ExoS Controls the Cell Contact-Mediated Switch to Effector Secretion in *Pseudomonas aeruginosa*^{▽†}

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Type III secretion is used by many gram-negative bacterial pathogens to directly deliver protein toxins (effectors) into targeted host cells. In all cases, secretion of effectors is triggered by host cell contact, although the mechanism is unclear. In *Pseudomonas aeruginosa*, expression of all type III secretion-related genes is up-regulated when secretion is triggered. We were able to visualize this process using a green fluorescent protein reporter system and to use it to monitor the ability of bacteria to trigger effector secretion on cell contact. Surprisingly, the action of one of the major type III secreted effectors, ExoS, prevented triggering of type III secretion by bacteria that subsequently attached to cells, suggesting that triggering of secretion is feedback regulated. Evidence is presented that translocation (secretion of effectors across the host cell plasma membrane) of ExoS is indeed self-regulated and that this inhibition of translocation can be achieved by either of its two enzymatic activities. The translocator proteins PopB, PopD, and PcrV are secreted via the type III secretion system and are required for pore formation and translocation of effectors across the host cell plasma membrane. Here we present data that secretion of translocators is in fact not controlled by calcium, implying that triggering of effector secretion on cell contact represents a switch in secretion specificity, rather than a triggering of secretion per se. The requirement for a host cell cofactor to control effector secretion may help explain the recently observed phenomenon of target cell specificity in both the *Yersinia* and *P. aeruginosa* type III secretion systems.

Pseudomonas aeruginosa is a frequent cause of hospital-acquired infections, including ventilator-associated pneumonia (9) and catheter infections in immunocompromised patients (45), as well as burn wound infections (31, 49). It is also the leading cause of the chronic lung infections afflicting patients with cystic fibrosis and is the leading cause of death in this patient group (37). Type III secretion is one of the most important virulence factors that this bacterial pathogen has at its disposal. The ability to secrete effector proteins is associated with higher rates of relapse and mortality in patients with ventilator-associated pneumonia (24, 29). It also has an important role in virulence in a wide variety of animal models of infection, including mouse models of acute lung infection and burn wound infection, as well as in hosts as distantly removed from humans as moth larvae and the slime mold *Dictyostelium discoideum* (43, 50, 52).

Four effector proteins (exoenzymes) in *P. aeruginosa* have been described to date: ExoS, ExoT, ExoU, and ExoY. ExoS and ExoT are highly homologous, bifunctional enzymes that contain amino-terminal Rho-GTPase-activating protein (GAP) domains with similar target specificities (both can stimulate GTPase activity of RhoA, Rac1, and CDC42) and carboxy-terminal ADP-ribosylation domains (3). The substrate specificities of the ADP-ribosylation domains differ markedly,

however, with ExoS targeting a wide variety of proteins, including small Ras-like GTPases (23, 41, 53), ezrin-radixin-moesin proteins (38), vimentin (10), and cyclophilin (14), and ExoT affecting CrkI and CrkII (63). ExoU is a phospholipase (59) and ExoY an adenylate-cyclase (76). Strains of *P. aeruginosa* differ in their complement of effectors. Interestingly, ExoS and ExoU appear to be mutually exclusive, since almost no strains which code for both effectors have been isolated (18). There appears to be some correlation of effectors with specific diseases. In particular, ExoS-producing strains appear to be more common in cystic fibrosis isolates (19), whereas ExoU-producing strains appear to be more common in isolates from keratitis patients (20).

Transcription of the type III secretion genes is induced by triggering of secretion (21). Secretion can be triggered in vitro by removing calcium from the medium or by contact with host cells (21, 33, 67, 69). Induction of gene expression is linked to secretion by virtue of a regulatory system comprised of four proteins. ExsA, the master regulator of the type III secretion genes, is an AraC-type transcription regulator that is required for the expression of the type III secretion genes (22). Its activity is controlled by the antiactivator ExsD (40). ExsD activity, in turn, is regulated by the type III secretion chaperone ExsC (12). The final regulator, ExsE, is a small secreted protein which interacts with ExsC (55, 68). When effector secretion is off, ExsE is thought to bind to and sequester ExsC. This in turn allows ExsD to bind to and inactivate ExsA. Upon triggering of secretion, ExsE is exported via the type III secretion machinery, freeing ExsC to bind to ExsD and activating ExsA (78). In this study we have used a green fluorescent protein (GFP) reporter to monitor triggering of *exoS* expression on cell contact.

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While the exact steps involved in the triggering of effector secretion on cell contact have not been well defined, we do know of several events that have to occur. First, the bacterium has to make contact with the cell, a process mediated by specific adhesins. In *P. aeruginosa* the type IV pili are important for this initial attachment step; however, they are not specifically required for type III secretion, since they can be replaced by the unrelated *Yersinia* ph6 adhesin (65). Subsequently, the type III secretion system (T3SS) has to be brought close to the plasma membrane. The translocator proteins PopB and PopD have to be inserted into the host membrane to form the translocation pore to which the type III secretion apparatus is docked (7, 11). Insertion of PopD, but not PopB, requires the needle tip protein PcrV (27, 28). In the closely related *Yersinia* T3SS, the PcrV homolog LcrV appears to be absolutely required for the insertion of the PopB homolog YopB but only partially required for YopD insertion (8, 27). Evidence has been presented that *Yersinia* secretes the translocator proteins (but not the effectors) into the culture medium during the infection process, an event that may be triggered by serum proteins in tissue culture medium (35, 36). However, at least in the case of YopB, it has been explicitly demonstrated that these secreted translocators cannot cross-complement a *yopB* null mutant, suggesting that the translocators that form the pore, to which a given apparatus is attached, have to be secreted and retained in close proximity to the needle (58). This is consistent with a recent observation that only minimal levels of secreted YopB and YopD are required for successful intoxication of cells (15). After the formation of the translocation pore and docking of the needle to the pore, effector secretion is triggered. In the closely related *Yersinia* T3SS, triggering of effector secretion has been proposed to be a function of sensing the lowered calcium concentration in the eukaryotic cell cytosol (35). Sensing of the host cell is thought to involve a conformational shift in the needle protein that is perpetuated from the site of contact down the needle to the base of the secretion apparatus (71). A somewhat different model has been proposed for triggering of type III secretion in *Shigella*. Here it has been proposed that the PopB homolog IpaB is, in fact, part of the needle tip complex (71). Insertion of IpaB into the host cell membrane is thought to result in a conformational shift in the needle tip which results in secretion of the remaining translocator, as well as the effector proteins. In support of this model, it has been reported that IpaB is surface displayed in *Shigella* (47, 71). To date, however, no one has been able to detect YopB or YopD at the tips of *Yersinia* T3SS needles (8, 44) or PopB or PopD at the surface of *P. aeruginosa* (unpublished observation), suggesting that the insertion of the translocator into the target membrane may proceed via a slightly different mechanism in these two classes of T3SSs.

Recent reports suggest that translocation of effectors by the *Yersinia* T3SS is controlled by a eukaryotic factor and modulated by effectors, YopE and YopK. Deletion of either gene results in an increase in effector translocation (1, 2, 32). YopK has been proposed to modulate the size of the translocation pore formed by YopB and YopD (32). YopE is a secreted toxin that exhibits Rho-GAP activity and displays some homology with the amino-terminal portions of ExoS and ExoT. YopE displays a somewhat different target specificity, however (77). Interestingly, the Rho-GAP domain of ExoS was able to sub-

stitute for YopE and reestablish Yop translocation control. The ADP-ribosylation domain of ExoS, however, had no effect in this assay (1).

In this study we have used the fact that expression of type III secretion-related genes is controlled by secretion of ExsE to study triggering of effector secretion on cell contact. Interestingly, we found that intoxication of cells by ExoS blocked subsequent induction of our reporter constructs, suggesting that triggering of effector secretion is controlled by a host factor that is inactivated by ExoS. Similar to the inhibition of Yop translocation by YopE in *Yersinia*, intoxication of cells by ExoS limits its own translocation into host cells. In fact, ExoS can also limit effector secretion when expressed in *trans* in a *Yersinia yopE* null mutant (1). Unlike in the *Yersinia* system, however, where only the Rho-GAP activity of ExoS was able to limit effector translocation when expressed in a *yopE* mutant, either enzymatic activity of ExoS could serve to limit translocation in *P. aeruginosa*. Triggering of *exoS* expression on cell contact depends on the presence of both PopB and PopD. These results suggest a hierarchy of secretion, in which translocators are secreted before effector proteins. In agreement with this hypothesis, we present evidence that secretion of translocator proteins is apparently not controlled by calcium. Interestingly, intracellular calcium levels in the host cell do not appear to control effector secretion either, suggesting that a signal other than calcium has to be responsible for the switch to effector secretion on cell contact. Taken together, these data allow us to propose a step-by-step model for triggering of effector secretion on cell contact. Triggering involves translocator insertion, docking, and subsequent switch of secretion specificity to allow secretion of effectors, a process that is controlled by a yet-to-be-identified eukaryotic factor.

MATERIALS AND METHODS

Bacterial strains, cells, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *P. aeruginosa* was grown in LB with 200 mM NaCl, 10 mM MgCl₂, and 0.5 mM CaCl₂ unless noted otherwise. Where indicated, calcium was removed from the medium by adding EGTA to a final concentration of 5 mM. Tissue culture cells were grown in RPMI supplemented with 2 mM glutamine and 10% fetal bovine serum (FBS) (RP10) in the presence of 5% CO₂ at 37°C. In some instances medium without FBS was used. A549 cells (lung epithelial cell line, ATCC CCL-185) were obtained from the laboratory of Susann Brady-Kalnay. Inhibitors were added 30 min before infection after replacing the medium with prewarmed RP10. Cycloheximide was added at a final concentration of 200 µg/ml, cytochalasin D at 10 µM, streptomycin O at 5 U/µl, alpha-hemolysin at 10 U/µl, and calcimycin at 10 µM.

Plasmid and strain construction. To construct pCTX2-*groE*-mCherry, a fragment containing the *groE* promoter and first two codons of *groES* was amplified (PgroE5, 5'-AAAAAactagtACGACCTGAACGCCGCTACGGA-3'; PgroE3, 5'-AAAAAgaattcCTTCATAGTCGTAACCTCTCCAAA-3' [restriction sites are lowercase]), as well as mCherry (CFP-5R, 5'-AAAAAgaattcATGGTGAGCAAGGGCGAGGA-3'; Cherry-3H, 5'-AAAAAaagctTTACTTGTACAGCTCGTCC-3'). The PCR fragments were digested with SpeI/EcoRI and EcoRI/HindIII, respectively, and cloned into SpeI/HindIII-digested pMiniCTX2 (30). The *groE*-mCherry reporter construct was then crossed onto the chromosome of PAO1F, and extraneous plasmid sequences were removed by FLP recombination using plasmid pFLP2 as described previously (30). Deletion of the three effector genes, as well as *exsE* and *pscC*, was performed using published constructs (55, 70, 74). Mutant constructs to introduce active-site mutations into the *exoS* gene on the PAO1F chromosome were generated by amplifying two flanking sequences using two outside primers (*exoS*5-2, 5'-AAAAAggtaccGCTTGCAAGGGTCCTGGCTGAACA-3'; *exoS*3-1, 5'-AAAAAaagctCCGTACCCTGCCGTACTGAAC-3') as well as the corresponding internal primers harboring the relevant mutations (lowercase) (SADR3-1, 5'-ATATCGAACTACAAGAATGatAAAGatATTCTCTATAACAAAGAAACCGA-3'; SADR5-2, 5'-TCGGTTCTTTGTGA

TABLE 1. Strains and plasmids

Strain or plasmid	Description	Reference
Strains		
RP1831	PAO1F (wild-type PAO1)	6
RP1949	PAO1F Δ exoS Δ exoT Δ exoY (Δ 3TOX)	This study
RP1871	PAO1F Δ pscC	This study
RP2230	PAO1F attB::groE-mCherry	This study
RP1879	PAO1F Δ popB	This study
RP1904	PAO1F Δ popD	This study
RP2231	PAO1F Δ exoS Δ exoT Δ exoY attB::groE-mCherry	This study
RP2233	PAO1F Δ pscC attB::groE-mCherry	This study
RP2234	PAO1F Δ exoS::GL3 Δ exoT Δ exoY attB::groE-mCherry	This study
RP2329	PAO1F Δ exoS::GL3 Δ exoT Δ exoY Δ popB attB::groE-mCherry	This study
RP2332	PAO1F Δ exoS::GL3 Δ exoT Δ exoY Δ popD attB::groE-mCherry	This study
RP1910	PAO1F Δ exsE	This study
RP2317	PAO1F Δ exsE Δ exoS Δ exoT Δ exoY	This study
RP2318	PAO1F Δ exsE Δ exoT Δ exoY ("exoS ⁺ ")	This study
RP2319	PAO1F Δ exsE Δ exoS Δ exoY ("exoT ⁺ ")	This study
RP2321	PAO1F Δ exsE Δ exoS Δ exoT ("exoY ⁺ ")	This study
RP2335	PAO1F Δ exsE Δ pscC	This study
RP2347	PAO1F Δ exsE Δ exoT Δ exoY Δ exoS-R146K ("exoS ^{GAP} -")	This study
RP2348	PAO1F Δ exsE Δ exoT Δ exoY Δ exoS-E379D/E381D ("exoS ^{ADPR} -")	This study
RP2349	PAO1F Δ exsE Δ exoT Δ exoY Δ exoS-R146K/E379D/E381D ("exoS ^{GAP} -/ADPR-")	This study
RP2648	PAO1F Δ exsE Δ pcrV2	This study
Plasmids		
pPSV35	Expression plasmid with <i>lacI^q</i> , <i>lacUV5</i> promoter, gentamycin resistance, <i>colE1</i> and <i>Pseudomonas</i> origins of replication, <i>oriT</i>	55
pP35- <i>pcrV</i>	<i>pcrV</i> cloned under control of <i>lacUV5</i> promoter	This study
pP35- <i>popB</i>	<i>popB</i> cloned under control of <i>lacUV5</i> promoter	This study
pP35- <i>popD</i>	<i>popD</i> cloned under control of <i>lacUV5</i> promoter	This study
pP33- <i>p_{exoS}</i> -GFP	<i>p_{exoS}</i> promoter controlling GFP expression, gentamycin resistance	54
pCTX2- <i>groE</i> -mCherry	<i>p_{groE}</i> controlling mCherry expression	This study
pEXG2	Allelic exchange vector, <i>colE1</i> origin, <i>oriT</i> , gentamycin resistance, <i>sacB</i>	55
pEXG2- <i>exoS</i> (GAP-)	pEXG2 with <i>exoSR146K</i> allele	This study
pEXG2- <i>exoS</i> (ADPR-)	pEXG2 with <i>exoSE379D/E381D</i> allele	This study
pEXG2- <i>exoS</i> (GAP-/ADPR-)	pEXG2 with <i>exoSR146K/E379D/E381D</i> allele	This study
pEXG2- Δ <i>pcrV</i> 2	pEXG2 with <i>pcrV</i> deletion allele (Δ 4-180)	This study
pEXG2- Δ <i>popD</i>	pEXG2 with <i>popD</i> deletion allele (Δ 3-268)	This study
pEX- Δ <i>popB</i>	pEXGW with <i>popB</i> deletion allele	65

TAGAGAATaTCTTTaTCATTCTGTAGTTCGATAT-3'; SGAP3-1, 5'-TGCGCAGCGGAGATGGGGCGCTGaaaTCGCTGAGCACCGCCTTGGCCGGCA-3'; SGAP5-2, 5'-TGCCGGCCAAGGCGGTGCTCAGCGAttCAGCGCCCCATCTCCGCTGGCCA-3'). Flanks were joined by splicing by overlap extension PCR (73) and cloned into the allelic exchange vector pEXG2 (55). After sucrose selection, the presence of the appropriate mutation was confirmed using test primers (SADRtest [5'-ATATCGAACTACAAGAAATGATAAAGAT-3'] or SGAPtest [5'-TGCCGGCCAAGGCGGTGCTCAGCGAtt-3']) in conjunction with the appropriate flanking primer. *pcrV* was cloned into plasmid pPSV35 as an EcoRI/HindIII fragment (*pcrV*2-5R, 5'-AAAAAgaattcTGGCTTGTGATCTGAGGAATCACGA-3'; *pcrV*2-3H, 5'-AAAAAgaattcTGGCTGGTTCATGGATACCTCA-3'). *popB* was cloned into plasmid pPSV35 as an EcoRI/HindIII fragment (*popB*5R, 5'-AAAAAgaattcTTAGGAGGCGCCCCATGAATCCGATAACGCTTGAA-3'; *popB*EX3, 5'-AAAAAgaattcGACGTCTCTCAGATCGCTGCCGGT-3'). *popD* was cloned into plasmid pPSV35 as an EcoRI/HindIII fragment (*popD*5R, 5'-AAAAAgaattcTTAGGAGGCGCCCCATGATCGACACGCAATATCCCT-3'; *popD*EX3, 5'-AAAAAgaattcCGCGCGGAGACGGCTCAGACCACT-3'). The *popD* deletion construct, pEXG2- Δ *popD*, was created by amplifying the two flanking regions (*popD*5-1, 5'-AAAAAgaattcTGGTTCAGCTTCGGCGGCTCAGCGGT-3'; *popD*5-2, 5'-AACTCGAGCCGCAAGCATGCTGAACGTGTGATCATGTGACGTCTCTCT-3'; *popD*3-1, 5'-TTCAGCATGCTTGGCGGCTCGAGTGTCTGAGCCGTCTCCGCGCGGAGGAA-3'; *popD*3-2, 5'-AAAAAgaattcAGGGTCAGTTGCGCTGCGAGAT-3'), which were subsequently stitched together by splicing by overlap extension PCR, digested with EcoRI and HindIII, and cloned into pEXG2.

Microscopy. A549 cells (10^5) were seeded 2 days ahead of time on glass coverslips placed in six-well plates and allowed to attach tightly to the substrate before the experiment was performed. On the day of the experiment, the cells

were washed twice with phosphate-buffered saline (PBS), and fresh medium was added to the wells. If indicated, cells were pretreated with toxins and inhibitors 30 min prior to infection, which was subsequently carried out in the continued presence of the given toxin or inhibitor. Overnight cultures of the appropriate strains of *P. aeruginosa* were used to infect cells for the indicated amounts of time. Subsequently, cells were gently washed twice with PBS (to prevent loss of rounded cells) and fixed with 4% paraformaldehyde in a 200 mM phosphate buffer (pH 7.4) (Poly Scientific) for 15 min at room temperature. Fixed slides were washed once with PBS, dabbed dry, and mounted on slides using Prolong mounting medium (Invitrogen). Slides were imaged using a Zeiss Axioplan2 microscope. While cell rounding was evident in cells intoxicated with ExoS and/or ExoT, cell lifting was minimized by allowing the cells to attach for 2 days and washing the cells gently after the infection (PBS was added to the wall of the well and allowed to wash over the cells, rather than added onto the cells directly). The similarity of cell numbers/field in slides with rounded and flat cells suggests that loss of rounded cells was minimal in the reported experiments.

For video microscopy 8×10^4 cells were seeded 2 days ahead of time in glass-bottom culture dishes. On the day of the experiment, the cells were washed twice with PBS, the medium was exchanged for fresh medium, and the cells were infected at a multiplicity of infection (MOI) of 25 (assuming 2×10^5 cells). Image collection was started 1.5 h postinfection. Images were collected at a rate of one image every 2 min using a 60 $\times$ objective on a Deltavision RT epifluorescence microscope with an automated stage (Applied Precision, Inc.) and captured using a charge-coupled device camera. Phase and fluorescent images were overlaid and assembled into movies using Softworx analysis software. Still images were cropped using Adobe Photoshop CS3 (Adobe, Inc.).

Translocation assay. The translocation assay was based on an established protocol (46). Two days prior to the experiment, 1.5×10^6 A549 cells were

seeded in 100-mm-diameter tissue culture dishes. On the day of the experiment, the cells were washed twice with PBS, and RPMI without FBS was added to each dish. For each strain three plates of cells were infected. Cells were infected at an MOI of 50 for 2 h and then washed twice with PBS and treated as follows. The first plate was not treated with protease (PBS control), and the second and third plates were treated with 1 ml PBS (Invitrogen; supplemented with Mg and Ca) plus 250 μ g/ml proteinase K for 20 min at room temperature. Cells were collected using a cell scraper, pelleted in a microcentrifuge, and resuspended in 75 μ l PBS with 2 mM phenylmethylsulfonyl fluoride. To the cells from the first and second plates, 75 μ l PBS with 1% Triton X-100 was added. To the cells derived from the third plate, 75 μ l of PBS with 1% sodium dodecyl sulfate (SDS) was added. The lysates were incubated at room temperature for 20 min, at which point the protein concentration was determined by the Bradford method (Bio-Rad). Equivalent amounts of protein were loaded on a 12.5% gel, separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose using a semidry transfer apparatus. Indicated proteins were detected by Western blotting. The anti-actin antibody (hybridoma supernatant, catalog no. JLA20) developed by J. J.-C. Lin was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City. Mouse-anti RpoB was purchased from NeoClone. Antisera against ExoS were a gift from Stephen Lory (Harvard Medical School). Antisera directed against PopB and PopD were a gift from Ina Attree (CEA Grenoble). All other antisera were raised against purified His-tagged proteins (Covance).

Secretion assay. Bacteria were diluted 1:300 into 3-ml cultures of "high-salt" LB (NaCl adjusted to 200 mM and medium supplemented with 0.5 mM CaCl₂ and 10 mM MgCl₂ [final concentrations]) and grown for 2 h, at which point secretion was induced by the addition of EGTA (5 mM final concentration). The cultures were grown for another 30 min, at which point the bacteria were pelleted by centrifugation. Supernatant proteins were precipitated with 10% (final concentration) trichloroacetic acid (TCA). Pellets were washed once with acetone, resuspended in sample buffer, and normalized according to the optical density at 600 nm (OD₆₀₀) of the culture. Samples were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with the appropriate antibodies.

In the case of the assay to reestablish calcium control, overnight cultures were diluted 1:300 into 5 ml "high-salt" LB with and without 5 mM EGTA. Bacteria were again cultured for 2 hours, at which point bacteria from 2-ml aliquots of each original culture were pelleted and resuspended in medium with or without 5 mM EGTA. The cultures were incubated for another 30 min, at which point the bacteria were pelleted and resuspended in sample buffer (cell pellet fraction). Supernatant protein was precipitated with TCA as noted above. Both cell pellet and supernatant fractions were normalized according to the OD₆₀₀ of the cultures.

RESULTS

While secretion of effectors via the T3SS can be triggered *in vitro* by removing calcium from the medium, the more relevant cue for triggering type III secretion in the context of an infection is most likely host cell contact. We set out to study triggering of type III secretion on host cell contact by utilizing a convenient property of *Pseudomonas aeruginosa*, the fact that triggering of effector secretion via the T3SS also results in up-regulation of all type III secretion-associated genes (both toxin and structural genes) (74). To this end we used one of two previously described reporter constructs: a plasmid-based reporter, in which GFP expression is controlled by the *exoS* promoter, and a chromosomal reporter, in which GFP and a translationally coupled copy of *lacZ* were integrated in the *exoS* locus, replacing the *exoS* open reading frame (54, 56).

In order to visualize all cell-associated bacteria, we further labeled our bacteria by creating a constitutively expressed reporter encoding an amino-terminal translational fusion of the first two amino acids of *groES* to the red fluorescent mCherry protein. The reporter was expressed from the *groES* promoter and inserted in the neutral CTX phage attachment site (see Fig. S1 in the supplemental material). All reporters were neutral with regard to T3SS function as assayed by the ability of

bacteria carrying the reporters to intoxicate epithelial cells (see Fig. S1 in the supplemental material). Our readout for cell contact-mediated triggering of type III secretion therefore is the percentage of cell-associated bacteria (red fluorescent) that are expressing GFP.

Initial experiments using a wild-type strain of *P. aeruginosa* failed to detect significant numbers of cell-associated bacteria expressing GFP. This was surprising to us, since induction of type III secretion gene expression on cell contact had been described previously (33, 69). However, triggering of *exoS* expression on cell contact was readily detectable when the experiment was performed with a *P. aeruginosa* strain lacking the genes for all three of the known toxin open reading frames, *exoS*, *exoT*, and *exoY* (Δ 3TOX) (Fig. 1A). We next performed time-lapse video microscopy to study triggering of *exoS* expression in real time. Interestingly, in these experiments both the wild-type and the Δ 3TOX bacteria readily turned on GFP expression on cell contact (Fig. 1B and C).

This apparent discrepancy is most easily explained by postulating that intoxication of cells by one or all of the known effector proteins prevents subsequent triggering of type III secretion. If this is the case, the first wild-type bacterium to contact a cell would still trigger type III secretion, inject its toxins, and up-regulate T3SS gene expression. Intoxication, however, would prevent subsequent triggering of *exoS* expression on cell contact by other bacteria, thereby accounting for the very low number of cell-associated GFP-positive bacteria in the fixed-cell experiments. Since the Δ 3TOX strain does not intoxicate the targeted host cell, the cell remains permissive for triggering type III secretion by bacteria that attach after the initial contact, and the percentage of GFP-positive bacteria would be expected to increase as we observed.

Intoxication of cells by ExoS prevents subsequent triggering of type III secretion on cell contact. To examine the hypothesis that intoxication of cells prevents subsequent triggering of effector secretion, we engineered three strains expressing only ExoS, ExoT, or ExoY. Expression was deregulated by deleting *exsE*, and all strains were tested to ensure that they expressed and secreted the appropriate toxin (Fig. 2C). Epithelial cells were preintoxicated with the indicated strains for 1 h, at which point the cells were washed twice with PBS and infected with the Δ 3TOX reporter strain bearing the *p_{exoS}*-GFP reporter plasmid. After 4 h of infection, the cells were washed and the ability of the reporter bacteria (readily distinguishable by virtue of the constitutively expressed mCherry reporter) to induce *exoS* expression was assayed. While preinfection of the cells with strains bearing mutation in a T3SS structural gene (Δ *pscC*) or a strain lacking the three known effectors (Δ 3TOX) had no apparent effect on the ability of the reporter to trigger *exoS* expression, preintoxication with a strain expressing all the toxins or just ExoS was able to completely inhibit subsequent triggering of *exoS* expression (Fig. 2A). These results could not be explained by an inability of the reporter bacteria to bind the epithelial cells (Fig. 2B). ExoT had an intermediate effect in these experiments that was not statistically significant, while ExoY did not prevent triggering of *exoS* expression by the reporter strain (Fig. 2A).

We next dissected which of the two enzymatic functions of ExoS is responsible for the negative effect on subsequent T3SS triggering. To this end, we engineered strains that constitu-

FIG. 1. Cell contact-dependent induction of *exoS* expression. (A) The *attB::groE-mCherry* chromosomal marker, as well as pP33-*p_{exoS}*-GFP, were introduced into wild-type PAO1F, a Δ *pscC* derivative, or a strain lacking all three exoenzymes (Δ 3TOX) and used to monitor induction of *exoS* expression over time. The cells were infected at an MOI of 25 for 1 h and washed twice with PBS, and the medium was replaced with fresh tissue culture medium. Subsequently, slides were removed at 1-h intervals, washed, and fixed with paraformaldehyde before being mounted for microscopic examination. Generally four fields, comprising 60 to 80 A549 cells and $\sim$ 100 to 300 cell-associated bacteria in total, were evaluated per strain and time point. The standard deviation represents the variation between fields. (B and C) Still images captured by live video microscopy of PAO1F/pP33-*p_{exoS}*-GFP (wild type) (B) and PAO1F Δ 3TOX/pP33-*p_{exoS}*-GFP (C) infecting A549 cells. Bar, 10 μ m. Arrowheads indicate bacteria that have made contact and induce GFP expression. The time postinfection in minutes is indicated above each panel.

tively expressed either wild-type ExoS or mutant forms of the protein in which Rho-GAP (R146K) (25), ADP-ribosylation (E379D/E381D) (51), or both activities had been inactivated. All proteins were readily secreted and expressed at similar levels (Fig. 3C). Interestingly, the Rho-GAP mutant and ADP-ribosylation mutant proteins were still able to prevent subsequent triggering of *exoS* expression in the reporter (Fig. 3A). Mutation of both active sites, however, resulted in a toxin that was unable to prevent induction of GFP expression in the reporter strain. This indicates that the repressive activity of ExoS is tied to its enzymatic activities and that at least one of the signaling pathways affected by ExoS is required for cell contact-mediated triggering of effector secretion.

Triggering of *exoS* expression is tied to host cell integrity but not to the low-calcium environment of the host cell cytosol. We further analyzed the cellular requirements for triggering of type III secretion by preintoxicating cells and monitoring the effect of intoxication on the ability of a reporter strain to induce *exoS* expression on cell contact.

Several of the enzymatic activities of *P. aeruginosa* exoenzymes affect the actin cytoskeleton, so we determined whether

cytochalasin D, which prevents elongation of F-actin, leading to a net disassembly of the actin cytoskeleton, prevents triggering of the T3SS. Cytochalasin D did not prevent induction of *exoS* expression on cell contact. If anything, induction of *exoS* expression was enhanced (Table 2). Cycloheximide pretreatment also did not prevent triggering of *exoS* expression, suggesting that the defect in triggering after ExoS intoxication is not due to the loss of a labile receptor protein.

On the other hand, intoxication of cells with either of two different pore-forming toxins, streptolysin O (cholesterol dependent, up to 30-nm-diameter pores) or alpha-toxin (not cholesterol dependent, 0.6- to 1-nm pores) (4), prevented triggering of the T3SS (Table 2), indicating that cells have to have an intact plasma membrane to allow triggering of the T3SS.

It has been proposed in the case of *Yersinia* that triggering of effector secretion on cell contact depends on sensing of the low calcium concentration in the host cell cytosol (35). We decided to test this hypothesis directly by using the ionophore calcimycin to shuttle calcium into the tissue culture cells and assay its effect on triggering of *exoS* expression (Fig. 4). Calcium was clearly shuttled into the cells as visualized using the calcium-

FIG. 2. Effect of preinfection on subsequent triggering of *exoS* expression. Cells were either mock infected or preinfected at an MOI of 50 for 1 h with PAO1F Δ pscC, PAO1F Δ exsE, PAO1F Δ exsE in which all three exoenzymes had been deleted (Δ 3TOX), or PAO1F Δ exsE in which two of the exoenzymes had been deleted so that only one *exo* gene remained (i.e., " Δ exsE exoS+" is Δ exsE Δ exoT Δ exoY). After the preinfection period, the cells were washed twice with PBS and the medium was replaced with fresh medium carrying the reporter strain (PAO1F Δ 3TOX *attB::groE-mCherry/pP33-p_{exoS}-GFP*) at an MOI of 25. The infection was continued for another 4 h, at which point the cells were washed twice with PBS and fixed with paraformaldehyde before being mounted for microscopic examination. Four to seven fields totaling ~40 to 70 A549 cells and ~120 to 250 cell-associated bacteria were analyzed for each condition. The error bars indicate standard deviations between fields. (A) Percentage of GFP-positive, cell-associated reporter bacteria (mCherry positive). The preinfection strains are noted on the x axis. Statistical significance was determined using Student's *t* test (*, $P < 0.05$). (B) Attachment of the reporter bacteria to cells (mCherry-positive bacteria/cell). The preinfection strains are noted on the x axis. (C) Secretion of effectors. The indicated strains were grown in LB. Secretion of effectors was triggered by removing calcium from the medium. Culture supernatants were collected after removing the bacteria by centrifugation, TCA precipitated, and resuspended in sample buffer. Samples were normalized based on the OD₆₀₀ of the culture, separated by SDS-PAGE, blotted, and probed with a mix of antibodies directed against ExoS, ExoT, ExoY, and PopN.

sensitive dye rhodamine 5N (K_d , 320 μ M; Invitrogen), but triggering of *exoS* expression on cell contact was unaffected.

The translocator proteins PopB and PopD are required for cell contact-mediated up-regulation of T3SS expression. Two models have been formulated with regard to triggering of type

FIG. 3. Influence of ExoS active-site mutations on the block in subsequent triggering of *exoS* expression by the reporter bacteria. The experiment was conducted as described for Fig. 2. Preinfection strains were derived from PAO1F Δ exsE and expressed only the indicated exoenzymes. Ten to 12 fields totaling ~150 to 200 A549 cells and ~300 to 700 cell-associated bacteria were analyzed for each condition. The error bars indicate standard deviations between fields. (A) Percentage of GFP-positive, cell-associated reporter bacteria (mCherry positive). The preinfection strains are noted on the x axis. Statistical significance was determined using Student's *t* test (**, $P < 0.01$). (B) Attachment of the reporter bacteria to cells (mCherry-positive bacteria/cell). The preinfection strains are noted on the x axis. (C) Secretion of effectors. Secretion was monitored as described for Fig. 2. The blot was probed with an anti-ExoS antiserum. The band above the ExoS band in the PAO1F Δ exsE supernatant is ExoT, which weakly cross-reacts with the anti-ExoS antiserum.

III secretion on cell contact. On the one hand, interaction of the needle tip or a needle tip-associated translocator protein with the plasma membrane or a putative receptor could result in triggering of the T3SS (as proposed for *Shigella* [71]). This in turn results in secretion of the remaining translocators, which are inserted into the plasma membrane, forming a pore to which the needle is subsequently docked. On the other hand, the translocators could be secreted prior to cell contact and

TABLE 2. Effect of toxins and inhibitors on cell contact-dependent *exoS* induction

Treatment	Medium ^a	Mean $\pm$ SD			
		% GFP-positive cell-associated bacteria	No. of bacteria/cell	% GFP-positive bacteria	
				No cells ^b	No cells + EGTA
PBS	RPMI	21.7 $\pm$ 4.4	3.7 $\pm$ 2.1	0.1 $\pm$ 0.1	55.5 $\pm$ 11.2
Alpha-toxin	RPMI	0.1 $\pm$ 0.2	30.9 $\pm$ 5.4	0.1 $\pm$ 0.2	62 $\pm$ 11.9
Streptolysin O	RPMI	0.4 $\pm$ 0.4	17 $\pm$ 3.4	0.1 $\pm$ 0.3	34.5 $\pm$ 2
Dimethyl sulfoxide	RP10	6.2 $\pm$ 4.5	13.5 $\pm$ 3.8	ND ^c	ND
Cytochalasin D	RP10	33.5 $\pm$ 7.9	8 $\pm$ 1.1	ND	ND
Cycloheximide	RP10	15.2 $\pm$ 8.1	6.4 $\pm$ 1.9	ND	ND

^a The experiments were performed in RPMI with 10% FBS (RP10) or without FBS (RPMI).

^b "No cells" indicates bacteria grown under tissue culture conditions in the indicated medium and with the corresponding toxin/inhibitor but in the absence of A549 cells.

^c ND, not determined.

inserted into the plasma membrane, and docking of the needle to the translocation pore results in a specificity switch and secretion of effector proteins (35). If the former is true, then induction of *exoS* expression on cell contact should be independent of at least one of the translocator proteins; if the latter is the case, then induction of the reporter should depend on both PopB and PopD. It was recently reported that triggering of *exoS* expression upon cell contact depends on PopD (67).

This does not, however, fully distinguish the two proposed models.

Mutants lacking either *popB* or *popD* were not able to trigger *exoS* expression on cell contact (Fig. 5A). The defect in *exoS* induction could be complemented by supplying the deleted gene on a plasmid. Loss of either translocator protein did not significantly affect attachment (Fig. 5B). Triggering of *exoS* expression on cell contact therefore depends on a functional

FIG. 4. Effect of the calcium ionophore calcimycin on triggering of *exoS* expression on cell contact. A549 cells were pretreated for 30 min with dimethyl sulfoxide (DMSO) or 10 μ M calcimycin and subsequently infected with the *exoS* reporter strain PAO1F Δ 3TOX *attB::groE-mCherry* Δ *exoS::GL3* at an MOI of 10 for 4 h. Cells were then washed twice with PBS lacking calcium and incubated for 10 min with 5 μ M rhodamine 5N in PBS (no calcium). Cells were washed again twice with PBS and fixed with paraformaldehyde before being mounted for microscopic examination. Four (DMSO) or five (calcimycin) fields totaling 78 (DMSO) or 90 (calcimycin) A549 cells and 298 (DMSO) or 465 (calcimycin) cell-associated bacteria were analyzed. (A) representative differential interference contrast (DIC), rhodamine channel, and GFP channel fluorescent images. (B) Percent GFP-positive cell-associated bacteria. (C) Average cell-associated (mCherry-positive) bacteria/cell. Error bars indicate standard deviations between fields.

FIG. 5. PopB and PopD are required for triggering of *exoS* expression on cell contact. (A) A549 cells were infected for 4 h (MOI, 10) with the reporter strain PAO1F Δ 3TOX *attB::groE-mCherry* Δ *exoS::GL3* or a Δ *popB* or Δ *popD* derivative thereof harboring either the vector plasmid (pPSV35) or the complementation plasmid (pP35-*popB* or pP35-*popD*, respectively). The infection was carried out in RPMI with 10% FBS and 10 μ M IPTG (isopropyl- β -D-thiogalactopyranoside). GFP expression was assayed by microscopy. Five to eight fields totaling 100 to 130 A549 cells and 500 to 1,000 cell-associated bacteria were analyzed for each strain. The error bars indicate standard deviations between fields. Statistical significance was determined using Student's *t* test (*, *P* < 0.05). (B) Attachment of the reporter bacteria to cells (mCherry-positive bacteria/cell). (C) PAO1F Δ *exoS::GL3*, a Δ *popB* derivative, and a Δ *popD* derivative were grown in "high-salt" LB. *exoS* expression was induced by removing calcium from the medium through the addition of EGTA (5 mM final concentration) and assayed by beta-galactosidase assay. Activity (averages from three assays) is depicted in Miller units, and error bars denote the standard deviations between replicates. w.t., wild type.

translocase. This observation differentiates induction on cell contact from induction under low-calcium conditions, which is independent of PopB and PopD (Fig. 5C) (67). As had been demonstrated for YopB in the *Yersinia* system (58), neither a *popB* mutant nor a *popD* mutant could be *trans*-complemented in a coinfection experiment (see Fig. S2 in the supplemental material), suggesting that both translocators have to be secreted in *cis*.

ExoS feedback regulates effector secretion. To confirm that ExoS controls effector secretion, we monitored translocation of wild-type ExoS into A549 cells. All experiments were performed with a deregulated strain lacking *exsE* to rule out the effect of changes in expression on the result of the transloca-

FIG. 6. Translocation of effectors into A549 cells. A549 cells were infected in medium lacking FBS for 2 h (MOI, 50). All strains were Δ *exsE*. *exoS*⁺ and *exoS*(G/A) were deleted for *exoY* and *exoT*, *exoT*⁺ was deleted for *exoS* and *exoY*, and Δ *pcrV* expressed all effectors. Cells were washed with PBS and treated with proteinase K where indicated, and proteins were extracted with Triton X-100 or SDS as indicated. Proteins were detected by Western blotting, and the relevant bands are identified on the right of each blot. Individual lanes are numbered below the blot.

tion assay. Cells were infected at a relatively high MOI (50) for 2 h. The cells were then washed and in some cases treated with proteinase K to digest protein associated with the outside of the cells. Translocated proteins were liberated by lysing the host cells with the detergent Triton X-100, which does not lyse *P. aeruginosa*. As a control, cells and cell-associated bacteria were lysed using SDS.

While a strain expressing the wild-type ExoS (*exoS*⁺) translocated only very little ExoS into the A549 cells (Fig. 6, lanes 1 and 2), a mutant expressing equal amounts of the GAP/ADP-ribosylation double mutant ExoS (G/A) translocated significantly more protein into the targeted epithelial cells (Fig. 6, lanes 4 and 5). Consistent with the preintoxication experiment, therefore, these data indicate that translocation of wild-type ExoS is self-inhibited in a manner that depends on its enzymatic activity. As expected (64), translocation depended on a functional needle tip protein (PcrV) (Fig. 6, lanes 10 and 11). ExoT exhibited an intermediate phenotype in this assay (Fig. 6, lanes 7 and 8), consistent with the intermediate phenotype observed in the preintoxication experiment (Fig. 2).

Calcium controls effector secretion but not translocator secretion in vitro. The requirement of PopB and PopD for triggering of *exoS* expression on cell contact suggests that translocators are secreted before effectors. In vitro, however, secretion of the effectors and translocators appears to be simultaneous upon depletion of calcium from the medium (75).

To test whether calcium in fact controls secretion of translocator proteins we performed experiments in which we reestablished calcium control of secretion after depleting calcium from the medium using EGTA. Wild-type PAO1 was grown in LB either in the presence of 0.5 mM Ca²⁺ or in the same medium supplemented with 5 mM EGTA. Once the bacteria had reached an OD₆₀₀ of ~0.2, the cultures were split in half, the cells were pelleted, and the bacteria from each culture were resuspended in medium with calcium or depleted of calcium with EGTA. The cultures were then incubated for another 30

FIG. 7. Calcium control of effector and translocator secretion. Wild-type PAO1 was grown to an OD_{600} of ~ 0.2 in either the presence or absence of calcium, and bacteria were subsequently spun down and resuspended in medium with calcium or lacking calcium and incubated for an additional 30 min before the cells were pelleted. The cell pellet and TCA-precipitated supernatant protein were separated by SDS-PAGE gel, transferred to nitrocellulose, and probed with the indicated antiserum. The four conditions are indicated above each lane: growth with calcium, spun down, and resuspended in medium with calcium (+/+); growth with calcium, spun down, and resuspended in medium without calcium (5 mM EGTA) (+/-); growth without calcium, spun down, and resuspended in medium with calcium (-/+); and growth without calcium, spun down, and resuspended in medium without calcium (-/-).

min at 37°C before pelleting the bacteria and precipitating supernatant proteins with TCA.

While secretion of “effector” class proteins (ExoS, ExoT, ExoY, ExsE, and PopN) was blocked immediately by resuspending bacteria that had been induced with EGTA in medium containing calcium, secretion of the translocator proteins (PopB, PopD, and PcrV) continued unabated (Fig. 7). Some secretion of PopD was apparent even in the culture that had

not been treated with EGTA at any point. These data suggest that calcium does not, in fact, control secretion of the translocator proteins and that the apparent triggering of translocator secretion witnessed in the standard experiment is likely a combination of up-regulation of translocator expression and an increase in the number of apparatuses. Additionally, degradation of secreted proteins by chelator-sensitive proteases (55) may also mask the secretion of translocators in the presence of calcium. In the case of *Yersinia*, it has been postulated that secretion of translocator proteins is stimulated by serum proteins, such as serum albumin (35). In our hands, however, translocators were secreted even in minimal medium lacking any protein (see Fig. S3 in the supplemental material), suggesting that secretion of translocator proteins is simply constitutive.

DISCUSSION

Type III secretion is a common virulence mechanism among gram-negative pathogens. One of the hallmarks of type III secretion is that toxins are delivered vectorially into the host cell cytoplasm in a contact-dependent manner. While elegant experiments with *Salmonella* have demonstrated that triggering of secretion is a very rapid process (60), the precise order of steps involved or, indeed, the involvement of host factors in controlling type III secretion is poorly understood.

Here we present data that support a model for cell contact-mediated triggering of effector secretion by *P. aeruginosa* (Fig. 8). We have presented data that secretion of translocator proteins (PopB, PopD, and PcrV) proceeds even in the presence of calcium. Cell contact, therefore, rather than triggering secretion per se, results in a switch in the secretion specificity, allowing secretion of effector class proteins (ExoS, ExoT, ExoY, PopN, and ExsE). While the data presented suggest that secretion of translocators is calcium independent, a closer look at the data, in particular for the deregulated *exsE* mutant (see Fig. S3 in the supplemental material), suggests that secretion of translocators may be more efficient in the absence of calcium. There could be several explanations for this observation. For one, it is known that removing calcium from the medium

FIG. 8. Model of translocon insertion and triggering of effector secretion on cell contact. The individual steps are depicted from left to right. V, PcrV; B, PopB; D, PopD. The question mark indicates a yet-unidentified eukaryotic factor (or factors) required for triggering of effector secretion, which is negatively controlled by translocated ExoS. The changes in the translocon and needle that are proposed to signal effector secretion are depicted as a thick border (PopB and PopD) or black shading of the needle.

stimulates cyclic AMP production, which in turn up-regulates type III secretion gene expression (74). In other words, even though the *exsE* mutant is deregulated, it may still receive a slight boost in expression by the cyclic AMP/Vfr pathway when grown in the absence of calcium. Consistent with this observation, *exoS* expression is slightly increased when *P. aeruginosa* is grown in the absence of calcium, even when *exsE* is deleted (55, 68). Alternatively, degradation of secreted protein by a chelator-sensitive protease could account for the apparent reduction of secreted PopB. An interesting third possibility is that the rate of secretion increases when effector secretion is triggered. There is some evidence to support this hypothesis, since the activity of the translocation ATPase in *Yersinia*, YscN (PscN in *P. aeruginosa*), is negatively regulated by the associated protein YscL (PscL) (5). YscL is required for type III secretion, and its level has to be carefully controlled since overexpression also negatively affects secretion (5). Interestingly, a 5-amino-acid deletion of the flagellar YscL homolog, FliH, was found to stimulate the ATPase activity of the flagellar ATPase, FliI, raising the possibility that YscN (PscN) ATPase activity could be activated as well as repressed (26).

These data also suggest that the current model of how YopN controls effector secretion in the closely related *Yersinia* T3SS may not apply to *P. aeruginosa*. A *yopN* mutant, just like a *P. aeruginosa* *popN* mutant, secretes effectors constitutively (13, 54, 64). It was proposed that YopN enters the secretion channel and blocks it by virtue of its C-terminal interaction with TyeA, which in turn is bound to a yet-to-be-identified component of the secretion machinery (19). Here we demonstrated that translocators are clearly secreted under conditions where secretion of PopN (the *P. aeruginosa* YopN homolog) is shut down, suggesting that PopN cannot be blocking the secretion channel (Fig. 7). This is likely also the case for *Yersinia*, since the proposed model is also not consistent with the finding that translocators but not effectors are secreted into the culture supernatant by *Yersinia* infecting tissue culture cells (34, 58). A simple model to explain these results would be that the PopN-Pcr1-Pcr2-PscB complex (YopN-TyeA-SycN-YscB in *Yersinia* spp.) occludes a component of the type III secretion machinery that allows access of effector-chaperone complexes to the secretion ATPase, while translocator-chaperone complexes gain access via a different route. Consistent with this hypothesis, it was recently demonstrated that certain *yscU* mutants fail to secrete translocators while retaining the ability to secrete effectors (62). Our assay to reestablish calcium control of secretion is a simple method to discriminate proteins secreted as "translocator" and "effector" class secretion substrates. It will be interesting to determine the molecular basis of the signals that allow the T3SS to differentially recognize translocators and effectors. In this context, it is tempting to speculate that the cognate chaperones may be involved in targeting translocators for secretion, since translocator and effector class chaperones differ structurally (48).

It was recently demonstrated that PopD is required for triggering of T3SS gene expression on cell contact (67). We have found that the switch to effector class secretion requires a functional translocation pore. Mutants lacking PopB or PopD fail to induce *exoS* expression on cell contact (which relies on the secretion of ExsE [55, 67, 68]). Our findings imply that the PcrV-dependent insertion of PopD into the host cell plasma

membrane itself cannot be the signal for triggering effector secretion, since PopD is still inserted in a *popB* mutant (28). As has been demonstrated for YopB in the case of *Yersinia* (58), neither *popB* nor *popD* mutants can be cross-complemented, arguing that insertion of the translocators has to occur in close proximity to the secretion needle. This is consistent with the previous finding that insertion of PopD requires the needle tip protein PcrV (27, 28). The simplest model for secretion control, therefore, is that after attachment to the cell, when the needle tip is brought into close proximity to the host cell, the translocators are inserted into the plasma membrane, forming the translocation pore. A yet-unknown host factor acts at the stage of either translocator insertion, docking of the needle to the translocation pore, or stabilization of the translocase. In our estimation the latter two possibilities are more likely, since PopB and PopD can form pores in model membranes in the absence of other cofactors (17). Interestingly, PcrV cannot bind to these pores (61). This could of course also be due to an inherently weak affinity of PcrV for the translocation pore, requiring either other cofactors or multiple simultaneous interactions as found in the assembled needle tip. The needle tip most likely harbors multiple PcrV molecules (8) that can interact with the translocation pore (purified PcrV, on the other hand, is a monomer [16]). We propose that it is docking of the needle tip to the translocation pore that results in a conformational change in PcrV, which is propagated down the needle and results in the activation of effector secretion. In this context, it should be noted that it has been proposed that removing calcium from the medium in vitro results in triggering effector secretion by causing a conformational change in the needle (66). It is therefore easy to envision how removing calcium from the needle in vitro essentially bypasses the need for a host-derived signal, which would originate at the assembled translocation apparatus, by switching the organization of the needle to a conformation that signals effector secretion.

Our findings are analogous to data from the *Yersinia* system, where it was found that translocation of effector proteins can be modulated by the action of the effector protein YopE (2). The amino-terminal portion of ExoS and ExoT (comprising the secretion signal, chaperone binding site, membrane localization domain, and Rho-GAP domain) are homologous to YopE. Interestingly, ExoS and ExoT can substitute for YopE with regard to control of effector translocation, suggesting that these two instances of translocation control are related (1). There are some differences between these systems, however, since a second protein, YopK, which has no homolog in *P. aeruginosa*, also contributes to translocation control in *Yersinia*. In our hands, both the Rho-GAP activity of ExoS and the ADP-ribosylation activity can serve to control effector translocation. This observation could be interpreted to mean that the signaling pathways affected by either activity of ExoS converge to control the activity of the host factor required for triggering effector secretion on cell contact. Alternatively, either activity could affect a separate process, both of which are required for the host factor to be active (e.g., down-regulating a step in phospholipid biogenesis and affecting phospholipid trafficking, either of which results in a change in plasma membrane phospholipid composition). Clearly, a better understanding of the nature of the host factor will be required to understand the role of ExoS in controlling the "effector switch."

The observation that either activity can control the switch to effector secretion is in contrast to the case for *Yersinia*, where only the Rho-GAP domain of ExoS was able to control effector translocation when expressed in a *yopE* null mutant. This discrepancy is perhaps due to differences in the cells used in these experiments (HeLa versus A549 cells). In the case of *Yersinia*, it has been suggested that injection of YopE generates a negative signal that shuts down effector translocation (1). Our results are more consistent with the interpretation that the switch to effector secretion requires a preexisting host factor, which is inactivated by ExoS. Permeabilization of cells with either streptolysin O or alpha-hemolysin, two pore-forming toxins with differing pore sizes and membrane requirements, abolishes triggering of effector secretion. Since these toxins kill the cell and allow release of small metabolites and nucleotides, it is unlikely that activation of a signaling cascade is responsible for the lack of *exoS* induction in this case. It has been described for both the *Yersinia* and *P. aeruginosa* systems that type III secretion can display target cell specificity (39, 42, 57). The absence of a cellular function required for activation of effector secretion offers a simple explanation for this phenomenon.

The nature of the signal or cellular function required for triggering of effector secretion is unclear. It has been proposed that the signal for triggering type III secretion is in fact the low-calcium environment of the host cell cytosol, which could conceivably be sensed once the translocation pore has formed and docked to the needle (35). Raising the intracellular calcium pool by using the ionophore calcimycin, however, had no effect on the ability of *P. aeruginosa* to induce *exoS* expression, suggesting that calcium plays no role in triggering of effector secretion on cell contact. In the *Yersinia* system YopE was demonstrated to prevent accumulation of actin at the site of bacterial attachment. In a *yopE* mutant, accumulation of actin was correlated with an increase in pore formation, and it was hypothesized that localized membrane ruffling resulted in the translocation pore being jarred loose from the needle (72). Accumulation of actin, however, is apparently not involved in controlling effector secretion, since addition of cytochalasin D did not prevent triggering of *exoS* expression.

Clearly, determining the molecular basis of triggering of effector secretion on cell contact will add an important facet to our understanding of type III secretion. As mentioned above, controlling the switch to effector secretion may underlie the observed target cell specificity. It is easy to imagine how being able to target specific cell types, or perhaps more importantly to avoid injecting toxins into innocuous cells, can help shape the course of an infection. A clearer understanding of the molecular principles governing the specificity of effector secretion on cell contact will allow us to design experiments to directly test its role in infection.

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