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N. N. Nickerson, S. S. Abby, E. P. C. Rocha, M. Chami, A. P. Pugsley. A Single Amino Acid Substitution Changes the Self-Assembly Status of a Type IV Piliation Secretin. *Journal of Bacteriology*, 2012, 194 (18), pp.4951 - 4958. 10.1128/JB.00798-12 . pasteur-01374952

HAL Id: pasteur-01374952

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Submitted on 2 Oct 2016

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A Single Amino Acid Substitution Changes the Self-Assembly Status of a Type IV Piliation Secretin

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Secretins form large multimeric complexes in the outer membranes of many Gram-negative bacteria, where they function as dedicated gateways that allow proteins to access the extracellular environment. Despite their overall relatedness, different secretins use different specific and general mechanisms for their targeting, assembly, and membrane insertion. We report that all tested secretins from several type II secretion systems and from the filamentous bacteriophage ϕ 1 can spontaneously multimerize and insert into liposomes in an *in vitro* transcription-translation system. Phylogenetic analyses indicate that these secretins form a group distinct from the secretins of the type IV piliation and type III secretion systems, which do not autoassemble *in vitro*. A mutation causing a proline-to-leucine substitution allowed PilQ secretins from two different type IV piliation systems to assemble *in vitro*, albeit with very low efficiency, suggesting that autoassembly is an inherent property of all secretins.

Secretins are large multimeric protein complexes in the outer membranes (OM) of Gram-negative bacteria (diderms) that form the portal to the extracellular milieu in type II secretion systems (T2SS), in type IV piliation and DNA uptake systems (T4PS), in needle formation by type III secretion systems (T3SS), and in filamentous phage secretion systems (reviewed in reference 29). Secretins share a domain organization comprising a relatively poorly conserved periplasmic N-terminal region, which confers substrate specificity (39) and interacts with inner membrane components of the secretion machinery (46), a more highly conserved secretin C domain (PFAM secretin, PF00263) involved in multimerization and membrane insertion (4, 19, 20), and, in at least some cases, a C-terminal region of variable length that is important for OM localization (33, 42).

Despite their similar primary structures and domain organization, secretins use different mechanisms for targeting and insertion into the OM. The archetypical secretin, the dodecameric PulD from the *Klebsiella oxytoca* (also called *K. pneumoniae*) T2SS (14), is targeted to the OM by a dedicated lipoprotein chaperone, the pilotin PulS, which binds to the intrinsically disordered C-terminal segment in PulD, the S domain (33, 42), and is itself targeted to the OM by the Lol machinery (8). Once docked at the OM, PulD assembly and insertion depend only on the core domain (the last repeat of the N-terminal domain and the C domain) (19, 21). Specific OM insertion factors, such as the Bam (beta-barrel assembly) machinery, are not required (7), as indicated by the ability of *in vitro*-synthesized PulD to insert into liposomes (19), the phenomenon referred to here as autoassembly. The closely related T2SS secretin OutD of *Dickeya dadantii* (*Erwinia chrysanthemi*) is also targeted and assembled by this mechanism (18, 19). The corresponding secretin XcpQ from the T2SS of *Pseudomonas aeruginosa* can also insert spontaneously into membranes *in vitro* (23), but its correct localization to the OM depends on a periplasmic protein, PA0943 (38). HxcQ, the secretin of a different *P. aeruginosa* T2SS, is a lipoprotein in which the lipid anchor is required for correct targeting to the OM but not for multimerization (44).

The BfpB secretin of an *Escherichia coli* T4PS is also a lipoprotein, but lipid modification is not required for targeting to the OM

(31). The secretin PilQ from the T4PS of *Neisseria meningitidis* requires a lipoprotein chaperone, PilW, for multimer stability (3) and the Bam complex for correct assembly in the OM (45) and cannot assemble *in vitro* (20). Likewise, PilQ from *P. aeruginosa* is also unable to assemble *in vitro* (23) and requires a PilW orthologue, PilF, for multimerization and OM localization (27), but it appears to be only minimally dependent on BamA and is negatively impacted by inactivation of the Lol pathway (23). In the *Myxococcus xanthus* T4PS, assembly of stable PilQ multimers is dependent on another PilW orthologue, Tgl, but targeting and membrane insertion of the complex have not been thoroughly investigated (35).

Stability and multimerization of the *Shigella flexneri* T3SS secretin MxiD are dependent on another lipoprotein, MxiM (37). Although PulS, PilF/PilW, and MxiM are all lipoproteins and have related functions, they have completely different structures (27, 36, 42, 43). Efficient assembly of the *P. aeruginosa* T3SS secretin PscC depends on both BamA and the Lol machinery, and this secretin does not autoassemble *in vitro* (23).

In contrast to all of these secretins, the pIV secretin from the *E. coli* filamentous bacteriophage ϕ 1 does not appear to need any accessory factor for targeting and assembly, although targeting is inefficient compared to that of other secretins (11, 24).

In this report, we extend the analysis of the capacity of T2SS and pIV secretins to autoassemble and correlate this capacity with their phylogenetic relatedness and their relation to T4PS and T3SS

Received 7 May 2012 Accepted 30 June 2012

Published ahead of print 6 July 2012

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Supplemental material for this article may be found at <http://jb.asm.org/>.

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doi:10.1128/JB.00798-12

secretins. We also describe attempts to identify mutations that allow autoassembly of T4PS secretins.

MATERIALS AND METHODS

Materials. Unless otherwise indicated, chemicals were of molecular biology grade and were purchased from Sigma. DNA polymerases, restriction endonucleases, and DNA modification enzymes were from New England BioLabs, Invitrogen, or Roche. Plasmid purification, PCR purification, and gel extraction kits were purchased from Qiagen and used according to manufacturers' recommendations.

Bacterial strains and growth conditions. Information on bacterial strains and plasmids is outlined in Table S1 in the supplemental material. *E. coli* K-12 strain PAP105 [$\Delta(lac-proAB)$ F' (*lacI^{q1} ΔlacZM15 proAB⁺ Tn10*)] was used for plasmid manipulations, and *E. coli* B strain BL21 (F⁻ *ompT hsdS gal endA*) (λ DE3) carrying pDIA17 (pACYC184 derivative carrying *lacI^q* under *tet* promoter control) was used for in cytotoxicity experiments (19). Cultures were grown under aeration at 30°C or 37°C in LB broth or LB with 10% M63 minimal medium (32) containing, when appropriate, 0.4% maltose to induce expression of *pul* genes encoding the T2SS machinery, and appropriate antibiotics (chloramphenicol, 25 $\mu\text{g}\cdot\text{ml}^{-1}$; ampicillin, 100 $\mu\text{g}\cdot\text{ml}^{-1}$; kanamycin, 50 $\mu\text{g}\cdot\text{ml}^{-1}$). For secretion assays, PAP105 carrying pCHAP8243 (a derivative of pCHAP231 [14] carrying the complete *pul* operon with a *pulD* deletion and *pulA* encoding a soluble nonacylated variant of PulA) and pCHAP3635 (*pulD⁺*) or its derivatives encoding PulD variants were grown to an optical density at 600 nm (OD₆₀₀) of approximately 1.5 and centrifuged to separate cells and supernatant.

Plasmid construction and mutagenesis. Constructed plasmids are outlined in Table S1 in the supplemental material, and primers used for cloning are listed in Table S2. *pilQ* amplified from *Neisseria meningitidis* strain 8013 genomic DNA using primers PNN30 and PNN31 and *pIV* amplified from f1 filamentous phage DNA using primers PNN59 and PNN60 were cloned into NdeI-BamHI sites of pIVEX2.3MCS (Roche Applied Science). DNA encoding T2SS secretins from *Legionella pneumophila* strain Paris (*lspD*), *Shewanella oneidensis* MR-1 (*gspD*), and *Xanthomonas campestris* pv. *campestris* (*xcsD*) were amplified with the appropriate primers (see Table S2) and likewise cloned into pIVEX2.3MCS. For reasons explained in Results, linear templates containing the essential T7 promoter elements were created for T2SS secretin genes from *Vibrio cholerae* El Tor O1 (*epsD*) and *Aeromonas hydrophila* subsp. *hydrophila* (*exeD*) using the LinTempGenSet kit (5 Prime) with the appropriate primers (see Table S2), thereby allowing their expression in the rapid translation system (RTS) without cloning.

Random-site mutagenesis of DNA encoding PilQ^{MC58} in pIVEX2.3MCS (Roche Applied Science) (pCHAP3783 [19]) was performed by error-prone PCR using the GeneMorph II EZClone domain mutagenesis kit (Stratagene) with *pilQ*-specific primers PNN5 and PNN6. Mutated DNA was electroporated into *E. coli* strain BL21(pDIA17), and clones of interest were selected based on their reduced growth at 37°C versus 30°C. Site-directed mutagenesis was performed with complementary pairs of mutagenic primers using an adapted two-step PCR based on the QuikChange site-directed mutagenesis kit protocol (Stratagene). All constructs were verified by sequencing.

In vitro synthesis reactions. To test their ability to self-assemble *in vitro*, secretins were synthesized in a 50- μl *E. coli* HY 100 RTS reaction mixture (5 Prime) in the presence of 100 μg of asolectin liposomes (Sigma) and 0.5 μg of vector DNA (pIVEX2.3MCS) at 30°C for 6 h (19). To check for multimer assembly and insertion into liposomes, the reaction mixture was diluted in 5 volumes of phosphate-buffered saline (PBS) before centrifugation in a Beckman TLA-55 rotor at 23,000 $\times g$ for 10 min at 20°C. The pellet was resuspended in 50 μl of the same buffer, and an aliquot was treated with phenol to dissociate multimers and then analyzed together with an untreated aliquot by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with or without prior heating to 100°C. Another aliquot of the resuspended pellet was centrifuged and resuspended in 4 M urea in the same buffer, incu-

bated for 15 min at room temperature, and centrifuged again. The urea-washed pellet was resuspended, and one aliquot of each fraction was prepared for analysis as described above.

SDS-PAGE and immunoblotting. Total cell extracts and aliquots from *in vitro* reaction mixtures were diluted in SDS sample buffer and, where appropriate, were heated at 100°C for 5 min. Secretin multimers were dissociated by phenol treatment (22), precipitated by acetone at -20°C, and resuspended in sample buffer. Proteins solubilized in sample buffer were separated by electrophoresis in Tris-glycine-buffered 10% polyacrylamide gels or 4-to-20% gradient gels (Bio-Rad) with SDS (SDS-PAGE) and either stained with Coomassie brilliant blue or electroblotted onto nitrocellulose membrane (GE Healthcare). Immunoblotting for PilQ from *N. meningitidis* (PilQ_{Nm}) was performed with either N-terminal or C-terminal specific antibodies (17), antibodies specific for PilQ from *P. aeruginosa* (PilQ_{pa}) (Stephen Lory), or PulD- and PulA-specific antibodies from the laboratory collection. Secondary antibodies conjugated to horseradish peroxidase (GE Healthcare) were detected by enhanced chemiluminescence (Pierce).

Electron microscopy. Liposomes were centrifuged at 23,000 $\times g$ for 10 min, resuspended in 4 M urea for 15 min to dissociate liposome aggregates and loosely bound material, diluted to 1 M urea by adding 20 mM Tris containing 250 mM NaCl (TN buffer), centrifuged at 250,000 $\times g$ for 30 min, and resuspended in TN buffer. For imaging of negatively stained samples, aliquots of 5 μl were adsorbed on carbon film-coated copper grids, washed with 10 droplets of pure water, and subsequently stained with 2% uranyl acetate. Images were recorded on a charge-coupled device (CCD) camera (2K by 2K pixel resolution; Veleta; Olympus) at a $\times 92,000$ magnification using a Philips CM10 transmission electron microscope operating at 80 kV.

Phylogenetic analysis of the secretin family. A selection of secretin sequences from a published data set (6) supplemented with additional sequences corresponding to the proteins examined experimentally were aligned by the Muscle program with default parameters (15). Informative positions were selected using the BMGE program using the following parameters (10): BLOSUM30 similarity matrix, a gap rate cutoff of 0.20, a sliding window size of 3, and an entropy score cutoff of 0.5. Phylogenetic trees were built with the maximum-likelihood search implemented in RAxML (40) (model LG+G+F). The tree with the highest likelihood was selected among 200 starting tree inferences. One thousand bootstrap replicates were constructed, and the relationships in these bootstrap trees were used to display supports on the branches of the selected maximum-likelihood tree.

RESULTS

The f1 filamentous phage pIV secretin can autoassemble. Previous studies revealed that the T2SS secretins PulD, OutD, and XcpQ assemble into membrane-embedded secretin rings when produced in an *in vitro* transcription-translation system supplemented with liposomes, whereas two PilQ secretins (T4PS) and PscC (T3SS) do not (19, 23). Here, we report that pIV secretin from f1 filamentous phage produced without its signal peptide behaves like PulD, OutD, and XcpQ in this system, forming SDS-, heat- and urea-resistant, phenol-sensitive multimers detectable by SDS-PAGE (Fig. 1A). In this experiment, the multimer (Mu) was identified by its dissociation following phenol treatment and the monomer (Mo) by its corresponding increased abundance. Neither of these two bands was detected when green fluorescent protein (GFP) DNA was used. Electron microscopy of *in vitro* synthesized pIV revealed membrane-embedded secretin-like rings (Fig. 1B).

Sequences of T2SS and filamentous phage secretins are closely related. The C domain of ~200 to 250 residues and encompassing the membrane-spanning and gate regions is the most conserved region of secretins (29). A secretin sequence data set

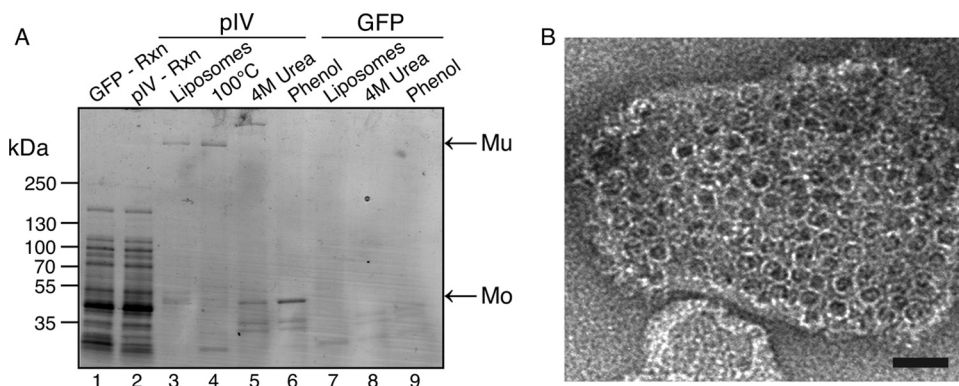


FIG 1 Phage pIV secretin can autoassemble and insert into liposomes *in vitro*. (A) Products (Rxn; lane 1 and lane 2) and centrifuged liposomes (pIV, lanes 3 to 5; GFP control, lanes 7 to 9) from *in vitro* synthesis reactions in which either pIV secretin or GFP control was synthesized were dissolved in SDS sample buffer and then separated by SDS-PAGE on a gradient gel and stained with Coomassie blue. Separate samples were heated in SDS to 100°C (lane 3) or were treated with 4 M urea (lanes 5 and 8) or phenol (lanes 6 and 9) prior to dissolution in SDS. Samples enriched for liposomes (lanes 3 to 9) correspond to five times more material than was in the total reaction mixture. Mu, pIV multimer; Mo, pIV monomer. We cannot explain the reduced mobility of the pIV multimer after urea treatment (lane 5). (B) Negatively stained liposomes with inserted pIV secretin multimers examined by electron microscopy. The average diameter of the secretin particles is 14 nm. Bar, 25 nm.

was constructed with secretins from T2SS, T3SS, T4PS, and filamentous phage secretion systems, and after poorly aligned regions had been filtered out, a maximum-likelihood phylogeny was constructed (Fig. 2). This tree revealed well-supported groups for the different secretin subfamilies: the T3SS secretins (100% bootstrap support), the T4PS secretins (100% bootstrap), and the T2SS secretins (99% bootstrap). Within the T2SS group, *X. campestris* XpsD-related secretins formed a clade (90% bootstrap) that was the first to emerge, followed by the filamentous phage secretins (98% bootstrap). This tree topology is consistent with a previously published tree (6) and groups filamentous phage secretins together with T2SS secretins. These observations suggest that autoassembly could be a specific characteristic of the T2SS-phage pIV secretin subfamily.

Spontaneous assembly and membrane insertion are general characteristics of T2SS secretins. We next tested whether other secretins of the T2SS-phage pIV subfamily (LspD from *Legionella pneumophila*, GspD from *Shewanella oneidensis*, ExeD from *Aeromonas hydrophila*, EpsD from *Vibrio cholerae*, and XcsD from *Xanthomonas campestris*) share with the previously tested members of this subfamily their capacity for autoassembly and membrane insertion. PCR-amplified DNA for each secretin without its signal peptide was cloned into pIVEX2.3MCS under the control of the T7 promoter. DNA encoding the secretins from *Aeromonas* and *Vibrio* could not be cloned into pIVEX2.3MCS. Previous studies have shown that *pulD* thus cloned is toxic except in the presence of multiple copies of the *lac* repressor gene *lacI* and, in cells grown at 30°C, a phenomenon referred to as incytotoxicity (21). DNA encoding the *Aeromonas* and *Vibrio* secretins could not be cloned even under these conditions, suggesting that they are even more toxic than PulD. Thus, DNA encoding these two secretins was produced as linear templates with the necessary T7 promoter elements.

SDS-PAGE (see Fig. S1 in the supplemental material) and electron microscopy (Fig. 3) demonstrated that all five newly tested secretins formed typical secretin rings that were associated with liposomes when produced in the *in vitro* transcription-translation system with lecithin liposomes. These and previous results are summarized in Table 1 and outlined on the phylogenetic tree

(Fig. 2). Thus, all tested T2SS and phage secretins can autoassemble, whereas the two previously tested T4PS secretins and the single T3SS secretin tested cannot (Fig. 2). The separation between these two groups of secretins is highly supported by the phylogenetic tree (99% bootstrap value). The autoassembly-competent secretins are thus closely related and have a common origin (see Discussion). These observations provide strong support for the idea that all secretins in the T2SS-phage secretion group are able to multimerize and insert spontaneously into liposomes.

Substitution of a single proline allows *in vitro* autoassembly of T4PS PilQ. Since secretins share ancestry, we reasoned that secretins that cannot autoassemble might have recently lost the ability to do so and might, through mutation, easily reacquire it. To test this hypothesis, we mutagenized DNA encoding a signal peptide-less form of PilQ from the T4PS of *N. meningitidis* strain MC58 (19) and expressed it in *E. coli*. *E. coli* cells producing PulD in this way grow well at 30°C but, because of the incytotoxicity phenomenon likely caused by the insertion of PulD into the inner membrane, grow poorly at 37°C (21). After screening a bank of mutants, we identified one that consistently grew less well than the parent strain at 37°C. The PilQ_{Nm} protein encoded by DNA in this clone was able to form multimers in the *in vitro* transcription-translation system (Fig. 4A). The efficiency of multimerization of this PilQ variant was difficult to establish because unassembled monomers were apparently degraded, giving rise to the multiple bands below that identified as the PilQ monomer. However, visual inspection of the immunoblot in Fig. 4A indicates that multimerization efficiency is considerably lower than that of PulD, which is estimated to multimerize at >50% efficiency under identical conditions.

This *pilQ* DNA carried two mutations, causing T359S and P613L substitutions. When the two mutations were created separately, incytotoxicity (poor growth at 37°C) and autoassembly *in vitro* were associated exclusively with the P613L substitution (Fig. 4A). Indeed, the T359S substitution appeared to diminish multimerization efficiency, since fewer multimers were observed with the T359S-P613L substitutions than with P613L alone (Fig. 4A). The P613L variant is referred to here as PilQ_{Nm}^{*}. Like other autoassembly-competent secretins, PilQ_{Nm}^{*} remained partially associated with the liposomes after a washing with 4 M urea (Fig.

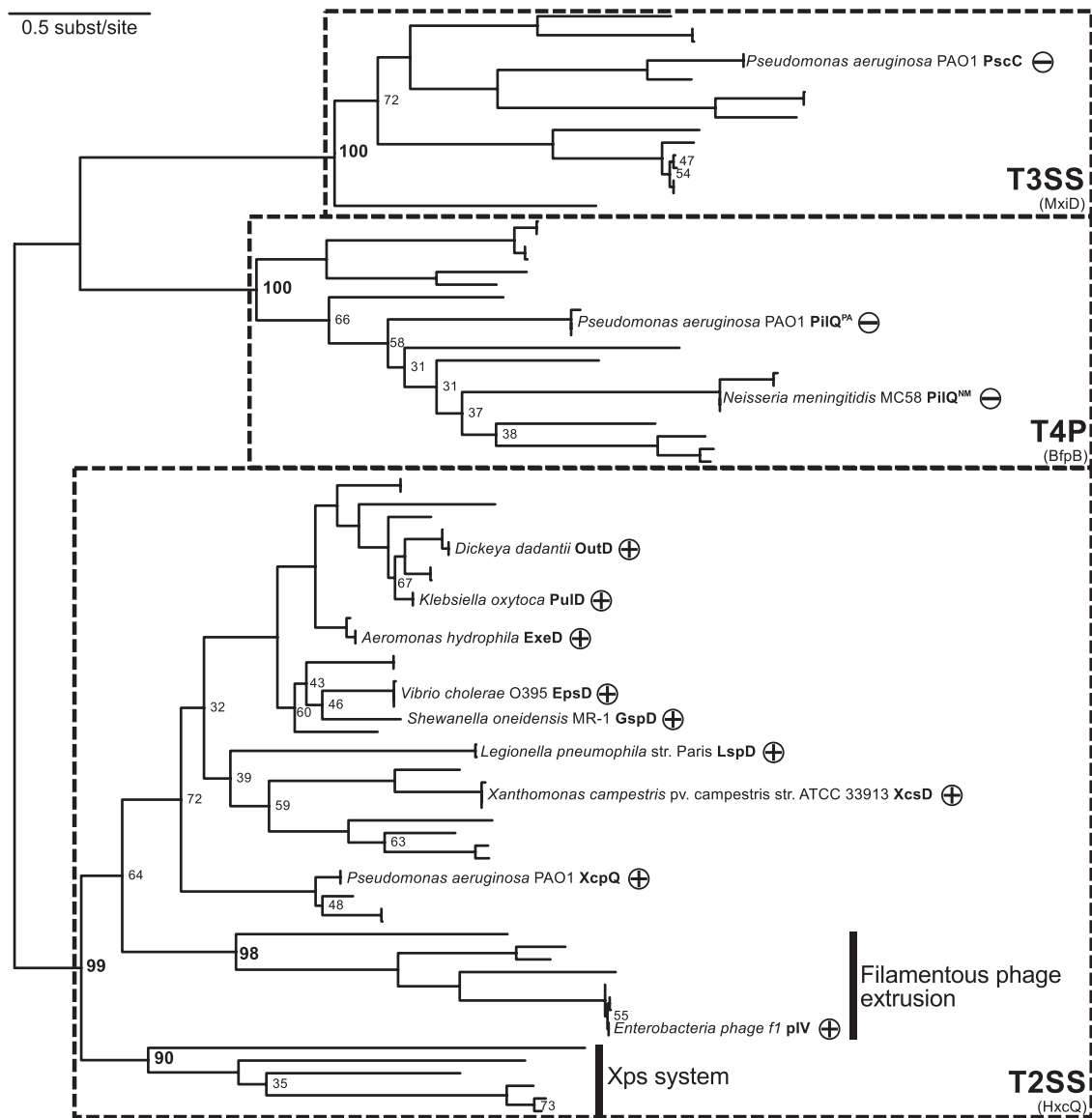


FIG 2 Phylogeny of the secretin family. The maximum-likelihood tree shows the relationships between secretins of the different machineries in which they are found. Species names and protein names are indicated for protein sequences tested experimentally in the *in vitro* synthesis system. + and −, secretins that are able and unable to multimerize and insert into liposomes *in vitro*, respectively. Bootstrap supports for the tree are displayed only when below 80%, except for branches corresponding to key relationships mentioned in the text (in bold). Proteins mentioned in the main text but not included in the analysis are given in parentheses below the name of the system to which they belong.

4B), was dissociated by phenol treatment (data not shown), and appeared as typical secretin rings when examined by electron microscopy (Fig. 5), confirming that PilQ_{Nm}* multimerizes and inserts into liposomes *in vitro*.

To test the specificity of the P613L substitution, proline 613 in PilQ_{Nm} was replaced by glycine, alanine, valine, phenylalanine, and cysteine. These substitutions all resulted in lower levels of autoassembly than P613L, except for the glycine substitution, which did not result in any detectable multimer formation but caused smearing that might indicate incomplete multimerization or multimer instability (Fig. 6A). Replacing the amino acid residues on either side of the proline by those found in the identical position in PulD (N612T and R614S) did not increase the efficiency of PilQ_{Nm}* autoassembly (data not shown).

The corresponding substitution in *P. aeruginosa* PilQ also promotes autoassembly. P613 is found in all analyzed secretins, irrespective of their ability to autoassemble. To test whether the autoassembly caused by the P613L substitution in PilQ_{Nm}* is specific to this particular secretin, the same substitution was constructed in PilQ_{Nm} from *N. meningitidis* strain 8013 (P605L). PilQ₈₀₁₃(P605L) behaved in the same manner as PilQ_{Nm}* (data not shown). The same proline-to-leucine change in *P. aeruginosa* PilQ (P562L), creating PilQ_{pa}*, also resulted in the ability to autoassemble *in vitro* (Fig. 6B).

The corresponding proline-leucine substitution in PulD reduces multimerization but does not drastically affect function. As mentioned above, proline at position 613 in PilQ_{Nm} is completely conserved in all secretins, including, paradoxically,

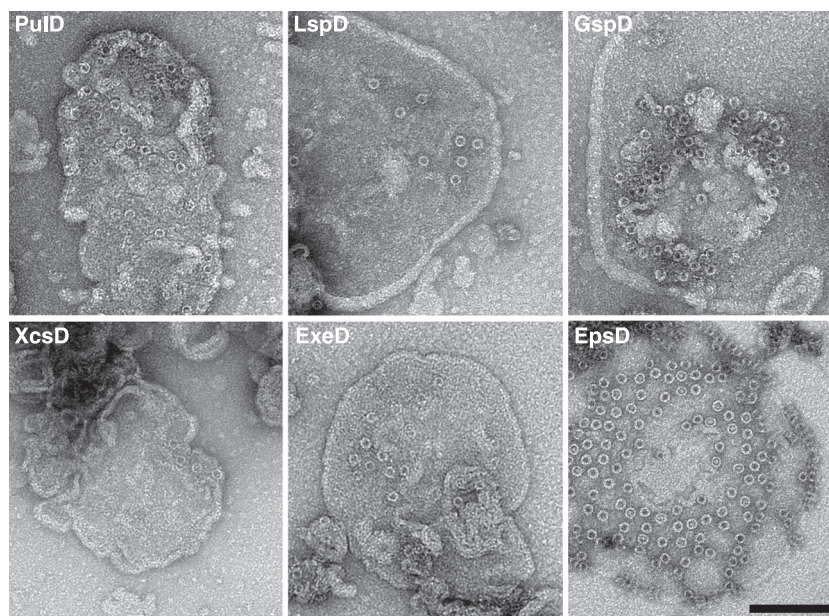


FIG 3 *In vitro* autoassembly is a general feature of T2SS secretins. Negatively stained liposomes from the respective samples were visualized by electron microscopy. Samples tested were from *Klebsiella oxytoca* (PulD), *Aeromonas hydrophila* (ExeD), *Legionella pneumophila* (LspD), *Shewanella oneidensis* (GspD), *Vibrio cholerae* (EpsD), and *Xanthomonas campestris* (XcsD). Bar, 100 nm.

those that are able to assemble spontaneously. To determine the role of this residue in these secretins, the proline-to-leucine substitution was made in full-length signal peptide-less PulD (PulD²⁸⁻⁶⁶⁰) and in a truncated version corresponding to the core and S domains (PulD^{28-42/259-660}). Both PulD(P443L) variants exhibited reduced *in vitro* assembly (see Fig. S2A in the supplemental material), but electron microscopy of purified PulD(P443L) secretin produced *in vitro* did not reveal any gross physical changes compared to wild-type protein (see Fig. S2B).

PulD(P443L) produced *in vivo* also exhibited less efficient multimerization (Fig. 7A, top) and caused cells to produce somewhat higher levels of the phage shock protein PspA, probably indicative of increased misrouting to the inner membrane (2, 20), than wild-type PulD (Fig. 7A, bottom). Despite its reduced capacity to multimerize, however, PulD(P443L) could substitute for wild-type PulD in complementation assays for pullulanase (PulA) secretion, albeit with reduced efficiency compared to the wild-type protein (Fig. 7B).

TABLE 1 Origins and *in vitro* assembly of analyzed secretins

Secretin (species)	Secretion system	Accession no.	<i>In vitro</i> multimerization ^a
PulD (<i>Klebsiella oxytoca</i>)	T2SS	P15644	+
OutD (<i>Dickeya dadantii</i>)	T2SS	P31700	+
XcpQ (<i>Pseudomonas aeruginosa</i>)	T2SS	NP_251795	+
pIV (f1 filamentous phage)	f1 phage	P03666	+
ExeD (<i>Aeromonas hydrophila</i>)	T2SS	P31780	+
LspD (<i>Legionella pneumophila</i>)	T2SS	YP_123599	+
EpsD (<i>Vibrio cholerae</i>)	T2SS	YP_001218221	+
XcsD (<i>Xanthomonas campestris</i>)	T2SS	NP_638771	+
GspD (<i>Shewanella oneidensis</i>)	T2SS	NP_715808	+
PscC (<i>Pseudomonas aeruginosa</i>)	T3SS	NP_250407	+
PilQ (<i>Neisseria meningitidis</i>)	T4PS	NP_274809	-
PilQ (<i>Pseudomonas aeruginosa</i>)	T4PS	NP_253727	-

^a *In vitro* multimerization and insertion into liposomes was tested in the cell-free coupled transcription and translation system and analyzed by SDS-PAGE and electron microscopy.

DISCUSSION

Secretins are key components of many trans-envelope OM machineries in diderms that allow proteins to reach the cell surface and beyond. The large complexes that they form incorporate a

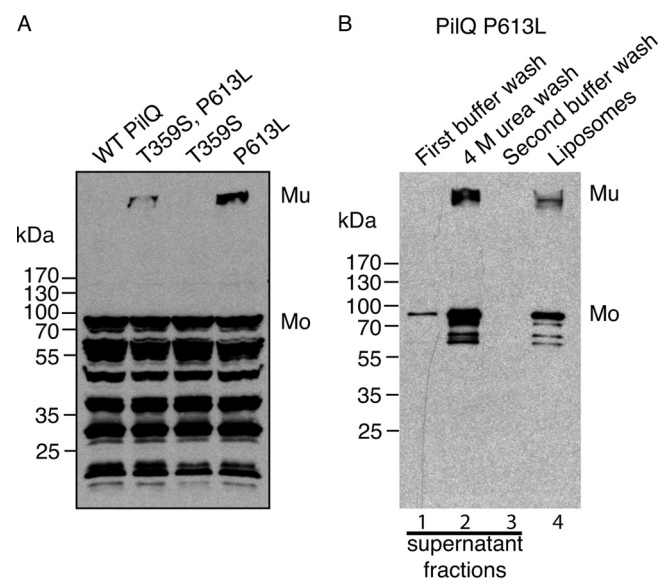


FIG 4 PilQ_{Nm}^{*} can multimerize and insert into liposomes spontaneously. (A) Total *in vitro* synthesis reactions of wild-type PilQ_{Nm} (WT) and its derivatives with the indicated amino acid substitutions were analyzed by SDS-PAGE and immunoblotting with PilQ_{Nm} antiserum. (B) Liposomes from the *in vitro* synthesis reaction of the PilQ_{Nm}^{*} mutant (P613L) were washed with PBS (supernatant fraction in lane 1) and 4 M urea treatment (supernatant in lane 2) and then again with PBS (supernatant in lane 3). The supernatant fractions and the washed liposomes (lane 4) were analyzed by SDS-PAGE and immunoblotting with PilQ_{Nm} antisera. Mu, PilQ multimer; Mo, PilQ monomer.

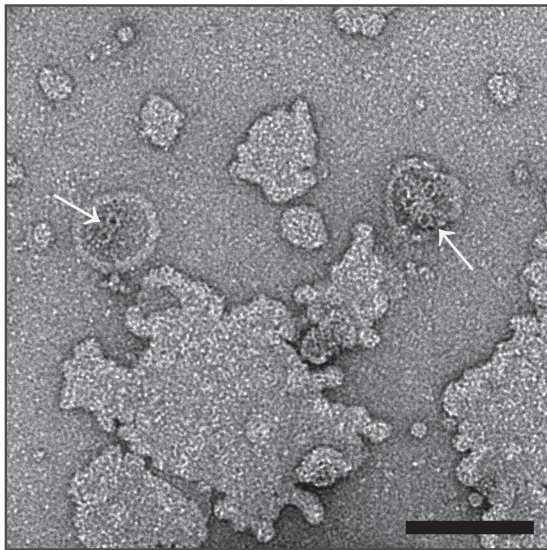


FIG 5 PilQ_{Nm}* inserted into liposomes. Negatively stained liposomes with inserted PilQ_{Nm}*P613L were visualized by electron microscopy. Arrows indicate the presence of PilQ_{Nm}* rings inserted into liposomes. Bar, 100 nm.

gated channel to allow proteins or filamentous bacteriophages to pass (secretion) or structures (needles, pili) to be assembled without disrupting the OM barrier. Although significant advances have been made, understanding secretin assembly and function has been hindered by the lack of high-resolution structural information on the core domain and relies mainly on biochemical and genetic approaches.

Secretins can be classified into one of two groups: those that can autoassemble and insert into membranes *in vitro* (T2SS and filamentous phage secretins) and those that cannot (T4PS and T3SS secretins) (19, 23; also this study). The phylogenetic tree of secretins cannot be rooted because of the lack of an outgroup sequence; hence, we cannot determine the branch of the tree in which the last common ancestor of all secretins is placed. Consequently, we cannot determine which system-specific secretin emerged first or if the original secretin performed multiple func-

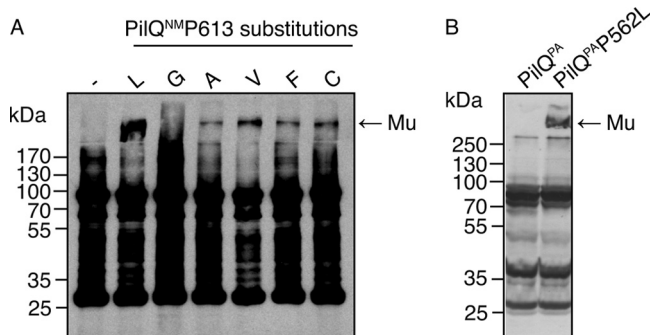


FIG 6 Effects of proline 613 substitutions in PilQ_{Nm} and of the corresponding P562L substitution in PilQ_{Pa} on autoassembly *in vitro*. (A) Total *in vitro* synthesis reactions producing wild-type PilQ_{Nm} or its variants were analyzed by SDS-PAGE and immunoblotting with *N. meningitidis* PilQ antisera. (B) Total *in vitro* synthesis reactions producing wild-type PilQ_{Pa} and PilQ_{Pa}(P562L) from *P. aeruginosa* were analyzed by SDS-PAGE and immunoblotting with PilQ_{Pa} antisera. Mu, PilQ multimer.

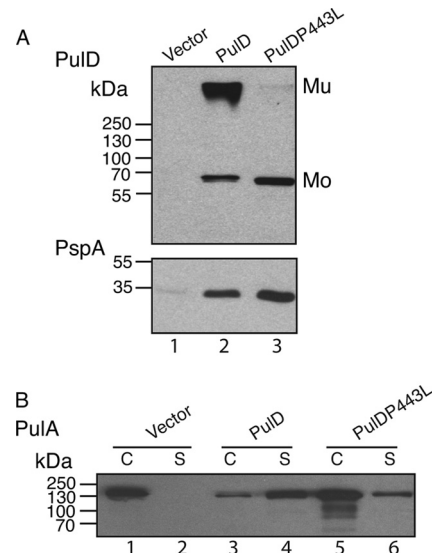


FIG 7 Effect of the P443L substitution on PulD assembly and function. (A) Total cell lysates of *E. coli* PAP105 in which either wild-type PulD or PulD(P443L) was produced. An equivalent of 0.05 absorbance unit of culture was separated by SDS-PAGE and immunoblotted with PulD- or PspA-specific antisera. Mu, PulD multimer; Mo, PulD monomer. (B) Total cell lysate (C) and culture supernatant (S) fractions of *E. coli* PAP105(pCHAP8243) producing all the Pul factors except PulD and a soluble variant of PulA (lanes 1 and 2), complemented with either wild-type PulD (lanes 3 and 4) or PulD(P443L) (lanes 5 and 6). An equivalent of 0.015 absorbance unit of culture was separated by SDS-PAGE and immunoblotted with PulA-specific antiserum.

tions. Of all of the machineries in which a secretin participates, only the T4PS has close homologs in monoderm (Gram-positive) bacteria and in archaea (5). It is therefore tempting to speculate that a secretin was added to this ancestral structure when it was acquired by diderms. However, the T4PS and T2SS are closely related and partially interchangeable (25), but self-assembling secretins are found only in the latter. Thus, in this scenario, the T4PS is the ancestor of the T2SS, T4PS secretins are the ancestors of T2SS secretins, and autoassembly evolved more recently than assisted assembly.

The filamentous phage secretins might have been acquired from a T2SS, an idea supported by the fact that at least one filamentous phage does not have its own secretin but instead uses the T2SS secretin of its host bacterium (12). This idea would be compatible with a potential selective advantage conferred by a minimal bacteriophage genome in which a secretin would be essential (for example, in *E. coli* strains that do not constitutively produce an endogenous secretin) but ancillary factors would not. However, this scenario does not explain all of the phenomena observed. For example, although genes for T2SS secretins are very frequently found sandwiched between *pulC* and *pulE* homologues, as in the *K. oxytoca* T2SS (13), suggesting highly conserved organization and function, they use different sets of specific cofactors for targeting to the OM (1, 26). This observation suggests that filamentous phage secretins, which do not have targeting and assembly factors, are ancestral and that different targeting cofactors evolved to facilitate efficient T2SS secretin targeting to the OM.

All secretins show substantial similarity in their C domains; in PulD, the C domain contains all the information needed for autoassembly and insertion (19–21). Presumably, sequence differ-

ences in this region explain the differing abilities of secretins to autoassemble. The fact that a single proline-to-leucine substitution in two different T4PS secretins enables them to assemble, albeit inefficiently, *in vitro* indicates that the structural or sequence differences underlying their ability or otherwise to autoassemble are likely quite minor. It might be significant that the P613G substitution in PilQ_{Nm} apparently caused an intermediate situation leading to the appearance of incomplete multimers seen as multiple bands when examined by SDS-PAGE (Fig. 6A), suggesting that flexibility (induced by proline or glycine) rather than side chains (present only in proline) at this position impedes autoassembly. However, this proline residue is completely conserved in all secretins, and thus, its presence or absence cannot be a primary determinant of autoassembly. We hypothesize that residues elsewhere in PilQ might prevent autoassembly and that the P613L substitution partially compensates for their effect, or that autoassembling secretins have overcome the deleterious effect of this proline residue on autoassembly by compensatory adjustments elsewhere in the C domain.

One intriguing question that we were unable to answer is whether the autoassembly variants of PilQ_{Nm}^{*} and PilQ_{Pa}^{*} are functional and have lost their dependence on their respective cofactors (PilW and PilF, respectively) for multimerization. PilQ_{Nm}^{*} was produced in insufficient amounts in a nonpolar *pilQ* mutant of *N. meningitidis* to be able to test its ability to multimerize and function *in vivo* (V. Pelicic, personal communication). Similarly, PilQ_{Pa}^{*} was unstable when produced in a *pilQ* mutant of *P. aeruginosa*, again precluding complementation tests (S. Lory, personal communication). These results led us to construct and test the corresponding substitution in PulD. Surprisingly, the P-to-L substitution actually caused decreased multimerization of PulD *in vitro* (see Fig. S1 in the supplemental material) and *in vivo* (Fig. 7), presumably because this proline residue plays a positive role in some aspect of secretin assembly.

Despite many efforts, it has not proven possible to isolate secretins in complex with other interacting components of the T2SS except pilotin (34) and GspC (28), suggesting that other interactions are transient. Thus, these secretins are not incorporated into a stable machinery and can be assembled independently of it, which can be easily achieved by autoassembly. This is also the case for filamentous phage secretions, for which the only known partner is an inner membrane protein (16). The requirement for the Bam complex to assist the assembly of *N. meningitidis* PilQ (45) might represent a further example of this targeting mechanism, since the core element of this system, BamA, is found only in the outer membrane, and thus, PilQ assembly can occur only at this membrane. Preliminary studies indicated that a soluble derivative of the *N. meningitidis* secretin chaperone PilW did not promote PilQ_{Nm} assembly in the *in vitro* assay, probably because the appropriate Bam complex was not present in the *in vitro* reaction. However, *P. aeruginosa* PilQ exhibits only a minimal Bam requirement (23). The situation for T3SS secretins is probably similar, for although they are an integral part of a stable multicomponent structure, they can assemble independently (9). Thus, all secretins share two major requirements: to efficiently target the OM to prevent insertion into the inner membrane, which could have deleterious consequences for the bacterium (as exemplified by secretin pIV) (2, 20, 24), and to avoid assembling into mixed multimers. Both of these challenges are met by using a variety of different targeting and assembly routes.

Clones encoding signal peptide-less variants of the *Vibrio* and *Aeromonas* T2SS secretins *epsD* and *exeD* could not be constructed, even under conditions of very tight repression, suggesting that these secretins are even more cytotoxic than PulD (21). EpsD and ExeD are closely related and belong to a subclass of T2SSs possessing GspA and GspB proteins that are thought to form a complex in the inner membrane that creates space in the peptidoglycan to allow the transport and assembly of the secretin (30, 41). One explanation for the elevated cytotoxicity might be that the EpsD and ExeD have a more open channel in their resting state than does PulD. Since GspA and GspB are required for efficient multimerization, they might also play a role in channel gating or in maintaining the OM barrier.

The ability of secretins to autoassemble in the OM might have been responsible for their impressive evolutionary success that is reflected by their presence in protein secretion, phage extrusion, bacterial appendage, and DNA uptake systems. Further studies will be required to extend the present analyses to other secretins and to study factors that permit the multimerization of secretins that are unable to autoassemble.

ACKNOWLEDGMENTS

We are grateful to Ingrid Guilvout and Séverine Collin for their helpful discussions and to members of the Molecular Genetics Unit for their support. We thank Andréa Dessen for the plasmid encoding soluble PilW, Vladimir Pelicic for *N. meningitidis* 8013 DNA and for performing PilQ_{Nm} complementation experiments, Stephen Lory for vector pHH41, for PilQ_{Pa} antibodies, and for performing PilQ_{Pa} complementation experiments, Marjorie Russel for PspA antibodies, Carmen Buchrieser for *Legionella pneumophila* strain Paris genomic DNA, Didier Mazel for *Vibrio cholerae* El Tor O1 genomic DNA, Tracy Palmer for *Shewanella oneidensis* MR-1 genomic DNA, and the Biological Resource Centre of Institut Pasteur (CRBIP) for strains of *Aeromonas hydrophila* subsp. *hydrophila* (CIP 76.14) and *Xanthomonas campestris* pv. *campestris* (CIP 74.23).

This work was supported in part by grants ANR-05-0307-01 and ANR-09-BLAN-0291. N.N.N. was supported by a postdoctoral fellowship from the Canadian Louis Pasteur Foundation during part of this work.

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