Control of type III secretion activity and substrate specificity by the cytoplasmic regulator PcrG

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Edited by Philippe J. Sansonetti, Institut Pasteur, Paris, France, and approved April 2, 2014 (received for review February 11, 2014)

Pathogenic Gram-negative bacteria use syringe-like type III secretion systems (T3SS) to inject effector proteins directly into targeted host cells. Effector secretion is triggered by host cell contact, and before contact is prevented by a set of conserved regulators. How these regulators interface with the T3SS apparatus to control secretion is unclear. We present evidence that the proton motive force (pmf) drives T3SS secretion in *Pseudomonas aeruginosa*, and that the cytoplasmic regulator PcrG interacts with distinct components of the T3SS apparatus to control two important aspects of effector secretion: (*i*) It coassembles with a second regulator (Pcr1) on the inner membrane T3SS component PcrD to prevent effectors from accessing the T3SS, and (*ii*) In conjunction with PscO, it controls protein secretion activity by modulating the ability of T3SS to convert pmf.

Many Gram-negative bacterial pathogens rely on a type III secretion system (T3SS) to promote disease by directly injecting effector proteins into the cytoplasm of host cells. This apparatus consists of a base that spans the bacterial envelope and a needle that projects from the base and ends in a specialized tip structure. The bacterium secretes two translocator proteins via the T3SS, which insert into the host cell membrane to form a pore, through which effector proteins are then transferred (1, 2).

One of the hallmarks of type III secretion is that export of effector proteins is triggered by host cell contact (3–5). The secretion apparatus is fully assembled before cell contact, but effector secretion is prevented through the concerted action of needle tip-associated proteins and regulators that control secretion from the bacterial cytoplasm.

In most systems, the needle tip protein prevents premature effector secretion, most likely by allosterically constraining the T3SS in an effector secretion "off" conformation (6–10). PcrG, the needle tip protein chaperone, as well as PopN, a member of the YopN/MxiC family of proteins, control effector secretion from the bacterial cytoplasm in *Pseudomonas aeruginosa*. PcrG's regulatory function is independent of its function in promoting the export of needle tip protein PcrV. Deletion of *pcrG* or *pcrV* results in partial deregulation of effector secretion, whereas removal of both genes results in high-level secretion of effectors (8). In some bacteria, the needle tip protein promotes its own export with the aid of a self-chaperoning domain, rather than with a separate export chaperone (11). Recent evidence suggests that in these systems, the needle tip protein itself also regulates effector secretion from the cytoplasm, in addition to its regulatory role at the T3SS needle tip (12). The mechanism of this regulation is unclear.

YopN/MxiC family proteins, PopN in *P. aeruginosa*, are T3SS regulators that are exported once effector secretion is triggered (13–17). These proteins control effector secretion from the bacterial cytoplasm (18–20). *P. aeruginosa* PopN and the closely related YopN associate with three other proteins that are required to prevent premature effector secretion (21–23). For PopN, these three proteins are Pcr1, Pcr2, and PscB. Pcr2 and PscB form a heterodimeric export chaperone, and Pcr1 is thought to tether the PopN complex to the apparatus (23). The prevailing model for explaining how PopN and related regulators control effector secretion is that they partially insert and plug the secretion channel while being tethered to the T3SS, either directly via a C-terminal interaction or indirectly via a C-terminal–associated protein, i.e., Pcr1 in *P. aeruginosa* (19, 20). The apparatus component with which these regulators interact is unknown, however.

Triggering of effector secretion results in the rapid injection of effector proteins into the host cell (4, 5). How this rapid burst of secretion is energized is a matter of some controversy. The flagellum, which also uses a type III secretion mechanism, uses the proton motive force (pmf) to catalyze the rapid export of flagellar subunits. In fact, secretion is possible in mutants lacking the flagellum-associated ATPase, FliI, if the associated regulatory protein, FliH, is eliminated as well (24–26). The pmf's contribution to the rate of secretion relative to the ATPase has been questioned in the case of virulence-associated T3SS (27), where removal of the ATPase results in a complete block of secretion (28, 29) that is not alleviated by deletion of the associated FliH homolog (30).

Here we present evidence that export via the *P. aeruginosa* T3SS is energized primarily by the pmf, thereby offering a unified model for how protein secretion is energized in all T3SSs. The cytoplasmic T3SS regulator PcrG controls both the access of effectors to the T3SS and, surprisingly, the secretion activity of the apparatus. These two functions are controlled by separate regions of PcrG. Control of secretion activity involves the central portion of PcrG as well as PscO, which regulate the pmf-dependent export of secretion substrates. Mutants that up-regulate translocator secretion without turning on effector export confirm that effector secretion is not blocked by physical obstruction of

Significance

Type III secretion systems (T3SSs) are nanomachines used by pathogenic bacteria to directly inject effector proteins into host cells and thereby promote disease. Effector secretion is actively prevented until it is triggered by host cell contact. We have determined how cytoplasmic regulators that prevent premature effector secretion interact with the apparatus to block effectors from accessing T3SS. We also demonstrate that the burst of secretion observed when effector secretion is triggered consists of two genetically separable components: access of effectors to the T3SS and a change in secretion activity. The latter is achieved by modulating the ability of the T3SS to convert proton motive force into protein export.

Author contributions: P.-C.L. and A.R. designed research; P.-C.L., S.E.Z., C.M.S., and J.T. performed research; P.-C.L. and A.R. analyzed data; and A.R. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1402658111/-/DCSupplemental.

the secretion channel. Instead, access of effectors to the T3SS is controlled by the C terminus of PcrG in conjunction with the PopN complex through an interaction with the inner membrane T3SS component PcrD. This protein complex likely blocks an acceptor site for effectors. Thus, PcrG is a multifaceted protein that, along with its export chaperone function, serves as a brake and a switch to control effector secretion.

Results

PcrG Controls Both Secretion Activity and Specificity of the Type III Secretion Apparatus. Our previous analysis demonstrated that the N-terminal 40 amino acids of PcrG are sufficient to bind to PcrG's cognate export substrate, PcrV, but have no regulatory role. In contrast, the C-terminal 45 amino acids of PcrG are sufficient to restore effector secretion control to near-WT levels in a *pcrG* null mutant strain (8). To characterize the regulatory activity of the C-terminal portion of PcrG further and identify residues that could be involved in contacting the apparatus, we analyzed the ability of truncated versions of PcrG, as well as of point mutants affecting a subset of conserved residues in the C terminus of the protein (Fig. 1*A*), to control effector secretion.

Point mutants and truncated versions of *pcrG* were fused to a version of *malE* that encodes a signal sequence-less maltose-binding protein (MBP), and then assayed for their ability to complement a *pcrG* deletion strain. Production of the MBP-PcrG fusion proteins was confirmed by Western blot analysis (Fig. 1*B*). Fusion of PcrG to the C terminus of MBP helps stabilize fragments of PcrG that otherwise would be rapidly degraded (8). To assay effector secretion control, we made use of a feature of the *P. aeruginosa* system whereby triggering of effector secretion results in up-regulation of T3SS gene expression owing to export of the negative regulator ExsE via the T3SS (31, 32). Thus, the triggering of effector secretion by removal of calcium from the medium or by cell contact can be assayed using a transcriptional reporter of effector gene expression, in our case *exoS*.

WT MBP-PcrG was fully functional and prevented triggering of effector secretion in the presence of calcium, as assayed using the *exoS-lacZ* reporter system (Fig. 1*C*). PcrG lacking the C-terminal 25 amino acids, PcrG(Δ71–95), was inactive, whereas the converse fusion, PcrG(Δ1–59), partially restored effector secretion control (Fig. 1*C*). PcrG(R85E/R86E), PcrG(M90A/R91A/G92A), and PcrG(I95E) were at least partially defective in terms of preventing effector secretion in the presence of calcium, highlighting the importance of the C terminus for effector secretion control.

To assess effector secretion control directly, and also to assay the effect of our *pcrG* mutations on the export of translocator proteins, which are secreted before effector secretion is triggered, we performed secretion assays in a Δ*exsE* Δ*pcrG* mutant strain background. Deletion of *exsE* results in constitutive, high-level expression of the T3SS and its substrates and minimizes the effect of changes in T3SS activation on gene expression and protein levels (31). A Δ*exsE* mutant strain assembles an equal number of secretion apparatuses in both the presence and absence of calcium (*SI Appendix*, Fig. S1). Effector secretion is still tightly regulated and has to be triggered, either by removing calcium from the medium or by cell contact (31, 32). We also removed *fleQ*, the master regulator of flagellar gene expression, to eliminate residual secretion of substrates via the flagellar T3SS. Removal of *pcrG* in the Δ*exsE* strain resulted in elevated secretion of both effectors and translocators (Fig. 1 *D* and *E*). Production of WT PcrG, as well as PcrG(A16R), which is unable to bind to PcrV (8), prevented effector secretion in the presence

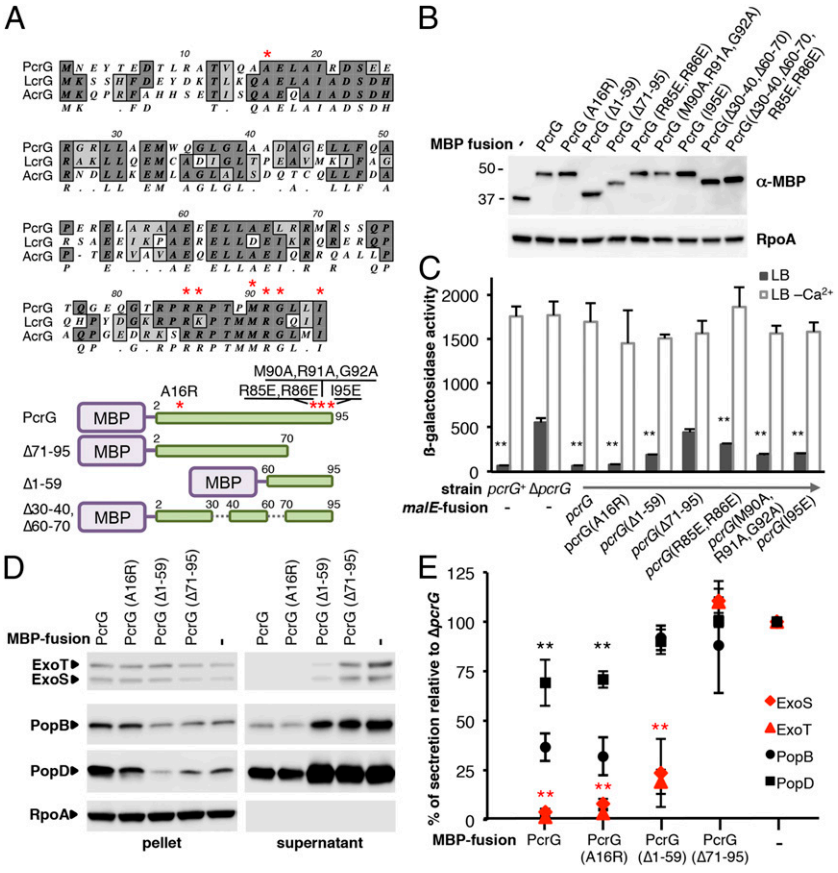

Fig. 1. PcrG controls secretion activity and specificity. (*A*) PcrG of *P. aeruginosa* PAO1 and homologs from *Y. pseudotuberculosis* YPIII and *A. hydrophila* AH3 were aligned using ClustalW (accession nos.: PcrG, AAG05094.1; LcrG, AAA27644.1; AcrG, AAS91818.1). Identical (dark gray) and similar (light gray) residues are indicated. PcrG point mutants (red asterisk) and truncations fused to MBP and assayed in *B* are shown below. (*B*) Production of MBP-PcrG fusion proteins was assayed in strain PAO1F Δ*pcrG* Δ*exoS*::GFP-*lacZ*, complemented with the indicated *malE-pcrG* fusions and induced with 10 μM isopropyl β-D-1-thiogalactopyranoside (IPTG). (*C*) Control of effector secretion with and without calcium was analyzed by the β-galactosidase assay using the same strain background and IPTG concentration. Statistical significance of differences compared with the vector control is noted. $^{**}P < 0.01$, Student *t* test. (*D*) PAO1F Δ*exsE* Δ*fleQ* Δ*pcrG* producing the indicated MBP-PcrG fusion protein (induced with 25 μM IPTG) was grown in the presence of calcium. Cell pellet and supernatant samples were probed with antibodies against effector proteins (ExoS and ExoT) or translocator proteins (PopB and PopD). RpoA served as a fractionation control. (*E*) Three independent secretion assays were quantitated. The secretion efficiency (i.e., supernatant protein/total protein) in strains producing the indicated MBP-PcrG fusions was determined for each protein by quantitating the band intensities and normalizing secretion to the Δ*pcrG* parent strain (set to 100%). Statistical significance of differences relative to vector control was determined for combined translocator secretion (black asterisk) or effector secretion (red asterisk). $^{**}P < 0.01$, Student *t* test.

of calcium and also reduced the amount of translocator proteins secreted (Fig. 1 *D* and *E*). PcrG lacking the C-terminal 25 amino acids, PcrG(Δ71–95), lost all regulatory control. Surprisingly, production of PcrG(Δ1–59) could significantly reduce effector export, but failed to reduce translocator secretion, suggesting that access of effectors to the apparatus and control of translocator secretion are genetically separable parameters (Fig. 1 *D* and *E*). Thus, the C terminus of PcrG affects the access of effectors to the T3SS without controlling translocator secretion. Control of both effector and translocator export requires all of PcrG, albeit not the ability to bind to PcrV.

The Central Portion of PcrG Controls Secretion Activity. Because PcrG is not secreted, it must control effector secretion by interacting with a cytoplasmic or inner membrane component of the T3SS. We identified potential binding partners for PcrG using a bacterial two-hybrid system (33). PcrG was fused to λ cI, and potential interaction partners were fused to the RNA polymerase α-subunit. Interaction results in cI-PcrG–dependent recruitment of RNA polymerase to a test promoter driving β-galactosidase expression. PcrG interacts more strongly with PscO than with any other cytoplasmic T3SS component in this assay (*SI Appendix*, Fig. S2*A*). PscO is homologous to the flagellar protein FliJ, which was recently linked to the pmf-dependent export of flagellar subunits (26).

To determine whether the interaction of PcrG with PscO involves the C-terminal 35 amino acids of PcrG that are important for effector secretion control (Fig. 1 *C* and *E*), we mapped the interaction by deletion analysis using the two-hybrid system. Residues 2–70 of PcrG were sufficient to bind to PscO in this assay, indicating that this interaction does not involve the C-terminal 25 amino acids of PcrG. Deletion of amino acids 1–20, 1–30, or 1–40 of PcrG progressively diminished binding to PscO; however, amino acids 2–40 of PcrG were not sufficient to allow binding to PscO, arguing that recognition of PscO involves the central portion of PcrG. Indeed, deletion of residues 30–40 and 60–70 of PcrG [PcrG(Δ30–40,Δ60–70)] resulted in a protein that can still interact with PcrV, but fails to bind to PscO (Fig. 2*A*). This result was confirmed by coprecipitation of proteins produced recombinantly in *Eshcerichia coli*. PcrG and PcrG(Δ71–95), but not PcrG(Δ30–40,Δ60–70), were able to coprecipitate a Myc-tagged version of PscO (*SI Appendix*, Fig. S2*B*). Binding of PcrG to PscO involves the central portion of PcrG and likely is not responsible for specificity control. Indeed, *pcrG(Δ30–40, Δ60–70)* was able to complement a *pcrG* null mutant to near-WT levels with regard to preventing effector secretion in the presence of calcium (Fig. 2*B*).

We next analyzed the ability of PcrG(Δ30–40,Δ60–70) to control translocator export in the Δ*exsE* mutant background. In the presence of calcium, export of the PopB, PopD, and PcrV translocator proteins was up-regulated relative to WT (Fig. 2*C*). In contrast, the level of effector export was significantly reduced compared with the *pcrG* null mutant strain (vector control) when providing PcrG(Δ30–40,Δ60–70). This phenotype closely mirrors the secretion phenotype seen when expressing PcrG(Δ1–59) (Fig. 1 *D* and *E*). Taken together, these data indicate that the *pcrG (Δ30–40,Δ60–70)* mutation results in selective up-regulation of translocator protein secretion over effector secretion.

Given that both PcrG and PcrV are needed to block effector secretion completely, we explored whether assembly of PcrV at the needle tip influences the *pcrG(Δ30–40,Δ60–70)* mutant phenotype. We assayed the ability of PcrG(Δ30–40,Δ60–70) to block effector export either in the absence of PcrV or in a strain producing PcrV(F279R), which is specifically defective for assembling at the needle tip (8). Whereas PcrG(Δ30–40,Δ60–70) was able to control effector export in a strain expressing WT PcrV, deletion of *pcrV* or prevention of PcrV assembly at the needle tip resulted in complete deregulation of effector secretion

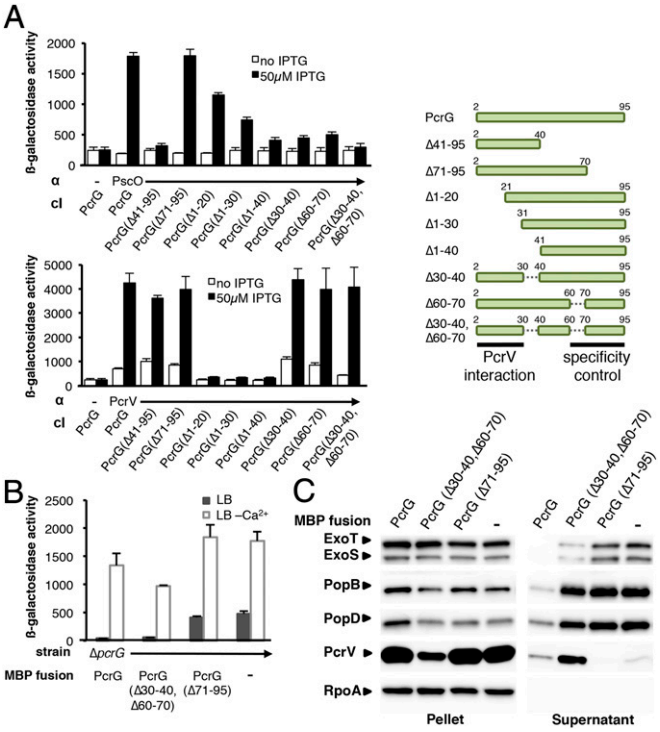

Fig. 2. PcrG interacts with the cytoplasmic T3SS component PscO. (*A*) Interaction-dependent recruitment of RNAP to the test promoter was monitored by the β-galactosidase assay. Production of the indicated α- and cI-fusion proteins was induced by adding IPTG to the medium. A schematic of the assayed PcrG mutants, as well as the regions of PcrG previously identified as important for binding to PcrV and control of effector secretion, is on the right. (*B*) Production of the indicated MBP-PcrG fusion protein was induced with 10 μM IPTG in strain PAO1F Δ*pcrG* Δ*exoS*::GFP-*lacZ*, and control of effector secretion was determined by the β-galactosidase assay. (*C*) Control of effector (ExoS, ExoT) and translocator (PopB, PopD, PcrV) secretion in strain PAO1F Δ*exsE* Δ*fleQ* Δ*pcrG* was evaluated by Western blot analysis. Bacteria were grown in the presence of Ca^{2+}, and expression of the indicated MBP-PcrG fusions was induced with 25 μM IPTG. RpoA served as a fractionation control.

(Fig. 3). Notably, combining PcrG(Δ30–40,Δ60–70) with the Arg85Glu/Arg86Glu double mutation resulted in complete loss of regulatory control, demonstrating that PcrG(Δ30–40,Δ60–70) still needs to associate with the apparatus via the regulatory interaction involving its C terminus (Fig. 3). Thus, we conclude that either PcrG(Δ30–40,Δ60–70) needs PcrV to assemble at the needle tip to be able to associate with the apparatus, or the *pcrG* (Δ30–40,Δ60–70) mutation causes a general increase in secretion, not just of translocator proteins, and hence results in high-level effector secretion when the apparatus is no longer held in the effector secretion "off" conformation in the absence of the needle tip.

PscO Controls Secretion Activity. After identifying PscO as an interaction partner of PcrG, we probed its involvement in secretion regulation by screening for *pscO* mutations that phenocopy the *pcrG(Δ30–40,Δ60–70)* mutant phenotype. To this end, we screened for *pscO* mutants that result in high-level expression of our *exoS-lacZ* reporter in a strain lacking *pcrV*, akin to the high- level expression seen when combining *pcrG(Δ30–40,Δ60–70)* and Δ*pcrV* (*SI Appendix*, Fig. S3*A*). Notably, this screen does not return misfolded or otherwise null alleles of *pscO*, because PscO is an essential component of the apparatus. We identified three independent mutations in *pscO* that resulted in up-regulation of effector secretion in the Δ*pcrV* strain background: G78E, E88K, and A92T (Fig. 4*A*). Although somewhat poorly conserved,

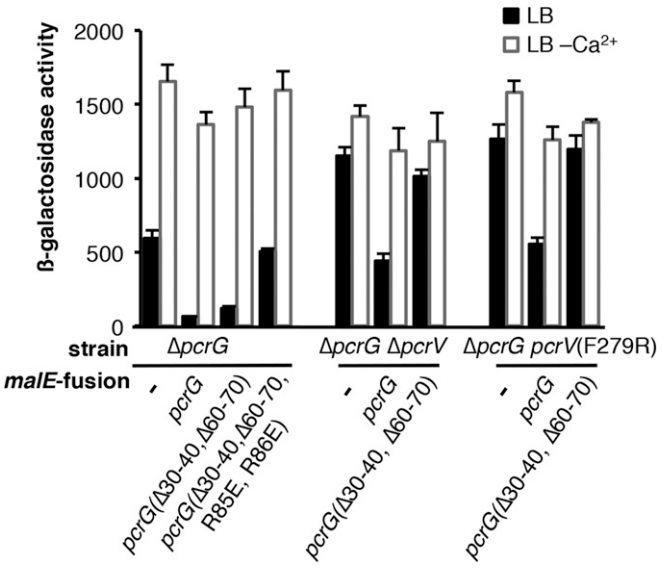

Fig. 3. PcrG(Δ30–40,Δ60–70) enhances effector secretion in the absence of PcrV at the needle tip. Control of effector secretion was evaluated by the β-galactosidase assay in strain PAO1F Δ*pcrG* Δ*exoS*::GFP-*lacZ*, as well as in Δ*pcrV* and *pcrV(F279R)* derivatives, transformed with plasmids encoding the indicated *malE-pcrG* fusions and induced with 10 μM IPTG.

these residues cluster together when mapped to the structure of the flagellar homolog FliJ, arguing that the mutations modulate a specific protein–protein interaction (*SI Appendix*, Fig. S4).

Interestingly, the residues that we identified are in close proximity to residues identified by Ibuki et al. (34) as being involved in the interaction between FliJ and FlhA, the flagellar homolog of the *P. aeruginosa* T3SS export apparatus component PcrD. The overlap suggests that our mutations may influence an interaction between PscO and PcrD. Indeed, two-hybrid analysis indicated that none of the *pscO* mutations affect the PcrG–PscO interaction, suggesting that they influence the interaction between PscO and another apparatus component (Fig. 4*B*).

We next examined the ability of these mutants to complement a *pscO* deletion strain with an intact *pcrV* allele. Although production of PscO(G78E), PscO(E88K), or PscO(A92T) resulted in complete deregulation of effector secretion in the absence of *pcrV*, it restored effector secretion control to near-WT levels in a *pcrV*$^+$ strain background (Fig. 4*A*). Combining the *pscO* mutants with a deletion in *pcrG* did not exacerbate the regulatory effect of the *pcrG* null (*SI Appendix*, Fig. S5). Expression of these *pscO* mutants in the *exsE* deletion background resulted in the hypersecretion of translocator proteins, akin to the hypersecretion seen with the *pcrG(Δ30–40,Δ60–70)* strain (Fig. 4*C*). None of the *pscO* mutations resulted in effector secretion in the presence of calcium. These data place PcrG and PscO in a pathway for controlling the secretion activity of the apparatus. Moreover, the complete deregulation that we observed when combining the *pscO* mutants or *pcrG*(Δ30–40,Δ60–70) with a deletion in *pcrV* suggests throttling of secretion as the primary means by which PcrG controls protein export in a *pcrV* null mutant.

PcrG and PscO Control Proton-Motive Force-Dependent Secretion. Convincing evidence has been provided that protein export via the flagellum relies on the pmf as an energy source. This mechanism has been called into question with regard to virulence-associated T3SSs, however. Although the addition of the protonophore carbonyl cyanide m-chlorophenylhydrazone abolished type III secretion in *Yersinia enterocolitica* (35), it has been argued that this is related to an indirect effect on ATP levels in the cell or to Sec-dependent export of T3SS apparatus components into the periplasm (27, 30). Interestingly, FliJ, the flagellar homolog of PscO, is thought to control the pmf-dependent export of proteins via the flagellar T3SS (26). This proposed function of FliJ raises the possibility that PcrG and PscO might

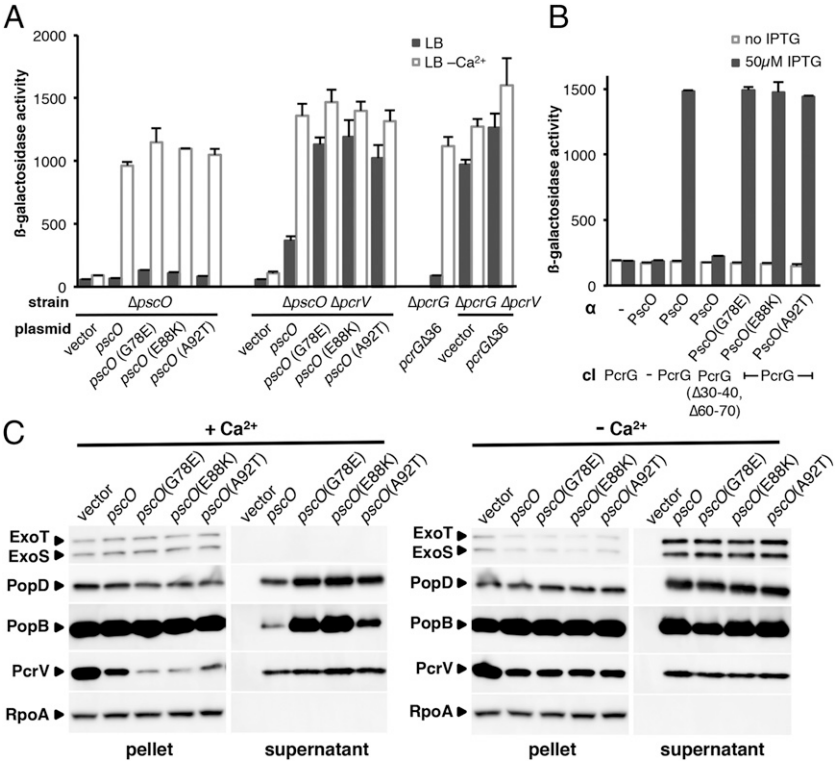

Fig. 4. Mutations in *pscO* result in the same phenotype as *pcrG*(Δ30–40,Δ60–70). (*A*) Control of effector secretion in strain PAO1F Δ*pscO* Δ*exoS*::GFP-*lacZ* and a Δ*pcrV* derivative, transformed with plasmids encoding the indicated *pscO* alleles and induced with 10 μM IPTG, was evaluated by β-galactosidase assay. (*B*) The interaction between PcrG and PscO mutants was assayed using a bacterial two-hybrid system. Protein–protein interaction-dependent activation of the test promoter was determined by the β-galactosidase assay. (*C*) Secretion of ExoS, ExoT, PopB, PopD, and PcrV was evaluated by Western blot analysis in strain PAO1F Δ*exsE* Δ*fleQ* Δ*pscO*, transformed with plasmids encoding the indicated *pscO* alleles and induced with 10 μM IPTG. RpoA served as a fractionation control.

not block translocator access to the apparatus but instead may modulate secretion activity by controlling the efficiency with which the T3SS uses the pmf to drive protein export.

If *pcrG(Δ30–40,Δ60–70)* and *pscO* mutants allow the T3SS to use the pmf more efficiently, then these mutations should render the export process more resistant to collapsing the pmf. Therefore, we assayed the ability of the *pcrG(Δ30–40,Δ60–70)* and *pscO*(E88K) mutations to modulate the sensitivity of translocator export to the addition of pmf inhibitors. The pmf consists of two components, a proton concentration gradient (ΔpH) and a difference in charge between the periplasmic and cytoplasmic faces of the membrane (Δψ). Benzoic acid is a weak acid that collapses the ΔpH component of the pmf. Valinomycin shuttles potassium into the cell, thereby balancing the difference in charge and collapsing the ΔΨ component of the pmf (26). Collapsing the pmf also interferes with T3SS-mediated export of effectors and translocators in *P. aeruginosa* (*SI Appendix*, Fig. S6*A*).

Disruption of either the pH gradient or membrane charge interfered with translocator secretion in the presence of calcium; however, both *pcrG(Δ30–40,Δ60–70)* and *pscO*(E88K) mutations resulted in increased resistance of translocator export to the addition of pmf inhibitors (Fig. 5*A*). The collapse of ΔpH and Δψ was monitored using the pH-sensitive GFP derivative pHluorin and the membrane charge-sensitive dye 3,3-Dipropylthiadicarbocyanine iodide [DiSC3(5)], respectively (Fig. 5*B*). Neither mutation affected the ability of benzoate and valinomycin to disrupt the pmf.

As a further control, we tested whether increasing secretion by increasing recruitment of translocators to the T3SS could account for the increased resistance to pmf collapse. The export of translocator proteins in the presence of calcium relies on additional translocator export signals (TES) that reside near the chaperone binding site (NTES) and the very C terminus of the protein (CTES) (36). Replacing the CTES of PopB with that of PopD, PopB(D-CTES), resulted in an approximately twofold increase in PopB secretion, presumably by enhancing recruitment of the translocator to the T3SS; however, the increased secretion of PopB(D-CTES) relative to WT PopB did not result in increased resistance of PopB export to pmf collapse (Fig. 5*C*). A greater affinity of translocator proteins for the T3SS cannot explain the resistant phenotype of the *pscO*(E88K) and *pcrG*(Δ30–40, Δ60–70) mutant bacteria.

To determine whether the differential decrease in translocator export in the WT strain compared with the mutant bacteria is related to a decreased intracellular concentration of ATP, we analyzed the effect of the addition of 12.5 mM sodium benzoate on ATP levels. This concentration of benzoic acid led to a significant reduction in translocator export in the WT bacteria, but not in the *pcrG(Δ30–40,Δ60–70)* or *pscO(E88K)* mutant strains. The addition of benzoic acid did not result in any significant decrease in the concentration of intracellular ATP in the WT or *pscO(E88K)* mutant bacteria ($P = 0.30$, Student t test). The intracellular concentration of ATP was somewhat reduced in the *pcrG(Δ30–40,Δ60–70)* mutant strain, providing further evidence that secretion activity is not limited by ATP under the conditions of this assay (*SI Appendix*, Fig. S6*B*). Thus, changes in ATP levels likely cannot account for the change in export. Similarly, dif-

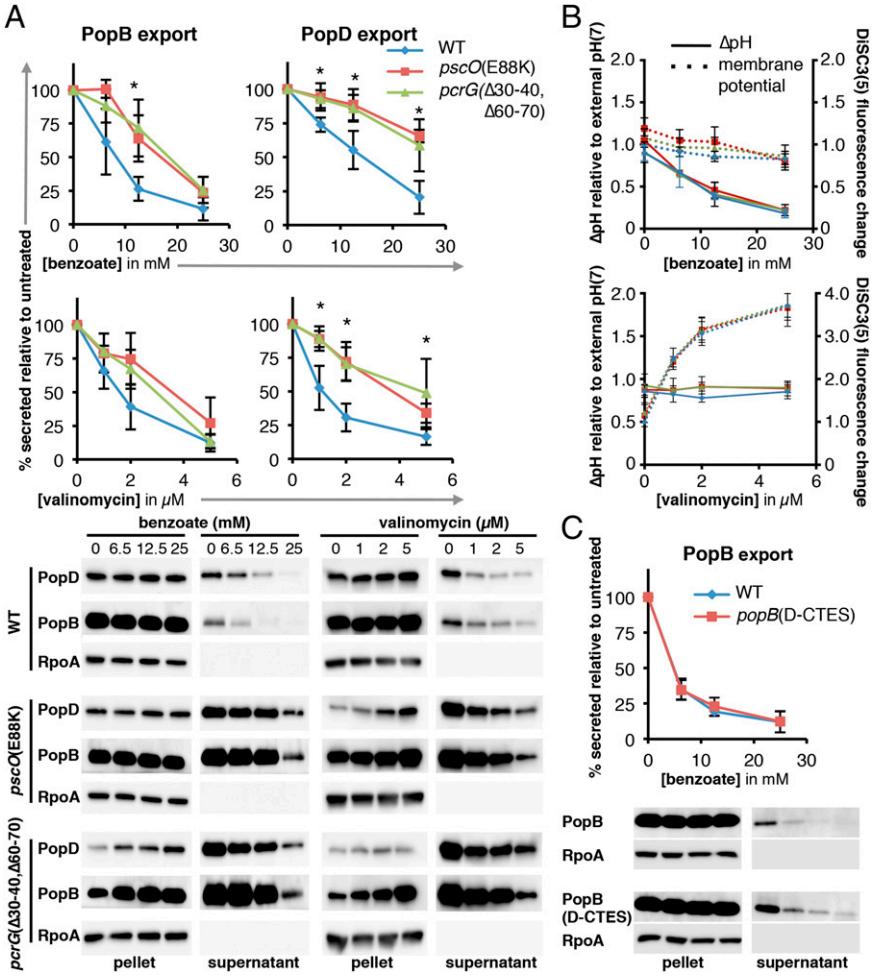

Fig. 5. Substrate secretion in *P. aeruginosa* with *pcrG*(Δ30–40,Δ60–70) or *pscO*(E88K) mutations is more resistant to collapse of proton-motive force. (*A*) Strain PAO1F Δ*exsE* Δ*fleQ* (WT), as well as *pcrG* (Δ30–40,Δ60–70) and *pscO*(E88K) mutant derivatives, were treated with potassium benzoate or valinomycin plus 200 mM KCl. Secretion efficiency of PopB and PopD was determined by Western blot analysis and normalized to an untreated control. Statistical significance of differences was analyzed by the Student t test comparing each mutant with WT at the indicated concentration of inhibitor. $*P < 0.05$. Representative blots of secretion experiments performed in the presence of benzoate or valinomycin are shown below the graphs. (*B*) Measurements of ΔpH and membrane potential of strains PAO1F Δ*exsE* Δ*fleQ* (WT), as well as *pcrG*(Δ30–40,Δ60–70) and *pscO*(E88K) mutant derivatives, treated with the indicated amount of benzoate or K^+/valinomycin. Intracellular pH was determined by producing the pH-sensitive ratiometric GFP derivative pHluorin, and is expressed as the difference in intracellular pH relative to pH 7. Membrane potential was assayed using the membrane potential-sensitive dye DiSC3(5) and is expressed as fold change in DiSC3(5) fluorescence relative to untreated WT cells. Color coding is the same as in *A*. (*C*) Export of PopB, in which the C-terminal export signal had been replaced with the more efficient export signal of PopD [PopB(D-CTES)], was measured in the presence of benzoate and compared with export of WT PopB. Representative Western blot data for this experiment are displayed below the graph. Data in *A*, *B*, and *C* represent the mean ± SD of at least three independent experiments.

ferences in Sec-dependent export of T3SS components cannot explain our data, given that our treatments did not affect apparatus number (*SI Appendix*, Fig. S6*C*). Treatment of cells with benzoate also did not reduce the export of proteins unrelated to type III secretion in our 30-min treatment period (*SI Appendix*, Fig. S6*A*). Taken together, our findings demonstrate that protein export through the T3SS is dependent primarily on the pmf. Moreover, PcrG and PscO control the activity of the apparatus by modulating the efficiency with which the T3SS uses the pmf for protein export.

The C Terminus of PcrG Controls Effector Secretion via Interaction with Inner Membrane T3SS Component PcrD. The C-terminal 25 amino acids of PcrG are critical for controlling effector secretion (Fig. 1). To identify the apparatus component that interacts with the C terminus of PcrG, we applied an in vivo cross-linking technique. Because PcrG has no native cysteines, we engineered a mutant in which proline 84 is replaced with cysteine. MBP-PcrG(P84C) is fully functional when produced in *P. aeruginosa* (*SI Appendix*, Fig. S7*A*). Interacting proteins were then trapped with a heterobifunctional cross-linking reagent, succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC), which cross-links cysteines to residues with primary amines with an 8.3-Å spacer arm. We next purified cross-linked proteins by amylose affinity purification (*SI Appendix*, Fig. S7*B*) and identified cross-linked proteins by mass spectrometry. The only apparatus-related protein thus identified was the inner membrane protein PcrD.

To confirm that PcrG can be specifically cross-linked to PcrD, we produced the MBP-PcrG(P84C) fusion protein in a strain in which the chromosomal copy of *pcrD* had been modified to produce PcrD tagged with two copies of the 11-aa VSV-G epitope. We detected a band corresponding to the expected molecular weight of the cross-linked MBP-PcrG-PcrD complex (~130 kDa) in the presence of the cross-linker (Fig. 6*A*). The cross-linked complex could be detected only with MBP-PcrG (P84C), and not with MBP fused to WT PcrG. Some non–cross-linked PcrD copurified with WT MBP-PcrG. The amount was increased in the cross-linked MBP-PcrG(P84C) sample, likely because members of the PcrD family form a nine-membered ring when incorporated into the type III secretion apparatus (37), which would result in non–cross-linked PcrD copurifying with the MBP-PcrG(P84C)-PcrD complex. We also observed a second, higher-molecular weight band in our assays (Fig. 6*A*). The nature of the 150-kDa cross-linked protein complex is unclear; however, the 130-kDa band likely represents the true MBP-PcrG-PcrD complex and not a breakdown product of the larger complex, given that it can be detected with anti-MBP antibodies and with PcrD tagged at either the N or C terminus. The same cross-linked protein complexes were also evident when PcrD was purified by immunoprecipitation and MBP-PcrG was detected by Western blot analysis (Fig. 6*B*).

If the C terminus of PcrG exerts its influence on effector secretion control by stabilizing the effector secretion "off" conformation of PcrD, then triggering of effector secretion would be expected to result in PcrG's dissociation from PcrD. Indeed, triggering of effector secretion by removing calcium from the growth medium prevented formation of the cross-linked MBP-PcrG-PcrD complex (Fig. 6*C*). Production of MBP-PcrG(P84C) in a strain lacking *pcrV*, which results in partial deregulation of effector secretion, diminished the level of the MBP-PcrG-PcrD complex. Deletion of *pcr1*, which completely deregulates effector secretion (*SI Appendix*, Fig. S8), resulted in complete loss of the MBP-PcrG-PcrD complex. Introduction of the R85E/R86E or I95E regulatory mutations into *pcrG* similarly prevented formation of the MBP-PcrG-PcrD complex. These data suggest that the C terminus of PcrG is released from PcrD on triggering of

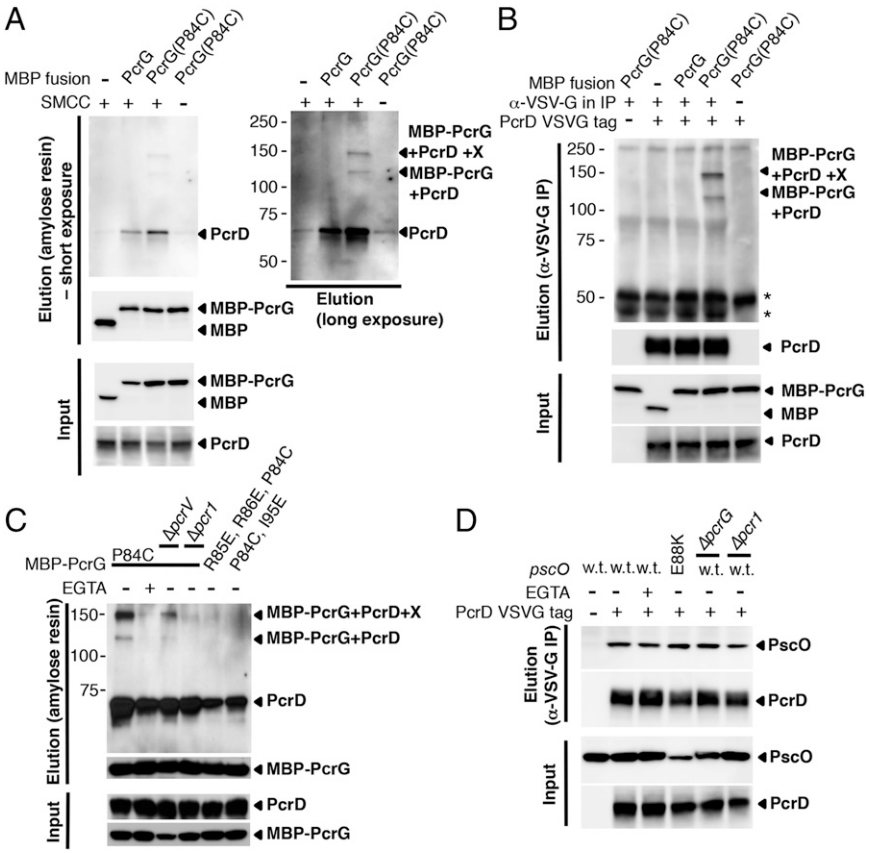

Fig. 6. PcrG and PscO interact with PcrD. (*A*) Production of the indicated MBP-PcrG fusions was induced with 10 μM IPTG in strain PAO1F Δ*exsE* Δ*pcrG pcrD*-VSV-G. Where noted, bacteria were treated with the heterobifunctional cross-linker SMCC. MBP-PcrG, along with any cross-linked proteins, was purified by amylose affinity purification and probed for the presence of PcrD (α-VSV-G) by Western blot analysis. (*B*) MBP-PcrG fusion production was induced with 10 μM IPTG in strain PAO1F Δ*exsE* Δ*pcrG pcrD*-VSV-G or strain PAO1F Δ*exsE* Δ*pcrG*. PcrD was immunoprecipitated using an α-VSV-G antibody, followed by Western blot analysis to detect PcrD (α-VSV-G) or MBP-PcrG (α-MBP). Nonspecific bands are marked with an asterisk. (*C*) The indicated MBP-PcrG fusions (P84C; P84C/R85E/R86E, and P84C I95E) were produced in strain PAO1F Δ*exsE* Δ*pcrG pcrD*-VSV-G or Δ*pcrV* or Δ*pcr1* derivatives, as indicated (10 μM IPTG). MBP-PcrG/PcrD complexes was purified and detected as described in *A*. (*D*) Myc-tagged alleles of *pscO* were expressed from a plasmid (10 μM IPTG) in strain PAO1F Δ*exsE* Δ*pscO* or PAO1F Δ*exsE* Δ*pscO pcrD*-VSV-G, as well as Δ*pcrG* or Δ*pcr1* derivatives thereof. Where indicated, calcium was chelated with EGTA. PcrD was immunoprecipitated using an α-VSV-G antibody. Copurification of PscO was detected by Western blot (α-Myc).

effector secretion and, conversely, that binding of PcrG to PcrD stabilizes the effector secretion "off" state of the apparatus.

FliJ, the flagellar homolog of PscO, is thought to influence pmf-dependent protein export in the flagellar system by interacting with FlhA, the flagellar PcrD homolog. Thus, we examined whether PscO could be coimmunoprecipitated with PcrD. A fully functional, Myc-tagged version of PscO (*SI Appendix*, Fig. S9) was coprecipitated with PcrD (Fig. 6*D*). The secretion state of the apparatus did not influence the interaction; PscO coprecipitated in both the presence and absence of calcium, as well as in a strain lacking the PopN complex component Pcr1. Notably, deletion of *pcrG* did not interfere with incorporation of PscO into the secretion apparatus, and neither did the introduction of the regulatory E88K mutation into *pscO*. These data suggest that PscO is a stable component of the T3SS and exerts its regulatory role through a conformational change rather than through a differential association with the apparatus.

PopN Complex Protein Pcr1 Assembles into a Regulatory Complex with PcrG and PcrD. Given that PcrG and PscO associate with PcrD, it stands to reason that PcrD is actively involved in the triggering of effector secretion. To further test this hypothesis, we used a selection to identify *pcrD* mutants that up-regulate effector secretion. To this end, we generated a strain in which a tetracycline resistance gene was integrated at the *exoS* locus. Triggering of effector secretion in this strain background resulted in tetracycline resistance (*SI Appendix*, Fig. S3*B*). Tetracycline selection was then used to identify *pcrD* mutant alleles that turn on effector secretion under normally repressive conditions. An important feature of the design is that PcrD is an essential component of the T3SS, and thus mutations that generally disrupt PcrD function will not answer the selection. We isolated three *pcrD* mutations: Y587H, Q626R, and M667T. Two mutants, Y587H and M667T, partially deregulated effector secretion, akin to the phenotype seen with a *pcrG* null mutant, whereas the Q626R mutation resulted in complete deregulation of effector secretion (Fig. 7*A*). On a threaded model of PcrD, Y587 and M667 are in proximity to each other. Residue Q626 is located near the tip of the ring formed by PcrD, facing the bacterial cytoplasm (Fig. 7*B*). Interestingly, Q626 is conserved among virulence-associated T3SSs, but not the flagellum, which has no equivalent to effector secretion control (*SI Appendix*, Fig. S10).

The strong deregulation by *pcrD*(Q626R) is reminiscent of the deregulation seen when deleting components of the PopN complex (*SI Appendix*, Fig. S8). By analogy to TyeA, which is hypothesized to tether YopN to the apparatus in the *Yersinia* Ysc T3SS (22, 38), Pcr1 is thought to tether PopN to the apparatus, thereby mediating the regulatory activity of the complex. Thus, we examined whether Pcr1 could be coimmunoprecipitated with PcrD from *P. aeruginosa* whole-cell extracts. For these experiments, we modified the chromosomal copy of *pcr1* to express a functional, HA-tagged version of Pcr1 (*SI Appendix*, Fig. S9). Pcr1 coprecipitated with PcrD (Fig. 7*C*). Unlike PcrG, Pcr1 was recovered regardless of the secretion state of the apparatus. Neither removal of calcium from the media nor deletion of *pcrG* and *pcrV* prevented the PcrD–Pcr1 association, suggesting that Pcr1 is not removed from the apparatus once effector secretion has been triggered. However, introduction of the *pcrD*(Q626R) mutation or deletion of *pscB* encoding a subunit of the PopN chaperone significantly reduced the PcrD-Pcr1 complex (Fig. 7*C*). These data argue that Pcr1 is assembled onto PcrD in association with the remainder of the PopN complex, and that this can be influenced by amino acid substitutions at position Q626 of PcrD.

Because both Pcr1 and PcrG appear to engage PcrD, we examined whether these two regulators interact as well. Two-hybrid analysis showed that Pcr1 and PcrG interact, but this interaction is abolished in the PcrG(Δ30–40,Δ60–70) mutant protein (*SI Appendix*, Fig. S11). We next tested whether a PcrG (L64C) mutant could be used to trap a complex between Pcr1 and PcrG in *P. aeruginosa*. Using SMCC, we identified a cross-linked complex between MBP-PcrG(L64C) and Pcr1, indicating that PcrG and Pcr1 are in close proximity to each other (Fig. 7*D*). Triggering of effector secretion somewhat diminished the amount of Pcr1 that was cross-linked to PcrG in this assay, it did not completely abolish the interaction, suggesting either that PcrG and Pcr1 also interact when not associated with the apparatus or that PcrG is not released from the apparatus on triggering of effector secretion, even though its C terminus dissociates from PcrD (Fig. 6*C*).

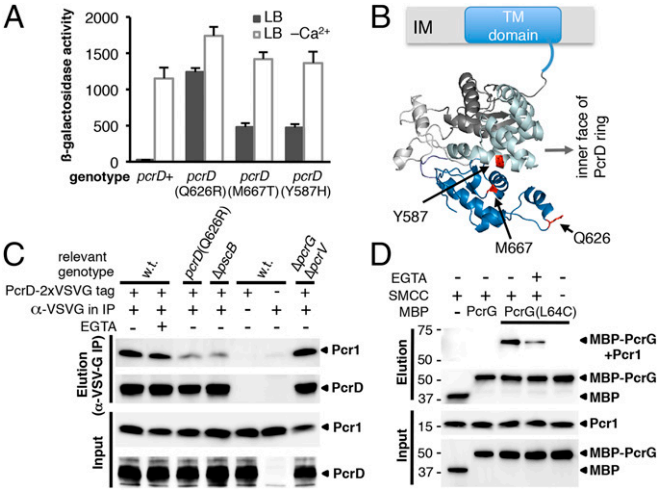

Fig. 7. PcrD serves as a platform for assembly of the regulatory complex. (*A*) Point mutations were introduced into the *pcrD* gene on the chromosome of PAO1F Δ*exoS*::GFP-*lacZ*. Control of effector secretion was analyzed by the β-galactosidase assay. (*B*) The cytoplasmic domain of PcrD (aa 379–702) was threaded into the structure of the *S. flexneri* homolog MxiA [Protein Data Bank ID code: C4A5 (37); threading was performed using the protein homology/analogy recognition engine V 2.0 (Phyre^2): (58)]. Subdomains D1 (dark gray), D2 (light gray), D3 (light cyan), and D4 (blue) are indicated, as are the positions of the three mutant residues (red, with side chain). The inner and outer faces of the ring structure formed by the PcrD are indicated, based on the MxiA nonamer (37). IM, inner membrane; TM, transmembrane domain. (*C*) The association between Pcr1 and PcrD was examined by coimmunoprecipitation in strain PAO1F Δ*exsE pcrD*-VSV-G *pcr1*-HA; derivatives harboring *pcrD*(Q626R), Δ*pscB*, or Δ*pcrGV* mutations; as well as PAO1F Δ*exsE pcr1*-HA. PcrD was immunoprecipitated using an α-VSV-G antibody and tested for coprecipitation of Pcr1 (α-HA). (*D*) Production of the indicated MBP-PcrG fusion was induced with 10 μM IPTG in strain PAO1F Δ*exsE* Δ*pcrG pcr1*-HA. Bacteria were treated with SMCC where noted. MBP-PcrG and cross-linked proteins were purified using an amylose resin, and MBP-PcrG/Pcr1 complexes were detected (α-HA).

Discussion

Control of effector secretion is a central feature of type III secretion systems. Here we investigated the mechanism by which the cytoplasmic regulator PcrG controls effector secretion in the *P. aeruginosa* T3SS. We found that PcrG controls two aspects of the secretion process: access of effectors to the apparatus and overall secretion activity. These two functions of PcrG are separable. PcrG and the cytoplasmic T3SS component PscO control the secretion by modulating the ability of the T3SS to use the pmf, which also indicates that the pmf is the primary energy source for type III secretion. Access of effectors to the T3SS is controlled by a protein complex that assembles on the inner membrane apparatus protein PcrD and involves the regulators PcrG and Pcr1. High-level secretion of translocator proteins, particularly in mutants that up-regulate secretion activity, demonstrates that effector access is not blocked by plugging the se-

cretion channel. Instead, we propose that the regulators block a specific acceptor site for effectors on the T3SS.

Both PcrG and the PopN Complex Assemble on PcrD to Control Effector Export. We used an in vivo cross-linking approach to identify interaction partners of the cytoplasmic regulator PcrG and isolated a cross-linked complex of PcrG(P84C) bound to the inner membrane component PcrD. Triggering of effector secretion prevented recovery of the PcrG-PcrD complex. PcrG lacking the last 25 amino acids, PcrG(Δ71–95), or with mutations in the C-terminal portion of the protein [e.g., PcrG(R85E/R86E)], was defective in controlling effector secretion, indicating that the C terminus of PcrG needs to contact PcrD to establish effector secretion control. The finding that triggering of effector secretion disrupts the PcrG–PcrD association suggests that PcrD undergoes a conformational change that results in release of PcrG. By extension, we propose that binding of PcrG to PcrD helps stabilize the effector secretion "off" conformation of PcrD. This regulatory function may extend to systems in which the needle tip protein self-chaperones its own export. Thus, the cytoplasmic regulatory function of IpaD, ascribed to its self-chaperoning domain (12, 39), may involve stabilizing the effector secretion "off" conformation of MxiA.

Consistent with the hypothesis that PcrD is directly involved in effector secretion control, we isolated three mutations in *pcrD* that up-regulate effector secretion. One mutant, Q626R, resulted in complete deregulation of effector secretion, akin to the phenotype of a mutant lacking the PopN complex. Indeed, Pcr1, a component of the PopN complex, could be coprecipitated with PcrD. The association of Pcr1 and PcrD was facilitated by the PopN chaperon PscB/Pcr2, as well as by residue Q626 of PcrD. We suspect that the association between Pcr1 and PcrD that we uncovered here is conserved across the T3SS. In fact, residue Q626 of PcrD, which modulates recruitment of Pcr1 to PcrD, is part of a motif that is highly conserved in virulence-associated T3SSs, but not the flagellar T3SS (*SI Appendix*, Fig. S10). This conservation extends to T3SSs in which the homologs of PopN and Pcr1 are fused, such as MxiC in *Shigella* spp. (40).

Our data indicate that PcrD serves as a platform on which PcrG and the PopN complex assemble and functions to integrate the regulatory input from these two sets of regulators. Pcr1 also could be cross-linked to PcrG, suggesting that these two proteins are close to each other. PcrD is an excellent target for regulatory control, considering that its homologs have been implicated in the recognition of secretion substrates in both the flagellar system and virulence-associated T3SSs (41–43). Affinities of chaperone/substrate complexes for the C-terminal domain of FlhA appear to dictate the order of export in the flagellar T3SS (44), and deletion of subdomain 2 of MxiA specifically interferes with export of the *Shigella* translocator protein IpaC (37).

How cytoplasmic regulators control effector secretion is largely unknown. Most models suggest that members of the MxiC/YopN family of proteins form a plug by partially inserting into the T3SS while being tethered to the apparatus (19, 20). Although translocator proteins are exported before effectors, secretion is low, raising the possibility that this export represents stochastic secretion events occurring before the secretion block is established. By identifying mutations that up-regulate translocator secretion without triggering effector export, we have demonstrated that the secretion apparatus is not plugged. Instead, we propose that cytoplasmic regulators block an acceptor site for effector proteins.

Control of pmf-Dependent Protein Export by PcrG and PscO. Until recently, it was assumed that T3SS-associated ATPase plays an important role in energizing export via the T3SS. The ATPase can recognize and unfold secretion substrates (45), and mutants that lack ATPase are type III secretion negative (28–30). This view was challenged by the observation that flagella, which use a type III secretion mechanism to export flagellar subunits, can be assembled in mutants lacking ATPase (FliI) and associated regulatory protein (FliH) in a pmf-dependent manner (24, 25). In a counterargument, it was noted that secretion in the absence of the ATPase is very poor, that the relative contribution of the pmf and ATPase-mediated unfolding to the rate of protein export remains unclear (27), and that the means by which export is energized may differ between the flagellum and virulence-associated T3SSs (30).

Here we demonstrate that mutations in two T3SS-associated genes, *pcrG* and *pscO*, increase secretion activity. This increased activity renders secretion more resistant to collapse of the pmf, suggesting that the increased secretion seen in these mutants is related to more effective utilization of the pmf. Thus, secretion via the virulence-associated T3SS of *P. aeruginosa* is likewise primarily pmf-dependent. A lack of export in mutants lacking the ATPase and associated regulatory protein, the equivalent of FliI and FliH in the flagellar system, could either reflect an assembly defect of the apparatus or suggest that secretion substrates in these systems are more dependent on unfolding by ATPase to enter the secretion channel.

Our data fit with observations in the flagellar T3SS, where pmf-dependent export has been tied to the association of FliJ, the flagellar homolog of PscO, with FlhA (PcrD) (26). FlhA itself is thought to mediate proton influx in the flagellar system (46). FliJ in turn interacts with the linker connecting the transmembrane domain to the C-terminal cytoplasmic domain of FlhA (41, 42), which conceivably could allow it to modulate proton influx. It stands to reason that PscO similarly interacts with PcrD to control pmf-dependent secretion. PscO did coprecipitate with PcrD, although more work is needed to determine whether this represents a direct interaction. Triggering of effector secretion did not result in dissociation of PscO from PcrD, nor did the activating E88K mutation, suggesting that PscO is stably associated with PcrD.

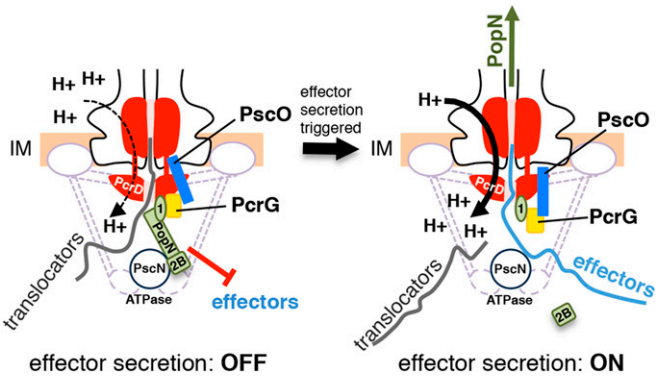

Fig. 8. Model of effector secretion control. The organization of the T3SS is based on the recent structures of *Salmonella* and *Yersinia* T3SS (52, 59). In the effector secretion off state, only translocator proteins are being secreted at a low level, controlled by the influx of protons via PcrD. The PopN complex [green: PopN, PscB (B), Pcr2 (2), and Pcr1 (1)] blocks access of effectors to the T3SS and is tethered to the apparatus via the interaction of Pcr1 with PcrD. PcrG (yellow) stabilizes the PopN complex and controls the activity of the apparatus via an interaction with PcrD. PscO (blue) binds to the linker domain that connects the inner membrane domain of PcrD to the C-terminal cytoplasmic domain and helps control secretion activity by modulating proton flow through PcrD. In the effector secretion on conformation, PopN is secreted, allowing effectors to access the secretion apparatus via the sorting platform consisting of PscK, PscL, and PscQ (pale purple). The activity of the apparatus is up-regulated, likely through a conformational change in PcrD and PscO, which allows for increased influx of protons.

Separation of activity control and effector access has been observed in other T3SSs, although it has not been interpreted in these terms, and the mechanistic underpinnings remain obscure. Deletion of *mxiC*, encoding the *Shigella* spp. homolog of PopN, results in premature secretion of effector proteins, but at a low level compared with complete induction of the system by Congo red (6). Slow-secreting, "constitutive on" needle-subunit mutants in *Shigella* similarly turn on effector secretion without the up-regulation of secretion rate seen in the presence of Congo red or in strains lacking the needle tip-associated translocator proteins IpaB and IpaD (47, 48). Conversely, small deletions in the needle tip protein IpaD can selectively up-regulate secretion of early export substrates, including the pore-forming translocator proteins IpaB and IpaC, but not late effectors (7). Because IpaB and IpaC proteins are secreted at low levels before triggering of effector secretion in a process termed leakage, these *ipaD* mutants modulate the secretion activity.

Although control of secretion activity and specificity likely are general features of T3SSs, the mechanism of activity control may vary among systems. In *Shigella*, for example, control of secretion activity appears to involve proteins associated with the needle tip, IpaD and IpaB. Perhaps here secretion is throttled by the tip structure through the formation of a plug that is removed by the insertion of IpaB into the host cell membrane (48). Ultimately, all type III secretion systems are faced with the same conundrum, highlighted by our work: the need to minimize the export of proteins before cell contact, allowing secretion of just enough translocators to permit sensing of host cell contact, versus the need to deliver a bolus of effectors quickly once effector secretion is triggered. The mechanism by which this problem is solved may vary among species.

A Model for Effector Secretion Control. Our data point to a model in which the access of effectors to the T3SS and secretion activity are controlled through PcrD and associated regulatory proteins (Fig. 8). We propose that the PopN complex assembles on the PcrD ring to prevent access of effectors to the T3SS without blocking the secretion channel. PcrG likely exerts its control by stabilizing the effector secretion "off" conformation of PcrD. On triggering of effector secretion, PopN is exported via the T3SS, thereby freeing the acceptor site for effectors. We have represented the PopN complex as spanning PcrD and ATPase, because PopN homologs require a secretion signal to prevent effector export (13, 19) and the secretion signal can mediate a specific interaction with the ATPase (13). Intriguingly, a F234S mutant of YopN did not require its secretion signal or chaperone binding domain to block effector export, suggesting that binding to the acceptor site precedes engagement of the ATPase (19). A better understanding of how effector export is prevented without interfering with translocator secretion will require a more detailed view of how export substrates are recruited to the apparatus. Identifying the interactions of secreted proteins with PcrD, as well as the temporal order with regard to the sorting platform (49) and ATPase (13, 45, 50), will be key in this endeavor.

Triggering of effector secretion also results in increased pmf-dependent secretion activity. The secretion activity is controlled through the concerted action of PcrG and PscO. Given that PscO does not dissociate from the apparatus on triggering of effector secretion, its effect on secretion activity likely involves a more subtle alteration in the conformation and activity of the proton channel. We depicted PscO as binding to the linker between the N-terminal transmembrane domain and cytoplasmic domain of PcrD. This location is in line with its role in controlling proton flux through the T3SS, but does not correspond with the recently proposed location of FliJ inserted in the flagellar ATPase (51). Recent structural findings have called this localization into question, however, because the ATPase is too far away from the FlhA linker domain to allow FliJ to interact with both (52). No density corresponding to FliJ could be discerned protruding from the ATPase either, but this may be related to the small size of the protein. FliJ promotes assembly of the ATPase hexamer in vitro (51). FliJ, as well as its homologs, also can interact with chaperone proteins for select secretion substrates (53–56); thus it may have three roles in T3SS function, each at a separate stage of the export process: assembly of the ATPase, facilitation of pmf-dependent protein export, and a chaperone sink.

In conclusion, we have presented evidence that protein export via T3SSs is regulated at the levels of both secretion activity and specificity. The inner membrane component PcrD serves as a platform on which regulators assemble to mediate their control. Cytoplasmic regulators do not plug the secretion channel; instead, PcrG stabilizes the effector secretion "off" conformation of the T3SS, and the PopN complex likely blocks an acceptor site for effectors. Secretion is energized by the pmf, and the secretion activity is modulated through the action of PcrG and PscO, which control the coupling of protein export to the pmf.

Materials and Methods

Strains, Plasmids, and Culture Conditions. Details of strain and plasmid construction are provided in *SI Appendix*. The *E. coli* strains used in this study were grown in LB (10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per L). *P. aeruginosa* was cultured in modified LB (LB-MC medium: 10 g of tryptone, 5 g of yeast extract, and 11.7 g of NaCl per L, supplemented with 10 mM $MgCl_2$ and 0.5 mM $CaCl_2$). Where indicated, effector secretion was triggered by removing calcium from the medium through the addition of EGTA (5 mM final concentration).

β-Galactosidase Activity Assay. Cultures were grown at 37 °C to early log phase in LB-MC medium, after which 1 mL of culture was added to 1 mL of prewarmed LB-MC medium ($+Ca^{2+}$ condition) and another 1 mL of culture was added to 1 mL of prewarmed LB-MC medium with 10 mM EGTA (5 mM final concentration; $-Ca^{2+}$ condition). The cultures were then incubated for an additional 2 h at 37 °C before being placed on ice for 10 min. Cells were permeabilized with chloroform and SDS, and β-galactosidase activity was measured as described previously (57). Activities are reported as average ± SD of three independent replicates.

Protein Secretion Assay. Overnight cultures were diluted and grown to midlog phase at 37 °C, after which the cultures were split and the bacteria pelleted. The cell pellets were resuspended in prewarmed LB-MC medium alone ($+Ca^{2+}$ condition) or in LB-MC medium with 5 mM EGTA ($-Ca^{2+}$ condition) and incubated for 30 min at 37 °C, then chilled on ice. The bacteria were then pelleted by centrifugation, and supernatant proteins were precipitated using trichloroacetic acid. Cell pellet and supernatant fractions were separated by SDS/PAGE, and proteins of interest were detected by Western blot analysis using a chemiluminescent detection system. Images were acquired by either exposing blots to film and scanning the film or using a GE ImageQuant LAS 4000 digital imaging system. Images were processed with the Levels function in Adobe Photoshop applied to the whole image before cropping.

Site-Specific Cysteine Cross-Linking. Cultures were grown at 37 °C to midlog phase in 200 mL of LB-MC medium. The bacteria were then pelleted by centrifugation and resuspended in cross-linking buffer (PBS with freshly prepared 1 mM PMSF and 0.5 mM SMCC). For cultures grown in the presence of calcium, the cross-linking buffer was supplemented with 0.5 mM $CaCl_2$. The cell suspensions were incubated at room temperature in the dark for 30 min, after which the bacteria were pelleted, washed once in MBP purification buffer (20 mM Tris pH 7.5, 100 mM NaCl, 10 mM β-mercaptoethanol, 1 mM EDTA, 1 mM PMSF, and 0.5% Nonidet P-40), and resuspended in 1 mL of MBP purification buffer before being lysed by sonication.

ACKNOWLEDGMENTS. We thank Dr. Simon Dove for providing the components of the α-cI two-hybrid system, as well as Dr. Dove and Dr. Piet de Boer for their critical reading of the manuscript. We also thank Dr. Joan Slonczewski for supplying plasmid pGFPR01. This work was funded by American Cancer Society Research Scholar Grant RSG-09-198-01-MPC (to A.R.). Flow cytometry at the Case Western Reserve University/University Hospitals Center for AIDS Research facility was supported by National Institutes of Health Grant P30 AI036219 (Dr. Jonathan Karn, PI).

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