A new class of hybrid secretion system is employed in *Pseudomonas* amyloid biogenesis

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Gram-negative bacteria possess specialised biogenesis machineries that facilitate the export of amyloid subunits for construction of a biofilm matrix. The secretion of bacterial functional amyloid requires a bespoke outer-membrane protein channel through which unfolded amyloid substrates are translocated. Here, we combine X-ray crystallography, native mass spectrometry, single-channel electrical recording, molecular simulations and circular dichroism measurements to provide high-resolution structural insight into the functional amyloid transporter from *Pseudomonas*, FapF. FapF forms a trimer of gated β-barrel channels in which opening is regulated by a helical plug connected to an extended coil-coiled platform spanning the bacterial periplasm. Although FapF represents a unique type of secretion system, it shares mechanistic features with a diverse range of peptide translocation systems. Our findings highlight alternative strategies for handling and export of amyloid protein sequences.

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The aggregation of amyloidogenic proteins is typically associated with human disease states such as Parkinson’s and Alzheimer’s. However, many organisms including bacteria produce extracellular amyloid fibres for beneficial purposes. Bacterial amyloid fibres are a major protein component of biofilms, and play important functional roles in bacterial persistence either in animal hosts or on surfaces. Bacterial biofilms are of high societal importance as they are a major cause of recurrent disease by allowing reservoirs of bacteria to persist in a host (animal/human) or the environment and also contribute to drug resistance. Gram-negative bacteria employ sophisticated multi-component secretion systems for amyloid assembly. Amyloidogenic, unstructured subunits cross the periplasm and are secreted through the outer membrane (OM), after which they self-assemble into cross β-strand fibrils upon interaction with an extracellular or membrane-embedded nucleator protein. The aggregation properties of these subunits require strict control until they are translocated to the bacterial extracellular surface. Bacterial amyloid fibres are a major protein component of biofilms, and play important functional roles in bacterial persistence either in animal hosts or on surfaces. Two major amyloid systems are known in Gram-negative bacteria: the well-studied curli machinery in *Escherichia coli* and the more recently discovered and genetically distinct Fap system in *Pseudomonas*. The curli machinery comprises seven proteins, encoded in two operons *csgBAC* and *csgDEFG*, of which the mechanistic roles in curli fibre formation have been largely documented. *CsgA* is the main component of the fibres along with *CsgB* present at low levels as a nucleator. The atomic structure of the CsgG pore-forming component was recently solved and illuminated the molecular basis of transport of CsgA across the membrane. The curli system is distinct from previously described secretion pathways and has been named the Type VIII pathway. The proteins encoded by the *Pseudomonas* fap operon, *fapABCDE*, are genetically distinct to curli proteins. However, the amyloid forming component FapC contains similar amyloid repeat sequence rich in asparagine and glutamine residues (GN-X-G-N-X2-G-A-N-X2-NOQ-X-N), and inhibitors of curli formation have also been shown to inhibit Fap fibril formation. FapC is the main fibre-forming component, with FapB and FapE present in the fibres as minor subunits. FapA and FapD appear to be accessory proteins with regulatory roles. FapA has been implicated as a chaperone of Fap amyloid secretion, as its absence alters the composition of Fap fibres, which become primarily composed of the minor component FapB. FapF is the membrane protein pore component through which the FapB, FapC and FapE substrates are secreted.

Unlike the well-characterised curli system, no structural information is available for any component of the Fap system. Therefore, to provide insight into mechanistic basis of Fap secretion, we solved the crystal structure of the transmembrane domain (TD) of the transporter FapF (*FapF*83–406), termed FapF<sub>83</sub> from hereon. We then used a combination of biophysical techniques including native mass spectrometry (MS), single-channel current recordings and circular dichroism in order to elucidate the role of the periplasmic N-terminal domain of full-length FapF (*FapF*1–406) and derive a structural model for the translocation complex. Based on this new insight we designed several mutants to probe the functional determinants of FapC secretion. Combining our structural data with computational and functional analyses we shed new light on the mechanism by which FapC is secreted across the OM. Furthermore, our findings contribute to a growing understanding of how bacteria can safely handle amyloidogenic polypeptides and provides the inspiration for new approaches in the control of bacterial biofilms.

**Results**

**Architecture of the trimeric FapF transporter.** The full-length FapF species (numbered 1–406 for the mature protein from uniprot accession C4IN73) did not afford highly diffracting crystals, despite exhaustive attempts to optimise protein micro-crystals. Therefore, a combination of limited proteolysis and secondary structure prediction was used to produce a series of constructs that generated improved crystals. Removing the N-terminal domain comprising a 39-residue helical region followed by a 42-residue disordered linker and adding an OmpA signal sequence and a hexahistidine tag to allow purification directly from the *E. coli* outer membrane (OM) led to larger, well-diffracting and reproducible crystals for FapF<sub>83</sub> (Fig. 1a). Incorporating a L273M mutation into this construct yielded selenomethionine-substituted crystals that produced sufficient anomalous signal for phase determination. The structure was solved by single-wavelength anomalous dispersion (SAD) to 2.5 Å resolution with an R<sub>free</sub> of 26% (Supplementary Table 1 and Supplementary Fig. 1).

The TD of FapF comprises a 12-stranded plugged β-barrel (FapF<sub>83</sub>), which forms a tightly packed trimer within the crystal (Fig. 1b and Supplementary Fig. 1). The trimer interface is extensive, with a prominent phenylalanine ladder (F325, F335 and F367) making hydrophobic contacts between protomers around the C3 axis. At the extracellular rim of the FapF trimer is the presence of two conserved ‘PTG’ motifs at the base of loop βS–β6 (residues 233–235 and 259–261, which introduces a distinct plait in the polypeptide chain (Fig. 1b).

A notable feature of the structure is the presence of a 13-residue helical plug between residues A89 and G101 that is inserted into the lumen of the pore from the periplasmic side with the C-terminal end pointing in and the N terminus out. Strikingly, the helix is immediately followed by a turn with a double phenylalanine motif (F102FF103) before the N terminus loops back into the periplasmic space (Fig. 1c). This hairpin structure constricts the pore and represents a closed conformation, which would not likely allow passage of molecules larger than water through the pore. An evolutionarily conserved salt-bridge interaction between E98 and K401 pins the plug to the internal wall of the barrel (Fig. 1c). The plug helix is also flanked on one side by a specific electrostatic interaction between E111 and R157 linking neighbouring strands within the barrel lumen (Fig. 1c). It is conceivable that a rearrangement of the three salt-bridge partners could facilitate movement of the helical plug and opening of the pore. Molecular simulations of the FapF<sub>83</sub> trimer in lipid bilayers (Fig. 1d) confirm that the plug is relatively rigid on a 100 ns timescale and no ion flux occurs (Supplementary Fig. 2). A substantial conformational change must therefore occur to open the channel before a polypeptide can be secreted, which is in stark contrast to secretion through the open CsgG barrel in which a single 9 Å ungated aperture is present (Supplementary Fig. 3).

Our crystal structure lacks the N-terminal region of FapF comprising the first 81 residues which would be predicted to lie within the periplasm. This region harbour a coiled-coil motif with a predicted preference for a parallel trimer and, consistent with the trimeric architecture of the FapF transmembrane domain observed in our crystal structure. To explore the multimeric nature of FapF further we performed native mass spectrometry of the truncated FapF<sub>83</sub> and full-length FapF (Fig. 2a). FapF<sub>83</sub> (FapF<sub>83–406</sub>) used for crystallography was observed to exist in a monomer/dimer/trimer equilibrium at \(1:2:1\) ratio in
C8E4 detergent solution (Fig. 2a). In contrast, for the full-length species (FapF1-406), the mass spectrum showed that the most abundant species was trimeric with a small amount of monomer and no dimeric species, suggesting that native FapF is indeed a trimer.

We then reconstituted both FapF constructs into planar DPhPC bilayers. Full-length FapF inserted stably into the bilayer and exhibited a pronounced current–voltage asymmetry, giving a current of 74 ± 5 pA at +100 mV and −106 ± 4 pA (n = 21) in buffer containing 0.1 mM KCl (Fig. 2b). The conductance of the full-length FapF trimer recorded in 1 M KCl was 4.95 ± 0.03 nS (Supplementary Fig. 4). All recorded channels exhibited a higher conductance at negative voltages, implying unidirectional insertion. FapFβ, which contains a plugged barrel in the crystal structure, required the presence of 4 M urea in the recording chamber in order to obtain insertion of stable channels in an open state for conductance measurements. Urea-induced channel opening in planar lipid bilayer current recordings has been previously reported for plugged OM TonB-dependent transporters25, and it is assumed that the urea unfolds the plug, opening an ion-conducting pathway through the barrel.

After the urea in the recording chamber was exchanged with fresh buffer, the conductance measured for FapFβ did not significantly differ from the full-length trimer: 73 ± 2 pA at +100 mV and −103 ± 4 pA at −100 mV in 0.1 M KCl (n = 10) (Fig. 2b). These observations suggest the plug is labile in the full-length FapF, which exists predominantly in an open state. The fact that we were unable to observe channel activity for

**Fig. 1** Overall architecture of the FapFβ transporter domain. a Sequence of FapF showing approximate domain boundaries and the construct used for structure solution which consisted of the OmpA signal sequence (SS) and an N-terminal His-Tag (6xH) followed by residues K83-F406 of FapF. b The crystal structure of FapFβ. View from the periplasm through the plane of the membrane and perpendicular to the plane. The lines of three closely packed phenylalanine residues are shown in green space-filling representation, and the small helix plug is shown in blue cartoon representation. Bound detergent molecules in the pore are shown as magenta ball-and-stick representation. The ‘PTG’ motifs (residues P233–G235 and P259–G261) and loop between are shown in red. c Closer view of the constriction site at the top of the helical plug. Conserved residues are shown in stick representation. The pore profile is shown as a surface where blue corresponds to bulk water (radius > 2.3 Å), green is single-file water (1.15–2.3 Å) and red is closed (< 1.15 Å). d The equilibrated position of FapF domain within a coarse-grained asymmetric LPS-containing lipid bilayer.
FapFβ in the absence of urea indicates a highly stable plugged conformation, and may suggest that the crystal structure corresponds to a final step in secretion in which the channels are stably closed. The removal of urea did not lead to a decrease in FapFβ conductance, which implies that the helical plug does not refold into the channel lumen. Irreversible plug denaturation was previously observed for the OM vitamin B12 receptor BtuB\(^{25}\). At applied potentials exceeding ±200 mV, both the full-length and FapFβ constructs exhibited voltage-induced closure in three steps (Fig. 2c), which has been previously observed in trimeric OM porins\(^{26}\). This further suggests that native FapF exists in the bilayer as a trimer. In further support of the FapF trimer being the native form, all attempts to generate a monomer by mutation of the trimer interface in order to generate a monomer, notably by disruption of the phenylalanine ladder (F325, F335 and F367) (Supplementary Fig. 5), prevented FapFβ from being isolated from the OM.

To investigate the structure of the putative coiled-coil region we performed circular dichroism (CD) spectroscopy. CD spectra were collected of both full-length FapF and a synthetic peptide D3–Q40, which corresponds to the predicted coiled-coil region (Fig. 3a). These data indicated a high helical content (64%) for the coiled-coil peptide alone (Fig. 3b) that was stable up to 80 °C. Furthermore, full-length FapF showed an increased helical content than would be expected for the FapFβ domain alone (Supplementary Fig. 6), consistent with the formation of the helical coiled-coil region. The oligomerisation state of the isolated coiled-coil region peptide was determined by size exclusion chromatography using a Superdex 200 HR 10/30 column coupled to multiple laser light scattering (SEC-MALS). The molecular mass of the D3–Q40 peptide (theoretical MW=4.5 kDa) in solution was determined to be 13.5 kDa±5% (Fig. 3c) which, together with the CD data, confirm a coiled-coil trimeric state of the N-terminal domain of FapFβ. The high stability of the N-terminal coiled-coil region would likely trimersise and localise within the periplasm, as predicted from our crystal structure of FapFβ. To provide further evidence we first immunoblotted for the N-terminal His-tag before and after cell lysis (Supplementary Fig. 7a). Only after lysis was the tag accessible to antibody detection. We also created an N-terminal FapF fusion to the globular β-lactamase and probed for ampicillin sensitivity, which has use to infer correct folding of fusion and periplasmic localisation\(^{27,28}\). Ampicillin resistance was observed for \textit{E. coli} cells, which expressed the FapF fusion, but not in those that produced the wild-type protein only (Supplementary Fig. 7b). The most likely explanation is that the N-terminal β-lactamase domain of the FapF fusion successfully folds and is retained with the periplasm.

To estimate the overall dimensions of full-length FapF we generated a structural model for the trimeric transporter. The N-terminal coiled-coil region (D3–Q40) was first modelled as an archetypal parallel trimer. The lowest energy model was chosen and shown to be stable by independent simulations (Supplementary Fig. 8). We then combined the N-terminal coiled
measurements for puri
grey
of full-length FapF coloured corresponding to the structural boundaries shown in Fig. 1a. The theoretical CCS of this model is shown.
Error bars
FapF trimer populations.
ed estimated by the BeStSel method.
(D3 barrel (Fig. 3g), and could potentially span the entire depth of highly elongated and mobile relative to the transmembrane that N-terminal coiled-coil domain together with the linker is
Coarse-grained molecular dynamics simulations demonstrate
model, in which the coiled-coil domain is further from the barrel. It adopts a more extended conformation than suggested by our sparse30. We observed electron density in the crystal structures stabilisation within the membrane, but structural information is
peptidoglycan layer.

Fig. 3 Biophysical characterisation of the FapF N-terminal coiled coil. a Far-UVCD spectra for the full-length FapF (dashed line) and the coiled-coil peptide (D3-Q40) at 20 °C (thick solid line) and 80 °C (thin solid line). b Secondary structure composition for full-length FapF and the coiled-coil peptide (20 °C) as estimated by the BeStSel method. c SEC-MALS profile of the coiled-coil indicating a monodisperse sample corresponding to the trimer. d Atomic model of full-length FapF coloured corresponding to the structural boundaries shown in Fig. 1a. The theoretical CCS of this model is shown. e Ion-mobility measurements for purified full-length FapF. These indicate a population of four main substate populations. f Collision cross-sections of the four different FapF trimer populations. Error bars are derived from an average of three different wave heights. g Motion of the N-terminal domain of full-length FapF observed during coarse-grained simulations. The trajectory was aligned to the barrel and the position of the N-terminal domain at 10 ns intervals are shown (grey=0 ns, green=10 ns, blue=20 ns, purple=30 ns)

coil trimer with our trimeric structure of the FapFβββ-barrel and plug (K83–F406) by introducing a disordered linker (P41–L82) to produce a complete model of full-length FapF (Supplementary Fig. 9). Our model predicts a cross-section of 6,878 Å2 (Fig. 3d). This value was compared with data from collision cross-section measurements using ion mobility mass spectrometry. The ion mobility data revealed the presence of four distinct conformations (Fig. 3e). The predominant form has a cross-section of 6,863–7,035 Å2 in excellent agreement with our model (Fig. 3b, and Supplementary Table 2). The remaining three conformational states, corresponding to larger collision-cross-sections, indicated that the full-length species can adopt more extended forms (Fig. 3f). This could indicate that the flexible linker region adopts a more extended conformation than suggested by our model, in which the coiled-coil domain is further from the barrel. Coarse-grained molecular dynamics simulations demonstrate that N-terminal coiled-coil domain together with the linker is highly elongated and mobile relative to the transmembrane barrel (Fig. 3g), and could potentially span the entire depth of the periplasm29, interacting with and/or anchoring beneath the peptidoglycan layer.

Lipid interactions are known to play a major role in protein stabilisation within the membrane, but structural information is sparse30. We observed electron density in the crystal structures that can be attributed to carbon chain density; however, only the bound detergent molecules shown in Fig. 1c were conserved between all three protomers (Supplementary Fig. 10). We performed molecular simulations of FapFβββ in a simple palmitoyl-oleoyl phosphatidylglycerol/palmitoyloleoyl phosphatidylethanolamine (POPG/POPE) membrane to further probe the influence of lipid binding on the behaviour of FapFβββ. The presence of POPG and POPE was found to disrupt the hydrogen bonding between strands at the plait near the PTG motifs. This leads to a larger gap between strands β5 and β6 (Supplementary Fig. 11). Furthermore, simulations of FapF in an asymmetric lipopolysaccharide (LPS)-containing membrane revealed putative LPS lipid-binding sites located on the extracellular loops of FapF (Fig. 1d and Supplementary Fig. 12). The presence of LPS-binding sites has recently been shown to be vital for biogenesis of Gram-negative OM proteins31. The conserved PTGs are interesting structural motifs as one of these induces a marked twist in the major extracellular loop β5–β6. It is conceivable that this forms part of an exit gate or regulatory role for secretion.

Coiled-coil and plug domains are critical for FapC secretion. The large N-terminal coiled-coil domain assists full-length FapF in forming highly stable trimers. Single-channel current recordings suggest an open conformation of the full-length trimer compared to the FapFβββ trimer, suggesting that all three plugs are readily released in the full-length trimer. Further, the associated
A second band is also produced of slight lower molecular weight which is detectable by formic acid (+) or water (−). In the absence of FapF, only intracellular FapC could be detected. While the ΔfapF mutant operon could be successfully complemented with full-length FapF, the N-terminal truncation FapFΔ could not support secretion, although FapFΔ was able to stably insert into the OM of E. coli (Fig. 4). This highlights a role for the N-terminal periplasmic regions in regulating secretion through FapF, likely by the controlling plug conformation. It is noteworthy that in secretion assays, two bands are visible for formic acid-treated FapC amyloid (Fig. 4). This has not been observed previously in immunoblots of Pseudomonas FapC secretion and therefore likely reflects a stable degradation product due to the presence of E. coli proteases. Furthermore, only small amounts of intracellular FapC can be detected mutant expressing the ΔfapF operon, suggesting that it is degrading by periplasmic proteases in E. coli. This is also observed for FapF mutants that do not secrete amyloid, suggesting that FapC monomer degradation is rapid. Some secretion-competent mutants display increased intercellular FapC, which indicates that amyloid secretion is perhaps less efficient and allow a temporary build-up of FapC (Fig. 4).

We next set out to shed light on the structural and functional importance of the helical plug interactions by generating FapF mutants for complementation of the ΔfapF strain that we anticipated would interfere with the packing and/or the dynamics of the plug domain within the pore. Immunoblotting for the His-tag of the mutant proteins confirmed their expression (Supplementary Fig. 13). Mutation of the salt-bridge interaction between E98 and K401, which pins the plug to the barrel wall, abolishes FapC secretion (Fig. 4 and Table 1). Two further conserved charged residues that engage in an interstrand salt bridge within the barrel lumen (E111 and R157) were also shown to contribute to FapC secretion: while the E111A mutant displays secretion activity, the R157A is secretion deficient. Reversing the charge in this salt bridge, with the double mutant E111R/R157E, restores secretion to normal levels, indicating the presence of an arginine residue in this area of the plug instead of the native aspartic acid. This is also observed for FapF mutants that do not secrete amyloid, suggesting that FapC monomer degradation is rapid. Some secretion-competent mutants display increased intercellular FapC, which indicates that amyloid secretion is perhaps less efficient and allow a temporary build-up of FapC (Fig. 4).

We also mutated the conserved double-phenylalanine residues (F102/F103) that stabilise the position of the plug within the barrel (Fig. 4 and Table 1). Interestingly, F103A FapF was fully functional, secreting FapC in an amyloid-competent form, whereas mutating the neighbouring F102 to alanine could not rescue amyloid secretion in the ΔfapF background. The critical F102 is packed within residue side chains in the barrel wall, anchoring the helix within the pore and defining the conformation of the hairpin within the barrel. In contrast, the dispensable F103 makes cation-π contacts with the residue R157. Steered

### Table 1 FapC secretion activity of FapF mutants described in Fig. 4

| FapF mutant | FapC secretion |
|-------------|----------------|
| E98A        | –              |
| F102A       | –              |
| F103A       | +              |
| E111A       | +              |
| R157A       | –              |
| E111R/R157E | +              |
| K401A       | –              |

periplasmic domain may regulate the interaction of the helix plugs with the interior of the β-barrels.

It has previously been shown that the whole fap operon can be expressed in E. coli, thereby enabling the bacteria to form Fap-dependent biofilms. We adapted this system to test mutant operons for secretion. Secrated FapC is rapidly transformed into the amyloid state outside the cell, whereas intracellular FapC remains in a monomeric state. Secrated FapC can therefore be detected using FapC-specific antibodies and western

**Fig. 4** Heterologous FapC amyloid production in E. coli. a Position of residues assessed in this work within the crystal structure. b Shown are secretion assays for the ΔfapF operon complemented with full-length, wild-type FapF, ΔfapF operon complemented with FapFΔ and various point mutations of FapF within the operon itself. These were treated with either formic acid (+) or water (−). If FapC is secreted to form amyloid, it is detected by αFapC as a band at ~23 kDa after formic acid treatment. A second band is also produced of slight lower molecular weight which is detectable by formic acid (+) or water (−). Approximate MW markers of 50 and 25 kDa are shown as indicated. Full, uncropped gels are shown in Supplementary Fig. 13. Mutation of the salt-bridge interaction between E98 and K401, which pins the plug to the barrel wall, abolishes FapC secretion (Fig. 4 and Table 1). Two further conserved charged residues that engage in an interstrand salt bridge within the barrel lumen (E111 and R157) were also shown to contribute to FapC secretion: while the E111A mutant displays secretion activity, the R157A is secretion deficient. Reversing the charge in this salt bridge, with the double mutant E111R/R157E, restores secretion to normal levels, indicating the presence of an arginine residue in this area of the plug instead of the native aspartic acid. This is also observed for FapF mutants that do not secrete amyloid, suggesting that FapC monomer degradation is rapid. Some secretion-competent mutants display increased intercellular FapC, which indicates that amyloid secretion is perhaps less efficient and allow a temporary build-up of FapC (Fig. 4).

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molecular dynamics simulations of FapFβ were performed to ‘pull’ the helix plug from the pore (Supplementary Fig. 14). These indicated that the F102 is a key anchoring point for the helix plug domain within the barrel lumen. Furthermore, unbiased molecular simulations of the F102A/F103A double mutant showed that the whole plug is more mobile, and in particular a helix begins to move out from the barrel in one of the three subunits within the simulation timescale of 100 ns (Supplementary Fig. 15), leading to an opening of the channel. Both mutations were introduced into FapFβ and purified as for the wild type. Suitable yields for crystallisation were not obtained for FapF F102A; however, a crystal structure was determined for FapF F103A mutant in which the same overall plug placement was observed within the pore as seen in the wild type (Supplementary Fig. 16).

Intriguingly, although R157A crystallises under the same condition as the wild type, we found the symmetry of the trimer was disrupted. The structure was solved at 3.2 Å. Despite the more limited resolution, it is clear that the helix plug remains in position. However, in a single monomer of each trimer there is a conformational rearrangement of side chains due to the void generated from removal of the large R157 side chain. The side chain of F103 is observed to shift (Supplementary Fig. 17), and extra density is observed that may be attributed to a detergent molecule binding in this region. While detergent binding is not necessarily relevant for the in vivo activity of FapF, these data together with simulations imply that each pore of the trimer can function independently.

FapD is a cysteine protease necessary for Fap secretion. Sequence analysis of FapD revealed sequence homology with the C39 family of cysteine peptidases, which are usually present in Type 1 secretion systems (T1SSs) for the transport and

![Figure 5](https://example.com/figure5.png)

**Fig. 5** FapD is a periplasmic C39 protease required for FapC secretion. **a** Comparison of C39 domains in peptidase-containing ATP-binding cassette transporters. **b** FapD homology model generated by i-Tasser from PDB id 4ry2 (green). The active site His and Cys are conserved and shown in stick representation. **c** A C38A mutant was unable to secrete FapC fibres demonstrating FapD functions as an active peptidase. Coexpression of the FapD knockout operon with a plasmid encoding wild-type FapD with an OmpA signal sequence restores FapC secretion and confirms FapD is active in the periplasm. The 25 and 50 kDa MW markers are shown. **d** Mapping of peptides observed in whole-cell lysates of *Pseudomonas* sp. UK4 expressing the complete fap operon to the Fap protein sequences. Regions with matching peptides are shown as red boxes and the signal peptides, which are absent in the mature proteins, are shown as blue boxes. For additional details, see the Supplementary Data 2. Peptides are observed for FapA-D throughout the sequence indicating no processing. No peptides were observed in the mature N-terminal of FapE, although sequence analysis indicate the possibility for several theoretical peptides of proper size. This indicates a proteolytical processing of FapE. For FapF the disordered linker region residue 60-100 is potentially processed whilst the putative coiled-coil region remains intact.
maturation of bacteriocin precursors (Fig. 5a, b). These peptidase domains associate with the cytosolic faces of ATP-binding cassette of the ABC transporters and usually cleave substrate polypeptides at a GG motif (and sometimes GA) before they are secreted through the periplasm. Although the FapFs OM secretion pore is distinct from all previously described secretion systems that have been structurally characterised thus far, it displays individual features found in a variety of bacterial importer and exporter families. The closest related structure in the Protein Data Bank is that of the COG4313 channel, Pput2725, which is an importer that belongs to the meta-degradation pathway of phenol. Despite the opposite directionality of transport, the first ‘PTG’ motif identified in EstA is also present in Pput2725 (Supplementary Fig. 19), and

**Discussion**

Although the FapFs OM secretion pore is distinct from all previously described secretion systems that have been structurally characterised thus far, it displays individual features found in a variety of bacterial importer and exporter families. The closest related structure in the Protein Data Bank is that of the COG4313 channel, Pput2725, which is an importer that belongs to the meta-degradation pathway of phenol. Despite the opposite directionality of transport, the first ‘PTG’ motif identified in EstA is also present in Pput2725 (Supplementary Fig. 19), and the target of FapD remains unclear and is the subject of further work. Although Fap proteins do not possess canonical C39 sequences (i.e., lv-x-GG-kag-y-ga), the presence of GG and GA motifs in FapE suggests that this gene product could be an alternative recognition target of FapD. Mutation of the active site cysteine in FapD (C38A) was sufficient to abolish FapC secretion, indicating that FapD is proteolytically active (Fig. 5c).

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The target of FapD remains unclear and is the subject of further work. Although Fap proteins do not possess canonical C39 sequences (i.e., lv-x-GG-kag-y-ga), the presence of GG and GA motifs in FapE suggests that this gene product could be an alternative recognition target of FapD. Mutation of the active site cysteine in FapD (C38A) was sufficient to abolish FapC secretion, indicating that FapD is proteolytically active (Fig. 5c).
this region has been proposed to be a lateral gate that would allow the uptake of hydrophobic substrates via the membrane. While the presence of a ‘PTG’ motif in FapF suggest a putative lateral gate, mutagenesis of the PTG demonstrates that these are not essential for amyloid export (Fig. 4) but could provide a recognition platform for attachment.

Perhaps the most striking similarity is between the TDs of the FapF protomer and the classical Type Va autotransporter (AT) family27, which are also characterised by a C-terminal, 12-stranded β-barrel with a helix-blocked pore in the closed state. Type Va ATs also carry a passenger domain fused to the N terminus of the β-barrel, which is released extracellularly after translocation by an autocataytic cleavage event38, 39. Several structures have been solved for Type Va ATs and perhaps the most the revealing comparison can be made between FapF and the pre- and post-cleavage states of EspP (Fig. 7)39. The β-barrels of these EspP structures overlay with a root-mean-square deviation of 3.9 Å over 191 backbone atoms of FapF. However, in the truncated FapF structure, the N terminus exits the barrel on the periplasmic side rather than passing completely through the pore as in ATs. Although unlikely considering the FapF periplasmic domain is a trimeric coiled-coil, we assessed whether the N terminus would pass completely through the β-barrel, by probing the cell surface exposure of the His-tagged N terminus of full-length FapF. Western blot analysis revealed that the His-tag is accessible to antibody only after cell lysis, confirming that the N terminus lies within the periplasm (Supplementary Fig. 7). Additionally, we produced a fusion protein of FapF with an N-terminally fused β-lactamase domain between the signal sequence and coiled-coil domain. The ampicillin sensitivity of cell growth was then used to probe both correct folding of the FapF fusion and the periplasmic location of the N terminus. Expression of the fusion protein conferred ampicillin resistance indicating that a functional, N-terminal β-lactamase domain was present in the periplasm (Supplementary Fig. 7).

It is interesting to note that our single-channel current measurements show that FapF5 exists in a closed state, while full-length FapF is open, indicating that the plug domain in full-length FapF is predominantly located outside of the pore. This observation is also borne out when comparing conductance measurements with those measured of the autotransporters. The conductance for the single barrel NaLP was found to be 0.15 nS in 1 M KCl with the helix present within the pore, which increased to 1.8 nS when the helix was removed46. Our value of 4.95 nS for the trimer, i.e., 1.6 nS for a single full-length FapF subunit in 1 M KCl, is indicative of a largely open channel. It is therefore likely that the periplasmic coiled-coil and linker domains play a role in regulating pore accessibility and capturing Fap substrates. Analogous to this is the role played by the cytoplasmic, coiled-coil bundle in voltage-gated sodium channels. A conformational change within this neck domain, proximal to the pore, provide a mechanism for channel activation41, 42. Furthermore, coiled-coil domains have been shown to function as a platform for translocated substrates. Most notably in the sucrose OM uptake channel ScrY33 for which it has been proposed that the trimeric coiled-coil spans the periplasm interacts with incoming maltooligosaccharides and routes them from the OM to the translocation system at the inner membrane. The FapF-coiled-coil may specialise in recruiting Fap substrates to the OM β-barrel for secretion.

We tentatively propose the following mechanism of how the Fap secretion might take place (Fig. 6). FapE is found extracellularly and remains associated with the Fap fibre as a minor component19, 44. FapE along with FapB are essential for subsequent secretion of FapC on the bacterial surface, suggesting a role in initiating the process. MS evidence showing that the N terminus of FapE is degraded during secretion, while both FapB and FapC remain intact, may reflect the removal of a FapF secretion targeting sequence. Sequence analysis of FapE also reveals a possible domain boundary between two well-conserved N-terminal and C-terminal regions, with a disordered linker region. The C-terminal portion of FapE possesses asparagine- and glutamine-rich sequences, akin to the amyloid repeats identified in FapB and FapC (Supplementary Fig. 18). In this speculative model, FapE could therefore represent an important subunit for initiating Fap secretion. The catalytic action of the C39-like peptidase FapD, most likely on the FapE substrate, may facilitate activation of the FapF translocator complex for secretion. This model would explain observations that mutation of either FapD or FapE abolishes Fap secretion44. It is also plausible that FapD-promoted cleavage of FapE occurs at the transmembrane–periplasmic domain junction which also bears GG motifs (Supplementary Fig. 20) and generates the closed state observed in our crystal structure. This could represent the final stage of the secretion process after subunits have been deployed from the periplasm and reassociate the OM barrier.

A prominent driving force for protein secretion across the bacterial OM (e.g., for AT passenger domains) is substrate folding on the extracellular side45. Furthermore, it has been shown that intrinsically disordered domains can be efficiently secreted despite the lack of folding as a possible driving force46, 47. The argument follows that electrostatic interactions with the periplasmic face at the base of the pore drive initial threading of the substrate into the hydrophilic lumen. The constriction subsequently allows for entropically favoured secretion by extracellular escape. This concept is highlighted in the curli functional amyloid secretion system16, in which amyloidogenic CsgA subunits are kept unfolded and monomeric in the periplasm and then captured at the base of the CsgG pore by a capping complex with CsgE and conformationally constrained. The CsgA subunit subsequently threads and is translocated through the pore driven by an entropic expansion into the extracellular milieu and lubricated by the hydrated, charged pore lumen. Although comparison to the curli translocator CsgG shows that FapF is structurally distinct (Supplementary Fig. 3), despite secreting similar substrates, a prominent role for electrostatic interactions and spontaneous conformational changes of a disordered substrate allowing progress down an entropy-driven free energy gradient may be a shared concept48, 47. This is supported by the recent observation that the curli inhibitor CsgC is also a potent inhibitor of FapC amyloidogenesis and that CsgC charge mutants with diminished capacity to inhibit CsgA folding and assembly into amyloid are equally ineffective against FapC48. Fap substrates could be recruited by the elongated periplasmic domains of the FapF trimer (similar to CsgE-mediated capture of CsgA), which may provide the necessary conformational restriction that facilitates threading and translocation of the disordered substrates through FapF.

The Pseudomonas Fap biogenesis system shares key features with several classified bacterial secretions systems, namely Type I in the deployment of a C39 peptidase, Type Va in the use of a 12-stranded AT-like barrel for translocation and Curli (or Type VIII) which export similar amyloidogenic substrates. Unique features not seen in OM peptide transporters include the trimeric nature of the AT-like barrel and a prominent role in regulating secretion for a periplasmic coiled-coil. The Pseudomonas Fap secretion system can therefore be classified as a new hybrid secretion machinery (Fig. 6). In summary, our structural and functional characterisation of the Fap system provides new insight into the molecular mechanism underlying the secretion of disordered amyloidogenic substrates at the OM.
Methods
Limited proteolysis. As described previously21, to improve the properties of pFapF for structural studies limited proteolysis was used to identify a stable fragment of the protein. Chymotrypsin, trypsin and subtilisin were used and the results indicated that stable fragments of pFapF could be produced of around 35 and 31 kDa. Bioinformatics predictions from DISOPRED6 and PSIPRED7 indicated that the N terminus has a disordered N-terminal domain of ~80 amino acids. Based on these predictions, constructs were designed with N-terminal truncations to remove the disordered region and produce a more structured protein for study. Two of these constructs were designed based on the predicted chymotrypsin and trypsin cleavage sites corresponding to the stable fragments produced by limited proteolysis and the other was based on bioinformatics predictions of the β-stranded topography. The corresponding region was then cloned into a series of homologues Pseudomonas P47, Pseudomonas UK4 and Burkholderia, of which Pseudomonas UK4 produced the most promising sample.

Cloning and expression. Pseudomonas UK4 FapFp, L273M and mutants, and full-length pFapF were extracted and purified as described previously22. Briefly, the N-terminal FapF (residues 83 to 406, FapFp) from Pseudomonas strain UK4 was cloned into a pRSF-1b vector with an OmpA leader signal sequence and an N-terminal His tag: MGKTAAJAVIALAGFTVQATSHHHHHHHHHHHHHHHH. This was transformed into LEMO21 cells (New England Biolabs), grown to OD600 of 0.6–0.8 at 37 °C in an aerobic bioreactor before overnight induction at 25 °C. To produce a plasmid containing a FapF β-lactamase domain (pFapF-βl), the full-length pFapF construct was modified by substitution to replace the HHHHHH with the β-lactamase domain from E. coli. A PET4Δ vector containing a constitutively expressed β-lactamase was used as a positive control for ampicillin resistance. Primers used are detailed in Supplementary Data 1.

Protein purification and crystallisation. Crystals were prepared as described previously22. Briefly, cells were harvested and resuspended in 20 mM Tris-HCl pH 8, 1 mM NaCl, 10% glycerol and phenylmethane sulfonyl fluoride, followed by lysis by cell disruption (Constant Systems) at a pressure of 25 kpsi and was prepared by centrifugation of the supernatant at 100,000 × g for 30 min, then a second spin at 100,000 × g for 2 h followed by resuspension of the pellet into 20 mM Tris-HCl pH 8, 200 mM NaCl and 1% N,N-dimethyldodecylamine N-oxide (LDAO; Sigma). pFapF was then purified from the OM fraction by nickel chromatography and detergent exchanged to 0.5% OEG40 and 100 mM NaCl. Samples were then analysed by SDS-PAGE and evaluated for purity.

Ramachandran statistics of main-chain dihedral angles (favoured/allowed/outlier (%) are wild-type pFapF 97.1/2.5/0.5, F102A 93.5/4.6/1.8, R157A 97.6/2.5/0.5. FapF secretion assays. The fap operon from UK4 was previously cloned and ligated into PMMB190Ap for heterologous expression in E. coli23. Mutations were introduced using Q5 site-directed mutagenesis (NEB) either into the operon directly, or into the full-length FapF construct in pRSF-1b for complementation assays. These were verified by sequencing and then maintained in E. coli DH5α. Plasmids were transformed into E. coli BL21, and plated in overnight culture on LB plates supplemented with 100 μg ml−1 ampicillin maintenance of PMBM190Ap or 100 μg ml−1 ampicillin and 50 μg ml−1 kanamycin for maintenance of PMBM190Ap and a complementing pRSF-1b construct. Agar was also supplemented with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) to induce expression of the Fap components for 48 h at 20 °C. Cells were harvested from plates grown in LB and resuspended in sterile 0.1 M Tris pH 8 before being frozen in liquid nitrogen. Two different samples were then thawed at 37 °C and resuspended in 10 mg ml−1 and 5 mg ml−1 E. coli DH5α. A pET46 vector containing an N-terminal His tag was used as a positive control for ampicillin resistance. Primers used are detailed in Supplementary Data 1.

Native mass spectrometry. The protein mixture was buffer exchanged to 0.5 M ammonium acetate pH 8.5 containing 0.5 M urea (7 M guanidine HCl) and dried down in a SpeedVac. The protein sample was then dissolved in Tris-buffered saline buffer containing 8 M urea, using a volume normalised according to the recorded OD280 of the original sample. Samples were then analysed by western blotting using an anti-His antibody.

N terminus accessibility assay. Cells were grown to an OD600 of 0.6 and induced using 1 M IPTG for 2 h at 37 °C. To produce an intact cell sample cells were diluted to OD600 of 0.2 in 2% paraformaldehyde in phosphate-buffered saline (PBS). To produce a lysed sample cells were resuspended in 10% sodium dodecyl sulphate in PBS and boiled for 5 min. Each sample was spotted onto a nitrocellulose membrane and analysed by western blotting using anti-his horseradish peroxidase conjugate.

Ampicillin sensitivity assay. Cultures of E. coli BL21 (DE3) harbouring pFapF, pFapF-βl or pET46 with pFapF were grown to an OD600 of 0.2 and induced overnight at 21 °C. Serial dilutions of each overnight culture were made in sterile LB media as indicated. Then, 5 μl of each dilution was spotted on both an LB Kan and an LB Kan/Amp plate and incubated overnight at 37 °C.

Native mass spectrometry. The protein mixture was buffer exchanged to 0.5 M ammonium acetate pH 8.5 containing 0.5 M urea (7 M guanidine HCl) and dried down in a SpeedVac. The protein sample was then dissolved in Tris-buffered saline buffer containing 8 M urea, using a volume normalised according to the recorded OD280 of the original sample. Samples were then analysed by western blotting using an anti-His horseradish peroxidase conjugate.

Single-channel recordings in planar lipid bilayers. Single-channel current recordings were carried out in 0.1 M KCl, 20 mM potassium phosphate pH 7.0. A solution of 1.2-diphtanoyl-sn-glycerol-3-phosphocholine (DPhPC) (Avanti Polar Lipids, Alabaster, AL, USA) dissolved in pentane (2.5 mg ml−1) was used to form a bilayer by the Montal–Muehler solvent-free method across a 100 μm diameter aperture in a 25 μm thick Tetlon film (Goodfellow)26. The film separated two 0.5 ml compartments designated ‘cis’ and ‘trans’—the cis compartment was connected to the ground and voltage, positive or negative, was applied to the trans compartment. A stock solution of FapF (0.5–1 μl of 2 mg ml−1) in 20 mM Tris-HCl pH 8.0, 150 mM NaCl and 0.1% (v/v) LDAO (Sigma) was added to the cis compartment and a potential of −200–200 mV was applied to induce insertion. Current flow was detected with two Ag/AgCl electrodes in 3 M KCl, 1.5% agarose bridges and ampliﬁed by a patch-clamp amplifier (Axopatch 200B, Axon Instruments, Foster City, CA, USA) connected to an Axon Instruments CV 200B headstage. Data were filtered with a 2 kHz low-pass Bessel filter and digitised with a Digidata 1322 A converter (Axon Instruments) at a sampling frequency of 10 kHz. Data analysis was performed with pClamp 10.3 software (Molecular Devices). All measurements were done at room temperature (20 ± 0.5 °C).

Molecular dynamics system setup. Simulations were performed using the GROMACS v4.6.3 (www.gromacs.org) simulation package27. The protein structure was converted to a coarse-grained (CG) representation using the MARTINI 2.2 force field28. The energy-minimised CG structure was centred in a simulation box with dimensions 100 × 100 × 100 Å3. A total of 250 POPE and 50 POPG lipids were
randomly placed around the protein and the system solvated and neutralised to a concentration of 0.15 M NaCl. An initial 1 ps of molecular dynamics simulation was applied in which a POPC/POPG lipid bilayer is spontaneously assembled around FapF to give an optimised position of the protein within the membrane. The endpoint of the CG bilayer self-assembly simulation was converted back to atomic detail using a fragment-based protocol for the lipid conformations, while retaining the original crystal structure of the protein. Equalisation of the atomic system was achieved through 5 ns of NPT simulation with the protein coordinates restrained, before the system was subjected to 100 ns of unrestrained atomistic molecular dynamics. For the simulations with LPS a preformed membrane was used and FapF incorporated using g_membed by aligning the phosphate groups of the model PE/PG membranes and the assembled LPS membrane. LPS simulations used additional 0.15 M Ca$^{2+}$ ions.

Coarse-grained simulations. The standard MARTINI force field and its extension to proteins was used to describe all system components. During the coarse-grained self-assembly simulation an elastic network was applied to the protein using force constant of 1,000 kJ mol nm$^{-2}$ and a cutoff of 1.0 nm. Temperature was maintained at 310 K using a Berendsen thermostat with a coupling constant of $\tau$ = 1 ps, and pressure was controlled at 1 bar using a Berendsen barostat with a coupling constant of $\tau$ = 1 ps and a compressibility of 5 x 10$^{-6}$ bar$^{-1}$. Electrostatics and van der Waals interactions in the CG simulations were shifted between 0.12 mm and 0.9 and 1.2 mm, respectively, using the standard MARTINI protocol. An integration time step of 2 fs was applied. Covalent bonds were constrained to their equilibrium values using the LINCS algorithm. All simulations were run in the presence of standard MARTINI water particles, and ions added to an approximate concentration of 0.15 M NaCl. CG LPS parameters were kindly provided by Syma Khalid (Southampton).

Atomistic simulations. Atomistic simulations were run using the GROMOS53a6 force field, and its extension to glycans. The system was solvated using the SPC water model, and ions added to give a neutral system with a NaCl concentration of 0.15 M. Systems contained ~140,000 atoms including 250 POPE molecules, 50 POPG molecules, 200 POPG bilayer, ~30,000 water molecules, and 1,250 sodium ions and 1,250 potassium ions. Periodic boundary conditions were applied, with a simulation time step of 2 fs. Temperature was maintained at 310 K using a V-rescale thermostat with a coupling constant of 0.1 ps, while pressure was controlled at 1 bar through coupling to a Parrinello–Rahman barostat with a coupling constant of 1 ps. Particle Mesh Ewald was used for long-range electrostatics and the LINCS algorithm was used to constrain covalent bond lengths. Steered molecular simulations were performed as described elsewhere, briefly a harmonic restraint with force constant of 500 kJ was applied to the centre of mass of the 13-residue helix plug with the lipid bilayer as the reference group. A pull rate of 0.5 mm s$^{-1}$ was used. Standard GROMACS tools were applied to analyse the simulation trajectories with VMD and PyMOL (The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC) used for visualisation. HOLE was used to generate pore profile surfaces.

Protein modelling. The full-length protein was modelled by generating an idealised trimeric coiled-coil. This was done using the converged results from the initial coarse-grained self-assembly simulation an elastic network was applied to the protein using force constant of 1,000 kJ mol nm$^{-2}$ and a cutoff of 1.0 nm. Temperature was maintained at 310 K using a Berendsen thermostat with a coupling constant of $\tau$ = 1 ps, and pressure was controlled at 1 bar using a Berendsen barostat with a coupling constant of $\tau$ = 1 ps and a compressibility of 5 x 10$^{-6}$ bar$^{-1}$. Electrostatics and van der Waals interactions in the CG simulations were shifted between 0.12 mm and 0.9 and 1.2 mm, respectively, using the standard MARTINI protocol. An integration time step of 2 fs was applied. Covalent bonds were constrained to their equilibrium values using the LINCS algorithm. All simulations were run in the presence of standard MARTINI water particles, and ions added to an approximate concentration of 0.15 M NaCl. CG LPS parameters were kindly provided by Syma Khalid (Southampton).

Circular dichroism. The coiled-coil peptide corresponding to residues D3 to Q40 was expressed in units of 10$^{-3}$ (deg cm$^2$ dmol$^{-1}$). CD spectra were averaged, corrected for baseline contributions, and the net spectra were plotted. CD spectra were measured from 200 to 260 nm, with intervals of 0.5 and 1 nm bandwidth. Samples were loaded into a quartz cuvette 100-QS with a path length of 1 cm. CD spectra were measured with the online server BeStSel. Denaturation was monitored by CD ellipticity at wavelengths of 222 to 260 nm, and time per point was set to 2 s with 5 repeats. The temperature was increased by 1 °C per min with a tolerance of 0.2 °C starting at 20 °C and ranging to 90 °C.

Mass spectrometry fingerprinting. The peptide fingerprinting was performed by an in-gel tryptic digestion followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) approach with biological duplicates. For this, 50 µl of each bacterial culture was lyophilised, treated with 100 µl of concentrated formic acid and lyophilised again. The sample was then resuspended in 50 µl of reducing SDS–PAGE loading buffer containing 8 M of urea. Insoluble material was pelleted by centrifugation for 1 min at 22,000g and 15 µl of supernatant was loaded on a 4–20% SDS–PAGE gel (ExpressPlus, GeneScript). Electrophoresis was carried out at 50 mA for ~15 min and the gel was stained with Coomassie Brilliant Blue G250. The part of the gel that stained positive for proteins were excised into 5 fractions and subjected to in-gel digestion and MS analyses, as previously described. The nLC-MS/MS setup consisted of a 13-mm i.d.3000 nano-LC (Thermo Fisher) coupled to a Q Exactive mass spectrometer (Thermo Fisher). Gradient time for each fraction was 40 min on a C18 columns (PepmapRSLC, C18, 75 µm x 50 cm, 2 µm, Thermo Fisher Scientific), resulting in 200 min for each sample. Protein identification and quantification were performed using MaxQuant. Carbamidomethylation of cysteines was defined as fixed, oxidation of methionines as variable modification. Digestion specificity was set to unspecific (all possible peptides of length seven and more are considered).

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Data availability. The authors declare that all the data supporting the findings of this study are available within the paper and its Supplementary Information or are available from the corresponding author on request. Atomic coordinates and structure factors files have been deposited in the Protein Data Bank under accession codes (FapF TDB-S065; FapF TDB-F103A-S067 and FapF TDB R157A-S068).

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Author contributions
S.L.R., W.J.H., J.-L.B. and S.M. designed experiments. S.L.R., W.J.H., S.L., U.M. and W.E. designed constructs and produced protein for crystal trials. S.L.R., R.M.L.M. and S.H. determined the crystal structures. J.-L.B. performed complementation secretion assays. D.S.C. and C.V.R. performed native mass spectrometry and ion mobility experiments. S.A.I. and H.B. performed and analysed single-channel conductance recordings. S.L.R. performed molecular simulations and structure modelling. F.S. performed circular dichroism and SEC-MALS. M.D., F.-A.H., D.O. and P.H.N. performed mass spectrometry fingerprinting and analysis. S.L.R. and S.M. wrote the manuscript with input from all authors.

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