Supramolecular assemblies underpin turnover of outer membrane proteins in bacteria

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Gram-negative bacteria inhabit a broad range of ecological niches. For *Escherichia coli*, this includes river water as well as humans and animals, where it can be both a commensal and a pathogen1–3. Intricate regulatory mechanisms ensure that bacteria have the right complement of β-barrel outer membrane proteins (OMPs) to enable adaptation to a particular habitat4–5. Yet no mechanism is known for replacing OMPs in the outer membrane, an issue that is further confounded by the lack of an energy source and the high stability6 and abundance of OMPs5. Here we uncover the process underpinning OMP turnover in *E. coli* and show it to be passive and binary in nature, in which old OMPs are displaced to the poles of growing cells as new OMPs take their place. Using fluorescent colicins as OMP-specific probes, in combination with ensemble and single-molecule fluorescence microscopy in *vivo* and *in vitro*, as well as molecular dynamics simulations, we established the mechanism for binary OMP partitioning. OMPs clustered to form ~0.5-μm diameter islands, where their diffusion is restricted by promiscuous interactions with other OMPs. OMP islands were distributed throughout the cell and contained the Bam complex, which catalyses the insertion of OMPs in the outer membrane6,7. However, OMP biogenesis occurred as a gradient that was highest at mid-cell but largely absent at cell poles. The cumulative effect is to push old OMP islands towards the poles of growing cells, leading to a binary distribution when cells divide. Hence, the outer membrane of a Gram-negative bacterium is a spatially and temporally organized structure, and this organization lies at the heart of how OMPs are turned over in the membrane.

We developed a strategy for following the localization and turnover of OMPs using colicins8,9, which circumvents the need for fluorescent protein fusions. Protein fusions are the method-of-choice for investigating the localization of cytoplasmic, inner membrane and periplasmic proteins11, but these generally inhibit OMP maturation. Colicins are species-specific bacteriocins that bind OMP receptors before translocating a cytotoxic domain into the cell6. ColE9 and Colla were used here as specific, high-affinity (~nM), non-covalent labels for the vitamin B₁₂ transporter BtuB and the iron siderophore transporter Cir, respectively12–14 (Fig. 1a). The colicins were engineered with disulfide bonds to block their import into bacterial cells12 and covalently modified with organic fluorophores (Alexa Fluor 488 (AF488) or tetramethyl rhodamine (TMR)). We first compared the distribution and turnover of ColE9TMR-labelled BtuB in the outer membrane with an inner membrane protein (IMP), green fluorescent protein (GFP)-labelled TatA, by confocal fluorescence microscopy following simultaneous, pulsed production of both proteins. These experiments were carried out over several rounds of cell division using *E. coli* JM83 cells expressing *btuB* and *tata-GFP* genes from arabinose-inducible promoters, in this instance using a variant of ColE9 in which the amino-terminal 83 amino acids are deleted (Δ¹⁻⁸³ ColE9TMR) to label BtuB. Unlike TatA–GFP, which showed a purely analogue diminution of fluorescence in the inner membrane as cells divided, BtuB–ColE9TMR distribution was binary in nature (Fig. 1b, f and Supplementary Video 1). The OMP migrated towards the old poles during the first cell division and thereafter was sequestered predominantly in two cells, which we designated repository cells. Repository cells retained the bulk of the transiently produced OMP (BtuB), leaving daughter cells to acquire new (unlabelled) OMPs. To ensure transient expression had not influenced OMP behaviour, we repeated these experiments but this time observed endogenous BtuB and Cir migration in JM83 cells, following labelling with the respective colicin (Supplementary Video 2). Starting from a single cell, the two OMPs behaved identically. Approximately half of each OMP moved towards the poles as the cell grew and divided and were then retained in two repository cells, while new daughter cells received little or no old colicin-labelled OMP.

We used a combination of laser scanning confocal fluorescence recovery after photobleaching (FRAP) and total internal reflection fluorescence microscopy (TIRFM) to determine the mechanistic basis of binary OMP partitioning in *E. coli*. Fluorescence recovery was rapid for TatA–GFP (<3 s at 37 °C), which is typical of IMPs15, whereas no recovery was observed for colicin-labelled BtuB or Cir even after 3 min (Extended Data Fig. 1), similar to the OMP OmpA16. From single-molecule tracking (SMT)-TIRFM, we established that BtuB and Cir have planar diffusion coefficients (D ~ 0.013 and 0.019 μm² s⁻¹, respectively) that are similar to those reported previously for IMPs and OMPs in *vivo* (Extended Data Fig. 2a, b, Extended Data Table 1 and Supplementary Videos 3 and 4), demonstrating that colicin-labelled OMPs diffuse in the membrane. However, mean square displacement (MSD) plots indicated that this diffusion was restricted to regions of ~0.5 μm diameter. Analysis of various *E. coli* mutants indicated that cell envelope structures such as cross-bridges to the peptidoglycan were not responsible for the restricted diffusion of OMPs (Extended Data Fig. 3 and Extended Data Table 1). However, Monte Carlo simulations of BtuB diffusion were consistent with its confinement being due to an increase in mass over time, such as through association with other OMPs (Extended Data Fig. 2c), a point we return to later.

We next investigated the distribution and co-localization of colicin-labelled BtuB and Cir in *E. coli* JM83 cells using TIRFM. BtuB–ColE9AF488 and Cir–CollaTMR clustered together in islands that were distributed throughout the membrane and moved to the poles as cells divided (Fig. 1c, d). Similar punctate appearance of trimeric porins in the outer membrane of *E. coli* has previously been reported17,18. OMP islands contained multiple copies of BtuB and Cir (mean ~7; Extended Data Fig. 4a) and their mean size was ~0.5 μm (Fig. 1e), equivalent to the...
OMPs biogenesis, with cell-to-cell variation in both number and distribution of new OMP islands.

OMPs are deposited in the outer membrane of Gram-negative bacteria by the Bam machinery although where or how this occurs is not known. We found that components of the Bam complex (BamA and BamC), which were labelled with fluorescently labelled antibodies, showed significant co-localization with BtuB- and Cir-containing OMP islands, and that this co-localization persisted as islands migrated towards the poles (Fig. 3a, b and Extended Data Fig. 4b, c). Importantly, new Bam-containing islands appeared in non-polar regions of dividing cells consistent with these regions being the major sites of OMP biogenesis (Fig. 2b).

The co-localization of different OMPs (BtuB, Cir and BamA) within islands and their restricted diffusion suggested promiscuous protein–protein interactions (PPIs) might be the underlying cause. To test this hypothesis, we reconstituted BtuB in a polymer-supported membrane (PSM) prepared from an E. coli lipid extract and followed the lateral mobility of single molecules after labelling with ColE9 TMR (see Methods). At low BtuB densities (~50 BtuB per μm²), similar to that estimated for the outer membrane (Fig. 4a, b and Supplementary Video 6) and with faster diffusion coefficients (D ~ 0.18 μm² s⁻¹). Over time, however, BtuB molecules began to diffuse more slowly suggesting self-association (Fig. 4b, panel 2 and Extended Data Fig. 5a). This interpretation was confirmed by experiments in which BtuB was added at a 1,000-fold higher concentration (Fig. 4b, panel 3). Addition of the trimeric porin OmpF at concentrations found in the outer membrane of E. coli (1,000-fold above that of BtuB) resulted in most of the BtuB–ColE9 TMR complexes exhibited Brownian diffusion with trajectories that extended beyond those observed in vivo (Fig. 4a, b, panel 1 and Supplementary Video 6) and with faster diffusion coefficients (D ~ 0.02 μm² s⁻¹ and confinement diameter ~0.4 μm, respectively; Fig. 4b, panel 4 and Extended Data Fig. 2d). Addition of maltose binding protein, which had been engineered with a single transmembrane helix (TM-MBP), to the PSM (Fig. 4b, panel 5; Extended Data Fig. 5b, d) or lipopolysaccharide (LPS) (Extended

**Figure 1** | Binary partitioning of OMPs in the E. coli outer membrane.

(a) Structures of colonies bound to their OMP receptors (Protein Data Bank (PDB) accessions 1U7W, 1CGH and 1FSJ for ColE9–BtuB model, and 2HDJ and 1CII for ColA–Cir) highlighting the positions of inactivating disulphide bonds and fluorophore labels. OM, outer membrane. (b) Distribution of fluorescently labelled OMP (BtuB-AΔ1-83, ColE9 TMR) and IMP (TatA–GFP) in E. coli JM83 cells following transient induction with arabinose and imaging by confocal microscopy (see Methods). In contrast to TatA–GFP, which distributed in a purely analogue fashion (see f), BtuB-AΔ1-83 ColE9 TMR segregation was binary. After four divisions two OMP repository cells (white arrowheads or asterisks) retained most of the original OMP. DIC, differential interference contrast. c-d, Left, TIRFM image (sum of 100 frames) of BtuB- and Cir-containing OMP islands in E. coli JM83 cells labelled with ColE9 TMR and ColA TMR, respectively. Right, automated ImageJ delineation of OMP islands. d, Top, false colour image of endogenous, colicin-labelling BtuB (green) and Cir (red) showing co-localization in OMP islands (same cell as in e). Bottom, as above but cells were grown for 1 h before imaging of cells undergoing division. Of the 130 OMP islands analysed before and after growth (n = 20 cells per experiment and in duplicate), 40 ± 9% and 39 ± 7% (mean ± s.e.m., respectively), contained both BtuB and Cir. All scale bars, 1 μm. e, Size distribution of BtuB- and Cir-containing OMP islands from c and d. Red line denotes the mean. f, Distribution of fluorescence, shown as a ratio of the two repository cells relative to total fluorescence, at each cell division for TatA–GFP (green bars) and BtuB-AΔ1-83 ColE9 TMR (red bars) from five confocal microscopy data sets. Data are mean and s.e.m. Diamond symbols denote expected ratios for an analogue mechanism.

**Figure 2** | Biogenesis of new OMP islands in the central regions of dividing E. coli cells forces old OMP islands towards the poles. a, Sequential insertion of OMPs in the outer membrane followed by two-colour confocal fluorescence microscopy. Growing E. coli JM83 cells were labelled initially with ColE9 TMR (red) (t = 0 min) and then with ColE9 AF488 (green) (t = 30 min), followed by a final growth phase (t = 60 min). Representative data shown from duplicate experiments. b, c, Confocal and TIRF microscopy images, respectively, of individual dividing E. coli JM83 cells from the 30-min time point (a). The mean fluorescence distributions shown below the panels are for 20 cells. *P < 0.1, Mann–Whitney test. Scale bars, 1 μm. Of the 165 OMP islands analysed by TIRFM at the 30-min time point (n = 20 cells per experiment and in duplicate) only 6.4 ± 6.2% showed overlap of old/new BtuB fluorescence, emphasizing that old OMP islands do not contribute to new OMP synthesis.
The Bam biogenesis machinery is located within OMP islands that emerge primarily in non-polar regions of the cell and migrate to the poles. a. TIRFM images (sum of 100 frames) of E. coli JM83 cells producing haemagglutinin (HA)-tagged BamA detected by Alexa 488-labelled anti-HA antibody (see Methods). ColE9TMR was used to detect endogenous BtuB before and after a 1-h period of growth. Of the 115 OMP islands analysed, 33 ± 9% (mean ± s.e.m.) contained both BamA and BtuB. b. TIRFM images (sum of 100 frames) for E. coli JM83 cells stained with Alexa 488-labelled anti-BamC antibody and ColE9TMR (BtuB). Temporal separation of labels by a 1-h period of growth showed old BtuB-containing islands localized primarily at the poles (red label), whereas new BamC-containing islands localized in non-polar regions (green label). The mean fluorescence distributions and co-localization histograms (error reported as s.e.m.) shown in the panels are from 20 cells in each case. *P < 0.1 (Student’s t-test); **P < 0.001 (Mann–Whitney).

OMPs engage in promiscuous protein–protein interactions. a. Representative single molecule trajectories (from duplicate experiments) of ColE9TMR-labelled BtuB reconstituted into a PSM overlaid onto a scaled outline of an E. coli cell (see Supplementary Video 6). Molecules exhibited Brownian diffusion, typically extending beyond the boundaries of an E. coli cell (green trace), mixed (orange trace) or confined diffusion (red trace). Scale bar, 1 μm. See Methods. b. Initially (at 5-min post-deposition of the PSM), and at concentrations found in the outer membrane of E. coli (denoted by 1×), BtuB molecules displayed predominantly Brownian diffusion (panel 1), but with faster diffusion coefficients than observed in vivo. Over time, however, BtuB exhibited slower diffusion suggesting self-association (panel 2), an interpretation that was confirmed by raising the BtuB concentration significantly (panel 3) which resulted in most of the molecules exhibiting restricted diffusion. The same effect could be elicited using an unrelated OMP, OmpF (panel 4), but not maltose binding protein (TM-MBP) tethered to the membrane through a single transmembrane helix (panel 5). c. Snapshots of a crowded bilayer (~30% and ~20% protein fractional area, for BtuB–OmpF and BtuB–BtuB simulations, respectively) from molecular dynamics simulations after 10 μs (see also Extended Data Figs 6 and 7 and Extended Data Table 2). OmpF is shown in yellow, BtuB in green. The blue inner square represents the simulation box (60 × 60 nm²) while the outer region represents the periodic replicas. Lipids in the unit cell were present in a ratio of 3:1 phosphatidylethanolamine (PE; grey) and phosphatidylglycerol (PG; red). d. Density plots of the frequency of occurrence of BtuB around OmpF (left) and of OmpF around BtuB (middle) observed in the BtuB–OmpF simulations, and of BtuB around BtuB (right) in the BtuB–BtuB simulations (see Extended Data Fig. 7). e. High frequency interaction sites at the interface between BtuB and OmpF. Dashed lines represent the position of the bilayer. The OmpF–BtuB interactions were predominantly mediated by hydrophobic and aromatic residues and, to a lesser extent, polar residues.
Data Fig. 5b, c) did not induce BtuB clustering, indicating that this effect was not simply due to crowding or LPS association, but involved interactions between OMPs. Recent atomic force microscopy data and molecular dynamics simulations indicate OMPs can self-associate in membranes24–25. Using coarse-grained molecular dynamics simulations of BtuB and OmfF/BtuB mixtures at high local concentration, we further showed that OMPs have a propensity to engage in both homologous and heterologous associations that slow their diffusion (Fig. 4c–e, Extended Data Figs 6 and 7 and Extended Data Table 2). Promiscuous OMP–OMP interactions were largely not, but exclusively, mediated by aromatic residues displayed from the surfaces of their β-barrels. In the case of BtuB, the same set of residues mediated both homologous and heterologous interactions (Fig. 4e). We propose that OMP islands are stabilized in vivo by such promiscuous PPIs.

In conclusion, we have shown that biogenesis and turnover of OMPs in the Gram-negative bacterium E. coli are intrinsically linked processes (see model in Extended Data Fig. 8). The resulting binary partitioning of OMPs ensures they are turned over rapidly, but also means old OMPs persist in repository cells, which implies bacterial populations have ‘memory’ of past OMP biosynthesis. The accumulation of old OMPs in repository cells could also be a factor in cellular aging as well as providing a route for the presentation of proteins at cell poles26–27. The co-localization of the Bam biogenesis machinery with the OMPs it has deposited in the membrane points to a high level of cellular organization and coordination in the outer membrane that has hitherto been unsuspected. Bam substrates include LptD (ref. 28), which inserts LPS into the outer leaflet of the outer membrane, and the β-barrels of autotransporters29, which are crucial for biofilm formation and adhesion to host epithelia during bacterial pathogenesis. Moreover, since Bam is also required for the surface exposure of the outer membrane lipoprotein RcsF through the pores of OMPs80, OMP and lipoprotein biogenesis may intersect within OMP islands.

Online Content

Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Author Contributions

P.R., O.B., J.P., M.S.P.S., C.G.B. and C.K. designed the experiments. N.A.C., assisted by C.G.B. and C.T., collected SMT-TIRFM data and C.T., assisted by N.A.C. and P.R., collected FRAP data for colicin-labelled OMPs using wild-type and deletion E. coli strains. P.R. conducted all SMT-TIRFM experiments on OMP islands. U.S. and C.G.B. designed and built the TIRF microscope at York used for all SMT-TIRFM experiments. O.B. and P.R. conducted all SMT-TIRFM experiments and data analysis. S.J.C. and C.G.B. designed and implemented software scripts used for different simulations and batch image analysis. C.T., P.R., N.A.C., N.G.H. and R.K. purified all the proteins used in the study. C.T., P.R. and N.A.C. labelled colicins with fluorophores. D.M.O. and T.J.G. constructed plasmids for colicin constructs and A.L.D. and M.C. thank the University of Oxford Micron facility and the University of York (Research Priming Fund) for financial support used to develop the TIRFM. P.R. acknowledges the late R. Saadia for his unwavering support. M.S.P.S. acknowledges support from the UK supercomputer ARCHER for molecular dynamics simulations. This work was supported by grants to C.K. and C.G.B. (BBSRC LoLa grant BB/G020671/1), J.P. (Deutsche Forschungsgemeinschaft SFB 944) and M.S.P.S. (BBSRC BB/L020558/1, Wellcome Trust WT092970MA).

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METHODS

Confirmation of single gene deletions in Keio collection strains. All Keio mutants33 of E. coli BW25113 used in this work (with deletions in lpp, ompA, pal, rfaC, tolA and tonB) were first validated by PCR, confirming the deletion strains contained the kanamycin resistance cassette within the open-reading frame of interest.

Colicin purification and fluorophore-labelling. Site-directed mutagenesis was used to introduce a soluble accessible cysteine (K469C) in the cysteic domain of a disulfide inactivated (Y324C, L447C) ColE9–Im9hap2 construct, cloned into pET-21a (ref. 32). An equivalent Y324C, L447C, K469C ColE9 construct in which the N-terminal 83 amino acids of the colicin had been deleted (Δ1–83 ColE9) was also generated. This intrinsically disordered region of the colicin contains important protein–protein interaction epitopes that are required for translocation into the cell10. Removal of this region had no effect on the localization of OMP islands on the turnover of OMPs. It was used primarily during long time courses where reduction of the inactivating disulfide bond was possible.

Standard recombinant molecular biology techniques were used to clone the colita gene into pET-21a with a carboxy-terminal His6-tag and to introduce an inactivating disulfide within the coiled-coil R-domain (L257C, A411C), analogous to that used to inactivate ColE9, in addition to a soluble exposed cysteine in the cysteic domain (K544C).

All recombinant proteins were expressed in E. coli BL21 (DE3) cells and purified by nickel affinity and size-exclusion chromatography using published procedures34. Elution from the nickel affinity column was achieved through guanidine denaturation of the ColE9–Im9hap complex and imidazole elution of ColIaHap-Cys469 and Cys544 within ColE9 and Colita, respectively, were labelled with a 20-fold molar excess of fluorophore (Alexa Fluor 488 maleimide (Invitrogen) or TMR-maleimide (Sigma)) in 20 mM potassium phosphate, pH 7.0, 2 M guanidine-HCl for 30 min at 37°C followed by dialysis against 20 mM potassium phosphate, pH 7.0, 0.5 M NaCl at 4°C. Dialysed proteins were further purified by size-exclusion chromatography (Superdex 200 HR 10/30, GE Healthcare) performed in the same buffer at room temperature and fractions containing fluorophore labelled protein analysed by SDS-PAGE, pooled, snap frozen and stored in aliquots at −20°C. All labelling and subsequent purification steps were performed in the dark. The labelling efficiency (typically >0.7 fluorophores/protein) was estimated from the spectrophotometrically determined fluorophore (Alexa Fluor 488, εmax = 71,000 cm−1 M−1; TMR, εmax = 80,000 cm−1 M−1) and colicin (ColE9, εmax = 46,075 cm−1 M−1; Colita, εmax = 59,360 cm−1 M−1) concentrations after correcting for absorption at 280 nm by the fluorophore (Alexa Fluor 488, ε280 = 0.11 × 106 cm−1 M−1; TMR, ε280 = 0.3 × 106 cm−1 M−1). Analysis of single molecule photobleaching characteristics for fluorophore-labelled colicins adsorbed on a quartz slide surface and viewed by TIRFM were consistent with labelling at a single position.

Confirmation of single gene deletions in Keio collection strains. All Keio mutants33 of E. coli BW25113 used in this work (with deletions in lpp, ompA, pal, rfaC, tolA and tonB) were first validated by PCR, confirming the deletion strains contained the kanamycin resistance cassette within the open-reading frame of interest.

Cell growth and OMP staining with colicins and antibodies. E. coli JM83 cells were grown at 37°C in LB broth to exponential phase, after which 200 μl of cells were transferred to 4 ml M9-glucose minimal media (0.1 mM CaCl2, 0.1 mM FeSO4, 2 mM MgSO4, 1 g l−1 NH4Cl, 0.05% (w/v) casamino acids, 0.0002% (w/v) thiamine, 0.4% (w/v) d-glucose) and grown until absorbance (A) at 600 nm of ~0.4. Then 200 μl of cells were pelleted by centrifugation (4,700 g, 3 min), with the pellet resuspended in 200 μl of fresh supplemented M9-glucose containing 300 μM fluorescein labelled colicin. Cells were pelleted again, resuspended in 500 μl fresh M9-glucose, before finally resuspending the pellet in 100 μl supplemented M9-glucose. For subsequent labelling of old and new OMPs, cells were grown for a further hour after the initial labelling such that the A600 nm had approximately doubled before a second staining was performed. Wash steps were repeated as above. For the transient expression of BtuB10 and TatA–GFP36 from pBAD plasmids, cultures were grown in the presence of 100 μg ml−1 ampicillin, and 0.2 mM L-arabinose added for 1 h before cells being used in confocal experiments.

For detection of BamA, JWD3 cells transformed with a pET17b plasmid expressing BamA-HA-L7 were grown in the presence of 100 μg ml−1 ampicillin and 50 μg ml−1 kanamycin, and prepared for microscopy as described for JM83 cells above. Insertion of an HA epitope into loop 7 of BamA was previously shown to be tolerated functionally and exposed on the external surface of bacteria37. BamA-HA-L7 was labelled in vivo, adding a 1:500 dilution of Alexa488 anti-HA antibody (Molecular Probes) and 1% (w/v) ultrapure BSA (Invitrogen) to 100 μl of JWD3 cells. After 15 min incubation with mixing by rotary inversion at room temperature, excess label was removed as described above for colicins.

For BamC labelling, a 1:1,000 dilution of mouse anti-BamC antibody37 (gift from S. Buchanan) was added to 100 μl of JM83 cells prepared as above in the presence of 2% (w/v) ultrapure BSA and incubated for 15 min with mixing by rotary inversion at room temperature. Cells were then washed twice by pelleting (4,700g, 3 min) and resuspended in 500 μl of fresh supplemented M9 media, before a 1:500 dilution of Alexa488 anti-mouse secondary antibody (Molecular Probes) was added in the presence of 2% (w/v) ultrapure BSA. After 15 min incubation with rotary inversion at room temperature, excess label was removed as described above for colicins.

For TIRFM, cells were fixed with 100 μl of fresh supplemented M9 media, before a 1:500 dilution of Alexa488 anti-mouse secondary antibody (Molecular Probes) was added in the presence of 2% (w/v) ultrapure BSA. After 15 min incubation with rotary inversion at room temperature, excess label was removed as described above for colicins. For qualitative labelling of colicins adsorbed on a quartz slide surface and viewed by TIRFM were consistent with labelling at a single position.

Membrane protein purification. OmpF and BtuB were purified according to previously published protocols38,39. The purified proteins contained some endogenous LPS carried through the purification. An artificial transmembrane domain (ALAALAALAALAALAALAALAALSKSR) was fused to the C terminus of maltose binding protein (TM-MBP) by insertion of the corresponding oligonucleotide (494 nm ). Fluorescence image was separated into two channels using an emission filter that depolarized the laser excitation, thus ensuring fluorophore orientation effects were minimised. The fluorescence image was acquired before and after each FRAP experiment to ensure the image of the bacterial cell remained in focus and adherent to the PDL coated surface. In all cases, DIC images were recorded using a 633 nm laser to prevent photobleaching of fluorescent probes.

In vivo SMT-TIRFM. JM83 cells were immobilised on poly-ε-lysine coated quartz slides (25 mm x 75 mm, cleaned in 1 M KOH) in supplemented M9 media containing 100 nM fluorescently labelled colicin. An ultra-thin sample chamber was formed by adding ~0.1 μg ml−1 of 5 μm silica beads to the media, overlaying with a 1 coverslip (22 mm x 64 mm) and sealing with nail varnish. An inverted epifluorescence microscope chamber equipped with a 100× oil immersion objective (Zeiss Plan-Apochromat, NA 1.4) was modified in-house for prism-coupled TIRFM. Illumination was provided by 488 nm (30 mW) and 561 nm (30 mW) optically pumped solid-state lasers (Sapphire LP, Coherent). A 2 wave plate was used to depolarize the laser excitation, thus ensuring fluorophore orientation effects were minimised. The fluorescence image was separated into two channels using an image splitter (Optosplit II, Cairn Research) with appropriate dichroic beamsplitter (FT580, Zeiss) and band pass filters for Alexa Fluor 488 (ET525/50m, Chroma) and TMR (HQ605/20M, Chroma). Background data sets were obtained by directly adsorbing fluorescently-labelled colicins to poly-ε-lysine coated quartz slides. Two-colour FRAP experiments (GFP and TMR) on a single bacterial cell were performed as follows: (1) acquire pre-bleach image with 543 nm laser illumination, (2) record complete FRAP sequence for TatA–GFP with 488 nm laser illumination, and (3) record post-bleach images with 543 nm laser illumination. The individual laser sources were used separately to track the two fluorophores. A DIC microscopy image was acquired before and after each FRAP experiment to ensure the image of the bacterial cell remained in focus and adherent to the PDL coated surface. For the transient expression of BtuB 100 μl of fresh supplemented M9 media, before a 1:500 dilution of Alexa488 anti-mouse secondary antibody (Molecular Probes) was added in the presence of 2% (w/v) ultrapure BSA. After 15 min incubation with rotary inversion at room temperature, excess label was removed as described above for colicins.
sequences with no saturation of the intensified CCD camera were selected. For R volume of BtuB and two of the most abundant proteins in the outer membrane, curvature of the cellular membrane was modelled by mapping planar diffusion trajectories onto a hemispherically capped cylinder of length 3 μm and radius 250 nm. The limited TIRF evanescent field depth was modelled by terminating, but not discarding, particle paths which diffused further than 150 nm from the closest observable plane. Molecular crowding in the outer membrane was simulated using excluded volume of BtuB and two of the most abundant proteins in the outer membrane, OmpA and OmpF. Each protein species was modelled in 2D as a series of single, non-overlapping, hard circles with radii of R_ompA = 1.3 nm, R_ompF = 3.1 nm and R_BtuB = 2.3 nm, and with relative populations of 333, 111 and 1, respectively. A random planar distribution of OmpA and OmpF was generated for each diffusing BtuB up to the percentage membrane occupancy, δ. BtuBs were randomly positioned and subject to the standard Monte-Carlo-based diffusion approach; however, only steps ending in vacant positions (no overlap of BtuB with OmpA or OmpF) were accepted. To model association of BtuB with the OmpF population in the outer membrane, the BtuB radius was increased randomly in a single step according to the following equation and association rate, k.

\[ R_{BtuB + OmpF} = \sqrt{R_{BtuB}^2 + R_{OmpF}^2} \]

Planar diffusion trajectories were subsequently mapped to the curved bacterial surface as described previously. For optimisation of the MSD simulated and parameters k and parameters D = (0.01–0.3 μm^2 s^{-1}) and δ (≤20%) were varied manually. Very similar parameters to those for OmpF-BtuB association were generated for OmpA-BtuB association, thus only those for OmpF-BtuF are shown in Extended Data Fig. 2c. Monte Carlo simulations that did not include promiscuous association between OMPs, but merely molecular crowding (δ ≤20%), did not reproduce the in vivo diffusive behaviour (data not shown), which is consistent with an increase in mass of the tracked diffusing species explaining the asymptotic experimental MSD observed in vitro and in vivo.

**Analysis of OMP islands by TIRFM.** JM83 cells were immobilised on poly-d-lysine and viewed using prism-coupled TIRFM as described above. 100 consecutive video frames were stacked to build an image that was then analysed. Only sequences with no saturation of the intensified CCD camera were selected. For each picture and each channel, we used ImageJ software to select only pixels that were three times more intense than background for further quantification. Intensity quantification within individual spots was obtained using ImageJ. Within each cluster or island, the total pixel intensity was normalised so the population corresponding to a single photobleaching step was fitted to 1 arbitrary unit (AU).

CoLocalizer Pro 2.7.1 software (CoLocalization Research Software, http://www.colocalizer.com) was used for co-localization of red and green channels. The co-localization value corresponded to the ratio between yellow pixels over the sum of yellow, red and green pixels on each overlapped picture. Distribution of fluorescence intensity along the x axis of bacteria was determined using a custom script implemented in MATLAB (version 2012a, MathWorks). Raw images were initially thresholded against a user-defined intensity (selection via a graphical user interface) to provide a binary image, approximately highlighting bacteria against the background. High frequency noise was reduced through application of the built-in MATLAB medfilt2 median filter. Individual bacteria were identified as continuous high intensity pixel regions, with pixels of a continuous region required to be within a 0.6 μm^2 area of each other. Further filtering was performed to remove bacteria less than 1 μm away from their neighbour to improve the quality of intensity profile analysis results. Bacterial poles were identified as corresponding to the two pixels in a continuous region with the largest separation. Intensity along the selected bacteria was measured 11 times between the bacterial ends-points, each at a uniform, spaced offset from the bacterial long axis in the range ±200 nm. The mean profile is calculated and normalized to the range 0–100 for comparison between bacteria.

**Confocal time-lapse microscopy.** For confocal microscopy, 200 μl of M9 containing 1% agaro (w/v) was introduced into a Gene Frame matrix that was previously adhered to a clean slide. The agar pad was formed by addition of a clean coverslip on top until solidification had occurred. Then, 10 μl of stained bacteria was added to the pad, which was sealed afterward using a clean coverslip. Cell growth was monitored at 37 °C and visualized through 100× oil-immersion objective (Zeiss Plan-APochromat NA 1.4) on a Zeiss LSM780 microscope. Individual cells were selected in a region of interest that was clear of other cells. Snapshots were taken every 30 min during 2–3 h time courses, using scan speed 7 and 1–2% of laser power (depending on laser line).

**Formation of polymer-supported membranes.** Modification of glass coverslips was performed as described previously. In brief, surfaces were first cleaned by plasma cleaning and then activated by silanization with pure (3-glycidoxypropyl)trimethoxysilane (Sigma) at 75 °C for 1 h. Subsequently, the surfaces were reacted with molten bis-amino-polyethylene glycol (PEG) with a molecular mass of 2,000 daltons (Da) (Rapp Polymere) for 4 h at 75 °C. Modification of the free amines with palmitic acid (Sigma) was carried out in the presence of an excess of diisopropylcarbodiimide (Sigma). Formation of proteoliposomes was carried out by detergent extraction in lipid-protein-detergent mixtures by addition of β-cycloexetrin. Lipids, proteins and lipopolysaccharides solubilized in Triton-X 100 and n-octyl-β-D-glucoside were combined as required before a twofold excess of heptakis(2,6-di-O-methyl)-β-cycloexetrin (Sigma) over the detergent was added. Reconstitution of BtuB was achieved by mixing an E. coli lipid extract with the protein in MES buffer pH 6.5 and adding a twofold molar excess of β-cycloexetrin. Experiments were conducted using either a defined E. coli polar lipid extract (PE 67%; PG 23.2%; cardiolipin (CA) 9.8%) or total E. coli lipid extract (PE 57.5%; PG 15.1%; CA 9.8%; unknown 17.6%) from Avanti LIPIDS. OMP mobilities in PSM experiments were essentially identical using these lipid extracts. So as to obtain a 1× final concentration of protein, a molar ratio of 1:100,000 (protein:lipid) was prepared and vortexed for 2 s before deposition on surfaces. After 10 min the proteoliposomes were added onto functionalized surfaces and incubated for 20 min for immobilization. The buffer was then exchanged for a solution of 10% (w/v) PEG with a molecular mass of 8,000 Da (Sigma Aldrich) to induce fusion of the proteoliposomes into a homogenous lipid bilayer. After 20 min, the surfaces were extensively washed with buffer to remove excess vesicles. While imaging, bilayer continuity was visualized by monitoring the homogeneous lateral diffusion of the lipids DiD in PE (red) or LPE-Chol (green) in the membrane. Before data acquisition, the TIRF microscopy was labelled by the addition of 100 nM C05 (Sigma Aldrich) after each 5 min, followed by removal of unbound ligand. Assuming all BtuB molecules were labelled, and single step photobleaching corresponded to single molecules, the density of BtuB at 1× concentration was comparable to that estimated in vivo (~50 molecules per μm^2).

**Tracking single molecules in polymer supported bilayers.** OMPs were tracked using TIRF microscopy carried out with an Olympus IX71 inverted microscope equipped with a quad-line total internal reflection illumination (TIR) condenser (Olympus), a 150× magnification objective with a numerical aperture of 1.45 (UAPO 150×/1.45 TIRF, Olympus) and a back-illuminated emCCD (Endscheide) cooled to −80 °C. TIR was illuminated by a 561-nm diode-pumped solid-state laser (Cobold Live, 200 mW) with 2 mW output power at the objective, whereas BODIPY and Dy647 were illuminated with 488 and 642 nm laser diodes (Omicron, 140 and 200 mW, respectively) with output powers of 5 and 4 mW, respectively. Fluorescence was detected using a quadband emission filter (446/523/600/677 HC Quadband Filter, Semrock) and recorded at 30 fps. All experiments were carried out using buffer complemented with oxygen scavenger and a redox-active photoprotectant (0.5 mg ml⁻¹ glucose oxidase (Sigma), 0.04 mg ml⁻¹ catalase (Roche Applied Science), 5% glucose (w/v), 10 -ascorbic acid and 1 μM methyl viologen) to minimize photo bleaching. Moderate bleaching of the sample was applied to be able to track single molecules.

All videos were analysed using custom software (PATRACK), implemented in Visual C++ and provided by P.-E. Milhiet and P. Dosset. The centre of each fluorescence peak was determined with sub-pixel resolution by fitting a...
two-dimensional elliptical Gaussian function. The two-dimensional trajectories of single molecules were constructed frame-by-frame, selecting particles that displayed a single bleaching step. Diffusion coefficient values were determined from a linear fit to the MSD plots between the first and fourth points according to the equation: MSD(t) = 4Dt.

We used a new algorithm within PATRACK based on a back-propagation neural network allowing automatic detection of Brownian, confined and directed motion modes within a trajectory. The ability of the software to accurately detect different diffusion modes was first established on simulated data and then evaluated on trajectories recorded from fluorescently labelled molecules in two different biological systems48,49.

**Statistical analysis of microscopy data.** All experiments were conducted at least twice (no significant differences were observed between data sets either by Student's t-test or non-parametric Mann--Whitney test) and in each case a representative set of data presented in the figures. Sampling sizes in experiments (cells, OMP islands, single molecule trajectories) were validated by very significant P values in a student t-test or a non-parametric Mann--Whitney test. In the analysis of OMP islands in post growth conditions by TIRFM, cells that were undergoing division were explicitly selected for imaging. No statistical methods were used to predetermine sample size.

**Molecular dynamics simulations.** Simulations were run using GROMACS (version 4) (http://www.gromacs.org) using a modified version of the MARTINI force field50. Structures of OmpF (PDB code 2OMF) and BtuB (PDB code 2GUF) were converted to coarse-grained models as described previously. In the model, each coarse-grained particle represents approximately four atoms in the atomistic structure. Protein molecules were represented with a single coarse-grained particle per backbone moiety, with variable numbers of sidechain particles. To maintain the β-barrel structure of OmpF and BtuB, an elastic network model was used, with a force constant 1,000 kJ mol⁻¹ nm⁻² applied between Cα particles within 7 Å of one another.

To mimic varied levels of crowding in the membrane, several systems of varying protein composition were inserted into a pre-equilibrated bilayer containing POPE and POPG in a 3:1 ratio. The lipid composition was selected to approximate that used in PSM experiments. Systems were solvated and sodium starting configuration we randomized the rotation of each protein around its centre of mass about the membrane normal. Systems were submitted to a force constant 1,000 kJ mol⁻¹ to relax the system. The lipid composition was selected to approximate that used in PSM experiments. Systems were solvated and sodium starting configuration we randomized the rotation of each protein around its centre of mass about the membrane normal. Systems were solvated and sodium starting configuration we randomized the rotation of each protein around its centre of mass about the membrane normal.

The factor of one half accounts for each interaction being counted twice. Similar to OmpF-BtuB interactions, the interaction frequency, int(res, resj), is normalized by the total number of interactions to give the proportional interaction frequency, Ii, for any two residues, resi and resj, on different monomers of BtuB. The proportional interaction frequency, Ii, of a single residue, res, of BtuB with OmpF—the values shown in red bars on the side of the interaction frequency matrix plots (Extended Data Fig. 7)—is then given by summing the proportional interaction frequencies of res over all residues of OmpF:

\[
I_i = \frac{\sum_{s} n_{res} \cdot n_{inter}(s)}{\sum_{s} n_{res}^{total}} \frac{\sum_{s} n_{inter}(s)}{n_{inter}^{total}}
\]

Similarly, a single residue function is defined for the proportional interaction frequencies of OmpF with BtuB, and residues of BtuB in their interaction with other BtuB monomers.

**Amino acid interaction propensities:** to assess the involvement of each amino acid in mediating BtuB–OmpF and BtuB–BtuB interactions, a residue interaction propensity is defined, adapted from the residue propensity matrix defined previously53 in the hallmark work on protein–protein interactions. For each amino acid, AA, the interaction propensity, IPAA, for the interaction of residues of BtuB with OmpF is given by comparing the proportion of BtuB interactions mediated by amino acid AA with the proportion of the BtuB surface it represents:

\[
IP_{AA} = \frac{\sum_{i} n_{AA} \cdot I_i}{\sum_{i} n_{inter} \cdot I_i}
\]

where Nres_BtuB and Nres_OmpF denote the number of residues in a BtuB and OmpF single protein, respectively.

Thus, the interaction frequency, int(resi, resj), between any two residues, resi in BtuB and resj in OmpF, is given by:

\[
\text{int}(\text{resi}, \text{resj}) = \frac{1}{\text{if the centroids of resi and resj are within 8 Å of each other}}
\]

where Nres is the total number of simulations being considered; \( \tau \) is the total simulation time (in ns); and Nres_BtuB and Nres_OmpF are the total number of BtuB and OmpF proteins, respectively, in simulation \( \sigma \). Only the last 100 ns of each simulation was used. The proportional interaction frequency, \( I_i \), is then given by normalizing the interaction frequency for any pair of residues, int(resi, resj) by the total number of interactions:

\[
I_i = \frac{\text{int}(\text{resi}, \text{resj})}{\sum_{j} \text{int}(\text{resi}, \text{resj})}
\]

where \( \text{int}(\text{resi}, \text{resj}) \) is the number of residues in a BtuB and OmpF single protein, respectively.

To look at interactions of BtuB with itself, int(resi, resj) is instead defined by:

\[
\text{int}(\text{resi}, \text{resj}) = \sum_{s} n_{res} \cdot n_{inter}(s)
\]

The factor of one half accounts for each interaction being counted twice. Similar to BtuB–OmpF interactions, the interaction frequency, \( I_i \), is normalized by the total number of interactions to give the proportional interaction frequency, \( I_i \), for any two residues, \( \text{resi} \) and \( \text{resj} \), on different monomers of BtuB. The proportional interaction frequency, \( I_i \), of a single residue, \( \text{res} \), of BtuB with OmpF—the values shown in red bars on the side of the interaction frequency matrix plots (Extended Data Fig. 7)—is then given by summing the proportional interaction frequencies of \( \text{res} \) over all residues of OmpF:

\[
I_i = \frac{\sum_{s} n_{res} \cdot n_{inter}(s)}{\sum_{s} n_{res}^{total}} \frac{\sum_{s} n_{inter}(s)}{n_{inter}^{total}}
\]

where \( n_{inter}^{total} \) is the number of total residues on the surface of BtuB, and \( n_{inter}^{total} \) is the total number of residues on the surface of BtuB. Similarly, residue interaction propensities are defined for the OmpF interaction with BtuB and interaction of OmpF with other BtuB monomers.

**Amino acid interaction propensities:** to assess the involvement of each amino acid in mediating BtuB–OmpF and BtuB–BtuB interactions, a residue interaction propensity is defined, adapted from the residue propensity matrix defined previously53 in the hallmark work on protein–protein interactions. For each amino acid, AA, the interaction propensity, IPAA, for the interaction of residues of BtuB with OmpF is given by comparing the proportion of BtuB interactions mediated by amino acid AA with the proportion of the BtuB surface it represents:

\[
IP_{AA} = \frac{\sum_{i} n_{AA} \cdot I_i}{\sum_{i} n_{inter} \cdot I_i}
\]

where \( N_{res}^{total} \) is the number of total residues on the surface of BtuB, and \( N_{inter}^{total} \) is the total number of residues on the surface of BtuB. Similarly, residue interaction propensities are defined for the OmpF interaction with BtuB and interaction of OmpF with other BtuB monomers.

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Extended Data Figure 1 | Visualizing the different diffusive behaviours of proteins in the inner and outer membranes of the same E. coli cell. Single E. coli JM83 bacterial cell visualized by laser-scanning confocal fluorescence microscopy at 37 °C. At the outer membrane (top row) BtuB labelled with ColE9TMR showed no fluorescence recovery after photobleaching a region of interest with a 543 nm laser (rectangle). By contrast, recovery of TatA–GFP fluorescence in the inner membrane of the same cell (bottom row) was observed within a few seconds after photobleaching an identical region with 488 nm laser. The unrestricted mobility of TatA–GFP in the inner membrane accounts for its analogue distribution during cell division (main text, Fig. 1b, f). This experiment was done in duplicate (one representative set of images is presented in the figure). Scale bar, 1 μm.
Extended Data Figure 2 | SMT-TIRFM defines the mobility of colicin-labelled BtuB and Cir in vivo. a, Top, Z-projection of fluorescence intensity (increasing from blue to red) of two Cir–ColIAF488 complexes on separate JM83 cells. Middle, the fluorescence intensity in consecutive images (30 f.p.s.) is displayed for a typical Cir–ColIAF488 fluorescent spot. The intensity and single-step photobleaching behaviour were consistent with a single membrane complex being tracked. Bottom, MSD was calculated for single Cir–ColIAF488 complexes (n = 41) that displayed single-step photobleaching behaviour, could be tracked for at least 1.7 s before photobleaching (error is reported as s.e.m.) and were not immobilized on the quartz surface (MSDend of trajectory > 0.004, determined for surface bound ColIAF488 in the same samples). The MSD value rapidly approached an asymptotic value that was consistent with restricted lateral diffusion. Linear regression of the MSD for the first five time delays (0.033–0.17 s) yielded the planar diffusion coefficient (D = 0.014 µm² s⁻¹). See Extended Data Table 1 for all fitted values (from a minimum of four experimental replicates).

b, Top, Z-projection of fluorescence intensity (increasing from blue to red) of a BtuB–ColE9AF488 complex on a JM83 cell. Middle, the fluorescence intensity in consecutive images (30 f.p.s.) is displayed for a typical fluorescent spot. The intensity and single-step photobleaching behaviour were consistent with a single membrane complex being tracked. Bottom, MSD was calculated for individual BtuB–ColE9AF488 complexes (n = 62) that displayed single-step photobleaching behaviour, could be tracked for at least 1.7 s before photobleaching (error is reported as s.e.m.) and were not immobilized on the quartz surface (MSDend of trajectory > 0.008, determined for surface bound ColE9AF488 in the same samples). The MSD value rapidly approached an asymptotic value that was consistent with restricted lateral diffusion. Linear regression of the MSD for the first five time delays (0.033–0.17 s) yielded the planar diffusion coefficient (D = 0.013 µm² s⁻¹). See Extended Data Table 1 for all fitted values (from a minimum of four experimental replicates).

c, Comparison of experimental MSD for BtuB–ColE9AF488 complexes from b (open circles) with mean output from Monte Carlo simulations (solid blue line) of 2D diffusion (D = 0.14 µm² s⁻¹, association time of 0.2 s (k⁻¹) and 15% volume occupancy) in a curved, crowded membrane typical of a rod-shaped bacterium, which is illuminated by an evanescent field (penetration depth = 150 nm). Here lateral D was identical to the value observed for monomeric BtuB diffusing in PSMs (Extended Data Fig. 5c). The lower apparent lateral D measured experimentally (≈ 0.013 µm² s⁻¹) indicates the promiscuous PPIs induce temporal corralling. The dashed (blue) lines are the upper and lower 99.75% confidence limits for the mean (n = 5,000 trajectories). See Methods for details of the Monte Carlo simulations.

d, MSD plots (± s.e.m.) for all confined trajectories (500 in each case) of BtuB–ColE9TMR complexes diffusing in PSMs, red data points in Fig. 4b (panels 3 and 4), main text. [BtuB] × 1,000 data (open circles) is compared to data for native levels of BtuB (closed circles) in the presence of native levels of OmpF (equivalent to [BtuB] × 1000). Fits to both sets of data yield values for D and confinement diameter, 0.02 µm² s⁻¹ and 0.4 µm, respectively, that are very similar to those observed in vivo.
**Extended Data Figure 3** Probing the roles of cell envelope systems in the restricted diffusion of OMPs. Laser-scanning confocal FRAP microscopy of E. coli cells labelled with ColE9AF488 for BtuB (a) and ColIaAF488 or ColIaTMR for Cir (b) was used in conjunction with gene deletions or chemical treatments to probe the involvement of various cell envelope systems in the restricted mobility of OMPs. The rationale for these experiments was twofold. First, structures or processes within the Gram-negative cell envelope could be responsible for the restricted mobility of OMPs (Extended Data Figs 1 and 2). Second, FRAP would provide a means of detecting the loss of restricted OMP mobility, resulting in the recovery of fluorescence after photobleaching, if these systems were perturbed. Each panel in the figure shows DIC microscopy images of the bacterial cell followed by pre-bleach, bleach (t = 0) and post-bleach (t = 3 min) fluorescent images of the same cell. All microscopy images are 3.03 × 4.17 μm². Top-to-bottom; CCCP treatment (0.1 mM) of E. coli JM83 cells assessed the impact of dissipating the proton-motive force across the inner membrane. E. coli BZB1107 cells (ompR vlamB ompF::Tn5) are deficient for the major outer membrane porins OmpF and OmpC. E. coli BW25113 rfaC (JW3596) is a deep-rough mutant in which the outer core of the LPS is truncated. E. coli BW25113 lpp (JW1667) is a deletion of Braun’s lipoprotein, an outer membrane lipoprotein that is one of the most abundant proteins in E. coli. ~40% of Lpp is covalently attached to the underlying peptidoglycan. E. coli BW25113 tonB (JWS195) is deleted for TonB, a protein that spans the periplasm and couples the proton-motive force across the inner membrane with transport of nutrients through outer membrane proteins such as BtuB and Cir. Colla depends on TonB for import into bacteria. E. coli BW25113 tolA (JW0729) is deleted for TolA, a protein that spans the periplasm and couples proton-motive force with stabilization of the outer membrane. ColE9 depends on TolA for import into bacteria. Other E. coli K-12 deletion strains tested (but not shown) include pal (JW0731), an outer membrane lipoprotein, and ompA (JW0940), both of which have domains that form non-covalent contacts with the peptidoglycan cell wall. As the data in the figure show, no mutation or condition resulted in the recovery of fluorescence in FRAP experiments from which we infer these systems/processes are not responsible for the restricted mobility of OMPs. Finally, we tested a mutation of ColE9 in which the first 83 amino acids of the colicin were deleted (D1–83 ColE9) but where the inactivating disulfide across the R-domain remained in place. The N-terminal 83 residues contain protein–protein interaction epitopes for OmpF as well as TolB in the periplasm. No change in FRAP behaviour (not shown) was observed demonstrating that interactions made by the colicin at the cell surface are not responsible for the restricted mobility of the OMP to which it is bound. These experiments were done in duplicate (one representative set of images for each condition is presented in the figure).
Extended Data Figure 4 | Estimation of the number of BtuB and Cir proteins within OMP islands. a, The scatter plots show the distribution of relative intensity of ColE9AF488 and ColIaTMR fluorescence signals when bound to their specific OMPs in bacteria or adhered to surfaces. The right-hand panels show representative TIRFM images that were used for the analysis. Fluorescence signals corresponded to a stack of 100 frames (30 f.p.s.) and were relatively stable in time and space. Scale bars, 1 μm. The mean value (± s.e.m.) for each distribution is shown by the histograms. We normalized the data for colicins adhered to bacteria using the mean intensity for surface-adhered ColE9AF488 and ColIaTMR (300 nM), assuming these correspond to one molecule (−1 AU). From this normalization, OMP islands on average contained ~7 labelled OMPs although values ranged from 1 to 19 labelled OMPs. The entire experiment was conducted twice, the replicate showing the same mean value of OMPs per island.

b, Co-localization of BamA with Cir. TIRFM images (sum of 100 frames) for E. coli JWD3 cells expressing HA-tagged BamA detected by Alexa488-labelled anti-HA antibody and Cir was detected by ColIaTMR labelling before and after a 1-h period of growth. BamA shows significant co-localization (31 ± 7% for 112 OMP islands visualized across 20 cells) with Cir within OMP islands. Owing to weak binding of the anti-HA antibody it was not possible to perform more detailed growth experiments as for BamC.

c, Co-localization of BamC with Cir/TIRFM images (sum of 100 frames) for E. coli JM83 cells stained with Alexa 488 labelled anti-BamC antibody and ColIaTMR showing co-localization within OMP islands that move to the poles in cells undergoing division. Temporal separation of BamC and the Cir labels by a 1-h period of growth, in which old BamC was first blocked with unlabelled antibody, showed that old Cir was localized primarily at the old poles (red label), whereas new BamC-containing islands appeared in non-polar regions of the cell (green label). The average fluorescence distributions and co-localization histograms (error is reported as s.e.m.) shown in b and c are from 20 cells in each case. *P < 0.1 (Student’s t-test); **P < 0.001 (Mann–Whitney test).
Extended Data Figure 5 | Influence of membrane components on diffusion in polymer-supported membranes. a, Analysis of the relative fluorescence intensity of BtuB–ColE9 TMR suggested significant self-association of BtuB, which was time and concentration dependent. The grey area corresponds to the range of intensities where single step photobleaching was observed and which were the molecules tracked in our experiments. b, Distribution of diffusion coefficient when tracking LPS BODIPY incorporated into a polymer-supported bilayer at a ratio of 17:1 ratio LPS:BtuB. High concentrations of BtuB or OmpF, but not of TM-MBP, induced significant trapping of some LPS molecules. c, Distribution of diffusion coefficient when tracking BtuB–ColE9 TMR incorporated into a polymer supported bilayer that contains a ratio of 17:1 or 0:1 LPS:BtuB. Addition of LPS did not alter significantly the diffusion behaviour of BtuB in these artificial membranes. d, Distribution of diffusion coefficients when tracking TM-MBP Cy5 incorporated into a polymer-supported bilayer. High concentrations of BtuB or OmpF, but not of TM-MBP, induced a slight but significant trapping of some TM-MBP molecules. All experiments were done in duplicate (one set are presented in the figure) and differences in diffusion coefficient determined using a non-parametric Mann–Whitney test.
Extended Data Figure 6 | Final snapshots of OmpF (yellow) and BtuB (green) positions for coarse grain molecular dynamics simulations. 

a–f, Each patch (−30 × 30 nm² for a–e, −60 × 60 nm² for f) contained four BtuB (a); nine BtuB (b); two OmpF trimers and two BtuB monomers (c); four OmpF and five BtuB (d); eight OmpF and eight BtuB (e); and eight OmpF and eight BtuB (f). In each case, the single unit cell is darkened and outlined in blue. Lipids are shown in grey (PE) and red (PG). See Extended Data Fig. 7 and Extended Data Table 2 for further details.

g, Propensity for BtuB (green) and OmpF (yellow) residues to be at the protein–protein interface based on simulations of BtuB–OmpF mixtures. A propensity greater than one indicates that a residue occurs more frequently at the interaction interface than on the protein surface. h, BtuB residue propensities for residue types at the interface between two BtuB monomers, based on simulations containing OmpF and BtuB (dark green), or just BtuB (light green). The two propensities are similar. Note that the sampling in terms of homo-interactions of BtuBs in the OmpF–BtuB simulation is less than in the simulation containing only BtuB owing to the starting positions of the proteins in OmpF–BtuB simulations, which favour hetero-interactions between BtuB and OmpF. See Methods for details about the calculation of the propensity values.
Extended Data Figure 7 | Residues that mediate BtuB–OmpF and BtuB–BtuB interactions in molecular dynamics simulations. a, b, Residues that mediate BtuB–OmpF (a) and BtuB–BtuB (b) interactions. The interaction matrix charts the frequency of interaction between any pair of BtuB and OmpF residues, as a proportion of the total number of interactions that occurred, from high proportional frequency (dark green) to low (white). Depicted here is a subset of the entire interaction matrix, showing only the residues which engaged in interactions with the other protein over a threshold value; any BtuB residue which had a proportional interaction frequency of more than $1 \times 10^{-3}$ with any OmpF residue is shown, and similarly for any OmpF residue. On each side, residues with interaction frequency values above approximately one-third of the maximum value of interaction are highlighted in bold. The bar plots show the proportional interaction frequencies of each single BtuB (side) and OmpF (top) residue, for the subset of residues that are shown in the interaction matrix. See Methods for a full mathematical explanation of the interaction value calculations. Bar plots are coloured according to the bar values, from high proportional interaction frequency (dark red) to low (white). This is consistent with the colour scheme in Fig. 4e. Note the matrix in b is symmetric.
Extended Data Figure 8 | Binary OMP partitioning and its relationship to biogenesis and organization of OMP islands. a, Model depicting the appearance of new OMP islands (green) within growing cells containing old OMP islands (red) in which OMP biogenesis has ceased. The TIRFM images of single cells shown alongside the different stages were taken from cell growth experiments (same experiments as shown in Fig. 2, main text). The model highlights how new OMP islands appear mostly at mid-cell, their creation in conjunction with cell elongation forces old OMP islands towards the poles. The result is binary turnover when the cell divides. The bulk of the old OMPs are retained at the poles of repository cells, which are created at every cell division. It remains unclear why OMP biogenesis ceases in old OMP islands. It is also unclear whether the architecture of the poles (for example, membrane curvature) has a role in retaining old OMPs or whether this is entirely a consequence of continued growth and biogenesis in daughter cells. Nevertheless, the outcome of such a mechanism is that within just two divisions cells appear that do not have any of the original old OMPs. The stochastic nature of the process occasionally results in some old OMP islands not partitioning with the old pole (for example, main text Fig. 2b, panel 3, TIRFM image), which likely explains why the process is not a pure binary mechanism (if it were, values of 100% would be expected in the histogram shown in Fig. 1f, main text). We speculate that the lack of intermixing between OMP islands, which is consistent with the absence of fluorescence recovery in vivo FRAP experiments (Extended Data Fig. 1), may be due to the very high density of OMPs (particularly porins) within islands separated by densely packed LPS. b, Model depicting the organization of an individual OMP island and the movement of an OMP within it. OMP islands contain one or more Bam complexes (see main text Fig. 3). Here, we show a single Bam machine having just deposited an OMP (green circle) in the OM. The new OMP diffuses laterally in the membrane but becomes increasingly restricted due to promiscuous interactions with other OMPs (grey circles), which is consistent the confinement experienced by OMPs in vitro and in vivo (main text Fig. 4b and Extended Data Fig. 2) and with Monte Carlo and molecular dynamics simulations (main text Fig. 4c, d and Extended Data Figs 2c, 6 and 7). Only a fraction of the OMPs presumed to be present within an OMP island are shown.
Extended Data Table 1 | Comparison of BtuB and Cir diffusion parameters from the present work with those reported for other Gram-negative OMPs and IMPs in the literature

| Protein                      | \( D \) (\( \mu \text{m}^2/\text{s} \)) | Confinement diameter (\( \mu \text{m} \)) | Reference |
|------------------------------|--------------------------------------|------------------------------------------|-----------|
| LamB                         | 0.15                                 | 0.03                                     | 55        |
| BtuB                         | 0.05, 0.10                           | NR                                       | 56        |
| OmpF                         | 0.006                                | 0.1                                      | 56        |
| BtuB (ColE9, JM83 cells)     | 0.013 (\( N = 62 \))                | 0.6                                      | Present work |
| BtuB (\( \Delta^{1-52} \) ColE9, JM83 cells) | 0.0081 (\( N = 51 \)) | 0.5                                      | Present work |
| BtuB (ColE9, BZB1107 cells) | 0.018 (\( N = 30 \))                | 0.5                                      | Present work |
| Cir (Colla, JM83 cells)      | 0.019 (\( N = 41 \))                | 0.6                                      | Present work |
| Cir (Colla, BZB1107 cells)  | 0.011 (\( N = 14 \))                | 0.5                                      | Present work |

NR, not reported

OMP and IMP data are shown above and below the grey bar, respectively. Diffusion coefficients (\( D \)) were obtained from linear regression of the first 4–5 time steps. See Methods and Extended Data Fig. 2 for details. Two strains were used for the single molecule studies reported in the present paper, the standard lab strain \( E. coli \) JM83 and a strain in which the major porins OmpF and OmpC had been deleted, \( E. coli \) BZ81107 (ref. 58). BtuB labelling was achieved using either full-length, fluorescently labelled ColE9 or a variant lacking the first 52 amino acids, a disordered region of the toxin that contains an OmpF binding site and a binding epitope for the periplasmic protein TolB\(^{10}\). Cir labelling was achieved using fluorescently labelled Colla. The single-molecule trajectories analysed for each condition represent a minimum of four experimental replicates.
Extended Data Table 2 | Summary of coarse grain molecular dynamics simulations for BtuB or OmpF systems and OmpF/BtuB mixtures at differing levels of crowding.

| Number of proteins | Dimensions (nm²) | Protein fractional ratio (%) | Simulation time (µs) | Diffusion coefficient (µm²/s) |
|--------------------|------------------|------------------------------|----------------------|------------------------------|
|                    |                  |                              |                      | BtuB                         |
| 1                  | 30 x 30          | 2                            | 10                   | 11.53 ± 0.33                 |
| 4                  | 30 x 30          | 9                            | 10                   | 8.16 ± 0.78                  |
| 9                  | 30 x 30          | 19                           | 10                   | 2.88 ± 0.94                  |
| 36                 | 60 x 60          | 19                           | 10                   | 6.71 ± 1.00                  |
| 0                  | 30 x 30          | 4                            | 10                   | ---                          |
| 2                  | 30 x 30          | 13                           | 10                   | 9.46 ± 0.78                  |
| 8                  | 60 x 60          | 13                           | 10                   | 13.94 ± 2.64                 |
| 5                  | 30 x 30          | 28                           | 10                   | 2.35 ± 0.06                  |
| 18                 | 60 x 60          | 29                           | 10                   | 2.25 ± 1.74                  |
| 8                  | 30 x 30          | 52                           | 10                   | 0.27 ± 0.00                  |

See Methods and Extended Data Figs 6 and 7 for details. Increasing the density of proteins decreased their calculated diffusion coefficients, although the magnitude of the change was contingent on the size of the system, in agreement with recent modelling studies on crowded membranes and with the PSM data shown in Fig. 4b. While diffusion coefficients are qualitatively in agreement with our experimental results the time scale remains too short to enable quantitative comparisons, as has been discussed in a number of other coarse-grained molecular dynamics studies.