A mRNA Signal for the Type III Secretion of Yop Proteins by *Yersinia enterocolitica*

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Pathogenic *Yersinia* species have a specialized secretion system (type III) to target cytotoxic Yop proteins during infection. The signals of YopE and YopN sufficient for the secretion of translational reporter fusions were mapped to the first 15 codons. No common amino acid or peptide sequence could be identified among the secretion signals. Systematic mutagenesis of the secretion signal yielded mutants defective in Yop translation; however, no point mutants could be identified that specifically abolished secretion. Frameshift mutations that completely altered the peptide sequences of these signals also failed to prevent secretion. Thus, the signal that leads to the type III secretion of Yop proteins appears to be encoded in their messenger RNA rather than the peptide sequence.

Secretion of Yop proteins during the pathogenesis of human or animal infections allows *Yersinia* species to evade phagocytic killing by macrophages (*1*). After establishing contact with specific host cells, *Yersinia* target some Yop proteins directly into the eukaryotic cytosol where these virulence factors exert their cytotoxic functions (*2–4*). This type III secretion of Yop proteins is thought to occur as a continuous translocation of polypeptide across the inner and outer membranes of the bacterial envelope (*5*). *Yersinia* export 12 different Yop proteins by this pathway (*1*); however, no common secretion signal within the amino acid sequences of these polypeptides has been identified (*6*, *7*). This feature clearly distinguishes type III secretion from other export pathways in which the secretion signals of substrate proteins are readily apparent on the basis of common peptide sequences, structures, or physical properties (*8–10*). To determine whether Yop proteins are marked for secretion by a covalent posttranslational modification, we purified, sequenced, and measured the mass of secreted YopE. The results indicated that YopE is not modified upon export by the *Yersinia* type III machinery (*11*).

Several other Gram-negative pathogens also target their cytotoxic proteins into eukaryotic host cells (*12*). Bacterial contact with the target cell induces expression of the otherwise tightly regulated export machinery and secretion substrates (*4*). Many components of the type III machinery are highly conserved among Gram-negative bacteria (*13*). Substrate proteins from one organism can be exported by heterologous pathogens, suggesting a universal mechanism for secretion (*14*). The NH_2-terminal 15 to 17 amino acids of Yop proteins have been proposed to function as a secretion signal; however, in the absence of a common peptide, it has been unclear how these signals can be universally recognized (*7*, *15*). We therefore sought to characterize the secretion signal through genetic and biochemical means. We studied two type III secretion substrates, YopE and YopN, in *Yersinia enterocolitica* by analyzing translational fusions to cytoplasmic neomycin phosphotransferase (Npt) (*16*).

To identify the minimal secretion signal of YopN, we fused NH_2-terminal coding sequences to Npt (*17*). Secretion of the hybrid proteins was measured by immunoblot analysis of the sedimented cells or medium of *Yersinia* cultures induced by temperature shift (37°C) and low calcium concentration (*18*). As reported for YopE (*7*, *15*), the first 15 codons of YopN still allowed secretion of the fused reporter protein (*19*), whereas truncating this signal to 10 codons abolished secretion (Fig. 1). Relative rates of polypeptide synthesis were analyzed by pulse labeling and compared with those of another type III secretion substrate, YopH (*20*). Truncating the YopE signal to 10 codons caused a reduction in synthesis of the fusion protein. This reduction was apparently caused by an inhibition of translation, because the relative amount of mRNA for this fusion protein was similar to that observed for the Npt hybrid containing the first 15 codons of YopE (*21*). We investigated whether the secretion signal of YopE functioned when moved from the NH_2-terminus by constructing a hybrid Npt protein that contained YopE fused to its COOH-terminal end. This hybrid was not secreted, indicating that the secretion signal is only functional when located at the translational start (Fig. 1).

To determine if single amino acid residues of the YopE and YopN signals were critically important for secretion, we individually replaced codons 2 to 15 with GCA or GCU, which both specify alanine. The alanyl substitutions had little effect on secretion of the hybrid Npt proteins (Table 1). However, as measured by pulse labeling, YopE signal mutants with substitutions at positions 2 and 15 were synthesized at lower rates (<50%), and GCA replacement at codon 4 ($YopE_{4S-A}$-Npt) completely abolished translation. We also individually mutagenized codons 2 to 15 of the YopE signal by substitution with GAG encoding glutamic acid. Substitution of hydrophobic or positively charged amino acids with this strongly acidic residue did not affect secretion of the mutant proteins, but replacement of codons 2, 3, 4, 10, or 12 caused a reduction in polypeptide synthesis (<50%).

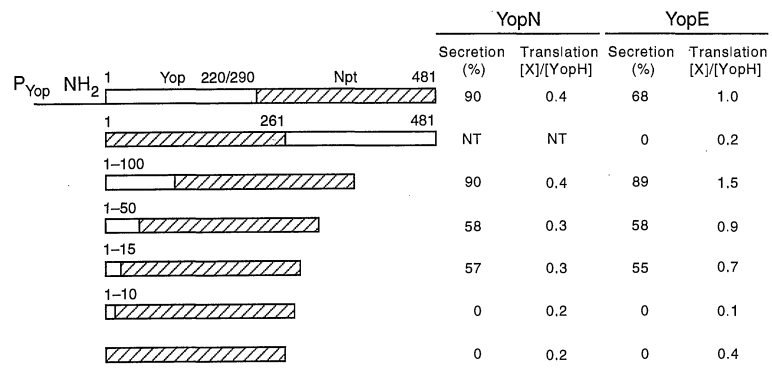

Fig. 1. Secretion signals located within codons 1 through 15 of YopE and YopN. Schematic diagram of hybrid proteins consisting of YopN, YopE, or their truncated derivatives with fused neomycin phosphotransferase (Npt). All constructs were expressed from their wild-type promoter (YopN or YopE) in *Y. enterocolitica*. Numbers refer to the respective codon positions. Secretion was measured by immunoblotting of the medium and cell sediment of induced *Yersinia* cultures and is reported as the percentage of total protein that is secreted. The relative synthesis of polypeptides [X]/[YopH] was analyzed by comparing immunoprecipitated substrates after pulse labeling with the amount of immunoprecipitated YopH. NT, not tested.

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Table 1. Scanning mutagenesis of the secretion signal of $YopE_{1-15}$-Npt (pDA46) and $YopN_{1-15}$-Npt (pDA85). Individual codons of the wild-type sequence were replaced with those encoding either alanine or glutamic acid. The secretion data were collected by separating culture medium from sedimented cells and immunoblotting with anti-Npt. The relative amount of synthesized fusion protein [X] was measured by pulse labeling and is reported as the ratio to another type III secreted protein [YopH]. ND, not determined.

Residue	YopE				YopN	
	Ala (GCA)		Glu (GAG)		Ala (GCU)	
	Secretion (%)	[X]/[YopH]	Secretion (%)	[X]/[YopH]	Secretion (%)	[X]/[YopH]
2	50	0.3	75	0.3	49	0.1
3	29	0.7	44	0.2	63	0.1
4	ND	0	33	0.2	51	0.2
5	42	1.0	53	0.4	44	0.2
6	47	0.5	53	0.4	62	0.2
7	56	0.6	50	0.5	46	0.2
8	38	0.8	53	0.4	55	0.2
9	62	0.4	51	0.4	54	0.1
10	63	0.4	46	0.3	47	0.2
11	43	0.4	39	0.4	61	0.2
12	68	0.5	51	0.3	63	0.2
13	57	0.5	38	0.7	55	0.1
14	48	0.6	55	0.4	63	0.2
15	47	0.3	57	0.4	58	0.2
WT	53	0.7			50	0.1

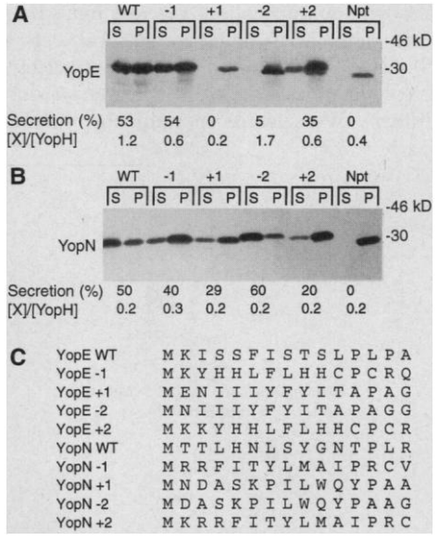

Fig. 2. Frameshift mutations of the secretion signals of YopN and YopE. Translational reading frameshifts were constructed by either deleting (−1, −2) or inserting nucleotides (A or G) (+1, +2) immediately after the AUG start codon of $YopE_{1-15}$-Npt (**A**) or $YopN_{1-15}$-Npt (**B**). The correct reading frame was restored by a reciprocal change at the fusion site with Npt. Secretion was measured by immunoblotting and is indicated as the percentage of secreted protein. Npt alone expressed from the YopE or YopN promoter was not secreted. [X]/[YopH] indicates relative levels of polypeptide synthesis as measured by pulse labeling and immunoprecipitation. The altered peptide sequences of the frameshift mutants are compared with those encoded by the wild-type secretion signals (**C**).

We sought to identify mutations that abolished substrate recognition of the type III machinery by drastically modifying the polypeptide sequence of the secretion signals. We constructed frame-shift mutations by inserting or deleting nucleotides immediately after the AUG start codon. The correct reading frame was restored by reciprocal nucleotide insertions or deletions at the fusion site with Npt. The secretion signals of both YopE and YopN tolerated several frameshift mutations, and the altered polypeptides were still secreted (Fig. 2). For YopE, deleting one nucleotide (−1) or adding two nucleotides (+2) did not prevent the secretion of hybrid proteins. In contrast, mutations shifting to the third reading frame (+1, −2) abolished secretion, and the Npt hybrids remained in the cytoplasm. This reading frame encodes a very hydrophobic NH_2-terminal peptide, a physical property that may interfere with its secretion by the type III machinery (Fig. 2C). For YopN, the +1, −1, +2, and −2 reading frame mutants all allowed secretion. To test whether frameshift mutations resulted in altered amino acid sequences, we purified one mutant protein (YopE −1) from the medium of *Yersinia* cultures and confirmed the predicted sequence by Edman degradation (*22*).

Because several frameshift mutations resulted in proteins that were secreted, we considered that the secretion signal might be located within the mRNA sequence. If this were true, nucleotide changes at the third position of codons that do not alter the protein sequence (*23*) might affect either secretion or translation of the hybrid Npt proteins. We tested codons 2 to 4 of YopN because these positions were sensitive to mutation in the secretion signal of YopE. Single-nucleotide changes at position 2, 3, or 4 of the YopN signal did not affect either translation or secretion (Table 2). However, combined mutations at codons 2 and 4 reduced the amount of mRNA translation, which was restored to wild-type amounts when the mutant RNA contained all three altered codons (Table 2). The reduced concentration of the mRNA with mutations at codons 2 and 4 is likely caused by its increased degradation rather than by an effect on transcription (Table 2).

Table 2. Nucleotide changes at the third position of codon triplets that do not alter the protein sequence of the secretion signal were introduced into $YopN_{1-15}$-Npt. Secretion was measured by immunoblotting of culture supernatants and cell sediment. The relative amount of synthesized fusion protein [X] was measured by pulse labeling and is reported as the ratio to another type III secreted protein [YopH]. [*x*]/[*yopH*] indicates relative levels of mRNA that were observed by RNA blot hybridization.

$YopN_{1-15}$-Npt	Codons 2 to 4	Secretion (%)	[X]/[YopH]	[*x*]/[*yopH*]
Wild type	ACG ACG CUU	50	0.1	0.7
Codon 2	ACC ACG CUU	52	0.1	0.2
Codon 3	ACG ACC CUU	39	0.1	0.3
Codon 4	ACG ACG CUG	64	0.1	0.3
Codons 2 and 3	ACC ACC CUU	46	0.1	0.4
Codons 3 and 4	ACG ACC CUG	48	0.1	0.3
Codons 2 and 4	ACC ACG CUG	40	0.04	0.1
Codons 2 to 4	ACC ACC CUG	45	0.1	0.5

Several mutations in the secretion signals of YopE and YopN either reduced or abolished synthesis of the recombinant proteins. These mutations may hinder Yop translation, for example, by interfering with ribosome binding or translational initiation. Alternatively, this mutational phenotype could represent a defect in the recognition of an mRNA signal that ultimately leads to the secretion of Yop proteins. Suppressor mutations that restore a translational defect of the $YopE_{4S-A}$-Npt mutant (pDA54, GCA replacing TCA at codon 4) should

alter the mutant codon, whereas mutations that suppress a signal-recognition defect could also be located at other positions involved in contacting the secretion machinery. We selected spontaneous mutants by plating *Yersinia enterocolitica* harboring pDA54 on agar medium containing neomycin (*24*). Nine independent mutants were analyzed by immunoblotting; each of them synthesized and secreted the Npt fusion protein in a manner similar to that observed for the wild-type construct. These isolates were intragenic suppressors that contained mutations located at codons 2 through 6 and 12 (Table 3). Transversion of the nucleotide at the third position of codon 12 (CCC to CCA) restored translation and thus secretion of the hybrid protein without an alteration of its amino acid sequence. This mutation was found in every suppressor isolate and was sometimes combined with mutations at codons 2, 4, or 5 or a deletion of codon 6. Although these results do not permit a definitive explanation, we think it is more likely that the mutational change at codon 4 abolished the recognition of an mRNA signal rather than causing a hindrance of translational initiation.

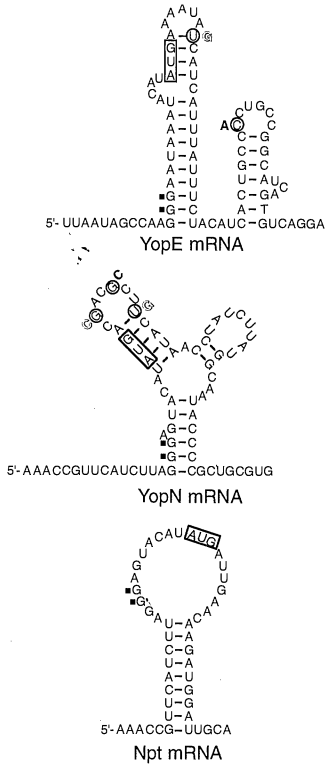

Fig. 3. Predicted RNA structures of the YopE and YopN secretion signals compared with that of the Npt mRNA. RNA sequences were subjected to folding analysis as described (*26*). The displayed structures show an area encompassing the Shine/Dalgarno ribosome binding site (filled squares), start codon (AUG, boxed), and downstream sequence of the YopE and YopN secretion signals [ΔG values (Gibbs energy) of −89.5 kj (YopE) and −102.9 kj (YopN)]. Nucleotides sensitive to mutation are circled. Mutations that abolished synthesis and secretion of reporter proteins are shadowed and their suppressors are indicated in bold.

Other secretion or protein-targeting signals do not tolerate such drastic mutational changes without a loss of function. The reason for this difference may reflect the mode of substrate recognition by the type III machinery. RNA may be the carrier of a signal that ultimately leads to the export of encoded Yop proteins. One possible mechanism is that the mRNA signals cotranslational secretion by the type III machinery. In support of this hypothesis, pulse-chase experiments of *Y. enterocolitica* cultures revealed that YopE was secreted during a short pulse with ^{35}S-methionine but not after the addition of unlabeled methionine, suggesting that secretion occurred during the ribosomal synthesis of YopE (*25*). Yop translation might be inhibited by an intrinsic property of the mRNA that can be relieved by its interaction with the secretion apparatus. Most mutations that affect recognition of an RNA signal would therefore abolish both secretion and translation. An uncoupling of secretion from translation might result from larger deletions of the signal that destroy its structure. We have incorporated some of the mutations described here in predicted RNA structures of the YopE and YopN secretion signals (*26*) (Fig. 3). Common to both structures is a stem loop that buries the AUG translational start in a base-paired duplex while positioning codons 2 to 4 within a loop. Mutations that abolished translation are located either within the predicted loop or its adjacent base pairs, that is, at positions typically recognized by RNA-binding proteins (*27*). Such an RNA structure would have to undergo dynamic changes because it would have to first assume an untranslatable fold, which could then be relieved by specific interaction with components of the secretion machinery.

Table 3. Spontaneous suppressor mutations of $YopE_{4S-A}$-Npt were selected by plating *Y. enterocolitica* harboring plasmid pDA54 ($YopE_{4S-A}$-Npt) on tryptic soy agar plates with neomycin (50 μg/ml). Plasmid was purified from individual colonies and transformed into W22703, and plasmid transformants were selected on chloramphenicol plates. Individual isolates were tested for resistance to neomycin [minimal inhibitory concentration (MIC) for 10^5 cells], relative concentration of mRNA ([*x*]/[*yopH*]), synthesis ([X]/[YopH]), and secretion of hybrid Npt proteins. Mutational changes of the suppressors were determined by DNA sequencing. ND, not determined.

Plasmid	Codons 2 to 7	Codon 12	MIC (μg/ml)	Secretion (%)	[X]/[YopH]	[*x*]/[*yopH*]
pDA46 wild type	AAA AUA UCA UCA UUU AAU	CCC	250	50	1.2	1.7
pDA 54 (4S-A)	AAA AUA GCA UCA UUU AAU	CCC	25	ND	0	0.7
1	AAA AUA GCA UCA UUU AAU	CCA	250	42	1.0	2.3
2	AAA AUA UCA UCA UUU AAU	CCA	250	46	0.8	0.8
3, 4	AAA AUA UCA GAA UUU AAU	CCA	500	46	0.9	1.6
5–8	AAA AUA GCA UAU AAU	CCA	500	43	1.0	1.9
9	AAG AUA GCA UAU AAU	CCA	500	49	1.0	1.5

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11. YopE was purified from the culture supernatant of *Y. enterocolitica* O:8 strain 8081 [D.A. Portnoy *et al.*, *Infect. Immun.* **31**, 775 (1981)] by ammonium sulfate precipitation (46%). The precipitate was solubilized in 6 M guanidine hydrochloride, 0.05 M phosphate buffer, 0.01 M dithiothreitol (pH 7.5) and separated by reversed-phase high-performance liquid chromatography on a C8 column (BDS Hypersil, 4.6 mm by 250 mm) with a linear gradient of acetonitrile from 5 to 95% (1%/min) in 0.1% trifluoroacetic acid. The NH_2-terminal sequence of purified YopE was confirmed by Edman degradation, and the molecule was subjected to electrospray ionization mass spectrometry. An average compound mass of 23,018.75 [mass-to-charge ratio (*m/z*) = 1212, 1280, 1355, 1440, 1645, 1772, 1919)] was observed, in agreement with a calculated compound mass of 23,016.10, given a standard error rate of 0.01% (±2 daltons).
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17. Two plasmids were present in *Y. enterocolitica* W22703: the virulence plasmid (pYV227) and a low-copy number plasmid expressing type III secretion substrates. The *yopE* and *yopN* genes were amplified by the polymerase chain reaction (PCR) from virulence plasmid as three separate DNA fragments, one containing the promoter and upstream untranslated sequences, a middle fragment specifying the open reading frame, and a downstream fragment harboring a putative transcriptional terminator. All fragments were assembled as cassettes and insert-

ed into pHSG575 [S. Takeshita *et al.*, *Gene* **61**, 63 (1987)]. To generate NH_2- or COOH-terminal fusions, we replaced the central Nde I to Bam HI cassette with two fragments joined at a Kpn I site. The *npt* gene [E. Beck *et al.*, *Gene* **19**, 327 (1982)] was amplified by PCR with abutted Kpn I and Bam HI sites. The YopE and YopN fusions were amplified with flanking Nde I and Kpn I sites. Fusions harboring the first 10 or 15 codons of Yop mRNA were created by annealing oligonucleotides with overlapping ends for cloning between the Nde I and Kpn I sites. All constructs were verified by DNA sequencing.

18. Overnight cultures of *Y. enterocolitica* were diluted 1:50 into fresh M9 medium with casamino acids and grown for 2 hours at 26°C before incubation for 3 hours at 37°C. A portion (30 ml) of the induced culture (absorbance at 600 nm of 0.5) was centrifuged at 17,000*g* for 13 min, and 20 ml of supernatant was removed and precipitated with 5% trichloroacetic acid (TCA). The remainder of the supernatant was discarded, and the sedimented material was suspended in water (750 μl). A portion (500 μl) of this suspension was precipitated with ice-cold 10% TCA (500 μl). All TCA precipitates were washed in acetone, dissolved in sample buffer, and analyzed by immunoblotting with rabbit antiserum. Immunoreactive species were identified as a chemiluminescent signal and quantitated by laser-densitometry scanning of developed x-ray films. Immunoblotting for cytoplasmic chloramphenicol acetyltransferase served as an internal control for correct fractionation of *Yersinia* cultures.
19. The location of pulse-labeled YopE-Npt and $YopE_{1-15}$-Npt was investigated with the membrane-impermeable reagent sulfosuccinimidobiotin (Pierce) [M. P. Lisanti *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 9557 (1988)]. The amount of fusion protein covalently modified with sulfosuccinimidobiotin was similar to that found to be soluble in the supernatant of centrifuged *Yersinia* cultures, suggesting that nonsecreted polypeptides remained within the bacterial cytosol. This result was confirmed by fractionating *Yersinia* cultures into medium, periplasm, cytosol, and membrane compartments [N. G. Davis, J. D. Boeke, P. Model, *J. Mol. Biol.* **181**, 111 (1985)] followed by immunoblotting. Similar amounts of fusion proteins were found to be soluble in the culture medium and the bacterial cytosol. Upon ultracentrifugation of cell extracts, a small amount of hybrid protein sedimented together with the membranes, which may result from its association with the type III secretion machinery.
20. Overnight cultures of *Yersinia* were diluted 1:20 into 20 ml of M9 minimal medium, grown for 2 hours at 26°C, and induced for 3 hours by temperature shift to 37°C. One milliliter of culture was labeled with 100 μCi of Pro-Mix for 1 min and precipitated with ice-cold TCA. The SDS-solubilized samples were immunoprecipitated with antiserum to purified YopH or Npt, separated by SDS–polyacrylamide gel electrophoresis, and quantified by PhosphorImager.
21. Portions of induced *Yersinia* cultures (1.5 ml) were centrifuged and sedimented cells were lysed in 0.1 ml of 1 mM EDTA, 5 mg/ml lysozyme. RNA was purified with the RNeasy (Qiagen). Total RNA (10 μg) was separated by formaldehyde-containing agarose gel electrophoresis and transferred to nylon membrane. The filter was hybridized with [^{32}P]cytidine 5′-triphosphate–labeled DNA sequences prepared by random primer labeling of restriction fragments corresponding to the full-length open reading frame of either *npt* or *yopH*.
22. Hybrid Npt proteins were purified with a fused COOH-terminal His_6 tag from the supernatant of induced *Yersinia* cultures. Briefly, protein from 1-liter culture supernatant was precipitated with 46% ammonium sulfate, collected by centrifugation, dissolved, and purified by chromatography on nickel-Sepharose. The NH_2-terminal amino acid sequence was determined by Edman degradation.
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24. W22703 cells [pYV227, pDA54 ($YopE_{4S-A}$-Npt)] (2×10^{10}), grown in Luria broth (LB) supplemented with chloramphenicol (20 μg/ml) at 28°C, were plated on tryptic soy broth (TSB) agar plates containing neomycin (50 μg/ml), chloramphenicol (20 μg/ml), and 5 mM EGTA. After 48 hours of growth at 30°C, nitrocellulose filters were placed on the surface of the plates, incubated for 30 min at room temperature, and probed with antibodies to Npt. Neomycin-resistant revertants arose at a frequency of 10^{-9} and were picked from the plates and patched onto fresh TSB agar supplemented with neomycin, chloramphenicol, and EGTA. Nitrocellulose filters were placed directly on the colonies and incubated in 1% SDS and lysozyme for 10 min. Colonies that reacted with antibodies to Npt were subsequently analyzed by immunoblotting for secretion of the Npt hybrid. Plasmid was isolated and transformed into W22703 to determine the linkage of the suppressor mutations to this DNA. Mutations were identified by DNA sequencing.
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