Reciprocal secretion of proteins by the bacterial type III machines of plant and animal pathogens suggests universal recognition of mRNA targeting signals

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Bacterial pathogens of both animals and plants use type III secretion machines to inject virulence proteins into host cells. Although many components of the secretion machinery are conserved among different bacterial species, the substrates for their type III pathways are not. The *Yersinia* type III machinery recognizes some secretion substrates via a signal that is encoded within the first 15 codons of *yop* mRNA. These signals can be altered by frameshift mutations without affecting secretion of the encoded polypeptides, suggesting a mechanism whereby translation of *yop* mRNA is coupled to the translocation of newly synthesized polypeptide. We report that the type III machinery of *Erwinia chrysanthemi* cloned in *Escherichia coli* recognizes the secretion signals of *yopE* and *yopQ. Pseudomonas syringae* AvrB and AvrPto, two proteins exported by the recombinant *Erwinia* machine, can also be secreted by the *Yersinia* type III pathway. Mapping AvrPto sequences sufficient for the secretion of reporter fusions in *Yersinia* revealed the presence of an mRNA secretion signal. We propose that 11 conserved components of type III secretion machines may recognize signals that couple mRNA translation to polypeptide secretion.

A search for factors that are necessary for the pathogenicity of Gram-negative microbes has identified many gene clusters that are closely related among different bacterial species (1). Several of these genetic loci encode type III secretion machines for the translocation of polypeptides across the bacterial double membrane envelope (1). Some mammalian pathogens such as *Yersinia*, *Salmonella*, *Escherichia coli*, *Pseudomonas*, and *Shigella* use type III machines for the injection of virulence factors into the cytosol of eukaryotic cells (2–7). A similar strategy is thought to be used by several plant pathogens; however, a direct demonstration of their protein injection has not yet been achieved (7). *Salmonella typhimurium* and perhaps other Gram-negative bacteria harbor two gene clusters that each specifies a type III machine (2). Mutants that abolish the function of individual type III machines arrest pathogenicity at distinct steps during *Salmonella* infection, indicating that protein secretion is needed for the continued successful interaction between the pathogen and its host (8).

Type III machines of pathogenic *Yersinia* species are encoded on a virulence plasmid and comprise 21 different *ysc* genes (*Yop secretion*) (9). Mutations in any one of the *ysc* genes abolish the type III secretion of 14 different Yop proteins (*Yersinia outer proteins*) by mutant *Yersinia* spp. (10). Yop proteins do not display amino acid sequence or physical similarity (9). Previous work revealed the existence of two independent type III export pathways for Yop proteins (11–13). The first pathway recognizes a secretion signal encoded in the first 15 codons of *yop* mRNA (13). This sequence can be altered by frameshift mutation without loss of function, suggesting that mRNA may provide a signal that leads to the coupling of *yop* translation and type III secretion of newly synthesized polypeptide (13–15). The second type III export pathway requires the presence of a bacterial Syc chaperone (*specific Yop chaperone*) (12, 16–18). For example, SycE binds as a homodimer to YopE amino acids 15–100 and maintains the polypeptide in a soluble state (19). The $SycE_2$:YopE complex interacts with the type III machine, thereby leading to the displacement of SycE and to the targeting of YopE into the cytosol of eukaryotic cells (19, 20).

Although all type III machines are believed to catalyze similar reactions, i.e., the translocation of polypeptides across the bacterial envelope or their injection into the cytosol of eukaryotic cells, only 9 of the 21 *ysc* genes are conserved among all known type III machinery components of mammalian and plant pathogens (1). Can the secretion substrates of one type III machine be recognized by the export apparatus of another pathogen? Rosqvist *et al.* (21) reported that *Yersinia* YopE can be secreted by *Salmonella* in the presence, but not in the absence, of SycE chaperone (21). Similarly, *Shigella* IpaB could be secreted by *Yersinia* in the presence, but not in the absence, of IpgC chaperone (21). These results suggested that the secretion chaperone-mediated export pathway may be functional in many, if not all, Gram-negative bacteria. Because mRNA signals are functional even in the absence of secretion chaperones, this result also implies that type III machines cannot recognize RNA signals of heterologous secretion substrates. However, another study reported the secretion of *Pseudomonas aeruginosa* ExoS by *Yersinia pseudotuberculosis*, which occurred without the concomitant expression of a presumed ExoS chaperone (22). Thus, the mechanism by which type III machines recognize heterologous substrates has not yet been resolved.

Recently, a cluster of *hrp* (*h*ypersensitive *r*esponse and *p*athogenicity) and *hrc* (*h*ypersensitive *r*esponse and *c*onserved) genes encoding the *Erwinia chrysanthemi* type III machine was cloned in *E. coli*, thereby allowing the secretion of *Pseudomonas syringae* export substrates AvrB and AvrPto (23). We asked whether the *Erwinia* type III machine recognizes *Yersinia* substrates and report the secretion of YopE and YopQ. The *Yersinia* type III machine exported the *P. syringae* secretion substrates AvrB and AvrPto. The secretion signals of AvrB and AvrPto were mapped to the first 15 codons. The AvrPto signal could be frameshifted without loss of secretion, indicating that type III machines of *Yersinia* and *Erwinia* can recognize heterologous secretion substrates by their mRNA signals.

Materials and Methods

Bacterial Strains and Plasmids. The strains and plasmids used in this study are listed in Table 1. *E. coli* strains were routinely grown in LB medium at 37°C for the isolation of plasmids and at 30°C

Abbreviations: cat, chloramphenicol acetyl-transferase; hrp, hypersensitive response and pathogenicity; hrc, hypersensitive response and conserved; npt, neomycin phosphotransferase; syc, specific Yop chaperone; yop, *Yersinia* outer protein; ysc, Yop secretion; IPTG, isopropyl-β-D-thiogalactoside.

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Table 1. Bacterial strains and plasmids used in this work

Designation	Relevant characteristics and use	Ref.
Y. enterocolitica		
W22703	0:9 serotype, Nal^r, wild-type isolate	35
E. coli		
DH5α	*supE44 ΔlacU169 (Φ80lacZΔM15)hsdR17 recA1 endA1 gyrA96 thi1 relA1*	36
P90C	*araΔ(gpt-lac)5*	37
Plasmids		
pDA219	For construction of Npt fusions driven by the tac promoter, Cm^r	14
pDA288	pDA219 carrying *avrB* codons 1–15 fused to npt	This work
pDA289	pDA219 carrying *avrPto* codons 1–15 fused to npt	This work
pDA291	pDA219 carrying *avrB* codons 1–44 fused to npt	This work
pDA294	pDA289 (−1 frameshift mutation)	This work
pDA295	pDA289 (+1 frameshift mutation)	This work
pDA296	pDA289 (−2 frameshift mutation)	This work
pDA297	pDA289 (+2 frameshift mutation)	This work
pFlag-CTC	For construction of C-terminal fusions to Flag peptide, Ap^r	Kodak
pYopE–Flag	pFlag-CTC carrying *yopE*	This work
pYopQ–Flag	pFlag–CTC carrying *yopQ*	This work
pML123	Broad host range expression vector, Gm^r	38
pAvrB–Flag2	pML123 carrying *avrB* Flag-tagged	23
pAvrPto–Flag	pML123 carrying *avrPto* Flag-tagged	23
pDA–AvrB15	pDA219 carrying *avrB* with codons 2–15 deleted	This work
pDA–AvrPto10	pDA219 carrying *avrPto* with codons 2–10 deleted	This work
pCPP2156	pCPP19 carrying *E. Chrysanthemi hrp* cluster, Sp^r	23
pCPP2368	pCPP2156:Tn5Cm that has HR− phenotype, Sp^r, Cm^r	23

in Luria medium for protein-secretion assays. The following concentrations were used for antibiotics: ampicillin (Ap), 25 μg/ml; chloramphenicol (Cm), 10 μg/ml; gentamycin (Gm), 10 μg/ml; kanamycin (km), 50 μg/ml; spectinomycin (Sp), 25 μg/ml. Npt fusions containing 15 codons and the frameshift-mutant derivatives were constructed as described (13). To construct the fusion of the first 44 codons of *avrB* to *npt*, DNA fragments were PCR-amplified with *Taq* DNA polymerase using primers AvrB-3 (5′-AACATATGGGCTGCGTCTCGTCAA-3′) and AvrB-4 (5′-AAGGTACCTAAGCATTGATCATA-GACCTC-3′) and pAvrB-Flag as the template. The PCR product was then digested with *Nde*I and *Kpn*I and ligated into pDA219 that had been cut with the same enzymes and purified using QiaQuik gel-purification columns (Qiagen, Chatsworth, CA). Plasmids carrying full-length *yopE* and *yopQ* were constructed by digestion of pDA36 and pDA243, respectively, by *Nde*I and *Kpn*I followed by ligation into the same sites of pFlag-CTC such that the resulting proteins carried a C-terminal Flag epitope tag.

Secretion Assays. *Yersinia enterocolitica* strains were grown overnight in tryptic soy broth at 26°C. Cultures were diluted 1:50 into 20 ml of fresh medium containing 5 mM EGTA and grown for 2 h at 26°C (OD_{600} = 0.2) before induction for type III secretion by a temperature switch to 37°C and incubation for 3 h (OD_{600} = 0.6). Where necessary, the *tac* promoter was induced by the addition of 1 mM isopropyl-β-D-thiogalactoside (IPTG) 30 min after the temperature shift. Twenty milliliters of induced culture were centrifuged at 7,000 × *g* for 15 min, and 15 ml of supernatant was removed and precipitated with 5% trichloroacetic acid (TCA). The remainder of the supernatant was discarded, and the pellet was suspended in 800 μl of water. A 600-μl aliquot of this suspension was precipitated with 600 μl of ice-cold 10% TCA. All TCA precipitates were centrifuged for 15 min at 17,000 × *g* and washed for 10 min on ice with acetone. Pellets were collected by centrifugation at 17,000 × *g* for 10 min and air-dried. Proteins were suspended in 150 μl of 0.5 M Tris·HCl/4% SDS (pH 8.0) and added to an equal volume of sample buffer. Proteins separated by SDS/PAGE were transferred to Immobilon P membrane (Millipore) and identified by their binding to polyclonal antibody followed by chemiluminescent detection. A dilution series of purified protein antigen was also immunoblotted to ensure that signal intensity corresponded to antigen concentration. Where indicated, the signals were quantified by densitometric scanning of the developed x-ray films.

E. coli colonies were inoculated into 5 ml of Luria medium containing the appropriate antibiotics and grown overnight at 30°C. Cells were washed twice in Luria medium, diluted to OD_{600} = 0.2, and grown at 30°C until OD_{600} = 0.6. When the OD_{600} reached 0.35, the culture was induced for the expression of type III substrates by adding IPTG to 1 mM. Cultures were centrifuged first for 15 min at 4,300 × *g*, and the pellet was saved. The supernatant was again centrifuged for 40 min at 17,500 × *g* to remove residual cells and precipitated with ice cold TCA to a final concentration of 20%. After incubation on ice for 2–4 h, the precipitate was collected by centrifugation for 40 min at 17,300 × *g*. The sediment was washed twice with cold acetone, air-dried, and solubilized by boiling in SDS sample buffer. The cell sediment was diluted into 4 ml of water, and 100-μl aliquots were mixed with sample buffer before boiling. Samples were separated by SDS/PAGE before immunoblotting with specific antisera. A dilution series of purified protein antigen was also immunoblotted to ensure that signal intensity corresponded to antigen concentration. Where indicated, the signals were quantified by densitometric scanning of the developed x-ray films.

Results

Secretion of YopE and YopQ by *E. coli* Expressing the *Erwinia* Type III Machine. Type III secretion of YopQ occurs solely via an mRNA signal, whereas YopE secretion can be initiated by two independent type III pathways (12, 14). One pathway recognizes the signal located in the first 15 codons of *yopE* mRNA (13). The second pathway absolutely requires binding of SycE to YopE amino acid residues 15–100 (12). Thus, in the absence of SycE chaperone, YopE can only be exported via the RNA signal, similar to the type III secretion of YopQ. We asked whether the type III machine of *Erwinia* can recognize *Yersinia* RNA signals and secrete YopE and YopQ. *Y. enterocolitica yopE* and *yopQ* were expressed by the IPTG-inducible *tac* promoter in *E. coli* DH5α (pCPP2156), containing the type III secretion machine of *Erwinia*. A Flag epitope tag was appended to the ORF of both *yopE* and *yopQ*, thereby permitting detection of polypeptides via peptide specific antibody. Both YopE–Flag and YopQ–Flag were secreted into the culture supernatant of *E. coli* expressing *Erwinia* type III genes (Fig. 1). Chloramphenicol acetyltransferase, a resident of the bacterial cytoplasm, was detected only in the sediment of the fractionated cultures (Fig. 1). An isogenic *E. coli* mutant DH5α (pCPP2368), carrying a transposon insertion in the *Erwinia hrc* cluster (23), failed to secrete either YopE–Flag or YopQ–Flag. Together, these results suggest that the mRNA secretion signals of YopE and YopQ are recognized by the *Erwinia* type III machine.

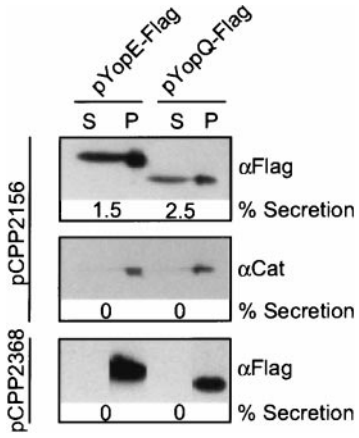

Fig. 1. The *Erwinia* type III machine secretes *Yersinia* Yops. *E. coli* DH5α (pCPP2156) expressing type III genes of *E. chrysanthemi* was transformed with plasmids that expressed either the *yopE* or the *yopQ* gene under control of the IPTG-inducible *tac* promoter. Cultures were induced for expression of Yops and centrifuged. Bacteria in the sedimented pellet (P) were separated from the culture medium in the supernatant (S). Supernatant samples were concentrated 60-fold to facilitate quantitation by SDS/PAGE. Proteins were analyzed by immunoblotting by using peptide antibody against the Flag epitope that had been appended to C-terminal *yopE* and *yopQ* sequences. The cytoplasmic protein, chloramphenicol acetyl transferase, supplied on an additional plasmid, was not secreted in these strains. As a control for type III secretion, *E. coli* DH5α (pCPP2368) carrying a transposon insertion in the *Erwinia hrc* gene cluster did not secrete YopE–Flag andYopQ–Flag. *Yersinia* YopQ and YopE harbor an mRNA signal within the first 15 codons that functions to couple mRNA translation and type III secretion of these polypeptides.

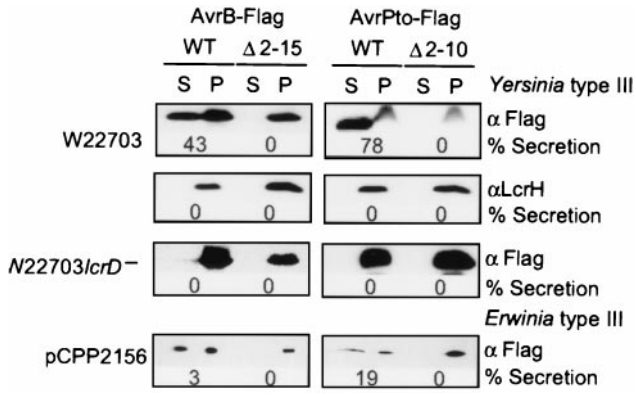

Fig. 2. *Yersinia* type III machines secrete *Pseudomonas* secretion substrates. *Y. enterocolitica* W22703 was transformed with plasmids carrying the *avrB* and *avrPto* genes under control of the constitutively expressed *npt* promoter. *Yersinia* cultures were induced for type III secretion by temperature shift to 37°C and chelation of calcium from the culture medium. Proteins were detected by immunoblotting with antibody against the Flag epitope, which had been appended to C-terminal *avrB* and *avrPto* sequences. *Yersinia* efficiently recognized the secretion signals of the *Pseudomonas* proteins, as 43% of AvrB and 78% of AvrPto were secreted, whereas the *Yersinia* cytoplasmic protein, LcrH, was not secreted. As a control for type III secretion, *Yersinia* carrying a null mutation in *lcrD* were unable to secrete AvrB or AvrPto. Deletion of the first 15 or 10 codons of AvrB or AvrPto, respectively, abolished secretion. The secretion of AvrB and AvrPto by the *Erwinia* type III machine cloned in *E. coli* was analyzed by concentrating culture supernatants 7.5 (AvrPto) and 80 fold (AvrB).

We wished to map the secretion signals for the recombinant *Erwinia* type III machine, and examined *yopE*, *yopQ*, and *avr* (see below) fusions to the neomycin phosphotransferase reporter (*npt*) gene. However, expression of *npt* fusions in *E. coli* DH5α carrying pCPP2156 and pCPP2368 proved to be toxic and caused leakage of cytoplasmic proteins into the supernatant of centrifuged cultures. Furthermore, our genetic characterization of export signals was hindered by the low efficiency of type III secretion in *E. coli* DH5α (pCPP2156). We routinely observed only 1–4% secretion for most secretion substrates, indicating that the cloned type III pathway of *Erwinia* was not fully induced under the conditions used. Because of these obstacles, we pursued the molecular characterization of secretion signals in *Y. enterocolitica*, a pathogen that secretes large amounts of proteins via the type III pathway (9).

Secretion of AvrB and AvrPto by the *Y. enterocolitica* Type III Machine. The *Erwinia* type III machine recognizes and secretes *P. syringae* export substrates, AvrB and AvrPto (23) (Fig. 2). We wondered whether *Pseudomonas* proteins can also be secreted by the mammalian pathogen *Y. enterocolitica* ,and we expressed AvrB–Flag as well as AvrPto–Flag under control of the constitutively expressed *npt* promoter in strain W22703 (Fig. 2). Forty-three percent of AvrB–Flag and 78% of AvrPto–Flag were found in the extracellular medium of *Y. enterocolitica* cultures. No type III secretion of AvrB–Flag and AvrPto–Flag was observed in a *Yersinia* mutant unable to express LcrD, a component of the type III machine that is located in the cytoplasmic membrane (Fig. 2). As a control, the cytoplasmic protein LcrH was found in the bacterial sediment but not in the culture supernatant of all *Yersinia* samples examined (Fig. 2).

The Secretion Signals of AvrB and AvrPto. To determine whether the *P. syringae* secretion substrates harbored mRNA signals similar to those of *Yersinia* Yops, we mapped and characterized the secretion signals of the *Pseudomonas* proteins. Fusion of the first 15 codons of *avrPto* to *npt* were sufficient to promote secretion of the hybrid Npt protein into the medium of *Yersinia* cultures (47% secretion) (Fig. 3*A*). Fusion of the first 15 codons of *avrB* promoted secretion of 25% of the Npt reporter. We also examined larger fusions and observed that the first 44 codons of *avrB* improved the efficiency of signal recognition as 42% of the Npt hybrid was located in the culture supernatant, similar to the secretion of full-length AvrB–Flag.

To examine whether these signals were absolutely necessary for the type III secretion of AvrB and AvrPto, we performed deletions of the first 10 or 15 codons of *Pseudomonas* mRNAs and expressed them under control of the IPTG-inducible *tac* promoter. Deletion of codons 2–15 of *avrB* mRNA, as well as deletion of codons 2–10 of *avrPto* mRNA, abolished all type III export of the mutant polypeptides by both the cloned *Erwinia* and the *Yersinia* type III machines (Fig. 2). To examine whether the *Pseudomonas* signals were encoded in the mRNA sequence, we focused on the secretion signal of AvrPto because it caused more secretion than the AvrB signal. Frameshift mutations were constructed by either inserting or deleting nucleotides immediately following the AUG translational start of *avrPto* mRNA. Each frameshift was corrected by reciprocal mutation at the fusion site with *npt*. The +1 frame shift mutation did not affect the efficiency of secretion, as 49% of the mutant protein was found in the culture supernatant (Fig. 3*B*). The −1 and +2 frameshift mutations of *avrPto* permitted 25% and 28% secretion of the mutant polypeptides into the culture medium of *Yersinia*. The −2 frame shift severely reduced expression of the mutant polypeptide, small amounts of which were detected in the bacterial cytoplasm but not in the culture medium. Together, these results revealed that the signals necessary and sufficient for the secretion of AvrB and AvrPto are located in the first 15 codons. Furthermore, the AvrPto signal is encoded in the mRNA sequence, because it can be altered by some frameshift mutations without loss of secretion.

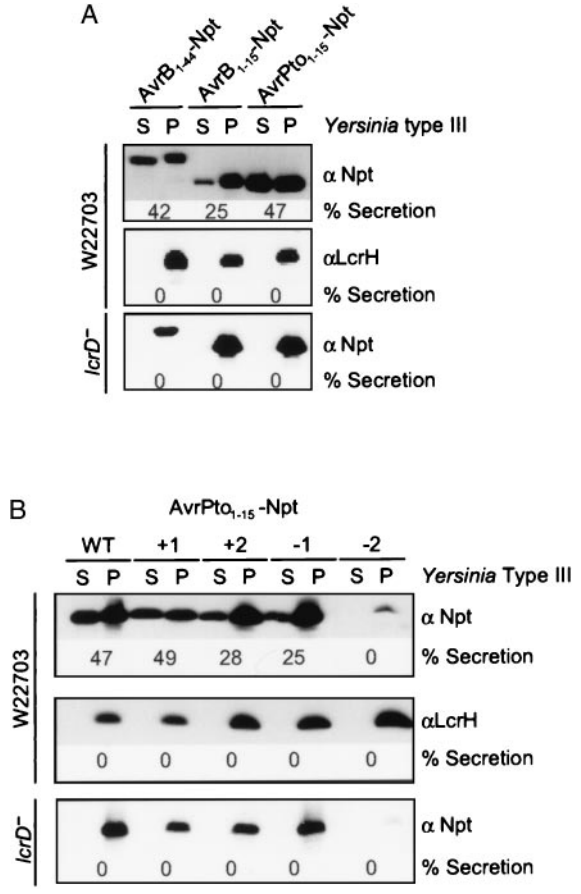

Fig. 3. *Pseudomonas* AvrPto is secreted by the type III machine via an mRNA secretion signal. (*A*) The secretion signal encoded by the first 15 codons is not only necessary but also sufficient for the secretion of reporter proteins. The first 15 codons of *avrB* and *avrPto* were fused to neomycin phosphotransferase (Npt) and secretion was measured in *Y. enterocolitica* W22703 with specific antibody to Npt. Both the first 15 codons of *avrB* and *avrPto* are sufficient to cause 25% and 47% secretion, respectively, of reporter fusions into the extracellular medium of *Yersinia* cultures. A fusion of the first 44 codons of *avrB* to *npt* caused an increase in secretion of the hybrid protein to 42%. (*B*) The secretion signal encoded within the first 15 codons of *avrPto* is recognized at the level of mRNA sequence, because it can tolerate frameshift mutations. Frameshift mutations were generated by nucleotide insertions (+1, +2) or deletions (−1, −2) immediately after the AUG translational start. Frameshifts were suppressed by reciprocal deletions and insertions at the fusion site with *npt*. The +1, +2, and −1 frameshift mutations did not interfere with *Yersinia* type III secretion because 49, 28 and 25% of the polypeptides were found in the culture supernatant. The −2 frameshift mutation abolished mRNA expression of the *npt* reporter.

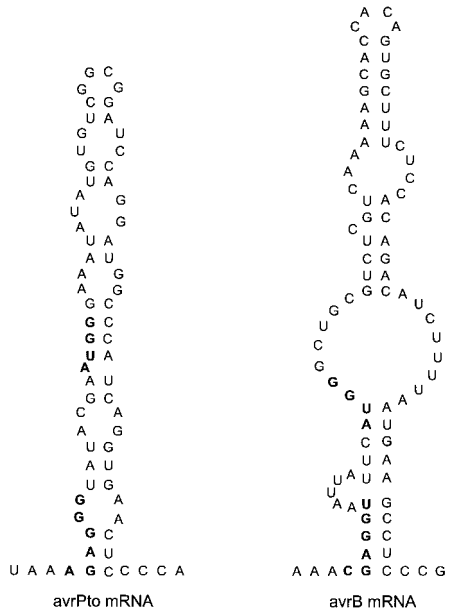

Fig. 4. Predicted RNA structures of the AvrPto and AvrB secretion signals. RNA sequences were subjected to folding analysis by using the Zuker program (24). The displayed structures show areas encompassing the ribosome-binding sites (bold), including the Shine–Dalgarno sequence (−13 to −8), start codon (+1 to +4), and downstream sequence of *avrB* and *avrPto* secretion signals. ΔG value (Gibbs energy) for *avrB* is −19.9 kCal (1 Cal = 4.18 J) and that of *avrPto* is −17.0 kCal.

Discussion

When cloned in *E. coli*, the type III gene cluster of *E. chrysanthemi* catalyzes the secretion of heterologous substrates from either *P. syringae* (23) or *Y. enterocolitica*. Characterization of the nature of the secretion signal of *Yersinia* Yops and *Pseudomonas* AvrPto revealed signals within the first 15 codons of the respective mRNAs. These signals can tolerate some frameshift mutations and still provide for the secretion of the mutant polypeptide chains, suggesting that the mRNA of secretion substrates is recognized by the type III pathways of *Erwinia*, *Yersinia*, and *Pseudomonas*. A computational analysis (24) of the mRNA sequences of *avrPto* and *avrB* revealed secondary structures similar to those previously described for *Yersinia yopE* and *yopN* (Fig. 4). The Shine–Dalgarno-binding site for 16S ribosomal RNA as well as the AUG translational start site, two elements required for translational initiation (25), are engaged in a hyphenated stem–loop structure. Codons 2–4 of *avrPto* and *avrB* are positioned within a loop and may function as recognition signals for the type III pathway (13).

The cloned *E. chrysanthemi* type III machine secretes both Avr and Yop proteins much less efficiently than either *Pseudomonas* or *Yersinia* spp. (9, 26). We think it is likely that the type III secretion pathway of the recombinant *Erwinia* machine is not properly induced, either because a specific signal is not provided in our experiments or because a regulatory gene(s) is absent from the recombinant *E. coli* strain (26). For example, *Yersinia* type III secretion is largely repressed by the addition of Ca^{2+} ions to the media or by temperatures below 30°C (27). We have tested whether *Erwinia* type III secretion is regulated by similar factors but could not detect conditions that improved secretion efficiency (data not shown).

Partial sequencing of the cloned *E. chrysanthemi* type III machine combined with its overall sequence and organizational similarity with the known *Erwinia amylovora* type III system suggests that 20 genes encode the *E. chrysanthemi* secretion machine (23), 11 of which are conserved in the *Yersinia* type III system (*yscCDJLNQRSTU* and *lcrD*) (9). Moreover, nine of these genes (*yscCJNQRSTU* and *lcrD*) are found in every type III system characterized to date, while two genes, *yscL* (*hrpE*) and *yscD* (*hrpQ*), are shared by only some of them (1). Thus, it is likely that type III machines comprising all 11 components will be able to recognize mRNA secretion signals of type III substrates. This hypothesis is in agreement with previous work on the recognition of substrates by heterologous organisms. For example, the *Salmonella* SPI-1 gene cluster lacks 1 of the 11 genes (*yscL*) (2) and is unable to recognize the RNA secretion signal of YopE (21). On the other hand, *P. aeruginosa* encodes all 11 of these genes (4), and substrates can be secreted without chaperones, suggesting RNA signaling (22).

Ten of the eleven conserved type III genes are homologous to genes that are required for assembly of the flagellar basal-body hook complex and for export of flagellin (*yscDJLNQRSTU* and *lcrD*) (1). The one gene not found in the flagellar system is *yscC*, an outer-membrane secretin of type III machines (28). Homologous secretins are also required for the type II secretion of proteins from the periplasm of Gram-negative bacteria across the outer membrane (29). Outer-membrane secretins are not needed to promote flagellar assembly (30). This type III pathway functions to polymerize translocated flagellin into a proteinaceous filament rather than releasing a secreted polypeptide into the extracellular milieu (31). We find the conservation of genes between type III secretion machines and the flagellar assembly complex compelling and speculate that the latter may also recognize mRNA signals to export polypeptides across the bacterial envelope.

If 11 components compose the core of type III machines, why does *E. chrysanthemi* use 9 additional *hrp* genes and *Yersinia* 10 additional *ysc* genes to direct protein secretion and translocation into host cells? This complexity is probably due to the large number of secretion substrates, the unique obstacles associated with protein translocation across host-surface barriers, and the differing destinations of the proteins that travel the pathway. For example, *Yersinia* secretes 14 different Yops, which are directed to distinct locations during infection (9). One group of Yops is injected into the cytosol of eukaryotic host cells (type III targeting) (20). A second group is secreted into the extracellular milieu, while yet another class of Yops remains associated with the bacterial surface and may be involved in regulating type III targeting and secretion (32). Future work will need to unravel all levels of substrate specificity of different Yops during infection. A similarly complex situation exists for some plant pathogens, which use type III machines to assemble a pilus-like structure (33) that is proposed to deliver Hrp proteins across the thick cell wall of plants into the target cell cytosol (34).

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