A bacterial secretosome for regulated envelope biogenesis and quality control?

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Abstract

The Gram-negative bacterial envelope is the first line of defence against environmental stress and antibiotics. Therefore, its biogenesis is of considerable fundamental interest, as well as a challenge to address the growing problem of antimicrobial resistance. All bacterial proteins are synthesised in the cytosol, so inner- and outer-membrane proteins, and periplasmic residents have to be transported to their final destinations via specialised protein machinery. The Sec translocon, a ubiquitous integral inner-membrane (IM) complex, is key to this process as the major gateway for protein transit from the cytosol to the cell envelope; this can be achieved during their translation, or afterwards. Proteins need to be directed into the inner-membrane (usually co-translational), otherwise SecA utilises ATP and the proton-motive-force (PMF) to drive proteins across the membrane post-translationally. These proteins are then picked up by chaperones for folding in the periplasm, or delivered to the \( \beta \)-barrel assembly machinery (BAM) for incorporation into the outer-membrane. The core hetero-trimeric SecYEG-complex forms the hub for an extensive network of interactions that regulate protein delivery and quality control. Here, we conduct a biochemical exploration of this ‘secretosome’ – a very large, versatile and interchangeable assembly with the Sec-translocon at its core; featuring interactions that facilitate secretion (SecDF), inner- and outer-membrane protein insertion (respectively, YidC and BAM), protein folding and quality control (e.g. PpiD, YfgM and FtsH). We propose the dynamic interplay amongst these, and other factors, act to ensure efficient envelope biogenesis, regulated to accommodate the requirements of cell elongation and division. We believe this organisation is critical for cell wall biogenesis and remodelling and thus its perturbation could be a means for the development of anti-microbials.

INTRODUCTION

The bacterial envelope is essential for survival against an extraordinary range of physical and chemical environmental stresses. This protection has enabled Gram-negative bacteria to invade all complex animal and plant hosts, and to occupy almost all areas of the planet, including even the most inhospitable of places. While the envelope provides a barrier against the defence mechanisms deployed against bacteria, it is also an area of weakness for targeting by antibiotics and synthetic drugs.

The Gram-negative envelope is composed of a periplasm, containing a matrix of polymeric peptidoglycan (PG), sandwiched between inner- and outer-membranes. These membranes and intervening space contain numerous proteins that need to be delivered, assembled and maintained at the right place (the inner- or outer-membrane, or periplasm), and time. Thus, the process is further complicated by the continuously changing state of the envelope, in response to changing environmental conditions and during cell division.

The classical view for this process involves the delivery of proteins to the inner-membrane – principally, the ubiquitous Sec machinery – for the passage of proteins across or into it. In the former case, secretion is usually achieved post-translationally by the cytosolic ATPase SecA in conjunction with the inner-membrane protein-channel complex SecYEG, facilitated by the ancillary

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Abbreviations: BAM, \( \beta \)-barrel assembly machinery; CL, cardiolipin; DDM, n-dodecyl-\( \beta \)-d-maltopyranoside; HRP, horseradish peroxidase; HTL, Holo-translocon; IM, inner-membrane; IMAC, immobilised-metal affinity chromatography; IP, immunoprecipitation; MW, molecular weight; OMP, outer-membrane protein; PG, peptidoglycan; PMF, proton-motive-force; SEC, size exclusion chromatography; TMT-MS, tandem mass tagging mass spectrometry.
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sub-complex SecDFyajC [1] and driven by the proton motive force (PMF) [2–4]. By contrast, inner-membrane protein insertion occurs co-translationally, also via SecYEG, together with the highly conserved YidC [5, 6].

The SecYEG core-translocon, SecDFyajC and YidC combine to form a larger assembly known as the holo-translocon (HTL), capable of engaging in specialised post- and co-translational translocation [7, 8], and providing protein substrates with alternative routes across or into the inner-membrane. Proteins destined for the periplasm, outer-membrane or beyond are recognised by the HTL and must travel through the centre of SecYEG, while α-helical membrane proteins slide through a lipid pool at the interface between SecYEG and YidC on their way to the bilayer of the inner-membrane [9].

Many of the proteins emerging into the periplasm are greeted by chaperones, such as SurA, Skp and DegP [10, 11], to facilitate folding, or degradation if they become mis-folded [12]. One class of such proteins are the outer-membrane proteins (OMPs) that primarily adopt a β-barrel trans-membrane domain. These are delivered across the periplasmic space to the β-barrel assembly machinery (BAM) complex in the outer membrane for insertion and folding [13, 14].

The idea of inner- and outer-membrane cross-talk for OMP transport has been proposed previously, involving SecYEG, SecA, SurA and BamA [15]. Later it was found that SecDF and YidC also participate (as part of the HTL) [16, 17]. We proposed this trans-envelope, and possibly transient, interaction between HTL and BAM provides a contiguous, unrestricted (e.g. by-passing SurA and BamA [15]. Later it was found that SecDF and YidC also participate (as part of the HTL) [16, 17]. We proposed this trans-envelope, and possibly transient, interaction between HTL and BAM provides a contiguous, unrestricted (e.g. by-passing the PG layer) pathway for OMPs directly from the inner- to the outer-membrane. Presumably, this co-operation enables the required passage of very large quantities of OMPs, while protecting the transiting unfolded polypeptide from proteolysis and aggregation, for high maturation efficiency. The assembly seems to be stabilised by cardiolipin (CL) [16], a phospholipid required for the conferment of PMF stimulated secretion of OMPs [18].

Considering the above, we sought to identify whether more proteins are directly involved in the interaction between the Sec and BAM complexes to facilitate envelope biogenesis, quality control and re-modelling during cell division. Such complexes may not have been distinguished before, e.g. by electron microscopy because the envelope is very crowded, or biochemically because of instability and plasticity. The experiments described below explore the existence of such a secretosome and some (but certainly not all) of its prospective constituents and clientele, as well as their roles.

**METHODS**

**Plasmids, strains and protein purification**

*E. coli* strains BL21 (DE3), C43 or ΔsecG (KN425 (W3110 M25 ΔsecG::kan)) (a gift from Professor Frank Duong) were used for all experiments. SecYEG and HTL were expressed and purified as described previously [7]. Briefly, SecYEG was expressed in *E. coli* C43 from pBAD (Amp<sup>r</sup>), solubilised from the membrane fraction with 1.5% (w/v) n-dodecyl-β-d-maltopyranoside (DDM, GLYCON Biochemicals GmbH) in 20 mM Tris pH 7.5, 130 mM NaCl, 10% glycerol (TSG) and purified using nickel-immobilised metal affinity chromatography (IMAC) followed by size exclusion chromatography (SEC) with an appended anion exchange column [19]. The HTL was expressed using the pACEMBL system (Amp<sup>r</sup>, Kan<sup>r</sup>, Cm<sup>r</sup>) and purified the same as for SecYEG except with 0.002% (w/v) cardiolipin supplemented into IMAC and SEC buffers.

**Production and solubilisation of membranes for co-immunoprecipitations and size exclusion chromatography**

Precultures of *E. coli* BL21 (DE3)/ BL21-C43 (DE3) [20]/ BL21-C43 ΔclsABC (DE3) [21], containing 100 ml 2xYT media were inoculated and grown overnight at 37 °C and 200 RPM. The following morning, 10 ml of pre-culture was used to inoculate 1 litre of 2xYT broth in a 2 litre flask and the culture was incubated in the same conditions as described above. When an OD<sub>600nm</sub> of 1.0 was achieved, cells were harvested by centrifugation (5000 g, 10 min, 4 °C) and resuspended in 20 ml of TSG. The sample was then passed through a cell disruptor (Constant Systems Ltd.) for lysis (two passes at 25 kPSI). Membranes were clarified from the sample by centrifugation (160000 g, 45 min, 4 °C), resuspended to 120 mg ml<sup>−1</sup> in TSG and stored at −80 °C for future use. Solubilisation was performed by the addition of DDM to 0.5% (w/v). After 1 h of gentle rocking at 4 °C, insoluble material was removed by centrifugation (160000 g, 45 min, 4 °C) and the soluble fraction taken for further analysis.

**Size exclusion chromatography**

A Superdex S200 10/300 GL size exclusion chromatography (SEC) column (Cytivia) was equilibrated in TSG buffer supplemented with 0.02% (w/v) DDM in the presence or absence of 0.002% (w/v) *E. coli* cardiolipin (Avanti Polar Lipids). With the flow rate set to 0.25 ml min<sup>−1</sup>, proteins solubilised from 60 mg of wet membrane pellet as described above were injected through a 0.5 ml loop. Then 8 ml after injection, fractions of 250 µl were collected for approximately 10 ml. For SecYEG, HTL and BAM controls, approximately 350 µg of purified protein was loaded using the same conditions as for the solubilised membranes. Protein standards were obtained from Merck.
Co-immunoprecipitations
For each reaction, 550 µl of solubilised membranes (66 mg of wet membrane pellet) were prepared as described above and 9 µl of purified monoclonal SecG antibody from our laboratory stocks was added. Samples were incubated overnight gently rocking at 4 °C. Then, 125 µl of Protein G Resin (Amintra) was prepared by washing 250 µl of the suspended resin in the manufacturer’s storage buffer in a microcentrifuge spin column three times with 500 µl of buffer containing 250 mM NaCl, 20 mM HEPES pH 8 (IP buffer) and centrifuge settings of 500 g for 1 min at 4 °C. After the final wash, the resin was resuspended in 500 µl of IP buffer supplemented with 2% bovine serum albumin and incubated overnight in the same conditions as the membranes.

The following morning, the resin was washed using the same procedure as described above, but resuspended in 250 µl of IP buffer on the final step. Then, 50 µl of material was removed from the solubilised membranes as a loading control and the resin and membranes were mixed and left to gently rock at 21 °C for 3 h. The resin was washed in a microcentrifuge spin column six times with 400 µl IP buffer containing 0.02% DDM in the presence or absence of 0.002% (w/v) E. coli cardiolipin. After the sixth spin, the resin was resuspended in 150 µl of IP buffer with DDM ± cardiolipin and 50 µl of the suspended resin removed for proteomic analysis. The remaining liquid in the spin column was once again removed by centrifugation and this time collected in a fresh tube for analysis by SDS-PAGE (last wash sample). Finally, bound proteins were removed from the resin by addition of 150 µl of 1 x LDS sample buffer followed by centrifugation, again collecting the sample in a fresh tube.

SDS-PAGE and immunoblotting
SEC samples were analysed by SDS-PAGE (NuPAGE 4–12%, Bis-Tris, 1.0 mm, Midi Protein Gel, 26-well) and transferred with a Power Blotter XL System (Invitrogen) onto 0.45 µm nitrocellulose blotting membrane (Cytivia Amersham Protran). Immunoblotting was performed by incubating with either purified mouse antibody (SecG, SecY, both diluted 1/10000, from our laboratory stocks) or rabbit antiserum (SecD from our laboratory stocks, or BamA, BamC and BamD, a gift from Harris Bernstein, or a single antiserum raised against both YigM/PpiD, a gift from Professor Daniel Daley, or PtsH, a gift from Professor Joen Luirink, all diluted 1/5000), followed by incubation with anti-rabbit or anti-mouse HRP-conjugated secondary antibody (Life Technologies). A homemade ECL kit was used for imaging. Images were acquired for 10 min with an Odyssey-Fc imaging system (LI-COR Biosciences) and densitometry performed with the Image Studio Light software (LI-COR Biosciences). Graphs were produced with the Prism eight software package.

Tandem mass tag quantitative proteomic analysis of SecG co-IPs
Immuno-isolated samples were reduced (10 mM TCEP, 55 °C for 1 h), alkylated (18.75 mM iodoacetamide, room temperature for 30 min) and then digested from the beads with trypsin (2.5 µg trypsin; 37 °C, overnight). The resulting peptides were then labelled with TMT eleven-plex reagents according to the manufacturer’s protocol (Thermo Fisher Scientific, Loughborough, LE11 5RG, UK) and the labelled samples pooled and desalted using a SepPak cartridge according to the manufacturer’s instructions (Waters, Milford, Massachusetts, USA). Eluate from the SepPak cartridge was evaporated to dryness and resuspended in 1% formic acid prior to analysis by nano-LC MSMS using an Ultimate 3000 nano-LC system in line with an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific).

In brief, peptides in 1% (v/v) formic acid were injected onto an Acclaim PepMap C18 nano-trap column (Thermo Scientific). After washing with 0.5% (v/v) acetonitrile 0.1% (vol/vol) formic acid peptides were resolved on a 250 mm×75 µm Acclaim PepMap C18 reverse phase analytical column (Thermo Scientific) over a 150 min organic gradient, using seven gradient segments (1–6% solvent B over 1 min, 6–15% B over 58 min, 15–32% B over 58 min, 32–40% B over 5 min, 40–90% B over 1 min, held at 90% B for 6 min and then reduced to 1% B over 1 min) with a flow rate of 300 nl min⁻¹. Solvent A was 0.1% formic acid and Solvent B was aqueous 80% acetonitrile in 0.1% formic acid. Peptides were ionized by nano-electrospray ionization at 2.0 kV using a stainless-steel emitter with an internal diameter of 30 µm (Thermo Scientific) and a capillary temperature of 275 °C.

All spectra were acquired using an Orbitrap Fusion Tribrid mass spectrometer controlled by Xcalibur 3.0 software (Thermo Scientific) and operated in data-dependent acquisition mode using an SPS-MS3 workflow. FTMS1 spectra were collected at a resolution of 120 000, with an automatic gain control (AGC) target of 200 000 and a max injection time of 50 ms. Precursors were filtered with an intensity threshold of 5000, according to charge state (to include charge states 2–7) and with monoisotopic peak determination set to peptide. Previously interrogated precursors were excluded using a dynamic window (60 s+/-10 ppm). The MS2 precursors were isolated with a quadrupole isolation window of 1.2 m/z. ITMS2 spectra were collected with an AGC target of 10 000, max injection time of 70 ms and CID collision energy of 35%.

For FTMS3 analysis, the Orbitrap was operated at 50 000 resolution with an AGC target of 50 000 and a max injection time of 105 ms. Precursors were fragmented by high energy collision dissociation (HCD) at a normalised collision energy of 60% to ensure maximal TMT reporter ion yield. Synchronous Precursor Selection (SPS) was enabled to include up to five MS2 fragment ions in the FTMS3 scan.
The raw data files were processed and quantified using Proteome Discoverer software v2.1 (Thermo Scientific) and searched against the UniProt *Escherichia coli* (strain B BL21-DE3) database (downloaded January 2020:4172 entries) using the SEQUEST HT algorithm. Peptide precursor mass tolerance was set at 10 ppm, and MS/MS tolerance was set at 0.6 Da. Search criteria included oxidation of methionine (+15.995 Da), acetylation of the protein N-terminus (+42.011 Da) and Methionine loss plus acetylation of the protein N-terminus (~89.03 Da) as variable modifications and carbamidomethylation of cysteine (+57.021 Da) and the addition of the TMT mass tag (+229.163 Da) to peptide N-termini and lysine as fixed modifications. Searches were performed with full tryptic digestion and a maximum of two missed cleavages were allowed. The reverse database search option was enabled and all data was filtered to satisfy false discovery rate (FDR) of 5%.

**RESULTS**

**Very large membrane protein complexes of the *E. coli* envelope stabilised by cardiolipin**

We began by analysing detergent extracts of total membranes of *E. coli* BL21 (DE3). Extracts were produced by solubilising membranes with the detergent dodecyl-maltoside (DDM) and subsequently fractionated by size exclusion chromatography. We know from previous findings that cardiolipin (CL) is required to stabilise the associated states of SecYEG-SecDFyajC-YidC (the holo-translocon) as well as the holo-translocon-BAM super-complex [22–24], so we reasoned that if even larger Sec-associated complexes existed in the envelope then they too would be stabilised by CL. Thus, size exclusion chromatography experiments were conducted with and without augmented CL in the column buffer (0.02% (w/v) DDM ±0.002% (w/v) CL). Interestingly, inclusion of CL during the chromatography did indeed enable the preservation of entities with very high apparent molecular weights of approximately 460–1700 kDa (calculated using calibration standards shown in Fig. S1, top panel), represented by a peak at 8.9 ml (Fig. 1a, light blue trace). In the absence of supplemented CL this peak was diminished, while the second peak at 10.2 ml, corresponding to lower molecular weight proteins and complexes (170–460 kDa), was enhanced (Fig. 1a, dark blue trace). These peaks contain a milieu of *E. coli* membrane proteins, with one population on average bigger than the other. Evidently, some of those in the larger population convert to a smaller size when CL is removed during chromatography, most likely due to complex destabilisation/dissociation, whilst being preserved by CL augmentation.

As noted, this CL-dependent complexation/stabilisation is a feature of the bacterial translocon, which we decided to explore further. The profile shown in Fig. 1a reflects the total protein extracted from the *E. coli* envelope, wherein the translocation components are only a tiny fraction. When the purified translocon complexes were applied to the same column their elution volumes were much larger (11.5–13.5 ml, corresponding to lower apparent molecular weights) than the main peaks of the total extract (Fig. 1a): purified SecYEG core-complex (Fig. 1a, magenta trace, peak at ~150 kDa, true molecular weight=74 kDa), HTL (Fig. 1a, red trace, peak at ~200 kDa, true molecular weight=249 kDa) and BAM (Fig. 1a, orange trace, peak at ~300 kDa, true molecular weight=209 kDa). The disparity between the apparent molecular weights of purified SecYEG, HTL and BAM and their true molecular weights is a common feature of membrane proteins due to the effect of shape, bound detergents and oligomerisation [19]. A further analysis was conducted in order to determine the elution volume (and relative size) of the native translocon containing complexes.

**A proportion of the envelope’s Sec translocons form part of a large assembly**

The total *E. coli* membrane extract liberates a major early eluting peak, suggesting a high proportion of the cell’s total membrane proteins are contained within an assortment of large membrane protein complexes, with many of them stabilised by CL. Given the Sec translocon is known to interact with many other proteins, we reasoned that the native assembly would be larger than the purified core- and holo-complexes, particularly in the presence of stabilising and complex promoting CL. Therefore, we took the gel-filtrated fractions and looked for the individual proteins by immunoblotting (Fig. 1b–j; Fig S1, available in the online version of this article-middle 3×3 panels). In these experiments nearly all of the endogenous CL would be rapidly removed from the membrane proteins, with one population on average bigger than the other. Evidently, some of those in the larger population convert to a smaller size when CL is removed during chromatography, most likely due to complex destabilisation/dissociation, whilst being preserved by CL augmentation.

In the absence supplementary CL, SecY and SecG of the core translocon and SecD of the HTL form similar elution profiles, mainly eluting between 10–12 ml corresponding to larger than expected molecular weights (approximately 130–370 kDa; Fig. 1b–d, blue traces vs red and magenta dashed lines). In the presence of CL (Fig. 1b–d, light blue trace), the elution profiles formed an additional shoulder between 9–10 ml (approximately 370–790 kDa), suggestive of stabilised even higher molecular mass complexes. Thus, in the presence of CL, there is an even greater disparity between the apparent molecular weights of the complexes contained in the crude and purified samples (Fig. 1b–d, light blue trace vs red and magenta dashed lines).

To verify that CL is indeed responsible for this effect, and exclude the possible interference of endogenous CL in the negative control, an experiment was conducted in its complete absence. A variant of BL21 (C43) [20], with all three biosynthetic genes (*clsA*, *clsB* and *clsC*) knocked out [21], thereby devoid of CL, was used for total membrane extraction. The extract was then analysed as above by gel filtration chromatography comparing the total absence of CL with the presence of an exogenous supplement (Fig. S1, lower left and middle panels). As can be seen in the absence of CL SecD migrates primarily in the lower molecular weight
fractions; while, as expected, the addition of CL induced a proportion to form part of a larger assembly. Therefore, CL does indeed stabilise a higher molecular weight assembly in detergent solution.

What other factors associate with SecYEG to account for the large disparity between the isolated translocons and the stabilised native version? The most obvious are the HTL components: SecDFyajC and YidC [7, 25], as shown above by co-elution of SecD with SecY and SecG (Fig. 1b–d). However, the elution volume of purified HTL is much larger than those of its constituents from the crude extract (Fig. 1b–d, red dotted line). Therefore, the assembled native constituents have a larger apparent molecular weight, suggesting that other factors are also associated.

Fig. 1. Size exclusion chromatography of E. coli total membranes. (a) Normalised A280nm traces of solubilised E. coli BL21 (DE3) total membranes purified on a S200 size exclusion column equilibrated in TSG with 0.02% DDM and with (+CL) or without (-CL) supplemented cardiolipin as indicated. Traces of purified SecYEG, BAM complex and HTL controls are also shown. Molecular mass standards were used to calibrate the column (Fig. S1) and their respective masses marked above the graph and indicated with dashed vertical lines. (b–j) Fractions from (a) were immunoblotted for proteins of interest (shown in the top left-hand corner of corresponding graphs). Blotting signal was quantified by densitometry and normalised to the maximum signal value. Both samples were analysed in triplicate (experimental repeats) and errors represent standard deviation. The peak of the elution profiles of the corresponding purified material from (a) are marked by magenta (SecYEG), red (HTL) and orange (BAM) dashed lines. Black dashed lines in (b–j) refer to elution peaks of molecular mass standards, with corresponding masses shown above the graphs.
Known Sec interactors form CL-stabilised high molecular weight complexes

Next, we analysed the size exclusion fractions for known interactors of the Sec-machinery. Further Western blots confirmed the presence of BAM (BamA, BamB and BamD) in the higher MW fractions at 9–10 ml (~370–790 kDa), which was also dependent on CL (Fig. 1e–g), consistent with our previous findings [16]. The periplasmic chaperones YfgM and PpiD have both been shown to associate with one another and with the lateral gate of SecYEG [26, 27]; both of them were detected at the higher molecular weight region of the chromatogram along with the HTL and BAM (Fig. 1h and i). Once again, this associated state was preserved by CL.

Finally, we looked for the inclusion of the AAA + ATP-dependent protease FtsH – a cytosolic inner-membrane anchored quality control factor required for the degradation of misfolded proteins [28, 29] and known to associate with SecY and YidC [30–33]. In this case there did not appear to be a CL dependence of its apparent molecular weight, but this could have been obscured by an earlier peak eluting close to the void volume at ~8.8 ml (Fig. 1j). This low volume peak may be due to instabilities and aggregation in the absence of supplemented CL. Indeed, when crude extracts were subject to immuno-precipitation with SecG antisera it was found that the recovery of FtsH, presumably associated with HTL et al., was enhanced with CL (Fig. S1, lower right panel).

The overlapping, CL-dependent elution profiles of the Sec components and its known interactors points towards the presence of large complexes in the E. coli membrane. The existence of a ‘secretosome’ is a very intriguing prospect (discussed previously [16]). Thus, we set out to understand more about its stability and additional interactors.

The secretosome is dependent on CL for stability

Constituents of the Sec and BAM translocons were also detected in fractions corresponding to lower MWs (Fig. 1), suggesting that the core-complex (SecYEG), the holo-translocon and the BAM complex also exist alone. This observation may also be in part due to the dissociation of the secretosome upon removal from the membranes and the extraction of specifically bound lipids – only partially ameliorated by the augmentation of CL. When the high molecular weight fractions (from between 10–11 ml of the +CL experiment) were pooled and reapplied to the size exclusion column, the elution profiles of SecG, SecD and BamA volume were highly dependent on CL (Fig. 2; Fig. S2). In the presence of CL the integrity of the higher MW complex containing the Sec and BAM complexes was maintained, while its omission resulted in a shift of all constituents towards the lower molecular weight regions of the chromatogram (higher volume). These experiments emphasise the importance of CL for maintenance/stabilisation of the higher organisation of these and other factors.

Additional ancillary factors of the secretosome

In order to identify additional interaction partners, we utilised immune-purification and tandem mass tagging mass spectrometry (TMT-MS). In these experiments we immuno-precipitated crude whole membrane extracts (± CL) with a monoclonal antibody raised against SecG – a non-essential constituent of the core-translocon SecYEG, and compared native E. coli membranes with those from a strain lacking SecG. Briefly, antibodies were incubated with the extracts which were then mixed with immobilised Protein-G resin. The resin was then stringently washed and the samples were prepared for TMT-MS. A ΔsecG strain (E. coli KN425 (W3110 M25 ΔsecG::kan) [34]), enabled us to control for non-specific effects of CL (Fig. 3a) and for non-specific binding to the antibody and resin (Fig. 3b–d). The three most significant interactors that were enriched even without CL were: YajC – a component of the SecDFyajC sub-complex; YfgM – known to interact with SecYEG [26, 27] (Fig. 1h) and YiaD – a suppressor of defective temperature sensitive bamD with an affinity for peptidoglycan (Fig. 3b). To the best of our knowledge, YiaD has not previously been identified as a Sec interactor.

The changes resulting from the addition of CL during the extraction were analysed by comparing results obtained from the ΔSecG and BL21 strains, both in the presence of CL (Fig. 3c), and of the BL21 strain in the presence and absence of CL (Fig. 3d).
As expected, supplementing with CL resulted in more proteins with an increased recovery. YajC and YiaD were shown to be more abundant, suggesting a CL dependent interaction with the secretosome. Inclusion of CL also resulted in the observation of enhanced quantities of SecY, likely due to the core translocon’s known dependence on CL for activity and stability.

Interestingly, HflC was detected, which is a modulator of FtsH (see also Fig. 1) and known interactor of YidC [33]. Two Sec substrates, OmpA and LptD, which are abundant β-barrel OMPs, were also identified. Their co-immunoprecipitation with SecG is likely due to their capture during transit through the secretosome; LptD is indeed prone to stalling in the BAM complex [35]. The noted CL enhanced recovery of both OmpA and LptD clients, along with the putative secretosome constituents YajC and YiaD (Fig. 3d), is suggestive of a CL-activated and stabilised complex. YajG was also identified when CL was retained in the extracts (Fig. 3c and d), which intriguingly is an uncharacterised lipoprotein, and product of the yajG-ampG operon, the latter of which is important for the regulation of PG recycling and remodelling [36–38].

There were a few other identified proteins that require explanation. The ribosome subunits are abundant in cells and are known to interact with SecYEG. Pectinesterase was the only hit in the negative control experiment and was ignored as a result. The

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**Fig. 3.** Tandem mass tag quantitative proteomic analysis of SecG co-IPs. Samples were prepared from SecG co-IPs of *E. coli* BL21 (DE3) and *E. coli* ΔSecG (W3110 M25 ΔsecG-kan) solubilised membranes in the presence or absence of supplemented cardiolipin. X axis units represent fold change of protein abundance between the two described experiments. Each sample was prepared in triplicate (experimental repeats). An arbitrary cut off is applied at log₂(fold change) = 1 and p-value=0.05, both indicated by dotted lines for clarity. Proteins with significant abundance changes (p-value <0.05 and log₂(fold change) > 1) are annotated on graphs.
appearance of subunits of the ATP synthase is a curiosity, as a complex with SecYEG was previously identified in mixtures of membrane protein assemblies ejected from native E. coli membranes [39].

Inexplicable hits in proteomic-MS are commonplace. Some may have been identified due to being trapped in the secretosome. Alternatively, others may have arisen due to the different background protein levels between experimental (BL21) and control strains (W3110 ΔsecG); for the latter a BL21 version was not available. False negatives are also a feature as some membrane proteins resist proteolysis and/or flight for MS. Moreover, extra caution was taken to ensure the protein G resin was stringently washed, and as a result proteins such as the BAM complex were detected, but not abundant enough to be accurately quantified.

YfgM, PpiD and BAM interact with the HTL

The TMT-MS did not identify an anticipated enrichment of SecDF, BAM or PpiD. So, to verify their membership of the secretosome the SecG co-immunoprecipitants from the extracted native membranes were subjected to Western blotting (Fig. 4a and b). SecD was detected and further enriched by the inclusion of CL, most likely due to HTL stabilisation (Fig. 4a, middle). The same CL enrichment was observed for BamA (Fig. 4a, right), supporting previous results that the BAM complex interacts with the translocon (HTL, but not SecYEG alone [7, 16]). The specificity of these interactions can be demonstrated through the analysis of membranes prepared from the ΔsecG strain, from which SecD and BamA were not co-immunoprecipitated (Fig. 4a, top). Similar experiments also recovered YfgM (highlighted also by TMT-MS; Fig. 3, c) and PpiD (Fig. 4b). In this case, the recovery was marginally increased in the presence of CL (Fig. 4c).

DISCUSSION

The experiments presented here all point to the existence of a very large assembly spanning the entirety of the Gram-negative cell envelope. The basic structures and function of this super-complex have already been discussed [15–17]. Beyond that we identify the involvement of additional components and speculate about the engagement of other factors for the formation of a ‘secretosome’: a dynamic and versatile hub for envelope biogenesis, quality control and remodelling (Fig. 5).

In this scenario, the basic activities of the core translocon SecYEG (Fig. 5a: 1) – protein secretion (Fig. 5a: 2) and membrane protein insertion (Fig. 5a: 3) – are streamlined, adapted or expanded by the association of various factors as required. The assembly of SecYEG with the ancillary components SecDFyajC and YidC forms the HTL (Fig. 5a: 4), which in all likelihood improves the efficiency of both core activities: YidC for membrane insertion (Fig. 5a: 5) and SecDF for secretion (Fig. 5a: 7) [5, 7, 40, 41]. Interestingly, a dynamic lipid pool at the interface between SecYEG and SecDFyajC-YidC is perfectly situated to facilitate trans-membrane helix insertion [9].

Chaperones also participate in the late stages of secretion, presumably in order to facilitate successful emergence and onward passage from the Sec-translocon, as well as folding and, where necessary, degradation. PpiD and YfgM, attached to the outer surface of the inner-membrane, and those of the periplasm, like Skp and SurA, presumably help facilitate the progression of proteins through and out of the secretosome (Fig. 5a: 8). While more work is needed to unravel the complex and dynamic interplay of the different chaperones at the secretosome, the variety of chaperones presumably have related, but subtly different activities. Indeed, SurA and Skp are at least partially redundant [10]. Perhaps the individuals, or various combinations of them,
are required for different outcomes, such as delivery of β-barrelled OMPs to the BAM complex at the outer-membrane (Fig. 5a: 9), or early exiting of globular proteins into the periplasm. Translocating proteins inevitably on occasion misfold and/or become trapped within the translocon [4, 42]. This could irreversibly block the secretosome and potentially increase the conductance of small ions, compromising the energy conserving function of the inner-membrane. If left unresolved this, and the loss of functional export sites, would prove catastrophic. Therefore, the conformational state of such a trapped complex might be recognised by factors, such as the chaperones mentioned above, whose recruitment could facilitate the clearance of denatured protein to nearby or associated proteases on either side of the membrane (e.g. DegP of the periplasm, or FtsH on the cytoplasmic face; Fig. 5a: 6). In this respect the confirmed presence of the AAA+ ATP-dependent protease FtsH in the higher molecular weight translocon assembly is interesting. Perhaps the secretosome can even be programmed for retro-translocation of trapped substrates back through the holo-translocon for cytosolic degradation by FtsH, associated at the cytosolic side of the protein-channel of SecYEG. Similarly, problematic blockages in the secretosome could be resolved by long-range conformational changes back to FtsH, for proteolytic degradation of SecY – self-destruction of the defunct assembly. FtsH has indeed been shown previously to act in this way [32].

The action of CL on the activation of SecA and SecYEG [18, 22], the stabilisation of the holo-translocon [7], the holo-translocon-BAM assembly [16] and the secretosome (shown here) is interesting because the lipid is known to localise in microdomains [43–46]. Thus, these CL-enriched islands could be sites for localisation of the translocon, by virtue of its affinity for this lipid over any other. These localised hubs could then recruit specific factors of the inner- and outer-membranes to form a bespoke secretosome. This idea resonates with a recently emerging concept of trans-envelope cross-talk for organisation and turnover of the residents of the inner- and outer-membranes [47, 48]. From the point of view of this analogy, it is striking that the outer-membrane islands involved in inter-membrane organisation are indeed enriched in BAM [48].

In this way we propose that specialised and dynamic secretosomes of varying composition localise, aided by inter-membrane communication, to form customised envelope biogenesis hot spots. Their activities could then be adapted accordingly for the provision of the required proteins to the inner-membrane, periplasm and outer-membrane. The high concentrations of CL at these sites, important for secretosome assembly, would also serve to activate SecA – conferring high levels of ATP and PMF driven translocation through the secretosome [18, 22]. The localisation of CL at areas of high membrane curvature, including division sites and the poles [44, 46, 49], could provide focal points for the localisation and activation of trans-envelope secretosome, establishing discrete sites for cell wall biogenesis. On this point, it has been shown that while most of SecA is generally membrane associated and mobile, a significant proportion of SecA is immobile (~25%) [50]. Perhaps the immobilised SecA is engaged with the secretosome at CL rich envelope biogenesis hubs. Indeed, we know that the affinity
of SecA for the holo-translocon is higher where there is an abundance of CL [7]. Interestingly, the formation of these static docking sites for SecA are dependent on the PMF [50], which ties in with the conferral of PMF stimulated protein translocation by CL [18], as well as the requirement of PMF for secretosome activity [16].

The absence of CL, such as in the E. coli ΔCL strain deployed here, must curtail many activities in the cell, including secretion, due to functional large assembly destabilisation. However, it is potentially off-set by elevation of the compensatory lipid phosphatidyl glycerol [51]. Note also, that the absence (or reduction by gel filtration) of CL merely destabilises the higher molecular weight assemblies, such that they disassemble in detergent solution. This is not to say that the higher molecular weight assemblies cannot form in vivo in membranes without it – presumably, they do so in a reduced or compromised, but not incapacitated, state.

The observed broad elution profile of the secretosome constituents we see is presumably due to the existence of various assembled combinations of SecYEG, SecDF-YajC, YidC, BAM and others. This might reflect the situation in native membranes wherein there may be many different requirements at a given time and location. Different super-complexes, with different activities, may be recruited or assembled on site and tuned according to functional requirement, whether that be for delivery of proteins to the periplasm, inner-membrane, outer-membrane and/or for regions in high demand of quality control. Presumably, the distribution of different complexes in the envelope will change during different stages of the cell cycle, which appears to be the case for the BAM complex [52]. Indeed, the activity of the BAM machinery at the septa is unhindered by nascent PG (enriched in penta-peptides); while elsewhere, mature PG (predominantly composed of tetra-peptides) inhibits its activity [53]. Therefore, the regulation, localisation and assembly of the secretosome components achieved by lipids, PG and inter-membrane interactions could enact the necessary envelope biogenesis at the septa (Fig. 5b).

On the subject of division, the HTL components YidC and SecG along with BAM were recently shown to localise adjacent to divisome proteins where they are presumably recruited to produce the high quantities of proteins required to enable cell division (Fig. 5b) [54]. This also aligns with a proteomics study demonstrating an interaction between SecY and the cell elongation/division protein RodZ, suggesting the secretosome directly interacts with the divisome [17]. The same study picks up interactions of RodZ and FtsH with BamA. Elsewhere, RodZ has also been shown to interact with SecA and FtsH [55], supporting links between the division and secretion apparatus. Furthermore, the activity of BamA is supported by an interaction with DolP [56], which in the context of cell division is interesting because DolP is recruited to the mid-cell in the latter stages of this process [57]. Another recent study implicates cross-talk between both the BAM machinery and YidC to the divisome [54].

Going back to the core features of the secretosome, i.e. the delivery and maturation of proteins to the inner-membrane, periplasm and outer-membrane, the trans-envelope association of the holo-translocon and BAM complexes brings together a couple of interesting motifs – the tetratricopeptide repeat (TPR; YfgM, BamD, BepA) and WD40 β-propeller (BamB). They could serve to recruit additional factors (e.g. SurA, Skp or DegP) necessary for transport, such as for retrieval into the periplasm for folding/degradation, or to facilitate outer-membrane insertion. Perhaps they provide staging posts in the thoroughfare for transiting proteins. This dynamic and adaptable trans-envelope organisation might be the prototype, also involving TPR and WD40 ladders, for widespread inter-membrane transport, including the mitochondrial (and chloroplast) inner- and outer-membranes, as well as between plastids and the ER.

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D.W.W. and I.C. conceived and designed experiments; D.W.W. conducted experiments; D.W.W. and I.C. wrote the manuscript; I.C. secured funding and led the project.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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