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The Secretion Signal of YopN, a Regulatory Protein of the *Yersinia enterocolitica* Type III Secretion Pathway†

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The type III secretion signal of *Yersinia enterocolitica* YopN was mapped using a gene fusion approach. *yopN* codons 1 to 12 were identified as critical for signal function. Several synonymous mutations that abolish secretion of hybrid proteins without altering the codon specificity of *yopN* mRNA were identified.

Many gram-negative bacteria employ a type III secretion mechanism to transport virulence factors across the bacterial envelope and, in some cases, even into host cells during infection (15). The 70-kb virulence plasmid of yersiniae harbors *ysc* genes, whose products are assembled to generate the type III machinery (10), as well as genes that encode substrates for the secretion pathway (29). Some substrates play a role in the injection of proteins into host cells (16, 17), whereas others travel the type III pathway into host cells (33). *Yersinia* type III secretion is regulated by environmental signals (for example, low calcium) that promote specific transport reactions (21, 29). Four *Yersinia* genes ensure proper activation of the type III machinery and the fidelity of transport into host cells: *yopN*, *sycN*, *tyeA*, and *yscB* (18–20, 40). SycN and YscB bind to the injection substrate YopN and promote its initiation into the type III pathway (7, 12). TyeA binds to the C-terminal portion of YopN, an interaction that prevents type III transport of YopN and of other injection substrates (7, 8).

The first approximately 15 amino acids of Yop proteins are sufficient to direct the type III secretion of fused reporter proteins (6, 24, 35, 36). Single amino acid replacements at each position of these secretion signals failed to detect residues that are critically important for function (3, 35), and no discernible similarity was identified among the first 15 amino acid residues of all Yop proteins (2, 3, 5). Several frameshift mutations, altering the reading frame of secretion signals but not that of the fused reporter gene, did not abolish secretion (3, 22). This unusual experimental result led to the RNA signal hypothesis, i.e., a property of *yop* mRNA may be responsible for the initiation of these polypeptides into the type III pathway (4).

yopQ harbors a minimal secretion signal within the first 10 codons (30, 31). The minimal secretion signal of *yopQ* does not tolerate frameshift mutations; however, the phenotype of such mutations can be suppressed by fusion of additional downstream sequences with linear signal dimensions, typically the first 15 codons. The function of the minimal secretion signal of *yopQ* can be abrogated by synonymous mutations that alter mRNA sequence without affecting codon specificity or amino acid incorporation into the polypeptide (31). Such mutations have pointed to codon 3, isoleucine, as a critical element in substrate recognition for *yopQ* (31). In silico analysis of the presumed secretion signals for all Yop proteins revealed the presence of isoleucine codons in twelve *yop* genes, whereas two genes, *lcrV* and *yopN*, represent an exception to this rule (28).

To identify the secretion signal of *yopN* we generated hybrid proteins by fusing DNA sequence specifying the *yopN* promoter, upstream untranslated *yopN* mRNA sequence, and portions of the *yopN* coding sequence to *npt*, encoding the cytoplasmic reporter protein neomycin phosphotransferase (Npt) (32). Unless YopN-Npt hybrids encompass the secretion signal for the type III pathway, fusion proteins would be expected to reside in the bacterial cytoplasm. Gene sequences for *yopN-npt* fusions were cloned on the low-copy-number plasmid pHSG576 (38). An NdeI restriction site was introduced downstream of the *yopN* untranslated region. Oligonucleotides specifying the desired sequence to be fused to *npt* were annealed and inserted between the NdeI and KpnI restriction sites (6). Recombinant plasmids were transformed into *Yersinia enterocolitica* strain W22703 (11). To measure protein transport, organisms were induced by growing bacterial cultures for 3 h at 37°C in calcium-depleted medium (M9 medium, containing 42 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.6 mM NaCl, 18.6 mM NH_4Cl, 0.01 mg of $FeSO_4$/ml, 0.001% thiamine, 1 mM $MgSO_4$, 0.4% glucose, and 1% Casamino Acids), a condition known to trigger type III secretion (25, 27). The cultures were then centrifuged, and the extracellular medium was separated with the supernatant (S) from the bacterial sediment in the pellet fraction (P). Proteins in both fractions were precipitated with trichloroacetic acid, washed in acetone, and suspended in sample buffer prior to separation on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After electrotransfer of proteins onto polyvinylidene difluoride (PVDF) membrane, YopN-Npt hybrids were detected by immunoblotting using specific rabbit antibody, horseradish peroxidase-conjugated secondary antibody, and chemiluminescence. As a control for proper fractionation of type III secretion substrates, the bacteria secreted YopE into the extracellular medium while chloramphenicol acetyltransferase remained in the bacterial cytoplasm (9) (Fig. 1). The hybrid $YopN_{1-15}$-Npt, generated through fusion of the first 15 codons of *yopN* to the *npt* re-

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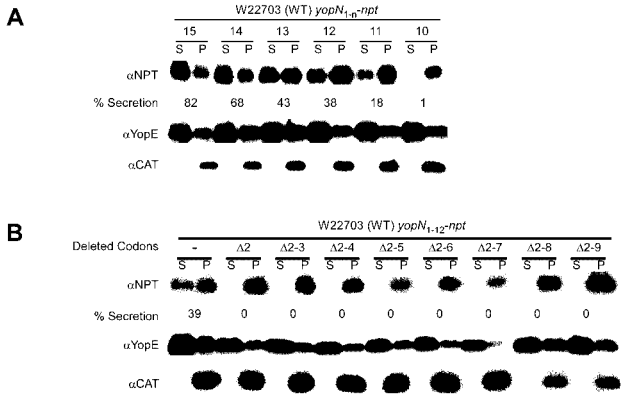

FIG. 1. Defining the minimal secretion signal of *yopN*. Successive 3′ *yopN* truncations were fused to the reporter gene *npt*, and recombinant genes were cloned into low-copy-number plasmids and then transformed into *Y. enterocolitica* W22703. *Yersinia* cultures were grown in M9 minimal media supplemented with Casamino Acids and were induced for type III secretion by temperature shift to 37°C and depletion of calcium ions. Protein in the extracellular culture supernatant (S) or the bacterial cell pellet (P) were precipitated with trichloroacetic acid and were analyzed by immunoblotting. (A) The percent amount of secreted YopN-Npt hybrids (i.e., the amount of secreted protein divided by the total amount of immunoreactive material and then multiplied by 100) is indicated underneath each panel. As a control for proper fractionation, the secretion of YopE, a type III secretion substrate, and chloramphenicol acetyltransferase, a cytosolic protein, was monitored with specific antiserum (αYopE or αCAT). (B) To determine the 5′ boundaries of the *yopN* secretion signal, codon 2 (Δ2), codons 2 to 3 (Δ2-3), and so forth were deleted and the secretion of YopN-Npt hybrids were measured. WT, wild type.

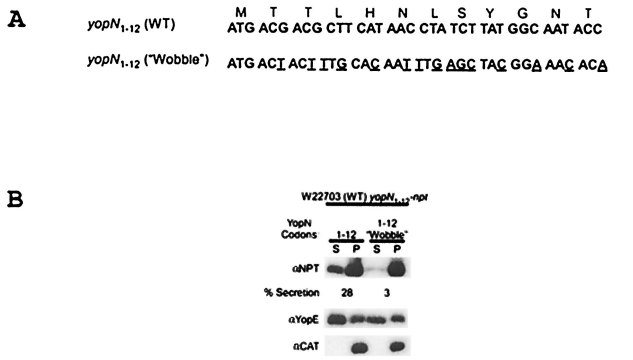

FIG. 2. Synonymous mutations in the minimal secretion signal of *yopN*. (A) Two signal fusions with the *npt* reporter gene were generated that carried either the wild-type (WT) mRNA sequence or a variant with 15 synonymous mutations that altered the nucleotide sequence of *yopN1-12* without affecting its encoded protein sequence. (B) Plasmid with cloned recombinant genes, $yopN_{1-12}$-*npt* or $yopN_{1-12wobble}$-*npt*, was transformed into *Y. enterocolitica* strain W22703. *Yersinia* organisms were induced for type III secretion, cultures were fractionated, and YopN-Npt secretion was measured as described in the legend to Fig. 1.

porter, was mostly secreted into the extracellular medium, as 82% of $YopN_{1-15}$-Npt was found in the culture supernatant after centrifugation (Fig. 1A). Fusion of shorter coding sequences of *yopN* to *npt* led to a reduction in the amount of secreted protein, as 68% of $YopN_{1-14}$-Npt, 43% of $YopN_{1-13}$-Npt, 38% of $YopN_{1-12}$-Npt, and 18% of $YopN_{1-11}$-Npt was observed in culture supernatants. Further truncation of the *yopN* gene sequence, generated via fusion of *yopN* codons 1 to 10 to *npt*, failed to promote type III secretion of $YopN_{1-10}$-Npt (Fig. 1A). These results suggest that the minimal secretion signal of *yopN* is encoded by codons 1 to 12, as the resulting $YopN_{1-12}$-Npt hybrid is initiated into the type III pathway with about a twofold-reduced rate compared to that of wild-type YopN (86%) (8) or $YopN_{1-15}$-Npt (82%). We wondered whether the minimal secretion signal of *yopN* is able to tolerate deletions of codons at the 5′ end without loss of signaling function. To test this, codon 2 (Δ2), codons 2 to 3 (Δ2-3), 2 to 4 (Δ2-4), 2 to 5 (Δ2-5), 2 to 6 (Δ2-6), 2 to 7 (Δ2-7), 2 to 8 (Δ2-8), or 2 to 9 (Δ2-9) were omitted from fused $yopN_{1-12}$-*npt*. All deletions of codons from the 5′ end of the *yopN* coding sequence eliminated the function of the type III secretion signal, and the $YopN_{1-12}$-Npt hybrids could not be found in culture supernatants (Fig. 1B). Together the results depicted in Fig. 1 suggest that the mRNA sequence of *yopN* codons 1 to 12 or amino acids 1 to 12 of YopN function as a secretion signal able to initiate hybrid reporter proteins into the type III pathway and that the boundaries of this linear element cannot tolerate truncations.

A mutant mRNA was generated that carried 15 nucleotide substitutions of the *yopN* secretion signal, each of which was predicted to not alter the codon specificity of the translational hybrid. When $yopN_{1-12}$-*npt* was expressed in *Y. enterocolitica* W22703, the hybrid polypeptide synthesized from wobble mRNA was not secreted (Fig. 2). The properties of the *yopN* secretion signal described here were examined with another reporter (portions of the *bla* gene encoding the amino acid sequence of mature, secreted β-lactamase), and the resulting hybrid proteins displayed similar transport properties (data not shown). Synonymous mRNA mutations with similar phenotypes have been generated in the minimal secretion signal of *yopQ* (31). Considering these earlier results, the data shown in Fig. 2 suggest that at least a portion of the minimal secretion signal of *yopN* may be decoded by a property of its mRNA nucleotide sequence. We sought to ascertain that *yopN1-12* and *yopN1-12 wobble* indeed generated the same polypeptide sequence. *Y. enterocolitica* strain W22703 cells, expressing plasmid-encoded $yopN_{1-12}$-*npt* hybrids, were lysed in a French pressure cell, and cytoplasmic protein was prepurified by ion exchange chromatography on a MonoQ column (Fig. 3A and B). Following separation on SDS-PAGE, proteins were electrotransferred to PVDF membrane and stained with Coomassie Brilliant Blue, and amino acid sequences were determined by Edman degradation (Fig. 3). The chromatography data in Fig. 3B, D, and F quantify phenylthiohydantoin amino acyl residues during each of the 11 sequencing cycles. The eluted compound of $YopN_{1-12}$-Npt following the first Edman degradation cycle was threonine (T) and not the initiator methionine (M) (Fig. 3B and D). Every phenylthiohydantoin amino acyl eluting after the initial cycle matched the residues predicted by the sequence of mRNA codons (Fig. 3B and D). Thus, these results suggest that *yopN1-12* and *yopN1-12 wobble* indeed generated the identical polypeptide sequence. A simple explanation for the position of threonine at the amino acyl end of $YopN_{1-12}$-Npt is deformylation and amino methionyl peptidase cleavage of YopN by f-Met peptide deformylase (Def) and methionine

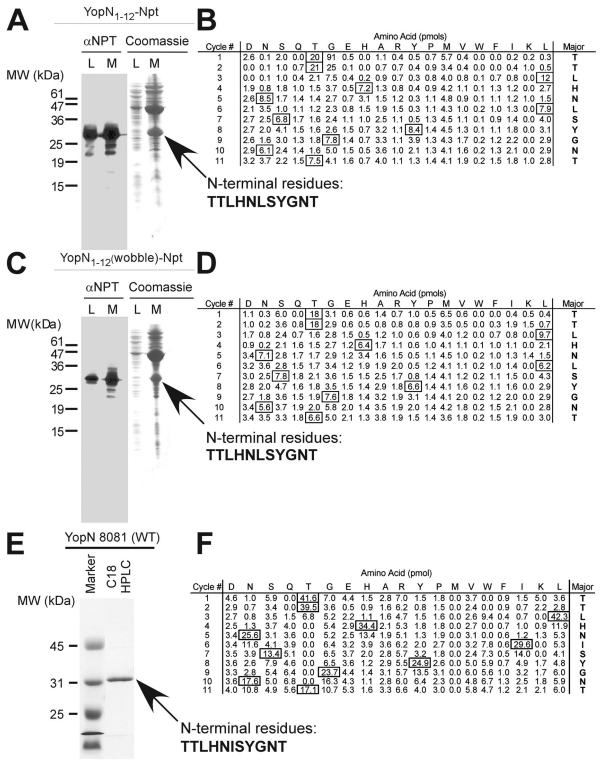

FIG. 3. Amino acid sequence analysis of peptides encoded by *yopN1-12* secretion signals. (A) $YopN_{1-12}$-Npt, synthesized from wild-type (WT) *yopN1-12* secretion signal, was purified from bacterial extracts generated via French pressure cell by chromatography on a MonoQ column. The crude lysate (L, for load) and eluate (M, for MonoQ) were subjected to SDS-PAGE and electrotransfer to PVDF membrane. The membranes were analyzed by immunoblotting with αNPT or were stained with Coomassie brilliant blue. The arrow points to the species that was subjected to Edman degradation. (B) Edman degradation profile reveals the elution of phenylthiohydantoin residues per sequencing cycle of the sample generated in panel A. High concentrations of glycine that eluted during each cycle were interpreted as sample contamination by electrotransfer buffer. (C) $YopN_{1-12}$-Npt, synthesized from the *yopN1-12 wobble* variant (see Fig. 2), was purified from bacterial extracts generated via French pressure cell by chromatography on a MonoQ column. The crude lysate (L) and eluate (M) were subjected to SDS-PAGE and electrotransfer to PVDF membrane. The membranes were analyzed by immunoblotting with αNPT or were stained with Coomassie brilliant blue. The arrow points to the species that was subjected to Edman degradation. (D) Edman degradation profile reveals the elution of phenylthiohydantoin residues per sequencing cycle of the sample depicted in panel C. (E) YopN was purified by reversed-phase high-performance liquid chromatography (HPLC) on a C_{18} column from the supernatant of centrifuged *Y. enterocolitica* strain 8081 cultures, separated on SDS-PAGE, and stained with Coomassie Brilliant Blue. The arrow points to the species that was subjected to Edman degradation. Molecular size markers (Marker) were included for size measurements. (F) Edman degradation profile reveals the elution of phenylthiohydantoin residues per sequencing cycle of the sample shown in panel E. MW, molecular size.

amino peptidase (MAP), respectively (23). Is deformylation and removal of methionyl caused by the secretion defect of $YopN_{1-12}$-Npt, perhaps because the polypeptide now resides in the bacterial cytoplasm and is substrate for Def and MAP (26), or does methionyl removal also occur prior to the secretion of wild-type YopN? To address this question, YopN was purified from the extracellular medium of *Y. enterocolitica* strain 8081 cultures by using reversed-phase high-performance liquid chromatography, and the peptide was then subjected to Edman degradation. Again, the first eluted residue of YopN following the initial Edman degradation cycle was threonine (T) and not methionine (M). Forsberg and colleagues made a similar observation for YopN of *Yersinia pseudotuberculosis* (14). These data therefore suggest that YopN may be modified by Def and MAP prior to the initiation of YopN into the type III secretion pathway. These results and previously published observations support the notion that type III substrates can travel the pathway in a posttranslational manner (1, 13, 34, 37).

To identify the nucleotide properties of *yopN* that are involved in secretion signaling, variants of *yopN1-12* were assembled from annealed oligonucleotides, cloned in frame to generate $yopN_{1-12}$*-npt* (see the supplementary material). Most nucleotide positions of *yopN1-12* tolerated transversions, i.e., replacement of purine nucleotides with pyrimidine nucleotides or reciprocal substitutions, without a measurable loss of secretion signal function (a fivefold reduction in protein transport was viewed as a significant loss of signaling) (Fig. 4). Using these criteria, single-nucleotide substitutions at seven positions (A4U, U11A, A14U, U20A, A21U, U25A, and C30G) of the 36-nucleotide sequence were sensitive to mutation. Many of the mutations analyzed in Fig. 4 introduced changes in codon specificity (amino acid substitutions) without causing a loss of function. With the exception of codon 7, where mutations at two positions affected signaling, only one of the three nucleotide positions at each of the codons 2, 4, 5, 9, and 10 was sensitive to mutagenesis. Because each codon tolerated at least one nonsynonomous nucleotide substitution, it appears that no single codon or amino acid of the *yopN* signal is absolutely essential for substrate recognition by the type III machinery. The mutational analysis described here identified some, but certainly not all, of the relevant nucleotide positions that may play a role in secretion signaling of *yopN1-12*. The reason for this limitation resides in the chemical nature of the changes that were introduced by the transversion mutations. Uracil was typically replaced with adenine or guanine was replaced with cytidine and vice versa to preserve the general uracil- and adenine-rich sequence character of *yop* secretion signals. More dramatic changes, for example, replacing uracil with guanine or replacing adenine with cytidine, may identify additional nucleotide positions that are sensitive to mutagenesis.

The experiment depicted in Fig. 2, reporting a secretion defect for *yopN1-12 wobble*, introduced 15 nucleotide substitutions into a signal that is comprised of 36 nucleotides. Our concern was that such a large number of mutations may have altered a general property of *yopN* mRNA, presumably affecting secretion in a manner that may not be pertinent to substrate recognition by the type III pathway. To analyze the *yopN1-12* secretion signal for synonymous mutations that alter nucleotide positions without affecting the specificity of individual codons, we tested all possible nucleotide triplets at each of the 11 positions that were accessible to mutagenesis. The data for this experiment are summarized in Table 1. Synonymous mutations at codons 2, 3, 4, 5, 6, 8, 9, 11, and 12 did not affect secretion signaling. However, two synonymous mutations at codon 7 eliminated secretion signaling. The wobble (X) position of the CUX leucine triplet must be occupied by a purine

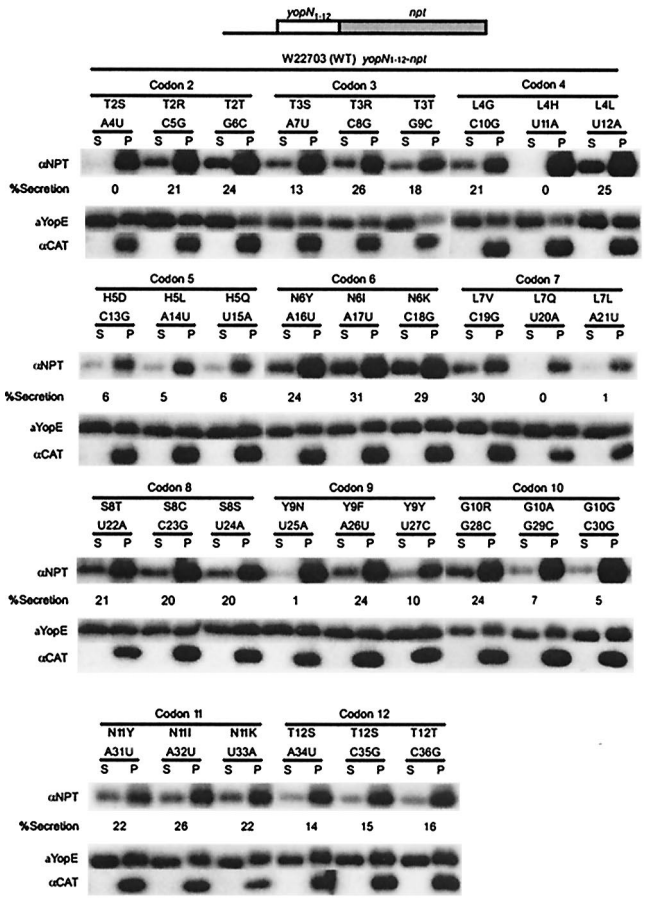

FIG. 4. Mutations within the minimal secretion signal of *yopN*. To identify nucleotide positions that are important for the function of the secretion signal of *yopN*, nucleotide transversions (purine and/or pyrimidine substitutions) were introduced into *yopN1-12.* The variant signals were fused to *npt*, *yopN*$_{1-12}$*-npt* was cloned on low-copy-number vectors, and recombinant plasmids were then transformed into *Y. enterocolitica* strain W22703. In the event that a stop codon would have been introduced by the transversion (codon 9), an alternate nucleotide was used for mutagenesis. Bacteria were induced for type III secretion, cultures were fractionated, and YopN-Npt secretion was measured as described in the legend to Fig. 1.

base, either adenyl or guanidyl, to provide for secretion signaling, as neither cytidyl nor uridyl was tolerated at this position (Table 1). The central nucleotide position of leucine codon 7, i.e., the uridyl in CUA, was also identified as important for secretion signaling, because U20A eliminated its function (Fig. 4). In contrast, the first nucleotide position (cytidyl) at *yopN* codon 7 could be replaced with uridyl, thereby representing a synonymous mutation, or with guanidyl, changing the codon specificity to valine, each of which did not produce a defect in secretion signaling. Replacement of the first nucleotide (C in CUA) with adenyl presumably may not affect secretion signaling, because the *yopN* gene of *Y. enterocolitica* strain 8081 harbors an AUA triplet at codon 7, incorporating isoleucine at position 7 of the YopN polypeptide (Fig. 3E and F) (10, 39). Three nucleotide triplets at *Y. enterocolitica* strain W22703 *yopN* codon 10, GGC, GGA, and GGU, promoted type III secretion, whereas the GGG triplet caused a significant reduction in secretion signaling.

TABLE 1. Synonymous mutations in the *yopN*$_{1-12}$ secretion signal and their effect on the secretion of YopN$_{1-12}$-Npt

Codon	Amino acid	Wild-type codon	Wobble codon	% Secretion
			ACC	24
2	Thr (T)	ACG	ACA	30
			ACU	26
			ACC	18
3	Thr (T)	ACG	ACA	25
			ACU	26
			CUA	25
			UUA	37
4	Leu (L)	CUU	UUG	35
			CUC	21
			CUG	30
5	His (H)	CAU	CAC	24
6	Asn (N)	AAC	AAU	17
			CUU	1
			UUA	35
7	Leu (L)	CUA	UUG	27
			CUC	1
			CUG	23
			UCA	20
			UCC	25
8	Ser (S)	UCU	UCG	24
			AGU	13
			AGC	16
9	Tyr (Y)	UAU	UAC	10
			GGG	5
10	Gly (G)	GGC	GGU	24
			GGA	16
11	Asn (N)	AAU	AAC	12
			ACG	16
12	Thr (T)	ACC	ACA	24
			ACU	22

Conclusions. Systematic mutagenesis of all nucleotides in the *yopN* minimal secretion signal identified 7 positions (A4U, U11A, A14U, U20A, A21U, U25A, and C30G) out of 36 nucleotides that were sensitive to mutation. One conclusion of this experiment is that the minimal secretion signal of *yopN* is astonishingly resilient to codon changes or amino acid substitutions. In fact, each of the 11 amino acids following the initiator methionine could be changed without loss of function. Further, three nucleotide positions that were critical for function showed that synonymous mutations at codons 7 or 10 can eliminate secretion of hybrid reporter proteins without altering the codon specificity of *yopN* mRNA.

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