2 h without the addition of IPTG. All cultures finally were centrifuged, and supernatant (S) and bacterial pellets (P) were precipitated with trichloroacetic acid and analyzed by immunoblotting for YscP, YopD, YopE, DHFR, and NPT. (*C*) Diagram displays the effects of no YopR-DHFR induction (no IPTG and no blockade), simultaneous induction (IPTG and 37°C at the same time), and delayed induction (IPTG 2 h after 37°C induction) on the secretion of YscP and YopE.

Initial efforts at addressing this question were fruitless and somewhat uninformative: fusion of effector Yops to reporter proteins generated impassable hybrids that were rejected from the type III pathway without blocking the secretion of other

substrates (25, 28). Secretion signals of YopE, YopN, and YopQ harbor unique features that may be decoded, at least in part, by signaling properties of their mRNA. These signal properties may enable type III machines to reject impassable hybrids from the pathway (45). In this report, we identified YopR fusions to impassable reporter molecules as the first hybrids capable of blocking type III secretion. Impassable YopR fusions block the entire pathway when present during type III machine assembly, presumably because the hybrids interfere with secretion of an essential early machinery component (YscP). In contrast, type III machines that are already engaged in transporting late substrates (effector Yops) cannot be blocked by impassable YopR hybrids. Thus, as YopR hybrids block the secretion of some, but not all, substrates, these data suggest that ordered recognition and initiation of substrates functions as an important mechanism of type III machine assembly.

YopR can be viewed as an early or middle substrate of the type III pathway. YopR is encoded by *yscH*, which is located in the *virC* operon (also specifying other machine components) (20). During tissue culture infection, YopR is secreted into extracellular media, and, although *yscH* is dispensable for assembly of the type III conduit (20), secreted YopR contributes virulence properties that aid in the pathogenesis of *Yersinia* infections (30). YopR harbors an N-terminal secretion signal that promotes type III secretion and is absolutely necessary for the type III blockade of YopR hybrids. This blockade may be caused by binding of YopR hybrids to YscN, the ATPase that is involved in substrate recognition and in generating energy for type III secretion reactions (38, 46). If one assumes that impassable hybrids capture translocase components involved in substrate recognition, YopR binding to YscN could represent an example for such interaction in bacterial type III machines.

The absence of a chaperone in YopR-GST/YscN complexes purified from *Yersinia* or the requirement for one in the reconstituted complexes *in vitro* seems surprising. Reports from several different type III systems suggest that chaperones are essential in mediating contacts between the ATPase and effectors (38, 41, 47). Another unique feature of the *Y. enterocolitica* secretion system appears to be the regulatory nature, blocking the synthesis or passage of other substrates, of tightly folded proteins fused to secreted proteins; similar fusion proteins in *Salmonella* have no effect on secretion (38). A more profound understanding of the regulatory context of secretion in these two systems will hopefully reconcile these observations.

Flagellar basal bodies, close relatives of type III machines, and *Yersinia* TTSS employ a morphogenetic switch to change substrate specificity (48, 49). Essential for morphogenesis is physical association of FlhB (YscU), a machine component, and FliK, a regulatory factor and early secretion substrate that determines the length of the flagellar hook (50). *Yersinia* YscP is the corresponding factor regulating needle length (21). Autoproteolytic cleavage of FlhB (YscU) flips the switch (51, 52), causing TTSS thenceforth to recognize only late substrates. Machines that have flipped the YscU switch and engage late substrates probably cannot recognize YopR as substrate and are therefore not blocked by YopR-DHFR. In contrast, impassable hybrids generated via fusion of effector Yops cannot block TTSS and are rejected from the secretion pathway without blocking type III machines (Fig. 6). Differences between YopR and effector hybrids (YopE, YopH, and YopQ) in blocking the pathway appear to be caused by differences in secretion signals and the mechanisms of substrate recognition for both TTSS and flagellar basal bodies (28, 53, 54).

Materials and Methods

Bacterial Strains. *Y. enterocolitica* strains W22703 (55) and EC2 [Δ(*yscM1/2*)] (56) were grown in brain heart infusion broth (BHI) or M9-casamino acids medium (M9-Ca) as described previously (28). Kanamycin (35 μg/ml) and chloramphenicol (30 μg/ml) were added to cultures to retain plasmids. *E. coli* DH5α was used for molecular biology experiments, and kanamycin (35 μg/ml) or chloramphenicol (30 μg/ml) was used for plasmid retention (57).

Molecular Biology Experiments. Moderate copy-number plasmid pJS95 is a pBBR1MCS-2 derivative (28). *yopH*, *yopQ*, and *yopR* genes were amplified via PCR by using *Taq*DNA polymerase with the primer set 5′YopH-NdeI (5′-AACATATGAACTTATCATTAAGCGATCTTCACGT-3′) and 3′YopH-BglII (5′-AAAGATCTGCTATTTAATAATGGTCGCCCTTGTCC-3′), 5′ YopQ-NdeI (5′-AACATATGTTTATTAAAGATACTTATAACATGCGTGCTT-3′), and 3′YopQ-BglII (5′-AAAGATCTTCCCATAATACATTCTTGATCGCAG-3′), 5′YopR-NdeI (5′-AACATATGACGGTTACCCTTAATAGAGGTTCCAT-3′) and 3′YopR-BglII (5′-AAAGATCTTGTATCCATATCAATTTGATGGCTGTTATGAA-3′), and they were ligated into pCR2.1 (Invitrogen, Carlsbad, CA). *yopH*, *yopQ*, and *yopR* were liberated from the resulting constructs by using NdeI and BglII, and each was ligated into pJS95 cut with NdeI and BglII to yield pJS108, pJS110, and pJS111, respectively. Smaller *yopR* hybrids were amplified with *Taq*DNA polymerase by using 5′YopR-NdeI and 3′YopR150-BglII (5′-AAAGATCTAATCGAGTTAGACGGTAATAGGATCA-3′), 3′YopR100-BglII (5′-AAAGATCT CAATACACTACGCAATTCAGGTAAATCTA-3′), and 3′YopR50-BglII (5′-AAAGATCT CAGAACTTCCCGTGTCTTTTCA-3′), and they were cloned into pCR2.1. These were digested with NdeI and BglII and cloned into pJS95 to generate pJS123, pJS124, and pJS125, respectively.

The low copy-number plasmid pDA46 is a pHSG576/pSC101 derivative (32). The secretion signal of *yopR* and variations thereof were generated by annealed oligo cloning and ligated into pDA46 cut with NdeI and KpnI.

gst was amplified from pGEX2TK with *Taq*DNA polymerase by using primers 5′GST-BglII (5′-AAAGATCTTCCCCTATACTAGGTTATTGGA-3′) and 3′GST-XbaI (5′-AATCTAGATTAAACAGATGCACGACGAGATC-3′) and cloned into pCR2.1. The recombinant plasmid then was digested with BglII and XbaI and cloned into pJS111 to yield pJS116 (*yopR-gst*). *gst* also was PCR-amplified from pGEX2TK with pfu DNA polymerase by using the primers 5′GST-NdeI (5′-GGGAATTCCATATGTCCCCTATACTAGGTTATTGGAAAATTAA-3′) and 3′ GST-XbaI 3 (5′-AATCTAGAGTCACGATGAATTCCCGGGGAT-3′). *yopR* was PCR-amplified with pfu DNA polymerase by using the primers 5′YopR-XbaI (5′-AATCTAGAATGACGGTACCCTTAATAGAGGTTCCAT-3′) and 3′YopR-HindIII (5′-AAAAGCTTTTATGTATCCATATCAATTTGATGGCTGTTATGAA-3′). The resulting PCR products were digested with NdeI/XbaI and XbaI/HindIII, respectively, and three-way ligated into pJS116 cut with NdeI and HindIII to yield pJS154 (*gst-yopR*).

For a full listing of the plasmids and primers used in this study, see Tables 1 and 2, which are published as supporting information on the PNAS web site.

Type III Secretion. Overnight, *Yersinia* cultures were diluted 50-fold into fresh brain heart infusion broth (BHI) supplemented with antibiotic, 20 mM oxalic acid, and 20 mM $MgCl_2$ (low-calcium conditions) or M9-Ca, grown for 2 h at 26°C, and then shifted for 3 h to 37°C to induce type III secretion. Where indicated, IPTG was added to cultures to induce expression of hybrid DHFR or GST. Fractionated cultures were analyzed for type III secretion.

Cytotoxicity Assay. HeLa cell tissue cultures, 4×10^5 cells, were grown on glass coverslips in a 12-well tissue culture dish and

infected as described above. After 3 h of infection, media were removed, and the cells were fixed with 3.7% formaldehyde in PBS for 20 min on a rotary shaker. Fixation was quenched with 0.1 M glycine in PBS for 5 min. Cells were permeabilized with 0.1% Triton X-100 in PBS for 30 min and then blocked for 15 min in PBS containing 5% skim milk and 0.05% Tween 20. Filamentous actin was labeled with 165 nM Texas red-conjugated phalloidin in PBS containing 5% skim milk and 0.05% Tween 20 for 20 min. The labeling solution was removed, and each well was washed four times with 1 ml of PBS, the buffer was aspirated, and samples were dried for 1 h. Coverslips were affixed to glass slides and visualized with an Olympus (Center Valley, PA) AX RL module microscope. Texas red-phalloidin visualization was achieved through excitation (591 nm) and emission (608 nm) with a U-MNG cube. Images were captured with a Hamamatsu Ocra digital camera.

Affinity Chromatography. Freshly diluted *Yersinia* cultures were grown at 26°C for 2 h. *gst* expression was induced by addition of IPTG, and type III secretion was induced by incubation at 37°C for 3 h. Bacteria were sedimented by centrifugation, suspended in lysis buffer (50 mM Tris·HCl/150 mM NaCl, pH 7.5), and lysed in a French pressure cell at 14,000 psi. Cleared lysates, obtained via ultracentrifugation at 100,000 × *g* for 60 min, were applied to glutathione-Sepharose chromatography (Amersham Biosciences). Columns were washed with 20 column volumes lysis buffer, and bound protein was eluted with lysis buffer containing 10 mM glutathione.

YscN Pull-Down. YscN was purified as reported previously (40). GST was purified from *E. coli* DH5α, whereas YopR fusions to GST (encoded by plasmids pJS116, pJS154, and pJS174) were induced in *E. coli* CA8000. Pellets from strains containing GST were subjected to French pressure lysis in the presence of 150 mM NaCl/50 mM Tris·HCl, pH 7.5. Lysate was clarified by centrifugation (10 min at 10,000 × *g*) and applied to glutathione-Sepharose beads. After thorough washing, the beads were taken up in the same volume of lysis buffer. Bead slurry (100 μl) was mixed with 50 μl of purified YscN in lysis buffer supplemented with 2 mM $MgCl_2$, 0.05% Tween 20, and 0.001% BSA. After 30 min of incubation at room temperature, the mixture was centrifuged (30 sec at 5,000 × *g*), supernatant was removed, beads were washed three times, and protein was eluted with sample buffer. The purified components were present at concentrations of 3.25, 2.06, 1.37, 1.36, and 0.21 mg/ml for bead slurries containing GST, YopR-GST, $YopR_{\Delta 2-15}$-GST, GST-YopR, and purified YscN, respectively.

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