Bacterial Secretins Form Constitutively Open Pores Akin to General Porins

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Proteins called secretins form large multimeric complexes that are essential for macromolecular transit across the outer membrane of Gram-negative bacteria. Evidence suggests that the channels formed by some secretin complexes are not tightly closed, but their permeability properties have not been well characterized. Here, we used cell-free synthesis coupled with spontaneous insertion into liposomes to investigate the permeability of the secretin PulD. Leakage assays using preloaded liposomes indicated that PulD allows the efflux of small fluorescent molecules with a permeation cutoff similar to that of general porins. Other secretins were also found to form similar pores. To define the polypeptide region involved in determining the pore size, we analyzed a collection of PulD variants and studied the roles of gates 1 and 2, which were previously reported to affect the pore size of filamentous phage f1 secretin pIV, in assembly and pore formation. Liposome leakage and a novel *in vivo* assay showed that replacement of the conserved proline residue at position 443 in PulD by leucine increased the apparent size of the pore. The *in vitro* approach described here could be used to study the pore properties of membrane proteins whose production *in vivo* is toxic.

Multidomain proteins called secretins form large outer membrane (OM) complexes that act as portals for protein (e.g., PulD, OutD, and XcpQ) and filamentous bacteriophage (e.g., pIV) secretion, for DNA uptake and type IV pilus (T4P) (e.g., PilQ) assembly, and for needle assembly and protein secretion in type III secretion systems (T3SS) in Gram-negative bacteria (1). Cryo-electron microscopy of the archetypical T2SS secretin PulD from *Klebsiella oxytoca* revealed how 12 protomers arrange in a barrel-like complex of dodecameric symmetry with an open, outward-facing ring connected to a second ring, creating a vestibule deep into the periplasm (2). A plug closes off the barrel near its center. Other secretins have a similar architecture (3–5), but the structure of the membrane-embedded part of the complex, including the plug, has not been reported at atomic resolution.

Other approaches have begun to reveal molecular details of secretin architecture. All secretins share a domain organization comprising a well-conserved C domain, which includes the aforementioned gated membrane channel, and a less-well-conserved N domain that consists of up to four globular domains named N₀ to N₃, all of which are present in the prototype secretin PulD (Fig. 1A). The N domain is located in the periplasm and interacts with inner membrane components of the secretion machinery (6–9). The atomic resolution structures of part of the N domains of several secretins have been solved by X-ray crystallography (for the T3SS, EscC [10], and for the T2SS, GspD [11] and XcpQ [12]) and nuclear magnetic resonance (NMR) spectroscopy (for T4P, EscC [13]). Some secretins, including PulD, possess a C-terminal extension (S domain) (Fig. 1A) that interacts with a dedicated chaperone (PulS in the case of PulD) that protects it from degradation and promotes correct localization (14, 15). In PulD, the C and S domains and the last of three periplasmic repeat domains (N₃) (Fig. 1A) are sufficient for targeting to and insertion as a multimer into the OM (16).

The plug in the ≥6-nm-wide secretin channel presumably

blocks the release of periplasmic proteins when the secretin channel is in its resting state, i.e., when proteins or phages are not being secreted and pili or needles are not assembled. However, the secretin channel is not necessarily tightly closed. Several studies investigated the ability of reconstituted (resting-state) secretins to form pores or channels in nonnative lipid bilayers. Data from conductance measurements were sometimes difficult to interpret. High currents were measured upon reconstitution of the *Pseudomonas aeruginosa* secretin XcpQ in artificial membranes (17). Large structural fluctuations in XcpQ were proposed to cause the observed nonuniform conductance, which was very high compared to that of porins, did not increase linearly with the applied potential, and even persisted when the applied potentials were high. The *Yersinia enterocolitica* secretin YscC formed stable conductance channels when reconstituted *in vitro* but did not facilitate uptake of β-lactam antibiotics *in vivo*, which is indicative of a narrow molecular weight cutoff (18). In contrast, PulD reconstituted into artificial bilayers only showed fluctuating conductance when a high voltage was applied across the lipid bilayer (19), suggesting that it is normally in a tightly closed conformation. However, while PulD produced *in vivo* in the presence of its chaperone PulS inserts in the outer membrane without dramatically modifying its permeability, in the absence of PulS, it mislocalizes into the inner membrane and induces a phage shock response (16). This

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FIG 1 (A) Linear representation of full-length PulD (PulD^{fl}) and the truncated form including residues 28 to 42 and 259 to 660 (PulD^{28-42/259-660}). N₁ to N₃ are repeat domains of the same module. Putative gate 1 and gate 2 regions in PulD are assigned in analogy with those described for pIV (21). (B) Alignment showing the conserved nature of P443 in the sequences of secretins involved in T2SS (PulD of *K. oxytoca* [NCBI accession no. WP_004872081.1], OutD of *D. dadantii* [YP_003883933.1], and XcpQ of *P. aeruginosa* [WP_003162836.1]), T3SS (YscC of *Y. pestis* [YP_001604484.1], PscC of *P. aeruginosa* [YP_005980859.1], and InvG of *S. enterica* [WP_000848107.1]), T4P (HofQ of *E. coli* [YP_001723327.1], PilQ₁ of *N. meningitidis* [WP_002227621.1], and PilQ₂ of *P. aeruginosa* [NP_253727.1]), and filamentous phage secretion (pIV of phage fl [P03666.1]).

phenomenon suggests that PulD forms a proton-permeable pore in the inner membrane. Electrophysiology studies also suggested that the pIV secretin channel is tightly closed *in vitro*, although it allows an *Escherichia coli* strain lacking maltoporin (LamB) to grow on small maltodextrins (20). The permeability of the pIV channel was increased by changes in the pIV sequence that allowed a LamB-deficient strain to regain its ability to import maltopentose (21). These sequence changes clustered mainly in two regions that were named gates 1 and 2 because of their proposed role in channel gating (21). To simplify the terminology, we will use the term secretin channel to define the wide-open conduit that permits protein or phage egress from the cell and the term secretin pore to define the (hypothetical) partially closed conduit. The existence and properties of a pore in a resting-state secretin complex are the subjects of this report.

Many membrane proteins, including PulD and some other secretins, can be efficiently synthesized and assembled into liposomes in a cell-free system (22–24). Here, we investigated the permeability properties of PulD, XcpQ, OutD, and pIV inserted into liposomes preloaded with small reporter molecules. A miniaturized version of the assay was used to screen a previously characterized PulD mutant library to identify residues influencing pore size (24, 25).

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* K-12 PAP105 [$\Delta(lac-pro)$ F' (*lacI*^{q1} $\Delta lacZM15$ *proAB*⁺ Tn10)], used for plasmid manipulations, and *E. coli* PAP7447 [MC4100(F' *lacI*^{q1} *pro*⁺ Tn10)], with *pulS*, *pulA*, and *pulC* to -O integrated into *malPp* and with a large deletion in *pulD*, were grown in LB medium at 30°C. *E. coli* strain NB190 (26) lacking cyanocobalamin

receptor BtuB [*proC22 trpE23 metE70 lysA23 rpoB308 thi-1 lacZ36 xyl-5 mtl-1 rpsL109 cyc-19 tsx-67 supE44* $\Delta btuB$ *arg* F' (*lacI*^{q1} $\Delta lacZM15$ *proAB*⁺ Tn10)] was maintained in LB medium at 30°C. Cyanocobalamin permeability assays with this strain were performed in 0.2% glucose M63B1 minimal medium (0.4% maltose in experiments with pCHAP1226) supplemented with all amino acids except methionine and cysteine. Ampicillin (100 μ g/ml), chloramphenicol (25 μ g/ml), and kanamycin (50 μ g/ml) were used when appropriate. Tetracycline was used at 5 μ g/ml to maintain the F' in NB190, PAP105, and PAP7447. *Staphylococcus aureus* RN4220 was grown in LB medium (27) at 37°C. Cloning experiments were all done in PAP105 at 30°C.

Gene constructions and plasmids. Plasmids and primers used in this study are listed in Tables S1 and S2, respectively, in the supplemental material. pCHAP3674 and pCHAP3675, carrying wild-type *fhuA* and *fhuA* Δ 322–355, respectively, were obtained by excision of the genes from pHK763 (28) and pHK226 (29), respectively, using EcoRI and HindIII and inserted into pBGS19 digested with the same enzymes.

pCHAP3909, encoding α -hemolysin with a C-terminal His tag, was obtained in a two-step cloning reaction. pCHAP3908 was first obtained by ligation of a BlnI and BglII fragment from pT7-HL (30) into pIVEX2.3MCS digested with the same enzymes. A hexahistidine tag was then introduced using primers ING163 and ING164 on pCHAP3908. The resulting fragment was cleaved using NdeI and BamHI and ligated into pIVEX2.3MCS cleaved with the same endonucleases.

Erwinia carotovora outD was amplified from pCPP2242 (31) with primers ING206 and ING207, digested with NdeI and BamHI, and ligated into pIVEX2.3MCS cleaved by the same enzymes to give pCHAP9718.

The linker deletion in *Dickeya dadantii* OutD was created stepwise. The outD fragments up- and downstream of residues 297 to 353 were amplified by PCR from pCHAP3794 using primers ING86 and DIS26 and primers DIS27 and ING91, in which DIS26 and -27 have overlapping termini. Both halves were assembled together by a second PCR using ING86 and ING91. The resulting amplicon was cloned into pIVEX2.3MCS after digestion with NdeI and SacI. Additional mutations introduced by the primers DIS26 and DIS27 were corrected by site-directed mutagenesis.

pCHAP9724 containing a P252L substitution in the filamentous phage secretin pIV was obtained by mutagenesis using pCHAP9147 (24) and primers DIS43 and DIS44.

PulD^{28-42/259-660} Δ Gate2 (pCHAP9715) was constructed from pCHAP3716 in two PCR steps. First, two fragments were generated using primers ING59 and DIS22 and primers ING62 and DIS23. In a second step, both amplified fragments were used as templates in a third reaction with primers ING59 and ING62. The resulting amplicon was inserted in the plasmid pIVEX 2.3MCS digested with NdeI and BamHI. PulD^{28-42/259-660} Δ Gate1 (pCHAP9722) was constructed by using the same procedure as the one described for PulD^{28-42/259-660} Δ Gate2 using primers DIS39 and DIS40 instead of DIS22 and DIS23.

Sac7d was amplified by PCR from pQUANTagen *sac7d-phoA* (32) using primers ING218 and ING219. The amplicon and pASK2C (IBA), encoding an OmpA signal peptide and a Strep tag at opposite sides of the multiple cloning site, were digested with EcoRI and PstI and ligated together to form pCHAP3978. The fragment containing the sequence for Sac7d with an N-terminal OmpA signal peptide and a C-terminal Strep tag was then subcloned in pASK12 (IBA) using the XbaI/HindIII restriction sites to give pCHAP9725.

All constructions were verified by DNA sequencing.

Preparation of liposomes. 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) (Avanti Polar Lipids) dissolved in chloroform was dried under a stream of nitrogen. The resulting thin lipid film was hydrated with different buffered solutions (50 mM Tris-Cl, pH 7.4) containing 50 mM disodium calcein, 10 mM carboxyfluorescein, or 100 mg/ml vancomycin. Carboxyfluorescein was added from a stock solution of 0.4 M in dimethyl sulfoxide. Multilamellar liposomes were probe sonicated three times for 2 min (with 1-min intervals on ice) at 50% duty cycle and at an output of 3

using a Sonics Vibracell sonicator. Liposomes were predominantly unilamellar when examined by electron microscopy. Nonencapsulated compounds were removed by dialysis (Spectra/Por membrane; molecular mass cutoff, 6 to 8 kDa) at 4°C against three changes of 2 liters of 50 mM Tris-Cl (pH 7.4). Liposomes were collected by centrifugation at $35,000 \times g$ for 15 min and resuspended in the same buffer to a final concentration of 25 mg/ml.

In vitro synthesis and purification of α -hemolysin. His-tagged α -hemolysin was synthesized for 24 h at 30°C by adding pCHAP3909 (10 μ g) to the RTS500 *E. coli* HY *in vitro* transcription-translation system (5 Prime). The reaction mixture was diluted in 25 mM phosphate buffer (pH 8) and applied to a Ni-nitrilotriacetic acid (NTA) column (1 ml; Qiagen) equilibrated with the same buffer. After washing with 10 column volumes of the same buffer, α -hemolysin was eluted with the same buffer containing 250 mM imidazole. Imidazole was removed by dialysis against the same buffer before the protein was concentrated on an Amicon Ultra-4 centrifugal filter (Millipore). Protein purity was verified by SDS-PAGE.

Calcein release by purified α -hemolysin. Calcein fluorescence was excited at 490 nm, and the emission was monitored at 520 nm in an Infinite F200 Pro Tecan spectrofluorometer. To initiate leakage, 3 μ g of α -hemolysin was added to 15 μ l of 50 mM Tris-Cl buffer (pH 7.4) containing 0.5 M sucrose (to enable comparison with MscL leakage) and 50 μ g of calcein-loaded liposomes. The reaction was performed in 384-well plates (black, flat bottom; Greiner) at 30°C with continuous shaking. The total calcein amount in the liposomes was indicated by the fluorescence observed upon the addition of 0.1% Triton X-100. Fluorescence (determined every 90 s) was plotted as the percentage of the total calcein present in the reaction mixture.

Calcein and carboxyfluorescein release during *in vitro* synthesis of α -hemolysin or secretins. Plasmid DNA (150 ng) and calcein- or carboxyfluorescein-loaded liposomes (50 μ g) were added to 15 μ l of the RTS100 *E. coli* HY reaction kit (5 Prime). *In vitro* synthesis was performed in 384-well plates (black, flat bottom; Greiner), and mixtures were incubated in a spectrofluorometer as described above at 30°C with continuous shaking. Calcein and carboxyfluorescein were excited at 490 nm and 485 nm, and the fluorescence emission was monitored at 520 nm and 513 nm, respectively. The fluorescence signal was measured every 15 min for 2 h or every 90 s for 2 h in initial experiments with α -hemolysin. The total amount of liposome-encapsulated dye was indicated by the fluorescence measured upon the addition of 0.1% Triton X-100 at the end of the reaction. Fluorescence was plotted as a percentage of total unquenched fluorescence.

Calcein release after *in vitro* synthesis of MscL. Plasmid DNA (150 ng) encoding MscL (33) was added to 15 μ l of the RTS100 *E. coli* HY reaction kit supplemented with 50 μ g calcein-loaded liposomes. The reaction mixture was incubated for 5 h at 30°C. Under these conditions, MscL inserts in the liposome in a closed conformation (isosmotic conditions) and no calcein leakage occurs. Following MscL synthesis, 2- μ l aliquots of the reaction mixture were diluted into 990 μ l of 50 mM Tris-Cl (pH 7.4) with or without 0.5 M sucrose. Calcein release is instantaneous and was measured immediately after mixing. The samples were excited at 490 nm, and emission scans were monitored from 500 to 600 nm in a cuvette with path length of 1 cm in a Varian Cary Eclipse fluorescence spectrophotometer. Buffer spectra were subtracted.

Vancomycin release. Plasmid DNA (150 ng) and 50 μ g of vancomycin-loaded liposomes were added to the *E. coli in vitro* transcription-translation system in a final volume of 15 μ l. Reaction mixtures were incubated for 1 h at 30°C. Ten microliters of the reaction mixtures was spotted onto Whatman paper discs that were placed onto LB agar inoculated with 100 μ l of *S. aureus*. Zones of growth inhibition around the discs were observed after 16 h of incubation at 37°C.

Cyanocobalamin uptake assay. Competent NB190 cells carrying pCHAP585 (encoding PulS) were transformed with pCHAP3674, pCHAP3675, pCHAP3635, or pCHAP9124. Experiments in the presence of all Pul proteins were performed with NB190 cells transformed with

pCHAP1226 and either pCHAP3635 or pCHAP9124. Cultures were grown in LB medium at 30°C. Cells from 1-ml aliquots of the culture were washed twice with 1 ml of M63B1 minimal medium (27) and resuspended in M63B1 (200 μ l at an optical density at 650 nm [OD_{650}] of 1). Aliquots of 100 μ l were spread on M63B1 agar plates supplemented with the appropriate antibiotics and carbon source, 40 μ l of 0.1 M X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside), and 40 μ l of 20-mg/ml IPTG (isopropyl- β -D-thiogalactopyranoside). Sterile filter paper disks soaked with either H₂O, 40 μ g methionine (Met), or 1 μ g or 2 μ g of cyanocobalamin (B₁₂) were placed on the agar plates and incubated for 36 h at 30°C before growth zones were observed.

Cell fractionation for Sac7d localization. *E. coli* PAP7447 cells transformed with pCHAP9725 (encoding periplasmic Sac7d) and pCHAP3635 (encoding PulDⁿ) or pCHAP9124 (encoding PulDⁿP443L) were induced at an OD_{650} of 0.3 with 200 ng/ml anhydrotetracycline for 4 h 30 min at 37°C. Cells were harvested and concentrated 6 times by resuspension in 10 mM Tris (pH 8.0)–20% sucrose supplemented with 0.1 mg/ml lysozyme, 0.1 mg/ml Pefabloc SC [4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride; Roche], and 0.5 mM EDTA. After 20 min of incubation at 4°C while shaking, spheroplasts and outer membranes were collected by centrifugation ($24,000 \times g$, 30 min, 4°C). Spheroplasts (and membranes) were resuspended in 10 mM Tris (pH 8.0) and broken by sonication. Cell debris was collected by centrifugation ($175,000 \times g$, 10 min, 4°C). Culture supernatant, periplasmic, and cytoplasmic fractions were trichloroacetic acid (TCA) precipitated and resuspended in a minimal volume before analysis by SDS-PAGE and immunoblotting.

SDS-PAGE and immunoblotting. Proteins were separated on 10% and 15% acrylamide-SDS gels and visualized after staining with Coomassie brilliant blue. For immunoblotting, the proteins were transferred onto nitrocellulose membranes and detected with specific antibodies (for PulD, PilQ, or pIV) and secondary antibodies coupled with horseradish peroxidase. Immunodetection of OutD was performed with anti-PulD antibodies. MscL and α -hemolysin were detected with anti-His antibodies coupled to horseradish peroxidase (Perkin-Elmer), and Sac7d was detected with Strep tag antibodies coupled with horseradish peroxidase (IBI).

RESULTS

Calcein efflux from liposomes is strictly dependent on the presence of a pore. The release of self-quenched fluorescent probes such as calcein from liposomes provides an efficient and practical way to follow pore formation (34). We exploited this approach to develop an *in vitro* system for studying the properties of pore- and channel-forming proteins that assemble into liposomes in a coupled transcription-translation system. To validate this approach, we first analyzed the ability of a bona fide channel-forming protein, α -hemolysin from *S. aureus*, to release calcein from liposomes. Purified α -hemolysin multimerizes and inserts spontaneously into liposomes to form a heptameric hydrophilic channel of about 2 nm in diameter (35). Mixing purified α -hemolysin with calcein-loaded liposomes caused a fluorescence increase resulting from decreased quenching (Fig. 2A). After 20 min, 80% of the trapped calcein was unquenched. The remaining 20% was unquenched only upon solubilization of the liposomes with Triton X-100, probably due to the presence of a small proportion of multilamellar liposomes or of calcein-loaded liposomes without α -hemolysin.

Calcein was also released from loaded liposomes when α -hemolysin was produced in a cell-free transcription-translation system in the presence of a plasmid encoding α -hemolysin. However, unquenching was delayed by about 15 min compared to when α -hemolysin was added directly to liposomes, likely reflecting the

FIG 2 (A) Permeabilization of calcein-loaded liposomes by purified α -hemolysin (black symbols) and by *in vitro*-synthesized α -hemolysin (white symbols). The released calcein is represented as a percentage of the total amount trapped in the liposomes. (B) Fluorescence emission spectra of calcein encapsulated in MscL-proteoliposomes diluted in the absence (black line; hypo-osmotic conditions) or presence (dashed line; isosmotic conditions) of 0.5 M sucrose. In these and all other leakage experiments using fluorescent dyes, the experiments were performed at least three times. Because the data from all experiments were consistent, only data from a single experiment are shown.

time required to synthesize and assemble sufficient α -hemolysin to form channels (Fig. 2A).

To verify that the observed calcein efflux reflected the formation of a pore or channel in the lipid membrane rather than leakage during protein insertion, calcein efflux was studied after *in vitro* synthesis of the *E. coli* protein MscL. MscL inserts into liposomes when produced in a cell-free system (33), but its channel (pore) opens only during an osmotic downshock (36). First, liposomes containing a closed MscL channel were prepared by *in vitro* synthesis (see Materials and Methods). The efflux of calcein was then tested under hypo- or isosmotic conditions. Because the intravesicular osmotic pressure of the calcein-loaded liposomes is close to that of 0.5 M sucrose (37), dilution of MscL calcein-loaded liposomes in the absence of sucrose would lead to channel opening (hypo-osmotic shock), while the MscL channel would remain closed when diluted in the presence of 0.5 M sucrose (isosmotic conditions). Indeed, only in the absence of sucrose was an increase in calcein fluorescence observed (Fig. 2B), indicating that calcein leakage occurs only in the presence of open pores. These data validate calcein efflux from liposomes as an indicator of open pore formation in the coupled transcription-translation-insertion assay.

PulD insertion into liposomes allows determination of its pore properties. The calcein release assay was next applied to a protein with unknown pore properties, the secretin PulD. Full-length PulD (PulD^{fl}) and a truncated variant of PulD comprising the third N domain repeat and the C and S domains (PulD^{28-42/259-660}) (Fig. 1) were synthesized *in vitro* in the presence of calcein-loaded liposomes. PulD^{28-42/259-660} has added advantages in *in vitro* synthesis compared to PulD^{fl}, as the latter is prone to degradation and multimerizes less efficiently than PulD^{28-42/259-660} (22, 38). We have already proven PulD^{28-42/259-660} to be useful for mutant screening with reduced multimerization (which was exploited again as described below) (24, 25). Both PulD^{fl} and PulD^{28-42/259-660} formed abundant multimers within 30 min (Fig. 3A) and triggered calcein release from the liposomes (Fig. 3B). A control synthesis reaction without plasmid DNA did not show any increase in calcein fluorescence (Fig. 3B).

Calcein leaked more slowly from liposomes during PulD synthesis than during α -hemolysin synthesis (Fig. 3B), suggesting either that the PulD pore is smaller than that of α -hemolysin or

FIG 3 (A) SDS-PAGE analysis and Coomassie blue staining of α -hemolysin, PulD^{fl}, and PulD^{28-42/259-660} multimers synthesized *in vitro* in the presence of calcein-loaded liposomes. Arrowheads indicate multimers. (B) Kinetics of calcein release from liposomes in the presence of α -hemolysin, PulD^{fl}, and PulD^{28-42/259-660}. Release in the absence of DNA is also shown (no synthesis). (C) Kinetics of carboxyfluorescein leakage from liposomes in the presence of α -hemolysin, PulD^{fl}, and PulD^{28-42/259-660}. The released calcein and carboxyfluorescein are represented as a percentage of the total amount trapped in the liposomes in panels B and C, respectively.

that PulD synthesis and insertion are slower or less efficient than those of α -hemolysin. To discriminate between these possibilities, synthesis and leakage were performed in the presence of liposomes loaded with carboxyfluorescein, a molecule about half the size of calcein (376 Da and 622 Da, respectively). Efflux of carboxyfluorescein occurred at similar rates in the presence of PulD, PulD^{28-42/259-660}, and α -hemolysin (Fig. 3C), suggesting that the slower leakage of calcein induced by PulD was indeed due to permeability differences between the PulD and α -hemolysin pores.

The relatively slow calcein release through PulD (compared to release via α -hemolysin) suggested that the molecular mass cutoff for the PulD pore is close to the size of calcein. We therefore tested the efficiency of release of vancomycin (1,449 Da) from PulD-permeabilized liposomes. Antibiotic release was assessed by growth inhibition of agar-plated *S. aureus* around a paper disc soaked with the synthesis reaction mixture. Bacterial growth was inhibited around reaction mixtures treated with Triton X-100 prior to soaking the paper discs (Fig. 4). Soaking discs with Triton X-100 alone did not inhibit growth (not shown). However, bacterial growth was not efficiently inhibited around discs soaked with PulD^{fl}, PulD^{28-42/259-660}, or α -hemolysin reaction mixtures, indicating that vancomycin could not leak through the pores formed by these proteins (Fig. 4).

Together, these results indicate that PulD in its resting state is not tightly closed but instead forms a pore that is permeable to molecules up to approximately 600 Da in mass. In addition, the similar leakage from liposomes with PulD^{fl} and PulD^{28-42/259-660} indicated that the N₀, N₁, and N₂ subdomains do not affect the PulD pore.

Secretins other than PulD form solute-permeable pores in liposomes. Having created a versatile assay to estimate the pore

FIG 4 Leakage of vancomycin from liposomes permeabilized with α -hemolysin, PulD^{fl}, PulD^{28-42/259-660}, and PulD^{28-42/259-660}P443L. Nonpermeabilized liposomes and liposomes solubilized by Triton X-100 were used as negative and positive controls for leakage, respectively.

sizes of channel-forming proteins, we next examined the permeation characteristics of other secretins that insert spontaneously into liposomes (24). Of all of the secretins examined, only OutD from *E. carotovora* caused calcein leakage at a rate similar to that of PulD (Fig. 5A). Calcein release was markedly slower through pIV and OutD from *D. dadantii* (even though multimerization efficiencies were similar to those of PulD^{28-42/259-660} and OutD from *E. carotovora*, respectively) (see Fig. S1 in the supplemental material), while calcein efflux through XcpQ was negligible (with multimerization levels similar to those of OutD) (Fig. 5A; see Fig. S1 in the supplemental material). Nonetheless, all self-assembly secretins tested allowed carboxyfluorescein efflux at rates similar to that for PulD, again suggesting that differences in calcein efflux reflect the formation by the different secretins of pores of different sizes, rather than differences in assembly rates (Fig. 5B). PilQ from *Neisseria meningitidis* does not multimerize *in vitro* (see Fig. S1 in the supplemental material) (24). In agreement with this observation, PilQ did not allow calcein or carboxyfluorescein leakage from liposomes (Fig. 5A and B).

The differences in calcein leakage facilitated by OutD from *E. carotovora* and *D. dadantii* were investigated further. According to a model based on GspD (3), a serine-rich linker in the N₃ domain of OutD from *D. dadantii* (8) faces the barrel lumen and could occlude the pore, explaining the comparatively faster calcein diffusion through the *E. carotovora* OutD pore, which lacks this linker. However, deletion of this linker by removing residues 297 to 353 did not increase the calcein leakage through *D. dadantii* OutD, excluding a major role for this linker in permeability (see Fig. S2 in the supplemental material).

Replacement of a conserved proline increases PulD permeability. Single-residue substitutions in gates 1 and 2 of the C domain of pIV were reported to change the permeability properties of this secretin in the *E. coli* OM (21). The PulD peptides whose

sequences correspond to gates 1 and 2 in pIV (Fig. 1A) were deleted from PulD^{28-42/259-660} to determine whether their absence increased permeability *in vitro*. Deletion of the region corresponding to gate 1 (residues 450 to 485, corresponding to residues 259 to 297 in pIV) significantly reduced the ability of PulD to multimerize and, therefore, pore formation (Table 1; see Fig. S3 in the supplemental material). Deletion of the region corresponding to gate 2 (residues 514 to 527, equivalent to residues 322 to 335 in pIV) also reduced multimerization but nevertheless facilitated calcein efflux (Table 1; see Fig. S3 in the supplemental material). However, due to the large difference in multimerization efficiency with respect to the wild-type protein, it is difficult to determine the precise effect of this deletion on calcein efflux. We therefore looked for substitutions specifically affecting permeability by assaying a previously described collection of PulD^{28-42/259-660} variants (24, 25) for changes in calcein efflux *in vitro* in the absence of large effects on PulD^{28-42/259-660} multimerization.

Many PulD^{28-42/259-660} variants did not show a measurable dif-

TABLE 1 Leakage of calcein trapped in liposomes upon synthesis and insertion of PulD derivatives in the *in vitro* transcription-translation system

PulD derivative
Leakage (% ^a)
D281N (70)
V336I (46)
I352F ^b (86)
T429I (89)
S434N (92)
P443L ^c (88)
N450Y (49)
V479F (95)
I494 M (56)
Q506E (78)
Q506L (49)
A512P (101)
G523D (100)
G538S (63)
Δ 514-527 (Δ Gate 2) ^b (87)
No leakage ^d
I322S ^b
T447I ^b
I569S ^b
F573L
V593M
Δ 450-485 (Δ Gate 1) ^b

^a Leakage level relative to that of unmodified PulD after 2 h.

^b This variant produced significantly lower yields of multimers (see Fig. S3 in the supplemental material).

^c Leakage kinetics are shown in Fig. 6A.

^d Leakage levels are comparable to those in mixtures without protein synthesis.

FIG 5 Calcein (A) and carboxyfluorescein (B) release by α -hemolysin and by different members of the secretin family (PulD, XcpQ, and OutD, T2SS; PilQ, T4P; pIV, phage secretion). Release is represented as a percentage of the total amount of trapped dye in the liposomes.

FIG 6 Kinetics of calcein release from liposomes permeabilized by PulD^{28-42/259-660} or PulD^{28-42/259-660}P443L (A) and by pIV or pIVP252L (B). The released calcein is represented as a percentage of the total amount trapped in the liposomes.

ference in calcein efflux compared to the unmodified pore (Table 1). Several variants that exhibited diminished multimerization did not leak calcein (Table 1; see Fig. S3 in the supplemental material) or carboxyfluorescein (not shown). One variant, PulD^{28-42/259-660}F573L, multimerized to wild-type levels without promoting leakage of calcein (Table 1; see Fig. S3 in the supplemental material) or carboxyfluorescein (not shown), indicating either that its pore was closed or that the protein was misfolded. One other variant, PulD^{28-42/259-660}P443L (Fig. 1B), exhibited a more rapid calcein efflux than unmodified PulD^{28-42/259-660}, even though its multimerization was impaired (24) (Table 1 and Fig. 6A; see Fig. S1 in the supplemental material). To investigate the effect of this substitution further, we tested whether PulD^{28-42/259-660}P443L facilitated vancomycin leakage from liposomes. Growth of *S. aureus* was inhibited around discs saturated with vancomycin-loaded liposomes from a PulD^{28-42/259-660}P443L synthesis reaction, indicating that the pore formed by this variant is more permeable than that formed by PulD^{fl} and PulD^{28-42/259-660} (Fig. 4).

To complement the *in vitro* *S. aureus* growth inhibition test, we developed a novel *in vivo* assay for OM permeability based on the inability of cyanocobalamin (1,579 Da) to permeate an OM that lacks the specific cyanocobalamin receptor/channel, BtuB (39), in an *E. coli* K-12 methionine auxotroph in which methionine biosynthesis is strictly cyanocobalamin dependent. Growth of this strain (NB190) producing PulD^{fl} (together with PulS to ensure targeting to the OM) or FhuA (the OM receptor and channel for ferrichrome) (28, 29) was assessed by the diameter of the growth zone formed around paper discs soaked with water, methionine, or cyanocobalamin. Figure 7 shows that growth occurred only in the presence of methionine when either FhuA or PulD^{fl} was produced, indicating that cyanocobalamin cannot permeate through the pores of FhuA or PulD^{fl}. However, the bacteria were able to grow around cyanocobalamin-impregnated discs in the absence

of methionine when producing a wide-open FhuA variant lacking the surface loop comprising residues 322 to 355 (28, 29) or when producing PulD^{fl}P443L, consistent with an increased pore size in PulD^{fl}P443L. We did not determine the upper permeability limit induced by the P-to-L substitution. However, it is unlikely that PulD^{fl}P443L forms a constitutively open secretin channel, since it did not facilitate the periplasmic exit of the 10-kDa protein Sac7d (see Fig. S4 in the supplemental material).

To examine whether other components of the secretion system would occlude the PulD pore *in vivo*, PulD^{fl} and PulD^{fl}P443L were produced in the presence of the entire T2SS. The presence of the entire T2SS and its substrate Pula in the strain producing PulD^{fl}P443L did not have any measurable effect on growth in the presence of cyanocobalamin, indicating that other Pul proteins did not occlude the PulD^{fl}P443L pore (see Fig. S5 in the supplemental material). In contrast, cells producing unaltered PulD^{fl} in the context of the complete T2SS grew (albeit less well than cells producing PulD^{fl}P443L) in the presence of cyanocobalamin (see Fig. S5 in the supplemental material), suggesting that cyanocobalamin can diffuse through PulD^{fl} when the T2SS is operating.

P443 is a highly conserved residue close to the region corresponding to gate 1 in pIV (Fig. 1B). We therefore replaced the corresponding proline in pIV (P252 in the mature protein) with leucine. The P252L substitution increased the rate of calcein efflux in pIV secretin (Fig. 6B). Thus, replacement of this proline in either PulD or pIV has an effect similar to that of the previously reported replacements in pIV gates 1 and 2 (21), further suggesting that this proline has a role in determining pore dimensions or permeability.

DISCUSSION

Liposomes with encapsulated fluorescent dyes have been widely used to test the permeability of reconstituted pore-forming proteins such as porins and toxins (34). Here, we took advantage of the fact that some secretins insert and assemble during cell-free synthesis (24) to study their ability to form pores, thereby avoiding detergent solubilization, purification, and reconstitution, which might alter the structure or properties of the pore or secretin channel. Such changes do indeed occur when PulD is solubilized in Zwittergent 3-14 (40), the detergent used in a study suggesting that PulD formed a closed pore (19). In addition, Zwittergent 3-14-solubilized PulD reconstitutes poorly into artificial liposomes, hampering reliable characterization of the PulD pore by electrophysiology and liposome swelling assays. Here, PulD inserted into liposomes during cell-free synthesis promoted calcein but not vancomycin efflux from liposomes, indicating that the PulD secretin allows the efflux of small solutes in its resting state. Several other observations support the presence of a pore in resting PulD, rather than complete closure of the PulD secretin

FIG 7 Uptake of cyanocobalamin (B₁₂) by an *E. coli* Δ btuB *metE* strain producing FhuA, FhuA Δ 322-355, PulD^{fl}, or PulD^{fl}P443L. Growth on methionine (Met)-soaked discs and growth on water-soaked discs were used as positive and negative controls, respectively.

channel suggested previously (19). First, mislocalization of PulD in the inner membrane is lethal (22), suggesting that PulD might form a pore in this membrane. Second, the permeability limit for PulD appears to be close to that of enterobacterial general OM porins, which allow the free diffusion of solutes under 600 Da (41). Consequently, PulD would not have any effect on OM permeability. Thus, there would not be any selective pressure to maintain secretins in a completely closed conformation. Third, other secretins also formed pores in this assay. The apparent variations in permeability cutoffs of the different secretins require further analysis. However, the apparently lower permeability of XcpQ than of other secretins might not be surprising, because the *P. aeruginosa* OM is less permeable than that of enterobacteria such as *E. coli* (42). Fourth, the observed permeability increase caused by the P443L substitution in PulD *in vitro* was also observed *in vivo*, validating that, at least for this variant, the conformation of the secretin is identical in both situations and thus that a pore is present. The corresponding substitution in the pIV secretin also appeared to increase pIV permeability.

If secretin pores are indeed open *in vivo*, the consequence for secretin channel gating is difficult to predict. The existence of such a pore could suggest that the loops occluding the secretin channel do not form a dense domain. A relatively small variation in local conformation might then be sufficient to open the secretin channel.

The methodology presented here provides a means of characterizing pore function by autoassembling secretins. The mutant bank assayed here was constructed based on cytotoxicity when the altered proteins were made in *E. coli* without a signal peptide. Most of these mutants are affected in multimerization, and so far, few have revealed evidence of altered secretin channel function. As noted elsewhere (25), mutants with constitutively open secretin channels cannot be isolated by *in vivo* methods. Indeed, *in vivo* multimerization would create large holes in the outer membrane (and also by self-assembly into the inner membrane), allowing cellular contents to leak out. However, an *in vitro* screen based on that described here could be used to identify such mutants without the need to passage the DNA *in vivo*. Consequently, the assay described could enable one to screen *in vitro*-generated mutant libraries for variants of any pore-forming protein in which the resulting increased permeability would be lethal for the cells producing it.

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