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1 **Dual function of OmpM as outer membrane tether and**
2 **nutrient uptake channel in diderm Firmicutes**

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25
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27

28 **Abstract**

29 The outer membrane (OM) in diderm, or Gram-negative, bacteria must be tethered to
30 peptidoglycan for mechanical stability and to maintain cell morphology. Most diderm
31 phyla from the Terrabacteria group have recently been shown to lack well-
32 characterised OM attachment systems, but instead have OmpM, which could
33 represent an ancestral tethering system in Bacteria. Here, we have determined the
34 structure of the most abundant OmpM protein from *Veillonella parvula* (diderm
35 Firmicutes) by single particle cryo- electron microscopy. We also characterised the
36 channel properties of the transmembrane β -barrel of OmpM and investigated the
37 structure and PG-binding properties of its periplasmic stalk region. Our results show
38 that OM tethering and nutrient acquisition are genetically linked in *V. parvula*, and
39 probably other diderm Terrabacteria. This dual function of OmpM may have played a
40 role in the loss of the OM in ancestral bacteria and the emergence of monoderm
41 bacterial lineages.

42

43

44 **Introduction**

45 The defining feature of diderm, or Gram-negative, bacteria is the presence of an outer
46 membrane (OM)^{1,2}. This complex cell envelope component is an asymmetric lipid
47 bilayer, usually containing lipopolysaccharide (LPS) in the outer leaflet and
48 phospholipids in the inner leaflet. The first formal definition of an OM was subsequent
49 to an electron microscopy analysis of the cell envelope of the diderm Firmicute
50 *Veillonella parvula* (Negativicutes class)³. However, most of our knowledge about the
51 OM derives from studies of Proteobacteria, especially *Escherichia coli*. While the OM
52 provides mechanical stabilisation to the cell⁴, diderm bacteria must tether the OM to
53 peptidoglycan (PG) for additional mechanical stability and to maintain cell morphology.
54 *E. coli* has three OM tethering systems: Braun's lipoprotein (Lpp) that covalently links
55 the PG with the OM^{5,6}, the lipoprotein Pal and the OM protein OmpA which associate
56 with PG non-covalently⁷⁻¹¹. Mutants of these systems exhibit cell envelope defects
57 and increased susceptibility to cell envelope stressors, such as detergents^{7,12-15}.

58

59 Recent phylogenetic analysis of the distribution of OM attachment systems throughout
60 the Bacteria Tree of Life has highlighted a striking bimodal distribution across the two
61 major clades in which Bacteria are divided, the Terrabacteria (including both classical
62 monoderms such as Bacilli and Clostridia, and diderms such as Cyanobacteria and
63 Deinococcus-Thermus) and the Gracilicutes (including only diderms such as
64 Proteobacteria and Bacteroidetes)¹⁶. Braun's lipoprotein was shown to be only present
65 in a subset of Proteobacteria, and Pal restricted to the Gracilicutes, together with the
66 Lol machinery for lipoprotein export to the OM. OmpA showed a patchy distribution,
67 being absent in many diderms. In contrast, the OmpM protein is a fourth OM tethering

68 system that appears to be widespread in diderm Terrabacteria but is completely
69 absent in the Gracilicutes¹⁶.

70

71 OmpM consists of an N-terminal periplasmic S-layer homology (SLH) domain
72 connected via a linker region to a C-terminal OM β -barrel¹⁷⁻²⁰. The SLH domain has
73 been mostly studied for its role in the building of S-layers in monoderm bacteria²¹. The
74 primary role of the SLH domain is binding to the cell wall. Similarly to the SLH domain
75 found in monoderm S-layer proteins²²⁻²⁴, the SLH domain of OmpM proteins of some
76 diderm Terrabacteria phyla such as Thermus and Cyanobacteria has been shown *in*
77 *vitro* to bind pyruvylated secondary cell wall polymers (SCWPs)²⁵⁻²⁷, whereas in
78 members of the Negativicutes (including *V. parvula*) it anchors the OM to PG modified
79 with polyamines (e.g. cadaverine and putrescine) on the D-glutamate of the peptide
80 stem^{18,28,29}.

81

82 Deletion of three out of the four OmpM paralogues in *V. parvula* resulted in a dramatic
83 phenotype where the OM detaches, the periplasmic space is greatly enlarged and
84 multiple cells share a single OM¹⁶. Complementation by the most abundant *V. parvula*
85 OmpM paralogue, OmpM1, reverted this phenotype back to wild type, demonstrating
86 *in vivo* that OmpM is responsible for tethering the OM to PG¹⁶. This dramatic
87 phenotype after deletion of OmpM-like proteins has been reported also in two other
88 representatives of diderm Terrabacteria, *Thermus aquaticus*³⁰ and *Deinococcus*
89 *radiodurans*^{31,32}.

90

91 These results led to the proposal that OmpM proteins represent an ancestral OM
92 tethering system in Terrabacteria and possibly the last bacterial common ancestor,

93 which united anchoring and nutrient acquisition functions¹⁶. The presence of this
94 unique OM tether in Terrabacteria might therefore have been involved in the multiple
95 OM-loss events inferred to have occurred specifically in this clade, perhaps via OmpM
96 mutations weakening (and eventually abolishing) the SLH-PG interactions¹⁶.

97

98 Due to its LPS component, the OM presents a permeability barrier for hydrophilic and
99 hydrophobic small molecules, both detrimental (*e.g.* antibiotics) and essential (*e.g.*
100 nutrients)^{33,34}. Controlled permeability of the OM is established by OM proteins, which
101 either mediate energized transport of specific nutrients in the case of TonB-dependent
102 transporters, or allow size-limited diffusion in the case of porins (<~600 Da for rigid
103 spherical molecules^{33,34}, although larger flexible molecules can also go through³⁵).
104 Porins are well-characterised in many Proteobacteria, but much less information is
105 available on small-molecule permeation in diderm Terrabacteria. Earlier work on the
106 OmpM-like protein Mep45 from the Negativicute *Selenomonas ruminantium*
107 suggested that this protein can transport nutrients via its β -barrel domain¹⁹, but
108 another study in *Synechocystis* (Cyanobacteria) claimed that its OmpM orthologues
109 form channels too small for most simple nutrients²⁰. In *V. parvula*, the role of OmpM
110 in nutrient acquisition has not been studied. In addition to the four OmpM paralogues,
111 there are two other putative porin genes in the *V. parvula* genome: an OmpA-like
112 homologue (*FNLLGLLA_00518*) and a porin (*FNLLGLLA_00833*)³⁶. Proteomic
113 analysis of the *V. parvula* OM showed that OmpM1, OmpM2 and FNLLGLLA_00833
114 are, respectively, the first, third, and sixth most abundant OM proteins³⁶, suggesting
115 that these proteins could play major roles in determining OM permeability.

116

117 A recent analysis has proposed that the barrels of most OmpM-like proteins have 16-
118 30 β -strands³⁷, and could therefore form pores that are large enough for small
119 molecule diffusion across the OM, a hypothesis supported by electrophysiology
120 experiments with *Deinococcus radiodurans* SlpA protein³⁸ and liposome swelling
121 studies on Mep45 from *S. ruminantium*¹⁹. The recently determined structures of SlpA
122 revealed that it forms a large trimeric complex composed of 30-stranded β -barrels, *i.e.*
123 much larger than those of the general *E. coli* porins OmpC and OmpF^{37,39}. However,
124 since *D. radiodurans* has an unusually complex cell envelope with an S-layer^{31,40,41}, it
125 is unclear how generalisable these findings are to OmpM proteins in other diderm
126 Terrabacteria, especially in Negativicutes which tether the OM to polyaminated PG
127 rather than pyruvylated SCWP.

128

129 Here we use single particle cryo-EM, X-ray crystallography, molecular dynamics
130 simulations, bioinformatic analyses and functional assays to show that OmpM1 from
131 *V. parvula* (VpOmpM1) is a general porin with similar structural and functional
132 properties to *E. coli* OmpF, with an additional mobile periplasmic region that can bind
133 *V. parvula* PG and take on different folds. We also show that the four OmpM
134 paralogues are likely the only porins in *V. parvula*, implying that nutrient acquisition
135 and OM attachment are genetically linked in diderm Firmicutes, and likely all diderm
136 Terrabacteria, via OmpM. Our results support an ancestral dual function of OmpM as
137 both an OM tether and nutrient uptake porin which may have supported life in early
138 bacteria.

139

140

141 **Results**

142 **VpOmpM1 is a trimeric porin with an extended periplasmic region**

143 We determined the structure of VpOmpM1 expressed in *E. coli* by single particle cryo-
144 EM (Figure 1a-d and Supplementary Table 1). Reconstructions up to 3.2 Å without
145 enforced symmetry revealed a trimeric arrangement of the protomers. The C-terminal
146 16-stranded β -barrels that reside in the OM form a classical three-fold symmetrical
147 porin assembly reminiscent of the well-characterised *E. coli* porins OmpF (EcOmpF)
148 and OmpC^{42,43}. However, the β -barrel lumens are constricted by inward-folded
149 extracellular loops 3 and 7 in VpOmpM1 (Figure 1b), rather than solely by loop 3 as in
150 the *E. coli* porins. The inter-protomer contact area, mediated by hydrophobic
151 sidechains on the outside of the β -barrel, completely excludes lipid/detergent and
152 solvent. The β -barrels exhibit other features common to known porin structures,
153 including an aromatic girdle at the membrane-solvent interfaces and strong density
154 surrounding the β -barrels that likely corresponds to the lipid moieties of LPS and
155 phospholipid or detergent (Figure 1b, d and Supplementary Fig. 1). An unusual feature
156 observed in the cryo-EM reconstructions was a weaker, elongated density that
157 corresponds to the N-terminal SLH domain at the distal part, connected to the β -barrel
158 via a triple coiled coil (Figure 1c, d). We term this periplasmic region of VpOmpM1 the
159 'stalk'. Only the region of the stalk proximal to the OM was resolved well enough for
160 model building, as the cryo-EM density deteriorates towards the SLH domain end of
161 the stalk. We speculated that the poor density is the result of movement of the stalk
162 relative to the β -barrels. Measurements of the blurry SLH domain density observed in
163 2D class averages suggest that the distal part of the stalk spans 50-60 Å (Figure 1c).
164 The VpOmpM1 trimer is pseudosymmetrical: while the β -barrels exhibit three-fold
165 symmetry, the stalk is tilted relative to the membrane plane normal (Figure 1d). The

166 pseudosymmetry was confirmed by enforcing C3 symmetry during data processing,
167 which resulted in a better-resolved β -barrel region (2.8 Å) but uninterpretable density
168 for the periplasmic stalk (Supplementary Fig. 2-4).

169

170 The *E. coli* cell wall does not contain polyaminated PG, the native ligand of VpOmpM1.
171 Therefore, to exclude the possibility that in our structure of VpOmpM1 purified from *E.*
172 *coli* the stalk region is misfolded due to the absence of polyaminated PG, we purified
173 a His-tagged OmpM1 construct expressed in *V. parvula* (native VpOmpM1) and
174 determined its structure by single particle cryo-EM to 3.3 Å resolution (Supplementary
175 Table 1, Supplementary Fig. 2-4). The β -barrel structures are virtually identical ($C\alpha$ -
176 $C\alpha$ root-mean-square deviation 0.239 Å), but there are differences in the relative
177 orientation of the stalk (Figure 1e). In reconstructions without enforced symmetry, the
178 α -helices forming the coiled-coil are not equivalent in either dataset, as they exit the
179 OM plane in different orientations (Figure 1f). The reasons for these structural
180 differences are not obvious. We speculate that intra- or inter-protomer hydrogen bonds
181 at the stalk-barrel interface could be responsible for the subtle conformational
182 differences. In the *E. coli* VpOmpM1 structure, the sidechain of R113 in one protomer
183 interacts with the sidechain of N155 (part of the periplasmic end of the β 2 strand) of
184 another protomer. This interaction is observed only in two inter-protomer interfaces,
185 but not the third one (Figure 1g). A similar interaction was observed in the native
186 VpOmpM1 structure: at two inter-protomer interfaces, R113 interacts with the carbonyl
187 oxygen of G115 and the sidechain of N116 (both part of the β -barrel end of the stalk)
188 (Figure 1h). Interestingly, the R113-N155 interaction was not observed in the native
189 VpOmpM1 structure, and the R113-G115/N116 interaction was not observed in the

190 VpOmpM1 structure from *E. coli*. It is possible that the two protein constructs differ
191 slightly in their lowest energy states which are observed in the averaged cryo-EM data.

192

193 We performed all-atom molecular dynamics (MD) simulations to investigate the
194 conformational space sampled by the stalk. We simulated the native VpOmpM1 model
195 to which an AlphaFold2⁴⁵ prediction of the unmodeled region of the stalk was grafted
196 and fit into the low resolution cryo-EM density (Figure 2a). The simulations revealed
197 substantial movement of the stalk in the periplasmic space (Supplementary Movie 1).
198 The SLH domain end of the stalk sampled a span of ~ 50 Å (Figure 2b) during the
199 simulations, in agreement with the cryo-EM data (Figure 1c). The coiled-coil does not
200 kink throughout the 1 μ s simulation (Figure 2c), and the movement of the stalk is due
201 to flexibility at the interface between the stalk and the β -barrels. We observed that the
202 inter-protomer hydrogen bonds identified in our static cryo-EM structures, R113-N155
203 and R113-G115, are broken and re-formed throughout the simulation (Figure 2d). The
204 R113 interaction with the sidechain of N116 was not stable, and R113 instead
205 interacted with the backbone carbonyl oxygen of N116 (Figure 2d). Similar results
206 were obtained from a replicate simulation with the AlphaFold2 graft model as well as
207 the native VpOmpM1 cryo-EM model by itself (Supplementary Fig. 5). The stalk would
208 presumably be bound to the relatively immobile PG in vivo, which would significantly
209 impair its mobility relative to the OM. We speculate that the flexible interface between
210 the stalk and the β -barrels would result in the ability of the OM to move slightly relative
211 to the PG, imparting mechanical resistance to the cell envelope.

212

213 **The stalk binds polyaminated peptidoglycan**

214 The SLH domain of OmpM binds polyaminated PG *in vivo*, as demonstrated in the
215 Negativicute *S. ruminantium*^{18,28}. Recent evidence for VpOmpM1 binding to PG comes
216 from fluorescence microscopy and cryogenic electron tomography experiments that
217 showed OM detachment when three *ompM* paralogues were deleted¹⁶. We tested
218 binding of recombinant full-length and barrel-only VpOmpM1 to sacculi isolated from
219 *V. parvula* and *E. coli* (Figure 3a). Only full-length protein binds to *V. parvula* sacculi,
220 as expected. Minor amounts of full-length VpOmpM1 were found in the wash fraction,
221 suggesting that the non-covalent PG-SLH domain interaction is reasonably strong. No
222 binding was observed to sacculi from *E. coli*, confirming that PG stem peptide
223 polyamination is required for binding.

224

225 The cryo-EM density for VpOmpM1 SLH domains was not sufficiently resolved for
226 model building due to movement of the stalk, as demonstrated by MD simulations. We
227 therefore used bioinformatics and structural comparisons to investigate where
228 polyaminated PG could bind within the AlphaFold2 predicted model of VpOmpM1 SLH
229 domains. Monoderm bacteria attach cell surface proteins to SCWPs using single-
230 chain proteins that have triple tandem SLH domain repeats, which fold into a core
231 three α -helix bundle with additional helices and loops packing against this bundle^{22,46–}
232 ⁴⁹. Available crystal structures of the SLH domains 1-3 of *Bacillus anthracis* surface
233 array protein (Sap) and *Paenibacillus alvei* S-layer protein SpaA are similar to the
234 predicted VpOmpM1 SLH domain^{46,47} (Figure 3b) with C α -C α r.m.s.d values of 1.0
235 and 1.3 Å, respectively. The main difference is that both Sap and SpaA have short
236 core helical bundles, but the VpOmpM1 central bundle extends into the long coiled-
237 coil that connects it to the β -barrels. Structures of SpaA bound to defined SCWP
238 ligands⁴⁷ show that the sugars bind in grooves between SLH repeats (Figure 3b) and

239 interact with conserved motifs therein: conserved tryptophan and glycine residues bind
240 the sugar moiety (W34 and G58 in VpOmpM1), and the arginine of the TRAE motif
241 forms a salt bridge with the ketal-pyruvate modification of the SCWP (residues 63-66,
242 TRYE, in VpOmpM1). It is not surprising that these motifs are conserved in most
243 OmpM-like proteins from diderm Terrabacteria because they are thought to bind
244 pyruvylated SCWP, but not in the Negativicutes (Figure 3c).

245

246 Negativicute OmpM proteins are thought to recognise the polyamine modification on
247 the α -carboxylate of the D-glutamate in the PG peptide stem. Crystal structures of
248 proteins bound to putrescine⁵⁰⁻⁵² and cadaverine^{53,54} indicate that aromatic residues
249 are involved in binding these polyamines via stacking interactions with the aliphatic
250 chain of the polyamine. We next investigated if the SLH domains are different between
251 OmpM and non-OmpM proteins (Figure 3c). We found that the tyrosine residue Y36
252 is highly conserved in the OmpM SLH domains from the Negativicutes and slightly
253 less in the other two lineages of diderm Firmicutes (Limnochordia and
254 Halanaerobiales), but it is not conserved in the SLH domains of OmpM homologues
255 from other diderm Terrabacteria as well as non-OmpM SLH domains (Figure 3c and
256 d). Also, Y65 of the TRYE motif is more conserved in the OmpM SLH domain of
257 Negativicutes than in the other groups of sequences.

258

259 Mapping these residues onto the predicted VpOmpM1 SLH domain structure reveals
260 that the conserved tyrosines are located away from the grooves between the SLH
261 chains that contain the putative PG disaccharide binding site (Figure 3e). We suggest
262 that the peptide stem containing the polyamine moiety specifically in Negativicutes
263 OmpM could extend away from the disaccharide binding groove to interact with either

264 of the conserved tyrosines. Alternatively, or perhaps additionally, PG interaction could
265 induce conformational changes that bring the tyrosine side chains closer to the binding
266 groove.

267

268 **The stalk domain of OmpM may exist in compact and extended conformations**

269 We generated a soluble construct encompassing almost the entire VpOmpM1 stalk
270 region (residues 22-107) in *E. coli* and determined its crystal structure to 1.7 Å (Figure
271 4a, Supplementary Table 2, PDB 8BZ2) in an attempt to verify the AlphaFold2
272 prediction for this region. Surprisingly, the crystallized stalk does not fold into a coiled-
273 coil but forms a compact trimer composed mainly of α -helices (residues 23-105). The
274 protomer interfaces form grooves on one side of the trimer that could potentially
275 accommodate ligands (Figure 4b). However, mapping of the previously identified
276 putative PG-binding residues onto the stalk crystal structure shows they are spread
277 across the structure (Supplementary Fig. 6), suggesting that this conformation does
278 not bind PG. The residues that form the coiled-coil in the extended AlphaFold2
279 prediction instead form helical hairpins and extensive intra-protomer interactions
280 rather than interact with the other chains in the asymmetric unit (Figure 4c). We
281 obtained a very similar structure, albeit at a lower resolution, with a longer construct
282 that encompasses the entire stalk region (residues 22-118). A DALI⁵⁶ search of the
283 PDB using the SLH model (PDB 8BZ2) showed that it has low structural similarity to
284 other proteins in the database (Supplementary Figure 5). The highest ranking hit was
285 a blood group antigen binding adhesin (PDB 5F7L⁵⁷) with a 5.7 Z-score and 2.5 Å C α -
286 C α RMSD.

287

288 The protomer fold of the stalk observed in the crystal structures aligns well with an
289 alternative, compact conformation predicted by AlphaFold2 (Figure 4d and e,
290 Supplementary Fig. 8). This was surprising as the confidence for this prediction was
291 low, yet it agreed with the experimental structure. However, the trimerization interface
292 in the compact stalk prediction is different: residues that are far away in the crystal
293 structure (e.g., M75) interact in the predicted compact conformation (Figure 3e). We
294 considered that this compact state could be an unstable high energy conformation
295 (hence the low prediction confidence), and that the stalk construct is stabilized in a
296 similar conformation in the crystallization condition. Notably, the soluble SLH domain
297 did not bind to *V. parvula* or *E. coli* sacculi (Figure 4f), unlike the full-length VpOmpM1
298 (Figure 3a). This suggests that the structure of the isolated SLH domain is different
299 than in the full-length protein, as observed by cryo-EM. Therefore, we do not believe
300 that the conformation observed in the crystal structure is the result of crystal packing.

301

302 All-atom MD simulation of VpOmpM1 with the compact stalk showed the stalk helix of
303 one protomer reaching towards the OM, partially occluding the β -barrel of another
304 protomer and forming salt bridges with its periplasmic turns (Figure 4g and
305 Supplementary Movie 2). Additionally, this conformation was stabilised by inter-
306 protomer hydrogen bonds in the stalk region (Supplementary Fig. 9). The role of this
307 interaction is unclear, but this simulation supports the dynamic nature of the stalk.

308

309 The two different stalk conformations of VpOmpM1 imply that either there is
310 conformational switching under the appropriate conditions (e.g., cell envelope stress,
311 presence of specific ligands), or that the protein commits to a particular conformation
312 during biogenesis. In one replicate of the all-atom MD simulations of the native

313 VpOmpM1-AlphaFold2 graft model (Figure 2a) we observed unfolding of the very N-
314 terminus of the SLH domain of one chain, which then proceeded to interact with
315 residues of the coiled-coil (Supplementary Movie 3, Supplementary Fig. 10). This
316 suggests some flexibility for the SLH domain, and perhaps *in vivo* this flexibility is
317 important for the SLH domain to find its PG ligand. The coiled-coil, however, remained
318 stable during the simulation. Protein melting temperature analysis by dynamic
319 scanning calorimetry suggested that the melting temperature of the stalk in the full-
320 length VpOmpM1 construct is $\sim 87^{\circ}\text{C}$ (Supplementary Fig. 11), which means that the
321 coiled-coil interaction is very stable and it is unlikely to unfold under physiological
322 conditions. Therefore, the more favourable hypothesis is that the VpOmpM1 trimer is
323 committed to either the extended or compact stalk conformation during biogenesis.

324

325 **OmpM paralogues are the only general diffusion porins in *V. parvula***

326 The β -barrel of VpOmpM1 consists of 16 anti-parallel β -strands connected by
327 extracellular loops and periplasmic turns similar to the *E. coli* porin OmpF (Figure
328 1b,c). Although sequence similarity of VpOmpM1 to *EcOmpF* is only 25%, the overall
329 structure is similar, but with some prominent differences in the extracellular loops
330 (Figure 5a and Supplementary Fig. 12). VpOmpM1 extracellular loops 3 and 7 fold into
331 the β -barrel to form the constriction. In *EcOmpF* and similar Proteobacterial general
332 porins the constriction is formed solely by loop 3 (Figure 5a). The eyelet (the narrowest
333 region of the pore) is formed by sidechains projecting from the β -barrel strands into
334 the lumen as well as sidechain and backbone atoms of the constricting loop residues
335 (Figure 5b). Analysis of the pore radius of experimental structure β -barrels by the
336 HOLE program⁵⁸ reveals a maximal constriction of $\sim 3.7 \text{ \AA}$ (Figure 5c). However, in
337 *EcOmpF* the extent of the constriction ($\pm 0.1 \text{ \AA}$) along the axis perpendicular to the

338 membrane plane is ~ 2.4 Å, and the VpOmpM1 constriction extends for ~ 7 Å. All-atom
339 MD simulations show that the pore constriction in VpOmpM1 is constant due to an
340 extensive interaction network restricting movement of the eyelet loops 3 and 7
341 (Supplementary Figure 13). In agreement with a previous report⁵⁹, we observed that
342 this is not the case in EcOmpF, where fluctuations in loop 3 can completely close the
343 pore.

344

345 An electric field exists across the eyelet of general porins as a result of asymmetric
346 distribution of charged residues^{60,61}, and a similar charge asymmetry is seen in the
347 eyelets of VpOmpM1 and EcOmpF (Figure 5b). We calculated the average dipole
348 moment of water molecules inside the channel during the MD simulations and used
349 this as a proxy for comparing the strength of the transverse electric field inside the two
350 porins. The largest average dipole moment magnitudes are observed in the
351 constriction regions (Supplementary Figure 14), and the direction of the dipole points
352 from the acidic residues towards the basic residues of the eyelet. This analysis shows
353 that the EcOmpF constriction has a stronger localised transverse electric field, while
354 VpOmpM1 has a weaker and more diffuse transverse electric field.

355

356 We tested the ability of VpOmpM1 to transport small molecules across membranes in
357 liposome swelling assays (Figure 6a). Notably, VpOmpM1 could transport lactate,
358 which is essential for *V. parvula* growth, and putrescine, which is used for modification
359 of the PG peptide stem. We also observed transport of the amino acids arginine,
360 lysine, glutamate, aspartate, glycine, alanine, leucine, and methionine. VpOmpM1
361 transported monosaccharides, even though *V. parvula* is asaccharolytic, but not
362 disaccharides, which underlines the non-specific, size-limited nature of transport. We

363 carried out experiments with a VpOmpM1 construct that lacks the stalk to test if the
364 stalk is involved in regulating transport, but this construct had similar transport
365 properties to the full-length protein (Figure 6a). The only exception was impaired
366 transport of arginine and alanine, and the reasons for this are unclear. VpOmpM1 had
367 a similar substrate permeation profile to EcOmpF but showed consistently lower
368 transport rates, which could be the consequence of a longer constriction region or
369 weaker transverse electric field (Figure 5c and Supplementary Figure 14). One notable
370 difference is that there was little transport of ampicillin, kanamycin and gentamicin by
371 VpOmpM1 compared to EcOmpF (Figure 6a), but the relevance of this for antibiotic
372 resistance *in vivo* is unclear.

373

374 We further characterised the channel properties of VpOmpM1 in bilayer
375 electrophysiology experiments. Initially, we obtained conductance value distributions
376 from recordings with multiple channel insertion events (Figure 6b and Supplementary
377 Fig. 15a,b). The resulting distributions were very broad and centred at 4.87 ± 1.86 nS
378 and 4.66 ± 1.88 nS for the full-length and barrel-only constructs of VpOmpM1,
379 respectively. The broad conductance distributions are likely due to multiple sub-
380 conductance states of the channel, as observed in recordings where the channels can
381 randomly close to different extent (Figure 6c and Supplementary Fig. 15c,d). We were
382 able to get more accurate conductance values from single channel recordings: $4.34 \pm$
383 0.46 nS (mean \pm SD, n=8) for full-length VpOmpM1 and 4.37 ± 0.28 nS (mean \pm SD,
384 n=8) for barrel-only VpOmpM1. In single channel recordings at higher voltages, we
385 occasionally observed classical trimeric porin behaviour where the three protomer
386 pores close sequentially (Figure 6c and Supplementary Fig. 15c,d). Using our setup
387 we obtained a conductance of 4.28 ± 0.19 (mean \pm SD, n=9) for EcOmpF, which is

388 similar to previously reported values^{62,63} and to VpOmpM1 conductance. Current-
389 voltage characteristics for both VpOmpM1 constructs and EcOmpF were again very
390 similar (Figure 6d).

391

392 We conclude that VpOmpM1 is a general diffusion channel in *V. parvula*, with similar
393 properties to EcOmpF. We expect that the VpOmpM2-4 paralogues also function as
394 nutrient uptake channels based on their sequence similarity¹⁶. We think it is unlikely
395 that the other two putative porins of *V. parvula*, FNLLGLLA_00518 and
396 FNLLGLLA_00833, also transport nutrients because they are predicted to only have
397 10 strands in their β -barrels (Figure 7a,b). Previous work on 10-stranded β -barrels has
398 shown that their lumen is occluded by amino acid residue sidechains and that they
399 cannot perform major transport roles^{65,66}. To verify this experimentally, we produced
400 the β -barrel region of FNLLGLLA_00518 in sufficient quantities for liposome swelling
401 experiments. It did not transport lactate and arabinose, and could only transport
402 putrescine and glycine at much slower rates than VpOmpM1 and EcOmpF (Figure
403 7c), in agreement with reported transport properties of small OM β -barrels^{67,68}.
404 Therefore, FNLLGLLA_00518 is not a general porin. We were not able to test the
405 transport activity of FNLLGLLA_00833 because the protein could not be expressed in
406 *E. coli*, but we expect this 10 β -strand OM protein to have very low substrate
407 permeation rates as well.

408

409

410 **Discussion**

411 Together, our results show that OmpM has a dual function as an OM tethering system
412 and a nutrient uptake channel. Our structures, simulations and functional data provide
413 insight into how these key OM-related functions are linked in diderm Firmicutes and
414 probably many other diderm Terrabacteria that possess OmpM-like attachment
415 systems and lack the well-characterised tethers present in the Gracilicutes.

416

417 Interestingly, structural data and MD simulations show that the extended stalk of
418 VpOmpM1 is highly mobile. The functional consequences of this mobility are not clear,
419 and neither is it clear why it may be preferable to a rigid connection between the PG
420 and OM. One possibility is that the stalk samples the local environment to find
421 polyaminated-PG. Although one would expect the polyamine modification to be
422 present on most peptide stems, PG spatial organization is not well understood, and it
423 is unclear what proportion of the modified peptide stems is easily accessible to the
424 SLH domains of VpOmpM1. Another possibility is that the flexible interface between
425 the stalk and the β -barrels imparts favourable mechanical properties to the cell
426 envelope, as having a somewhat flexible PG-OM tether would allow the OM to deform
427 when external mechanical forces are applied.

428

429 The combination of structural, functional and bioinformatics data suggests the
430 possibility that the stalk exists in multiple conformations, despite the fact we did not
431 observe any particle populations with a compact stalk in our cryo-EM datasets.
432 Unravelling the roles of both potential states of the stalk is a challenging problem that
433 will likely have to be resolved using *in vivo* studies. Based on structural comparisons
434 with SCWP-binding SLH domains from monoderm bacteria and our PG binding assay

435 results, we can conclude that the extended stalk state observed in cryo-EM
436 reconstructions of VpOmpM1 is the PG-binding state.

437

438 The stalked OmpM-like porins represent a new variation of SLH domains. The three
439 copies of the SLH domain from separate protein chains come together to form three
440 likely identical ligand-binding sites, whereas previously characterised triple SLH
441 domains are encoded in a single chain and their ligand-binding grooves have different
442 affinities for SCWPs due to differences in binding motif residues in each SLH
443 repeat^{47,49,69,70}. The functional consequences of having equivalent or non-equivalent
444 ligand binding sites are not clear in the context of protein anchoring to the PG/SCWP.

445

446 SlpA from *D. radiodurans* is the only other OmpM-like protein for which structures have
447 been determined³⁷⁻³⁹. SlpA, like VpOmpM1, plays an important role in maintaining cell
448 envelope integrity^{16,31}. VpOmpM1 is similar to SlpA in its overall architecture: both
449 proteins are trimers and have an N-terminal SLH domain connected via a coiled coil
450 to a C-terminal β -barrel domain. However, the VpOmpM1 β -barrel trimer is
451 approximately the size of a single SlpA β -barrel (Supplementary Fig. 16).
452 Consequently, the pore inside the SlpA β -barrel, although constricted by extracellular
453 loop insertions, is much larger than in VpOmpM1 and is ~ 14 Å in diameter at the
454 narrowest point. VpOmpM1 clearly imposes a size filter for diffusion across the OM
455 (Figures 5 and 6) and prevents leakage of large periplasmic contents from the cell.
456 SlpA has been shown to transport nutrients³⁸, but it is unclear how escape of
457 periplasmic material via the massive SlpA β -barrel pore is prevented *in vivo*. Other
458 components of the complex and unusual cell envelope characteristics of *D.*
459 *radiodurans* could partially obstruct the SlpA pore^{40,71}. A superoxide dismutase

460 (DR_0644) was observed bound at the top of the stalk and the β -barrel interface in
461 SlpA structures^{37,39}, and there are no homologues of this protein coded in the *V.*
462 *parvula* genome. The authors of one study noted that large (28-30 strand) β -barrels in
463 OmpM-like proteins appear to be confined to the Deinococcus-Thermus phylum³⁷.
464 Thus, the unusual β -barrel structure of SlpA and the superoxide dismutase partner
465 protein might be adaptations of this phylum to resist environmental extremes, such as
466 oxidative stress, radiation, vacuum conditions, and high temperatures⁷².

467

468 VpOmpM1, on the other hand, could be more representative of OmpM proteins in
469 mesophilic diderm Terrabacteria. In fact, the β -barrel of Mep45, the OmpM homologue
470 from the Negativicute *S. ruminantium*, has been shown to form a diffusion channel
471 with similar properties to VpOmpM1, albeit with a larger estimated diameter (11.6 Å)
472 than that observed for VpOmpM1 (7.4 Å)¹⁹. Cyanobacterial OmpM homologues have
473 been shown to form small channels that facilitate transport of inorganic ions²⁰. Our
474 VpOmpM1 structures suggest that OmpM homologues from these and perhaps most
475 diderm Terrabacteria might be similar to proteobacterial porins, unlike *D. radiodurans*
476 SlpA. Our results indeed show that nutrient acquisition and OM tethering are
477 genetically linked via OmpM in *V. parvula* and likely in all Negativicutes and other
478 diderm Terrabacteria.

479

480 In conclusion, these data support the recent hypothesis that OmpM is an ancestral
481 system that may have been present in the last common bacterial ancestor, possibly
482 consolidating two key OM-related functions, tethering and nutrient uptake¹⁶.
483 Therefore, deletion of or mutations in *ompM* may have promoted loss of the OM due
484 to the concurrent loss of these fundamental cell functions. Although we envisage that

485 OmpM plays similar roles in all diderm Terrabacteria, experimental characterisation of
486 OmpM orthologues from diverse members of this clade will be required to fully capture
487 the conservation and variation of this ancient OM tethering system.

488 **Methods**

489 **Bacterial strains, culture conditions and strain manipulation**

490 Bacterial strains used in this work are listed in Supplementary Table 3. *E. coli* strains
491 were genetically manipulated using standard laboratory procedures⁷³. When needed,
492 the following compounds were added to *E. coli* cultures at the following concentrations:
493 ampicillin (liquid media) or ticarcillin (solid media) – 100 mg/l, chloramphenicol – 30
494 mg/l (liquid media) or 25 mg/l (solid media), kanamycin – 50 mg/ml, apramycin – 50
495 mg/l, diaminopimelic acid – 300 µM.

496

497 *V. parvula* was manipulated as described previously^{16,74,75}. When needed, the
498 following compounds were added to *V. parvula* cultures at the following
499 concentrations: chloramphenicol – 25 mg/l, anhydrotetracycline – 250 µg/l. The
500 anaerobic conditions were generated using the GenBag Anaer generator
501 (Biomérieux), or the GP Campus anaerobic chamber (Jacomex). The anaerobic
502 chamber was filled with a H₂/CO₂/N₂ (5%/5%/90%) mixture.

503

504 **Plasmids, primers, and DNA manipulation**

505 All plasmids and primers used in this study are listed in Supplementary Tables 4 and
506 5, respectively. Cloning was performed using either NEBuilder HiFi DNA Assembly
507 Master Mix (New England Biolabs) or standard restriction cloning methods.
508 Chemically competent *E. coli* DH5α or TOP10 cells⁷⁶ were used for transformation of
509 cloning products or plasmids. *V. parvula* genomic DNA was extracted according to a
510 protocol previously described for *Streptomyces* gDNA extraction⁷⁷ from stationary
511 phase cultures in SK medium⁷⁸. PCR reactions for cloning applications were carried
512 out using Phusion HiFi Master Mix (Thermo Fisher Scientific) according to

513 manufacturer's protocol. PCR reactions for the control of constructs were carried out
514 using the DreamTaq Green MasterMix (Thermo Fisher Scientific) or the EmeraldAmp
515 GT PCR Master Mix (Takara Bio). Primers were obtained from Merck or Eurofins
516 Genomics. PCR products were purified using the NucleoSpin Gel and PCR Clean-up
517 kit (Macherey-Nagel). Restriction enzymes were of the FastDigest family of products
518 (Thermo Fisher Scientific). Digestion products were isolated on agarose gels and
519 purified with the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel). Plasmid
520 isolation was performed with NucleoSpin Plasmid kit (Macherey-Nagel). Sequence in
521 silico manipulation was carried out using SnapGene (GSL Biotech,
522 www.snapgene.com) and Geneious (Dotmatics). Primers were designed with
523 NEBuilder (New England Biolabs, nebuilder.neb.com). Construct Sanger sequencing
524 was performed by Eurofins (eurofinsgenomics.eu).

525

526 **Construction of expression vectors**

527 For full length VpOmpM1 expression in *E. coli* we inserted the VpOmpM1 CDS without
528 the signal peptide into the pB22 vector (adding *E. coli* TamB signal peptide and seven
529 histidine residues to the N-terminus). Briefly, we amplified the VpOmpM1 fragment
530 with the JW203/JW202 primer pair using *V. parvula* SKV38 gDNA as a template and
531 cloned it into pB22 digested with XhoI/XbaI, yielding the pJW46 vector. Similarly, the
532 β -barrel portion of VpOmpM1 (pJW45) and FNLLGLLA_00518 (pB22-00518_21-200),
533 and full-length FNLLGLLA_00833 (pB22-00833) were cloned into pB22. The *ompM1*
534 region coding for the stalk (residues 22-107) was amplified from *V. parvula* SKV38
535 gDNA with the primer pair stalk_F/R, digested with NcoI and XhoI, and cloned into
536 pET28b yielding a C-terminal His₆ fusion. For the expression of full length VpOmpM1
537 in *V. parvula*, we inserted the C-terminal His-tagged *ompM1* coding gene containing

538 the native ribosome binding site into pRPF185 using the JW172/JW206 primer pair
539 and *V. parvula* SKV38 gDNA as a template, yielding vector pJW48. The vector was
540 then transferred by conjugation into the $\Delta ompM1-3$ *V. parvula* mutant strain as
541 described previously¹⁶.

542

543 **Protein expression and purification in *E. coli***

544 *E. coli* C43(DE3) $\Delta cyoABCD$ (Cyo complex deletion to improve purity) cells were
545 transformed with a pB22 plasmid carrying either the full-length VpOmpM1 (pJW46),
546 barrel-only VpOmpM1 (pJW45), barrel-only FNLLGLLA_00518 (pB22-00518_21-200)
547 or full-length FNLLGLLA_00833 (pB22-00833). After overnight incubation at 37°C,
548 transformants were picked from Lysogeny Broth (LB)-ampicillin plates and used to
549 inoculate a starter LB-ampicillin culture incubated at 37°C with shaking for 2 h. Flasks
550 with 1 l LB were inoculated with 8-12 ml of the starter culture and incubated at 37°C,
551 160 rpm until OD₆₀₀~0.5-0.6. Protein expression was induced by supplementing the
552 cultures with 0.1% arabinose, followed by a further 3-4 h incubation at 37°C with
553 shaking. Cultures were harvested and cell pellets were stored at -20°C. Cell pellets
554 were thawed, resuspended in cold 20 mM Tris-HCl pH 8.0, 300 mM NaCl (TBS) and
555 supplemented with DNase I. Cells were lysed by passing the cell suspension once
556 through a cell disruptor (Constant Systems) at 23 kpsi. The lysate was clarified by
557 centrifugation at 30,000 x g, 4°C for 30 min. The membranes were isolated from the
558 clarified lysate by ultracentrifugation at 140,000 x g, 4°C for 50 min. The membranes
559 were solubilised in 2.5% Elugent (Millipore) in TBS for 1 h at 4°C. Insoluble material
560 was pelleted by centrifugation at 44,000 x g, 4°C for 30 min. The solubilised fraction
561 was passed through a ~4 ml chelating sepharose column charged with Ni²⁺ ions. The
562 column was washed with 20 column volumes of TBS with 30 mM imidazole and 0.15%

563 lauryldimethylamine oxide (LDAO), and bound protein was eluted with TBS
564 supplemented with 200 mM imidazole and 0.2% decyl maltoside (DM). The eluate was
565 concentrated using an Amicon Ultra filtration device (50 kDa cut-off membrane),
566 loaded on a HiLoad Superdex 200 16/60 column and eluted in 10 mM HEPES-NaOH
567 pH 7.5, 100 mM NaCl, 0.12% DM. Fractions were analysed by SDS-PAGE, pooled,
568 concentrated by filtration (100 kDa cut-off membrane) and flash-frozen in liquid
569 nitrogen. Protein samples were stored at -80°C.

570

571 *E. coli* BL21 (DE3) cells were transformed with the pET28b-SLH_22-107 plasmid for
572 production of the VpOmpM1 stalk region. Expression cultures were set up as above,
573 except kanamycin instead of ampicillin was used in all media, and protein expression
574 was induced by adding 0.4 mM isopropyl β -D-1-thiogalactopyranoside. Protein
575 purification was performed as above, omitting the ultracentrifugation and solubilisation
576 steps, and without detergents in buffers. A 10 kDa cut-off membrane was used for
577 concentrating protein.

578 *E. coli* BL21 (DE3) cells were transformed with the pBAD24-EcOmpF plasmid for
579 production of EcOmpF. Protein expression and purification was performed as for the
580 pB22 constructs up to the membrane isolation stage. Given that pBAD24-EcOmpF
581 doesn't encode a His-tag, inner membranes were selectively solubilised in 20 mM
582 HEPES-NaOH pH 7.5 and 0.5 % (w/v) sodium lauroyl sarcosinate for 30 min at room
583 temperature with stirring. The insoluble fraction containing the outer membranes was
584 recovered by ultracentrifugation for 30 min at 140,000 x g. The sarcosinate wash and
585 ultracentrifugation steps were repeated once. Outer membranes were solubilised in
586 20 mM HEPES-NaOH pH 7.5, 50 mM NaCl and 1.5% LDAO for 1 h at 4°C. The extract
587 was clarified by ultracentrifugation and loaded on a 1 ml ResourceQ anion exchange

588 column (Cytiva). The column was washed and eluted with a 0-500 mM NaCl gradient.
589 Fractions containing EcOmpF were concentrated and subjected to size exclusion
590 chromatography on a Superdex200 10/300 GL column (10 mM HEPES-NaOH pH 7.5,
591 100 mM NaCl, 0.05% LDAO). The protein was further purified on a MonoQ 5/50 GL
592 anion exchange column (Cytiva), and detergent was exchanged to 0.12% DM via a
593 final size exclusion chromatography run.

594

595 **Expression and purification of VpOmpM1 from *V. parvula***

596 15 l of *V. parvula* was grown overnight in anaerobic SK media containing 1.2% sodium
597 lactate and supplemented with 700 µg/l sodium resazurin as an oxygen indicator.
598 Briefly, media was mixed from sterile components, boiled to remove oxygen from the
599 solution (until resazurin appeared colourless) and flushed for 15 minutes with N₂ to
600 remove oxygen from the bottle head volume, before being sealed with a pierceable
601 lid. Anaerobic bottles were inoculated from overnight starter cultures at an OD of 0.04
602 and supplemented with 25 mg/l chloramphenicol and 250 µg/l anhydrotetracycline.
603 Cultures were grown at 37°C overnight with 180 rpm agitation. Cells were harvested
604 by centrifugation at 8,000 x g for 10 minutes. Pellets were pooled to represent 1.5 l of
605 original culture each and resuspended in 5 ml HEPES pH 7.4. 100 µl 3.5x10⁴ U/ml
606 benzonase and a small spatula of lysozyme was added and sample was incubated on
607 ice for 15 minutes. Cells were lysed by two rounds of French press and the debris was
608 pelleted by centrifugation for 90 minutes at 15,000 x g at 4°C. The supernatant was
609 carefully transferred to an ultracentrifuge tube containing 2 ml of a 50% sucrose
610 cushion and centrifuged for 4 hours at 35,000 x g. The volume at the sucrose
611 supernatant interface was extracted, resuspended in 20 ml of HEPES pH 7.4 and

612 pelleted at 35,000 x g for 4 hours. Pelleted membranes were frozen in liquid nitrogen
613 and stored at -80°C.

614

615 *V. parvula* membrane solubilization and the protein purification procedure was the
616 same as for VpOmpM1 expressed in *E. coli*, except that the flow-through from the
617 chelating sepharose column was put through the column again due to poor binding of
618 protein of interest to the resin. The final size exclusion chromatography step was done
619 on a Superdex 200 10/300 Increase column.

620

621 **Cryo-EM structure determination**

622 Purified VpOmpM1 from *E. coli* (11.6 mg/ml) or *V. parvula* (8 mg/ml) was applied to
623 glow discharged Quantifoil R 1.2/1.3 copper 200 mesh holey carbon grids. The grids
624 were immediately blotted and plunge-frozen in liquid ethane using a Vitrobot Mark IV
625 (ThermoFisher Scientific) device operating at 4°C and 100% humidity. Data were
626 collected on a FEI Glacios microscope operating at 200 kV using a Falcon 4 direct
627 electron detector (ThermoFisher Scientific) at the University of York (Supplementary
628 Table 1). A total of 4,284 and 6,505 movies with the *E. coli* and *V. parvula* samples,
629 respectively, were recorded in electron event representation (EER) mode at 240,000
630 magnification, corresponding to a pixel size of 0.574 Å.

631

632 All image processing was done in cryoSPARC v3.3.2^{79,80}. Data processing workflows
633 are depicted in Supplementary Fig. 2. Movies were motion corrected using patch
634 motion correction, and CTF parameters were estimated using patch CTF estimation.
635 For VpOmpM1 expressed in *E. coli*, 2D classes generated from manually picked
636 particles were used for template-based picking. 838,051 particles were extracted in

637 600 pixel boxes and Fourier cropped to 300 pixel boxes corresponding to a pixel size
638 of 1.148 Å. Three rounds of 2D classification were used to discard bad particles,
639 followed by generation of an ab initio 3D map using a stochastic gradient descent
640 algorithm with 3 classes and 145,518 particles. All three classes were very similar,
641 and a single class was used as a template in non-uniform refinement with the whole
642 particle stack either without symmetry (C1) or with C3 symmetry enforced, with per-
643 particle defocus and CTF group parameter (beam tilt and trefoil) refinement enabled.
644 The 145,518 particle stack was subjected to heterogeneous refinements against the
645 C1 map from non-uniform refinement and three decoy templates, or against the C3
646 map and the same three decoy templates. Particles were re-extracted in 600 pixel
647 boxes with a pixel size of 0.574 Å. The final particle stacks (96,280 particles for the
648 C1 map; 119,001 particles for the C3 map) were subjected to non-uniform refinement
649 either with C1 or C3 symmetry enforced. The native VpOmpM1 dataset was processed
650 similarly, except that two rounds of 2D classification were performed, four classes
651 were used to make the ab initio map, four decoy templates were used in
652 heterogeneous refinement, and the pixel size remained 1.148 Å throughout. Local
653 resolution estimates, gold-standard Fourier shell correlations, and particle angular
654 distribution plots for all maps are shown in Supplementary Fig. 3.

655

656 The final B-factor-sharpened maps from non-uniform refinement were used to build
657 the models. A de novo model was built into the C3 map using Buccaneer⁸¹ (part of
658 CCPEM package⁸²), followed by cycles of manual building in COOT⁸³ and real space
659 refinement in Phenix⁸⁴. The C3 model was then docked into the C1 map, manually
660 extended in COOT and real-space-refined. The model built into the C1 map of
661 VpOmpM1 expressed in *E. coli* was docked into the native VpOmpM1 C1 map,

662 manually adjusted in COOT and real-space-refined. Models were validated using
663 MolProbity⁸⁵. Model refinement statistics are shown in Supplementary Table 1, and
664 representative map-to-model fits are shown in Supplementary Fig. 4.

665

666 **Crystal structure determination**

667 The purified VpOmpM1 stalk construct was concentrated to ~20 mg/ml. Sitting drop
668 vapour diffusion crystallisation screens were set up using a Mosquito robot (SPT
669 Labtech). Crystals grew in 0.1 M citric acid pH 3.5, 2.0 M ammonium sulphate at 20°C.
670 Crystals were cryo-protected in mother liquor supplemented with ~20% PEG400 and
671 flash-cooled in liquid nitrogen. Diffraction data were collected at the synchrotron
672 beamline I03 at Diamond Light Source (UK) at a temperature of 100 K (Supplementary
673 Table 2). The dataset was processed with XIA2⁸⁶, scaled with Aimless⁸⁷, and the
674 space group was confirmed with Pointless⁸⁸. Data quality was evaluated in Xtriage⁸⁴.
675 Arcimboldo⁸⁹, part of the CCP4i2 suite⁹⁰, was used for ab initio phasing. The initial
676 model was extended by Buccaneer and subjected to cycles of manual building and
677 refinement in Phenix⁸⁴ (Supplementary Table 2).

678

679 **Isolation of sacculi**

680 Peptidoglycan was isolated using the protocol of Wheeler et al.⁹¹. Briefly, cells were
681 grown to exponential phase, harvested and boiled for one hour in 4% SDS with
682 agitation. The resulting insoluble sacculi were isolated by ultracentrifugation at
683 150,000 x g for 2 h. The pellet was then washed by 8 rounds of resuspension in milliQ
684 H₂O and ultracentrifugation. Proteins were removed from the sample by incubating the
685 cell pellet with 100 µg/ml trypsin in digestion buffer (50 mM Tris-HCl pH 7.0, 10 mM
686 CaCl₂) overnight at 37°C with agitation. To inactivate and remove the trypsin, sacculi

687 were again boiled in 4% SDS and washed to remove residual SDS. Sacculi were then
688 resuspended in MiliQ H₂O and lyophilized in preweighed Eppendorf tubes.

689

690 **Peptidoglycan binding assay**

691 Dry *V. parvula* and *E. coli* sacculi were resuspended in binding assay buffer (10 mM
692 HEPES-NaOH pH 7.5, 150 mM NaCl) at 20 mg/ml. Sacculi suspensions were
693 sonicated for 10 min in an ultrasonic bath. Binding assays with full-length and barrel-
694 only VpOmpM1 were carried out in 200 µl aliquots, each containing binding assay
695 buffer with 1 mg/ml sacculus suspension, ~12 µg protein, and 0.12% DM. Binding
696 reactions were incubated at 20°C for 2 h. The sacculi were pelleted by centrifugation
697 in a benchtop microfuge (21,000 x g) for 15 min at 8°C. The supernatant was saved
698 (the soluble fraction), the pellet was resuspended in binding buffer, and the sacculi
699 were pelleted again. The second supernatant (the wash fraction) was saved, and the
700 pellet was resuspended in binding buffer (the pellet fraction). Samples of the soluble,
701 wash and pellet fractions were boiled in SDS loading buffer and analysed by SDS-
702 PAGE on 12% FastCast gels (BioRad). The SLH domain binding assay was carried
703 out with the following modifications. 36 µg of protein was used and the detergent was
704 omitted. The resuspended pellet fraction was split into two 100 µl aliquots, and one
705 aliquot was supplemented with 25 U mutanolysin (Sigma) and incubated at 37°C for
706 30 min. The mutanolysin digestion was included to ensure that no SLH domain that
707 could be bound to PG was prevented from entering the gel due to insolubility of the
708 sacculi. The SLH binding assay was analysed by SDS-PAGE using pre-cast Bis-Tris
709 4-12% gels (Invitrogen) in MES running buffer.

710

711 **Computational Modelling & Simulation**

712 **VpOmpM1 model building.** Computational models of trimeric full-length VpOmpM1
713 were predicted using AlphaFold2⁴⁵. Two unique structures were predicted: one with
714 an extended coiled-coil stalk, and one with a compacted stalk region (Supplementary
715 Fig. 6). The AlphaFold2 predicted structure with the extended stalk displays no tilt of
716 the stalk relative to the z-axis, in contrast to the reconstructed cryo-EM density. To
717 generate a full-length VpOmpM1 structure with a tilted stalk consistent with the
718 experimental data, the extended stalk of the AlphaFold2 prediction was fit into the low
719 resolution cryo-EM density and grafted to the well-resolved experimental structure
720 using ChimeraX⁹².

721

722 **System generation.** Three different models of VpOmpM1 were used to build protein-
723 membrane systems: full-length VpOmpM1 with the grafted extended stalk; full-length
724 VpOmpM1 with the compact stalk (AlphaFold2 predicted structure); and truncated
725 VpOmpM1 from the reconstructed cryo-EM density (from residue L100 onwards). For
726 each protein model, the β -barrel domain of the VpOmpM1 trimer was embedded in a
727 model *Escherichia coli* outer membrane using the CHARMM-GUI Membrane Builder
728 module⁹³. The inner leaflet consisted of 90% 1-palmitoyl-2-oleoyl-sn-glycero-3-
729 phosphoethanolamine (POPE), 5% 1-palmitoyl-2-oleoyl-sn-glycero-3-
730 phosphoglycerol (POPG), and 5% 1',3'-bis[1-palmitoyl-2-oleoyl-sn-glycero-3-
731 phospho]glycerol (cardiolipin), and the outer leaflet consisted of 100% LPS (R1 core,
732 5 x O1 O-antigen units). This asymmetric bilayer system was solvated in 150 mM KCl,
733 with additional calcium ions to neutralise the LPS headgroups (~115 mM in each
734 system). The system composition is detailed in Supplementary Table 7.

735

736 **Atomistic Molecular Dynamics Simulations.** All simulations used the
737 CHARMM36m forcefield⁹⁴ with the TIP3P water model⁹⁵. Simulations were carried out
738 using the GROMACS simulation package (version 2021.2)⁹⁶. For all simulation steps,
739 a cut-off of 1.2 nm was applied to Lennard-Jones interactions and short-range
740 electrostatics using the potential shift Verlet scheme. Long-range electrostatics were
741 treated using the particle mesh-Ewald (PME) method⁹⁷. Atoms were constrained using
742 the LINCS algorithm to allow the use of a 2 fs timestep in NPT equilibration and
743 production phases⁹⁸. The three systems were energy minimised in 5000 steps using
744 the steepest descent method⁹⁹. The subsequent systems were equilibrated in six
745 phases (two NVT and four NPT phases) in which the protein and lipid headgroups
746 were subjected to position restraints with varying force constants (Supplementary
747 Table 6). All equilibration phases used the Berendsen thermostat¹⁰⁰ to bring the
748 system to 303.15 K (coupling constant of 1.0 ps). NPT equilibration phases used semi-
749 isotropic Berendsen pressure coupling scheme¹⁰⁰ to equilibrate with a pressure bath
750 of 1 bar ($\tau_p = 5.0$ ps, compressibility of 4.5×10^{-5} bar⁻¹).

751

752 These equilibrated systems were then simulated. The extended stalk system was
753 simulated in duplicate for 1 μ s, and the compacted and truncated stalk systems were
754 each simulated as a single replicate for 1 μ s. These production simulations utilised the
755 Nosé-Hoover thermostat¹⁰¹ (coupling constant of 1.0 ps) and the semi-isotropic
756 Parrinello-Rahman barostat¹⁰² ($\tau_p = 5.0$ ps, compressibility of 4.5×10^{-5} bar⁻¹).
757 Trajectories were analysed using GROMACS tools and MDAnalysis utilities^{103,104}.
758 Water orientation was analysed using a locally-written python script (available with
759 deposited data on Zenodo). Molecular graphics and supplementary animations were
760 generated using VMD (version 1.9.4a51)¹⁰⁵ and Molywood (version 0.22)¹⁰⁶.

761

762 **OmpF Simulation.** To compare the dynamics of the constriction regions, EcOmpF
763 was also simulated. The crystal structure of trimeric EcOmpF (PDB ID: 2OMF⁴²) was
764 embedded in a model *E. coli* outer membrane and solvated using CHARMM-GUI, then
765 equilibrated as described for the VpOmpM1 systems above. The system composition
766 is shown in Supplementary Table 8. This system was simulated once for 500 ns under
767 the same conditions as the above VpOmpM1 simulations.

768

769 **Bilayer electrophysiology**

770 Electrophysiology measurements were carried out in a custom-made cuvette at the
771 centre of which was suspended a 25 μm thick Teflon film (Goodfellow Cambridge Ltd)
772 with a 80-100 μm aperture and 1.25 ml electrode chambers either side of the film. The
773 Teflon film aperture was made lipophilic by painting with 1.5-3 μl 1% hexadecane in
774 hexane solution on both sides, and the hexane was allowed to evaporate for at least
775 30 min. Electrophysiology buffer (10 mM HEPES-KOH pH 7.5, 1 M KCl) was added to
776 both cuvette chambers, and 3 μl of 5 mg/ml 1,2-diphytanoyl-sn-glycero-3-
777 phosphocholine (DPhPC) dissolved in *n*-pentane was added. The *n*-pentane was
778 allowed to evaporate for at least 5 min. DPhPC bilayers were formed using the method
779 of Montal and Mueller¹⁰⁷. Pure, concentrated protein (7-15 mg/ml) was serially diluted
780 in 1% Genapol X-100 (Sigma). 0.3-0.5 μl of diluted protein was added to the *cis*-
781 (ground side) chamber, and diluted with electrophysiology buffer as required to
782 promote protein insertion into the bilayer. Protein insertion events were detected as
783 sudden jumps in current in constant-voltage mode. All measurements were carried out
784 using Ag/AgCl pellet electrodes attached to an Axopatch 200B amplifier headstage,
785 and a Digidata 1440A digitiser. The cuvette and headstage were enclosed in a custom-

786 made Faraday shield during recording. Clampex software was used for recording.
787 Clampfit software was used to analyse data.

788

789 **Liposome swelling assays**

790 Liposome swelling assays were carried out using the method of Nikaido and
791 Rosenberg¹⁰⁸ with modifications. Lipid solution (4 mg/ml phosphatidylcholine and 0.46
792 mg/ml dihexadecyl phosphate in chloroform) was dried under an air stream, and
793 completely dried under vacuum for at least 2 h. 80 µl of lipid solution was used per
794 condition, *i.e.* per protein to be reconstituted. The dried lipids were resuspended in
795 100 µl deionised water per condition. Equimolar amounts of protein were added to the
796 resuspended lipid so that the total protein quantity was 15-30 µg, followed by
797 immediate vortexing. All conditions were supplemented with DM containing buffer to
798 ensure that equal amounts of detergent were present in all samples. DM-containing
799 buffer was added to control liposomes. Solutions were sonicated in a water bath for 1-
800 1.5 min until translucent, and dried under vacuum overnight. The following day the
801 proteoliposomes were rehydrated in 200 µl 10 mM HEPES-NaOH pH 7.5 and 12 mM
802 stachyose per condition for 1-2 h at room temperature. Control liposomes were used
803 to determine the concentration of each substrate that is isosmotic to the intraliposomal
804 milieu (*i.e.* there is no swelling of the control liposomes), usually between 7.5 and 15
805 mM substrate in 10 mM HEPES-NaOH pH 7.5. Substrate concentrations used can be
806 found in Supplementary Table 9. The decrease in A_{400} due to liposome swelling was
807 measured after adding 200 µl of the substrate solution to 15 µl of the proteoliposome
808 suspension. Readings were taken on a Perkin Elmer Lambda 35 spectrophotometer
809 in 1 s intervals for 30 s. A line was fit to the absorbance data corresponding to the
810 linear phase of swelling (2-15 s), and the slope of the line was recorded as the swelling

811 rate. Control liposome swelling rates were subtracted from all proteoliposome swelling
812 rates. All data were normalized to EcOmpF-containing liposome swelling in the
813 presence of arabinose.

814

815

816 **Data availability**

817 Electron microscopy maps have been deposited in the Electron Microscopy Data Bank
818 with the accession codes EMD-16328 [[https://www.ebi.ac.uk/pdbe/entry/emdb/EMD-](https://www.ebi.ac.uk/pdbe/entry/emdb/EMD-16328)
819 16328] (VpOmpM1 from *E. coli* in C1), EMD-16333
820 [<https://www.ebi.ac.uk/pdbe/entry/emdb/EMD-16333>] (VpOmpM1 from *E. coli* in C3)
821 and EMD-16332 [<https://www.ebi.ac.uk/pdbe/entry/emdb/EMD-16332>] (VpOmpM1
822 from *V. parvula* in C1). Atomic coordinates have been deposited in the Protein Data
823 Bank under accession codes 8BYM [<https://doi.org/10.2210/pdb8BYM/pdb>]
824 (VpOmpM1 from *E. coli* in C1), 8BYT [<https://doi.org/10.2210/pdb8BYT/pdb>]
825 (VpOmpM1 from *E. coli* in C3), 8BYS [<https://doi.org/10.2210/pdb8BYS/pdb>]
826 (VpOmpM1 from *V. parvula* in C1), and 8BZ2 [<https://doi.org/10.2210/pdb8BZ2/pdb>]
827 (VpOmpM1 stalk crystal structure). The following atomic coordinates used for
828 comparison purposes were downloaded from the Protein Data Bank:

829 3PYW [<https://doi.org/10.2210/pdb3PYW/pdb>], 6CWH
830 [<https://doi.org/10.2210/pdb6CWH/pdb>], 3POQ
831 [<https://doi.org/10.2210/pdb3POQ/pdb>], 5F7L [<https://doi.org/10.2210/pdb5F7L/pdb>],
832 2OMF [<https://doi.org/10.2210/pdb2OMF/pdb>], 2ZFG
833 [<https://doi.org/10.2210/pdb2ZFG/pdb>], 8AGD [<https://doi.org/10.2210/pdb8AGDpdb>].

834 Trajectories and run input files for the Molecular Dynamics simulations are available
835 on Zenodo (<https://doi.org/10.5281/zenodo.8239075>). Source data for
836 electrophysiology, liposome swelling assays, and HOLE and MD analyses are
837 available. Other data presented in this paper, constructs and strains are available on
838 reasonable request.

839

840 **Code availability**

841 The water orientation analysis script is available on Zenodo
842 (<https://doi.org/10.5281/zenodo.8239075>).

843

844

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1124

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1142

1143 **Author contributions**

1144 A.S. made constructs, purified proteins, determined cryo-EM and crystal structures,
1145 carried out functional assays, and wrote the manuscript with input from all authors.
1146 Y.Z. purified proteins and carried out functional assays. J.W. made constructs and
1147 strains and prepared the dataset for ConSurf analysis. R.E.S. grew *V. parvula*,
1148 prepared membrane pellets, and isolated sacculi. K.E.N. performed simulations,
1149 supervised by S.K. S.P.B. collected preliminary electrophysiology data. A.B. collected

1150 X-ray diffraction data and managed the Newcastle Structural Biology Laboratory.

1151 B.v.d.B. made constructs, purified and crystallised proteins. S.G., C.B. and B.v.d.B.

1152 conceived and supervised the project.

1153

1154 **Competing Interests**

1155 The authors declare no competing interests.

1156

1157 Figure Legends

1158

1159 **Figure 1. Cryo-EM structures of VpOmpM1 expressed in *E. coli* and *V. parvula*.**
1160 **a** Schematic depicting VpOmpM1 (UniProt A0A100YN03) domain arrangement and
1161 boundaries. SP, signal peptide; SLH, S-layer homology; CC, coiled coil. Generated
1162 using IBS 2.0⁴⁴. **b** Structure of recombinant VpOmpM1. The cryo-EM density is shown
1163 on the left and the model is shown on the right as viewed towards the OM from outside
1164 the cell. **c** Representative 2D class averages. White arrowheads point to the diffuse
1165 SLH domain density. The edge of each square is 344.4 Å long. **d** Side view of the
1166 cryo-EM density at high (left) and low (right) contour level. The grey bar represents
1167 the outer membrane (OM). The diffuse grey density around the membrane region in
1168 the high contour view likely corresponds to lipid or detergent. **e** Superposition of
1169 structures from recombinant VpOmpM1 (in colour) and native VpOmpM1 purified from
1170 *V. parvula* (white). **f** Superposition of coiled-coils of protomers from the recombinant
1171 (yellow, pink, blue) and native (grey, white, black) VpOmpM1 structures. The structural
1172 alignment was performed on the β -barrel region only (not shown). Left – side view,
1173 right – view from the periplasm towards the OM. **g, h** Inter-protomer contacts at the β -
1174 barrel-stalk interface in the recombinant (**g**) and native (**h**) structures. The protomer
1175 (A, B, C) for each sidechain is indicated in superscript.

1176

1177 **Figure 2. All-atom molecular dynamics simulations with native VpOmpM1.** **a** The
1178 AlphaFold2 prediction for the stalk (grey) was grafted onto the N-terminus of the first
1179 residue (L100) modelled into the experimental density of native VpOmpM1 (in colour).
1180 R64 (bottom of SLH domain) and R113 are shown as space filling models. **b** Bird's-
1181 eye-view of the β -barrel region from outside the OM (left) and a plot of the stalk end
1182 projection (centre of mass of the R64 C α atoms) over the 1 μ s simulation (right). The
1183 observation point in the left and right panels is equivalent. **c** Frequency distribution of
1184 stalk lengths (R64-R113 C α -C α) observed throughout the simulation. **d** Distances
1185 between the R113 sidechain and interacting sidechain atoms throughout the
1186 simulation. The dotted line denotes an inter-atomic distance of 3 Å. The equivalent
1187 plots for a replicate simulation and a simulation without the AlphaFold2 graft are
1188 presented in Supplementary Fig. 5.

1189

1190 **Figure 3. Analysis of the putative peptidoglycan binding site within the SLH**
1191 **domain.** **a** Binding assay of recombinant full-length and barrel-only VpOmpM1 to
1192 sacculi (PG) isolated from *V. parvula* and *E. coli*. After incubation of protein with PG,
1193 the insoluble PG was pelleted by centrifugation, washed by resuspending in buffer,
1194 and pelleted again. The supernatant from the binding reaction (S), the wash (W) and
1195 the resuspended PG pellet (P) were boiled and analysed by SDS-PAGE. Three
1196 independent binding experiments were performed and yielded similar results. **b** Close
1197 up view of the VparOmpM1 SLH domains predicted by AlphaFold2, the crystal
1198 structure of SLH domains 1-3 from *Bacillus anthracis* Sap (surface array protein) (PDB
1199 3PYW)⁴⁶, and the crystal structure of SLH domains 1-3 from *Paenibacillus alvei* SpaA
1200 in complex with the monosaccharide 4,6-pyr- β -D-ManNAcOMe (space-filling
1201 representation) (PDB 6CWH)⁴⁷. Views were generated from a superposition. **c**
1202 Multiple sequence alignment results from different subsets of SLH domain-containing
1203 proteins mapped onto the sequence of VpOmpM1 SLH domain and coloured by
1204 conservation using ConSurf⁵⁵ (colour key). Row 1 - OmpM homologues from
1205 Negativicutes; row 2 – representative OmpM homologues from all diderm Firmicutes;
1206 row 3 – representative OmpM homologues from diderm Terrabacteria, excluding

1207 Firmicutes; row 4 – SLH domains from non-OmpM from diderm Firmicutes. The arrows
1208 point to Y36 and Y65, which could be important for binding polyaminated PG. **d**
1209 ConSurf results mapped onto the predicted SLH domains of VpOmpM1 (same
1210 sequence subsets and colour key as **b**). Arrows point to the grooves between SLH
1211 protomers. Y36 is shown in space-filling representation. The datasets used for
1212 ConSurf analysis correspond to the datasets used in a previous study¹⁶ that were
1213 subsampled using custom scripts. **e** Conserved residues shown as stick models on
1214 the predicted SLH structure, coloured by chain, except for Y36 and Y65 which are in
1215 purple. SpaA bound to monosaccharide (PDB 6CWH) was superposed onto the
1216 VpOmpM1 SLH structure; the monosaccharide is in grey, the SpaA protein model is
1217 not shown.
1218

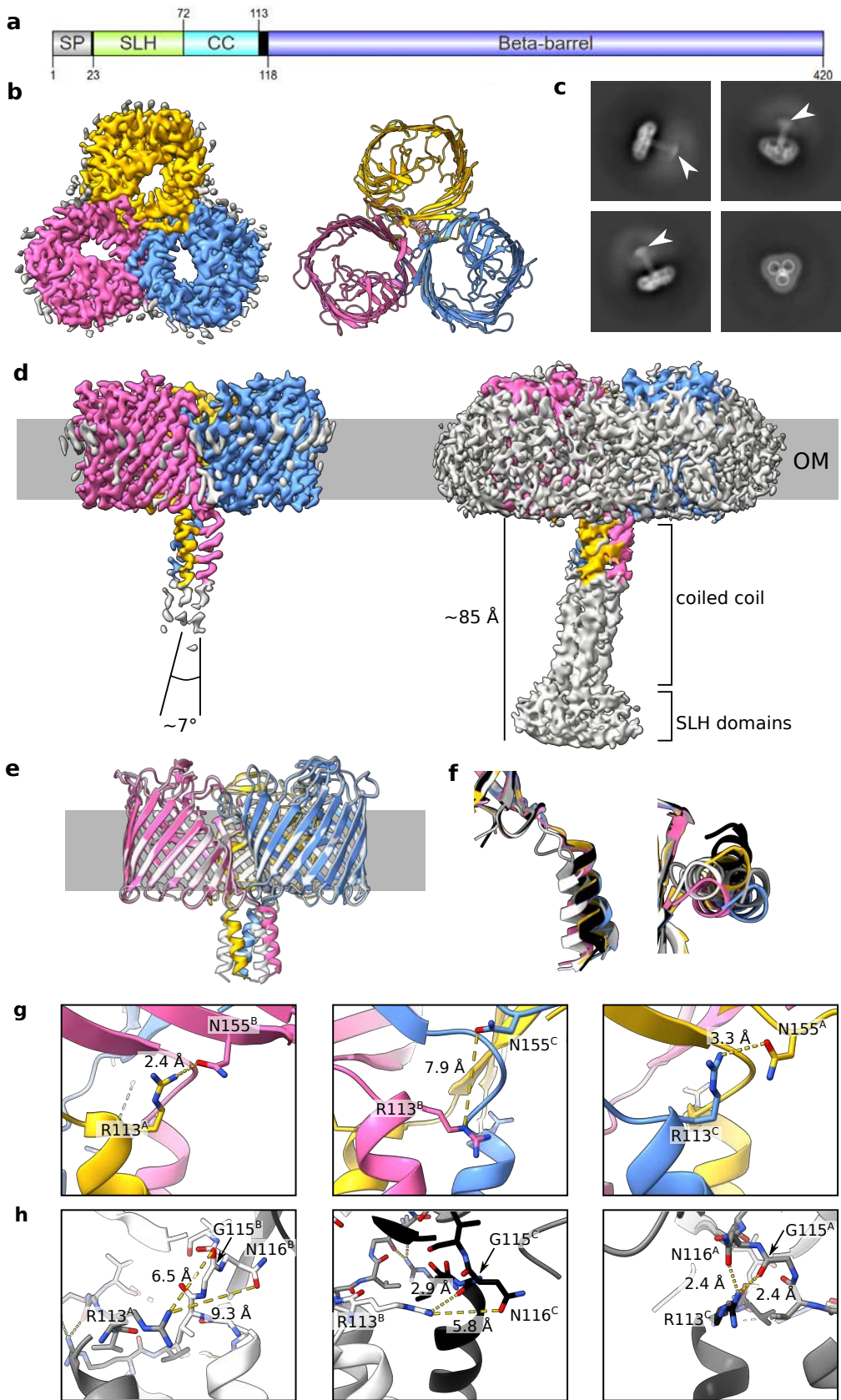
1219 **Figure 4. Crystallography, AlphaFold2, and binding assay show evidence of**
1220 **alternate stalk conformations.** **a** Crystal structure of VpOmpM1 stalk trimer at 1.7 Å.
1221 Residues 23-105 were resolved. One chain is in rainbow: N-terminus is blue, C-
1222 terminus is red. **b** Electrostatic surface presentation of the stalk crystal structure. (Left)
1223 Putative view from the OM and (right) from the periplasm. **c** Comparison of the stalk
1224 crystal structure (left) and AlphaFold2 predicted extended conformation (middle). Only
1225 residues 23-105 are displayed for both. (Right) Residues 64-105 of the stalk crystal
1226 structure that form the extended coiled-coil in the AlphaFold2 prediction are in grey. **d**
1227 Alternative, compact stalk conformation predicted by AlphaFold2. **e** Close up view of
1228 the compact stalk prediction with a single chain from the crystal structure (black)
1229 superposed ($C\alpha$ - $C\alpha$ r.m.s.d. 0.78 Å) (left). (Middle) Top view down the section marked
1230 by the dashed line. (Right) Stalk crystal structure. The sidechain of M75 is shown in
1231 green space-filling representation in each panel. **f** Binding assay of recombinant
1232 VpOmpM1 SLH domain to sacculi (PG) isolated from *V. parvula* and *E. coli*. After
1233 incubation of protein with PG, the insoluble PG was pelleted by centrifugation, washed
1234 by resuspending in buffer, and pelleted again. The PG was then resuspended and split
1235 into two aliquots, one of which was incubated with mutanolysin (Mut). The supernatant
1236 from the binding reaction (S), the wash (W) and the resuspended PG pellet without
1237 (P) and with Mut treatment (P_{Mut}) were boiled and analysed by SDS-PAGE. Three
1238 independent binding experiments were performed and yielded similar results. **g** All-
1239 atom MD simulation of AlphaFold2 predicted compact stalk model over 1 μ s. The SLH
1240 domain of one of the protomers (pink) reaches towards the OM and interacts with the
1241 periplasmic turns of the β -barrel of another protomer (blue). The salt bridges shown
1242 on the right had occupancies of 5.8%, 3.44% and 1.89% (top to bottom) throughout
1243 the simulation.
1244

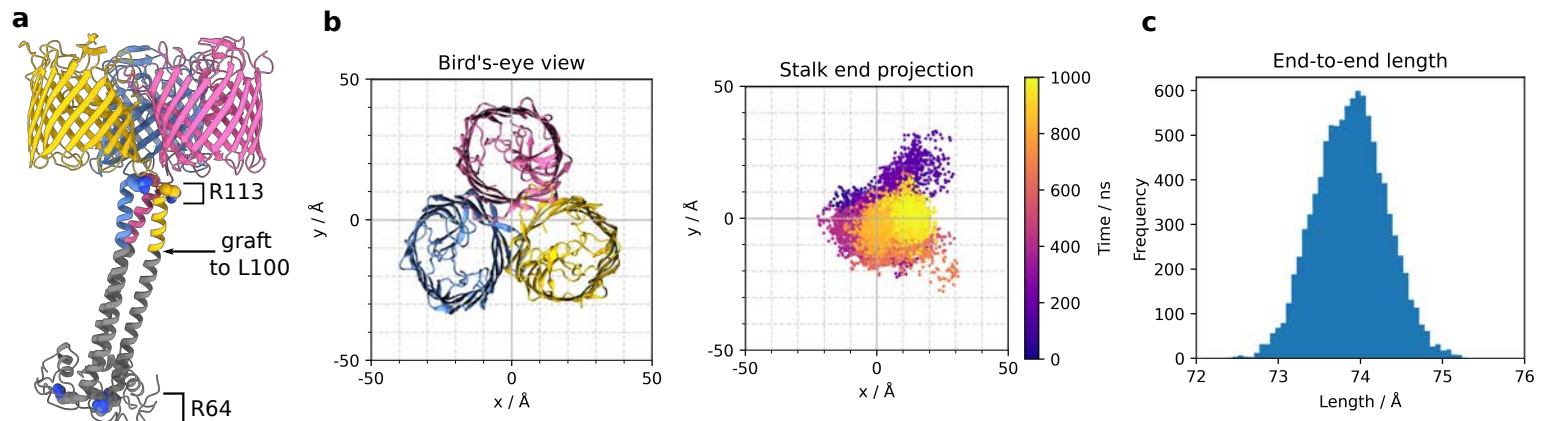
1245 **Figure 5. VpOmpM1 and EcOmpF eyelet comparison.** **a** β -barrel from the C3 cryo-
1246 EM reconstruction of VpOmpM1 (top) and an EcOmpF protomer (PDB 3POQ)⁶⁴
1247 (bottom). The views are generated from a superposition. VpOmpM1 loops 3 and 7 are
1248 in green and orange, respectively; EcOmpF loop 3 is in black. Residues forming the
1249 eyelet are shown in stick representation. **b** Close-up view of the eyelet regions of
1250 VpOmpM1 (top) and EcOmpF (bottom). Colours as in **a**. Positively and negatively
1251 charged residues lining the eyelet are annotated with a blue plus or red minus sign,
1252 respectively. **c** HOLE⁵⁸ profiles calculated from aligned experimental structures of
1253 VpOmpM1 β -barrel and EcOmpF. The z-coordinate is perpendicular to the membrane
1254 plane and its origin is at the narrowest part of the VpOmpM1 eyelet.
1255

1256 **Figure 6. VpOmpM1 channel properties.** **a** Liposome swelling in the presence of
1257 indicated substrate. Liposomes with embedded full-length VpOmpM1, VpOmpM1
1258 barrel-only or EcOmpF were tested. Swelling rates were normalised to the rate of
1259 swelling of EcOmpF-containing liposomes in the presence of arabinose. Each
1260 condition was measured in technical triplicates (except EcOmpF with fructose, where
1261 $n=2$) from the same proteoliposome preparation. Error bars show mean \pm SD. **b**
1262 Bilayer electrophysiology conductance distribution plots obtained from recordings with
1263 multiple insertion events (see Supplementary Fig. 15 for representative traces). Full-
1264 length VpOmpM1 distribution is centred on 4.87 ± 1.86 nS (SD, $n=117$); VpOmpM1
1265 barrel-only distribution is centred on 4.66 ± 1.88 nS (SD, $n=269$). **c** Representative
1266 bilayer electrophysiology recordings of full-length VpOmpM1 (top) showing multiple
1267 sub-conductance states and of the barrel-only construct (bottom) showing classical
1268 trimeric porin sequential channel closure at high voltage. **d** Current-voltage
1269 characteristics from single channel recordings of full-length VpOmpM1 ($n=4$), barrel-
1270 only VpOmpM1 ($n=4$), and EcOmpF ($n=3$). Individual data points are shown as open
1271 black circles and means as coloured dots. The error bars show the SD.

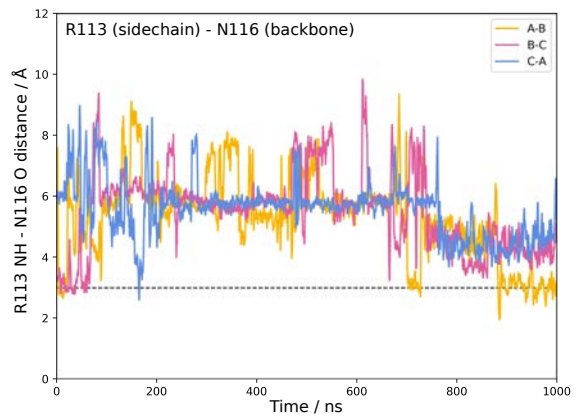
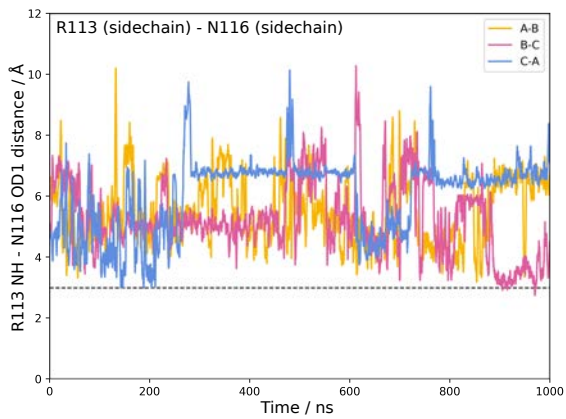
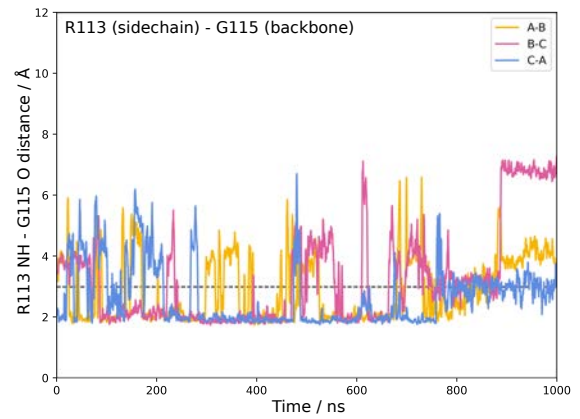
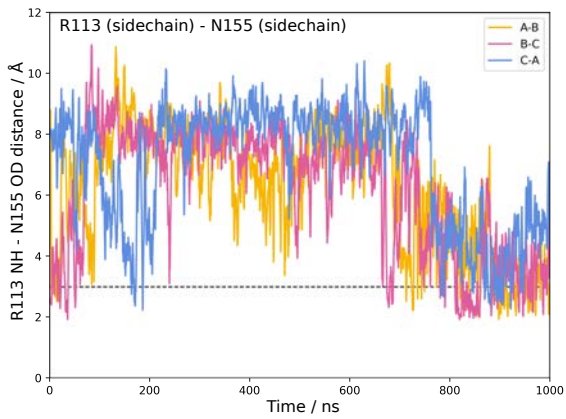
1272
1273 **Figure 7. *V. parvula* OM proteins other than OmpM paralogues are unlikely to be**
1274 **general diffusion channels.** **a** AlphaFold²⁴⁵ models of the FNLLGLLA_00518
1275 (OmpA-like) and FNLLGLLA_00833 proteins. The colours represent the per residue
1276 confidence of the prediction (pLDDT). **b** Molecular surface models of *V. parvula* OM
1277 proteins and EcOmpF as viewed from outside the cell. **c** Comparative liposome
1278 swelling assays with FNLLGLLA_00518 β -barrel, VpOmpM1 β -barrel, and EcOmpF.
1279 Each condition was measured in technical triplicates from the same proteoliposome
1280 preparation. Bars show mean \pm SD.

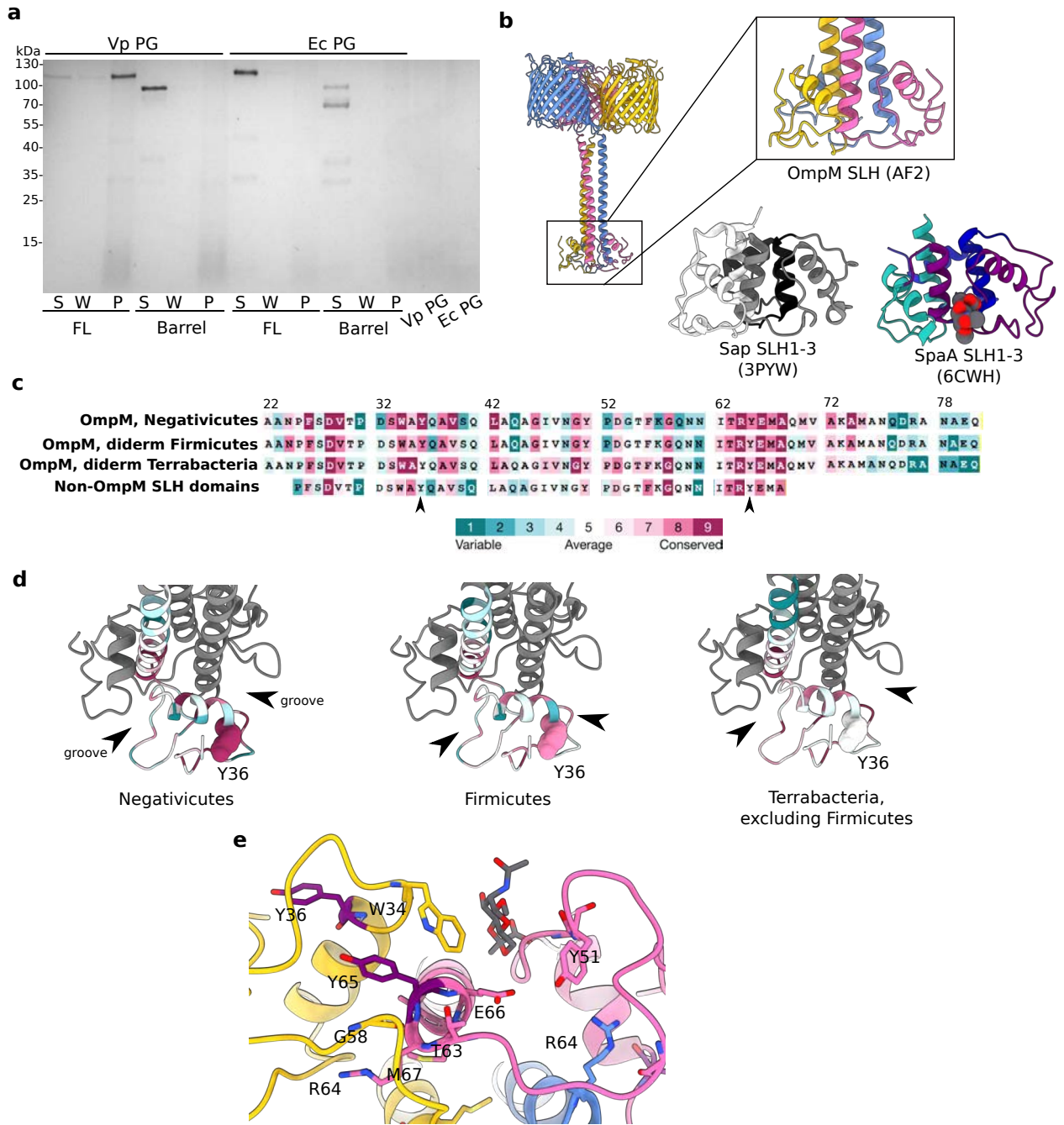
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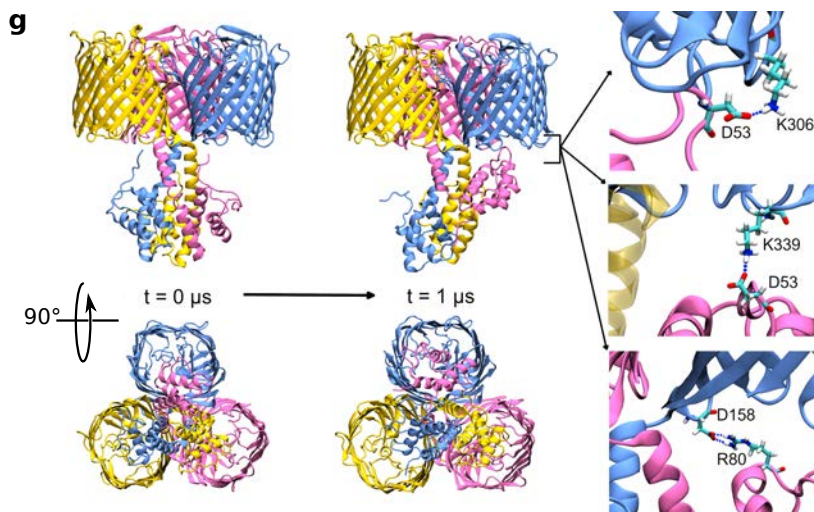
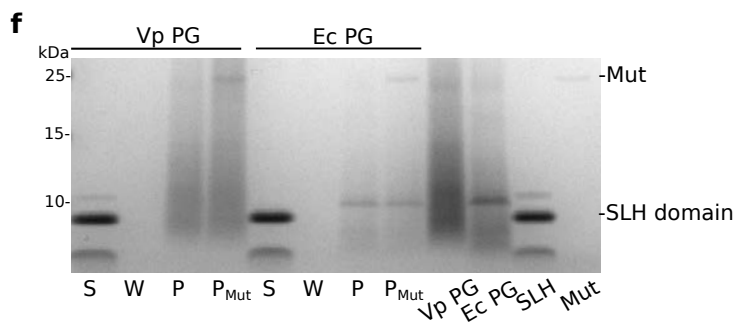
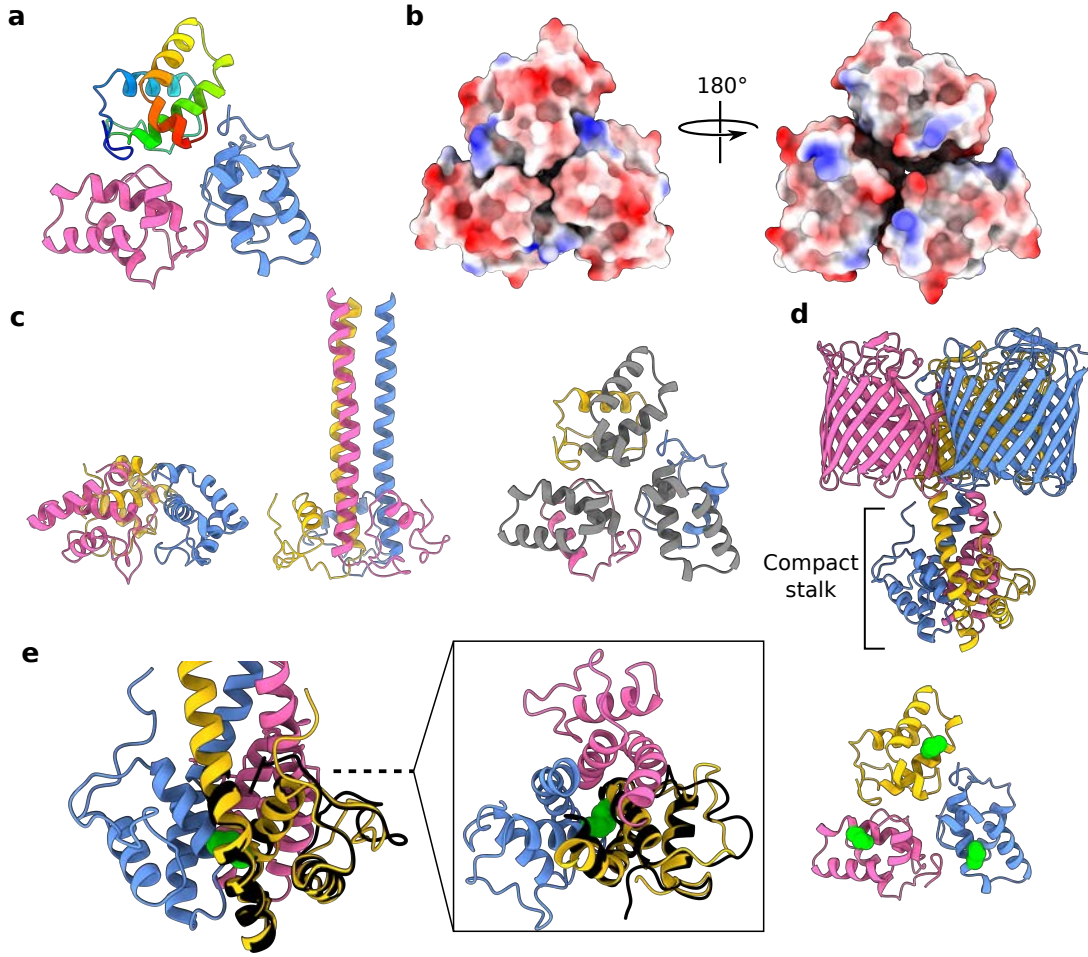


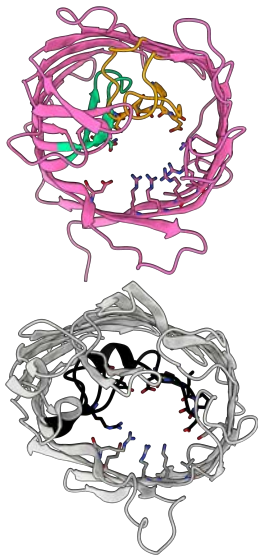
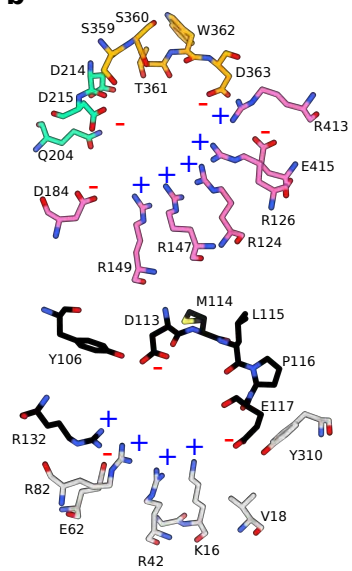
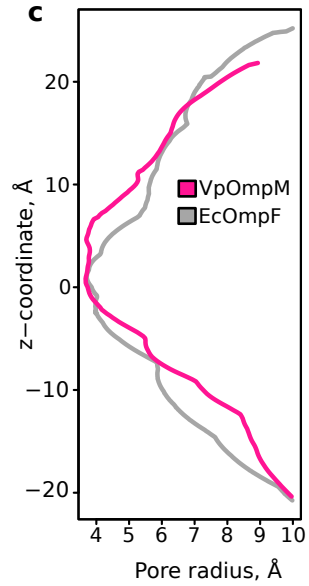


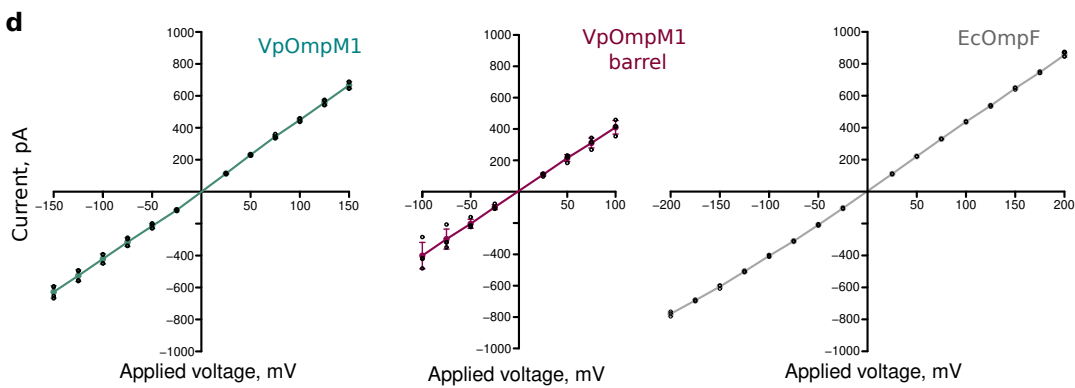
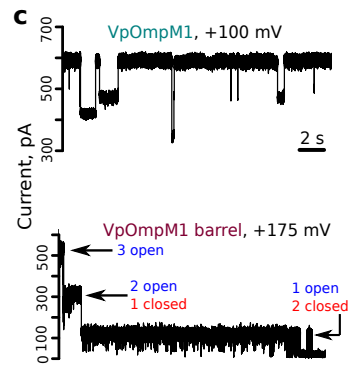
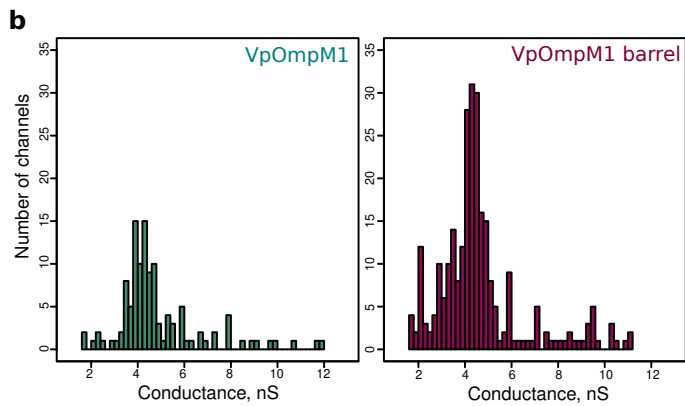
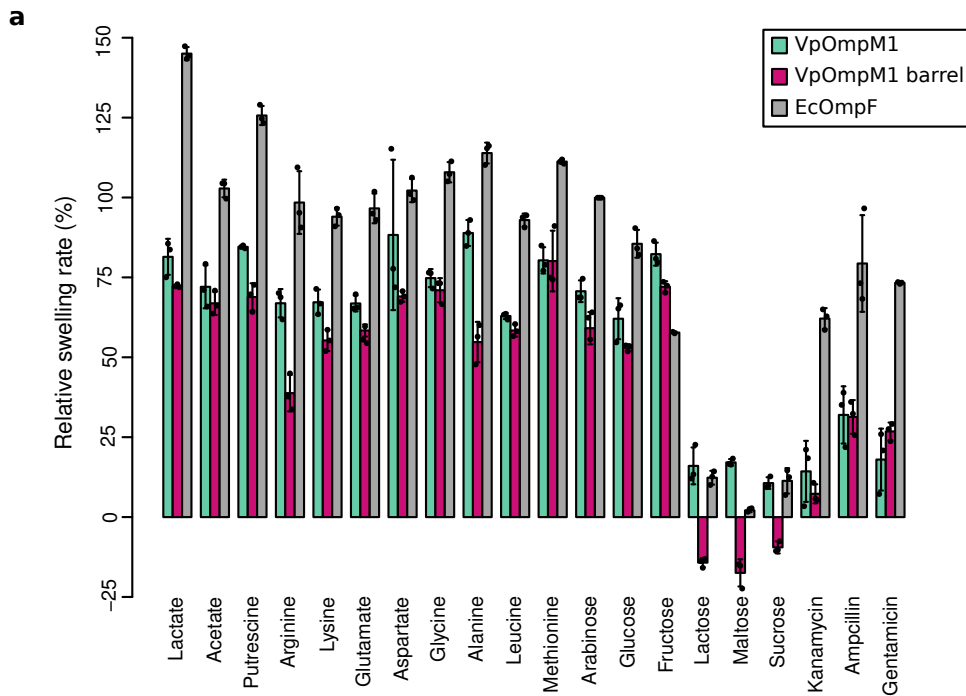
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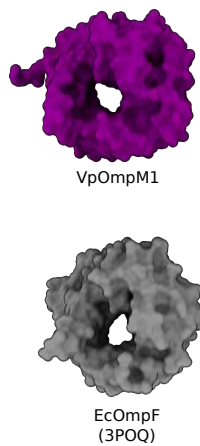
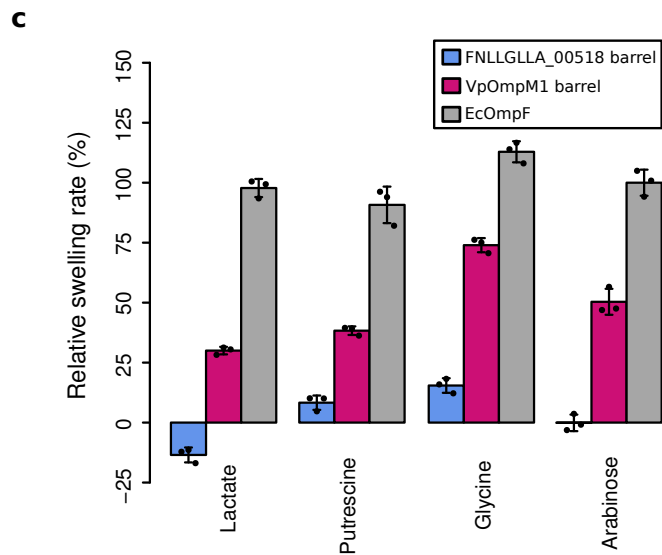
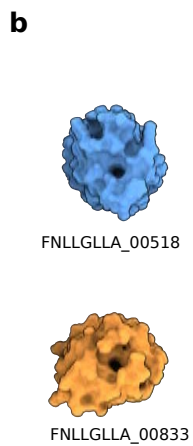
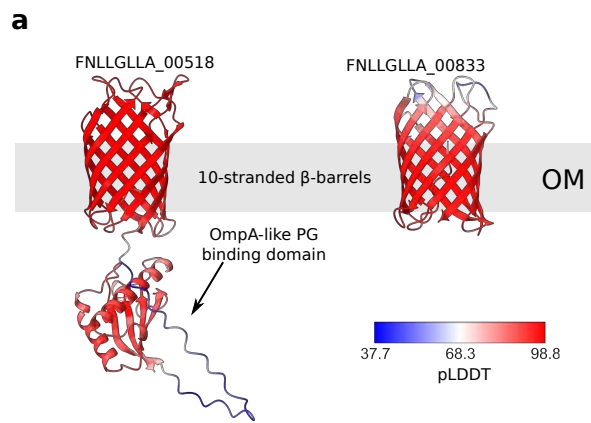


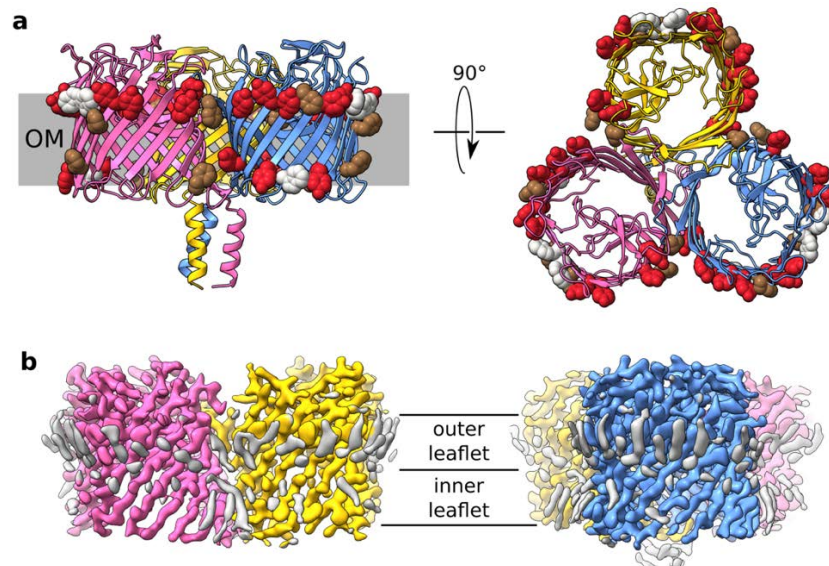




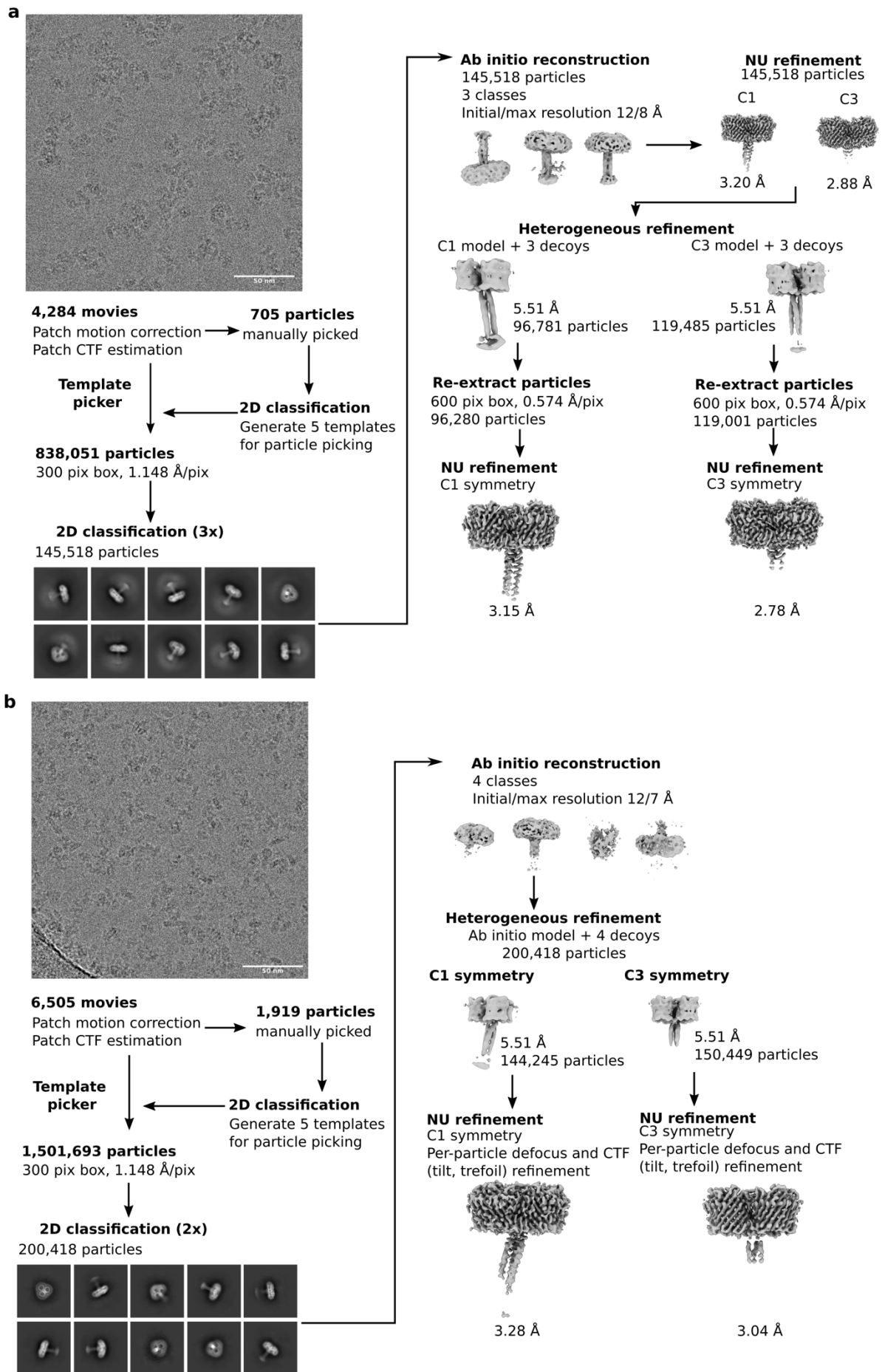
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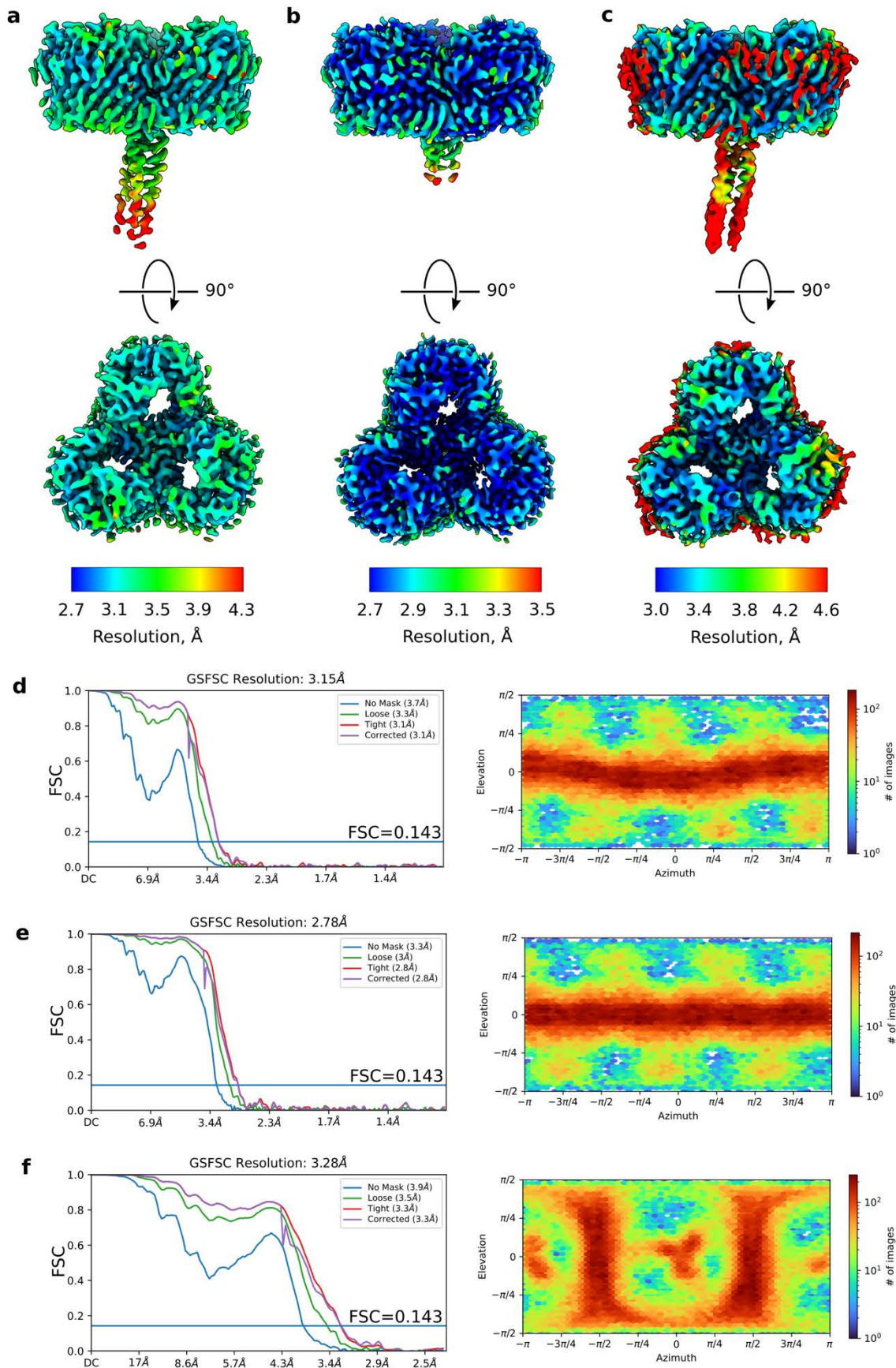




Supplementary Figure 1. VpOmpM1 aromatic girdle and putative lipid density. **a** Aromatic residues forming the aromatic girdle shown in space-filling representation on the cartoon of C1 VpOmpM1 reconstruction. Brown, phenylalanine; red, tyrosine; grey, tryptophan. **b** Cryo-EM density of the C3 VpOmpM1 reconstruction. Protein density coloured by chain; grey, putative lipid or detergent density.

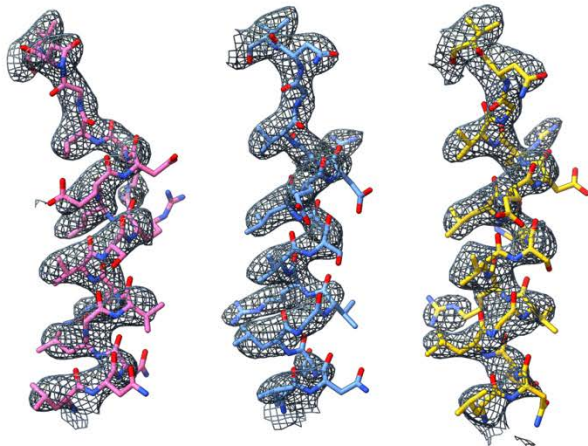


Supplementary Figure 2. Cryo-EM data processing. Cryo-EM data processing workflows for VpOmpM1 expressed in *E. coli* (a) and purified from *V. parvula* (b), showing representative motion-corrected movies, 2D class averages, and intermediate and final cryo-EM maps. A total of 4,284 and 6,505 movies were collected of grids with VpOmpM1 expressed in *E. coli* and *V. parvula*, respectively.



Supplementary Figure 3. Cryo-EM map local resolution estimates, FSC curves, and angular distribution plots. **a, d** VpOmpM1 purified from *E. coli*, C1 symmetry. **b, e** VpOmpM1 purified from *E. coli*, C3 symmetry. **c, f** VpOmpM1 purified from *V. parvula*, C1 symmetry.

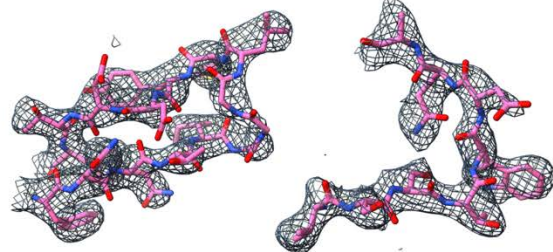
a VpOmpM1 from *E. coli*, C1 symmetry



Chain A, $\alpha 1$

Chain B, $\alpha 1$

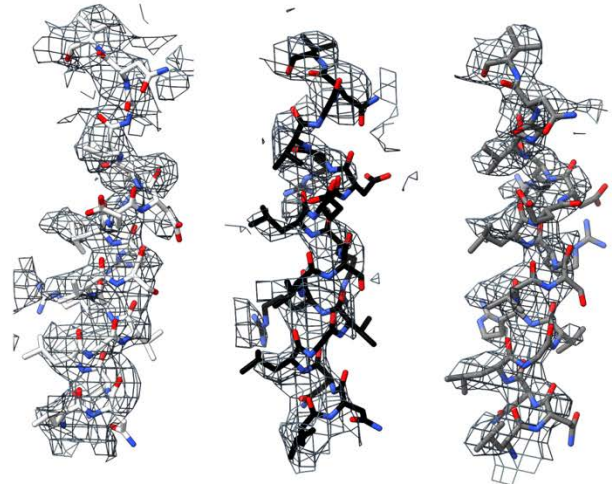
Chain C, $\alpha 1$



Chain A, L3

Chain A, L7

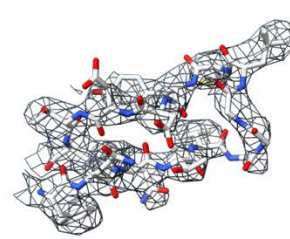
b VpOmpM1 from *V. parvula*, C1 symmetry



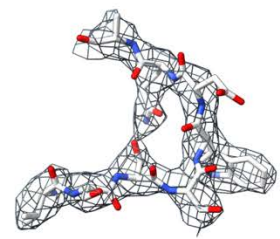
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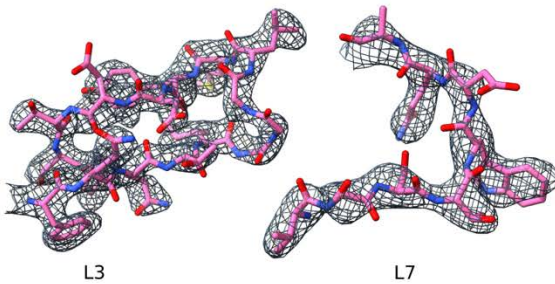


Chain A, L3



Chain A, L7

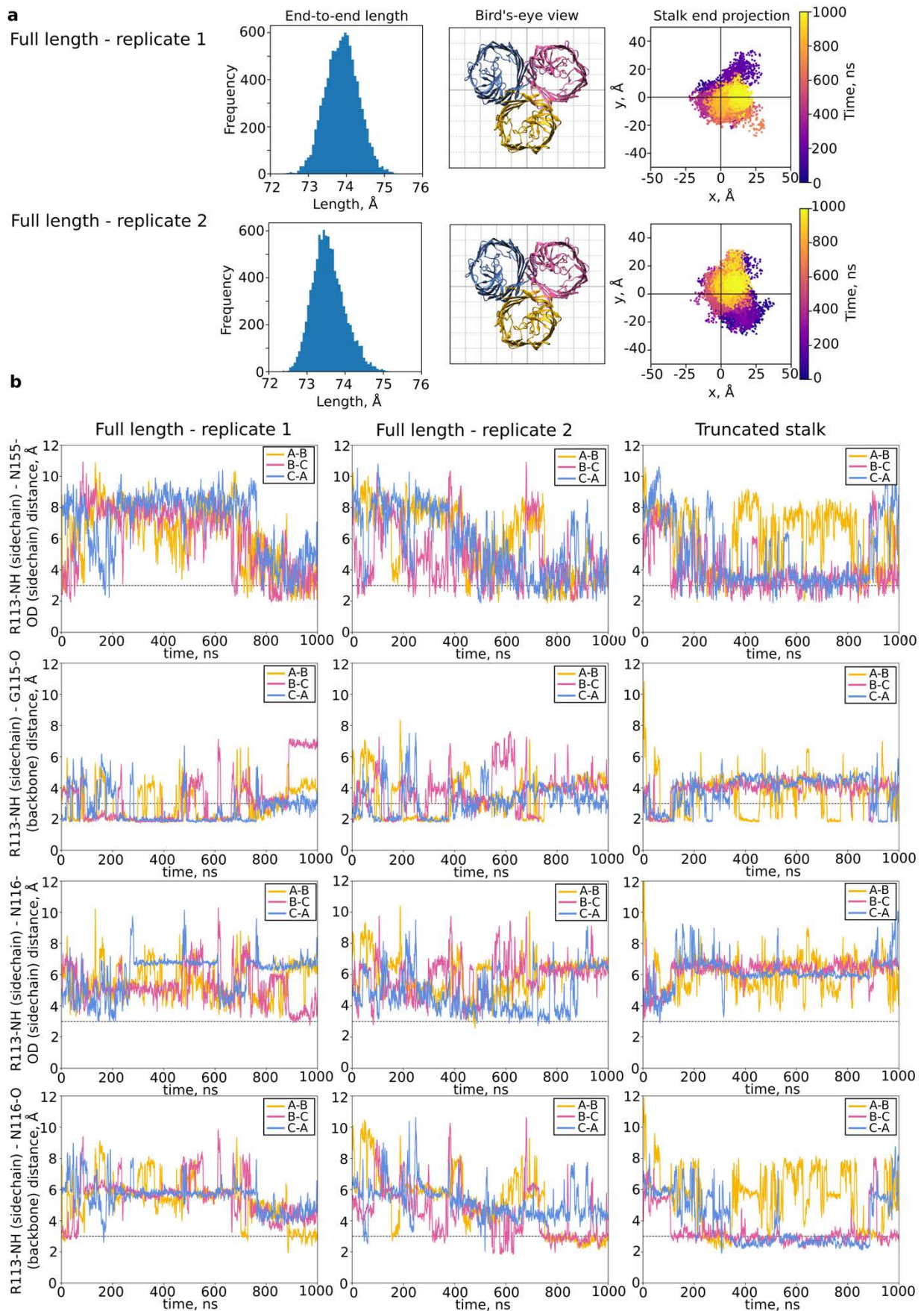
c VpOmpM1 from *E. coli*, C3 symmetry



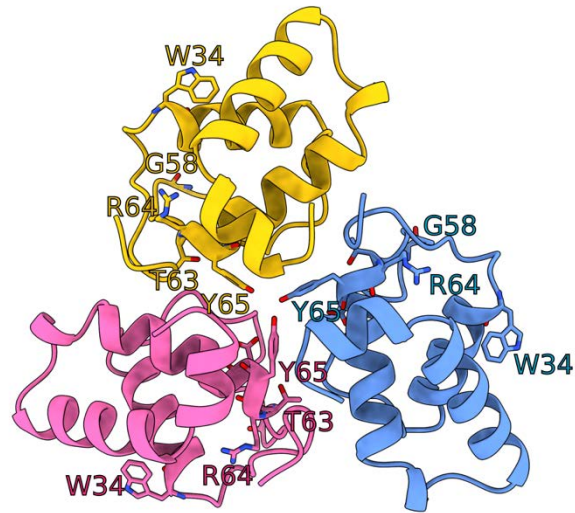
L3

L7

Supplementary Figure 4. Cryo-EM representative map-to-model fits. Densities and models for the stalk coiled-coil helix for each protomer and the eyelet loops 3 and 7 from a single protomer are shown for VpOmpM1 purified from *E. coli* (a) and from *V. parvula* (b) in C1 symmetry. Only the eyelet model fit for VpOmpM1 purified from *E. coli* with applied C3 symmetry is shown (c), as it was not possible to build a model into the stalk density in the symmetrized map. Protein models are shown in stick representation. Cryo-EM density is represented by a mesh.



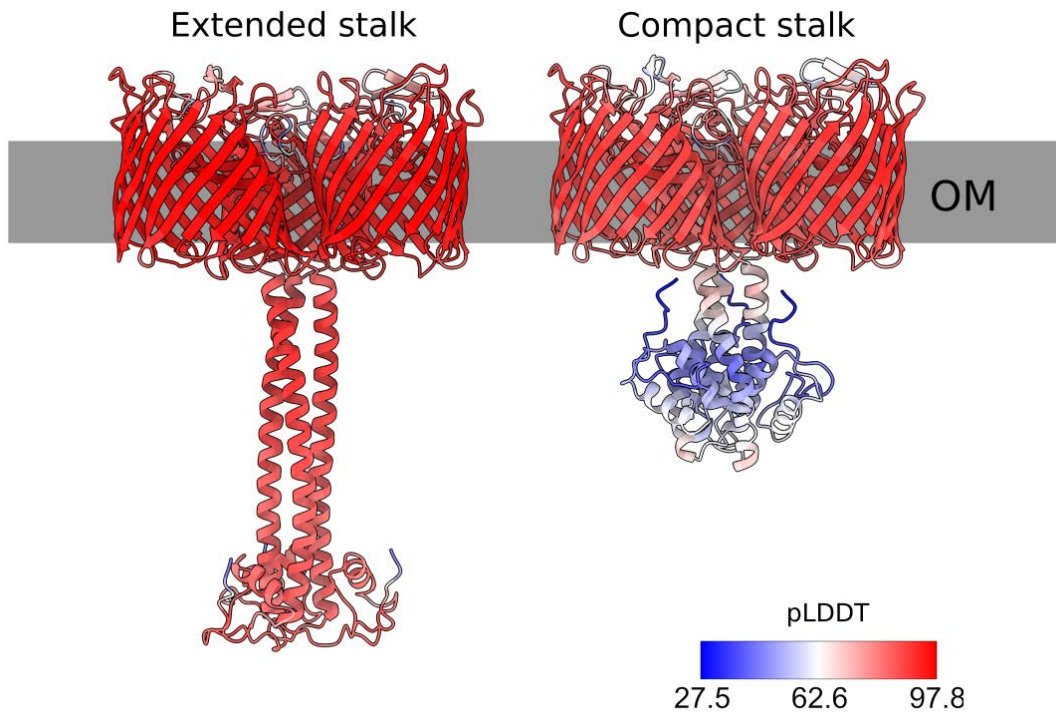
Supplementary Figure 5. All-atom molecular dynamics simulation replicates. **a** Stalk length and end projection plots for the two replicates of all-atom simulations with the native VpOmpM1 and AlphaFold2 graft model (full-length). The bird's-eye view coordinates correspond to the plot coordinates in the stalk projection plot. **b** Hydrogen bond distances throughout the simulation. The 'truncated stalk' simulation was performed with the native VpOmpM1 model without the AlphaFold2 graft. Data for replicate 1 shown in this figure are the same as the data shown in Figure 2.



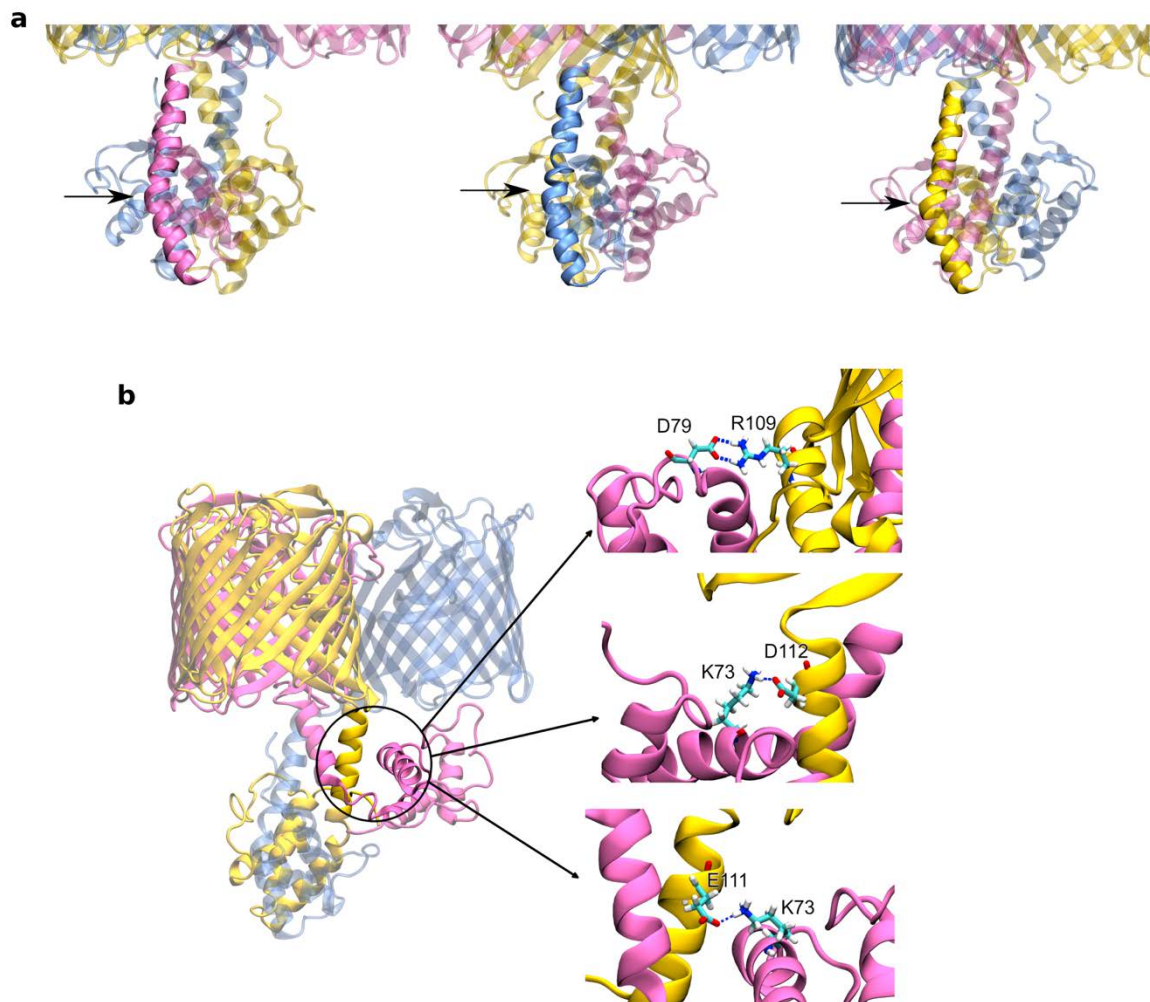
Supplementary Figure 6. Putative PG-binding motifs in the SLH crystal structure. Residues that are part of motifs conserved in SLH domains are shown in stick representation. Motif residues are not located in intra-protomer grooves as seen in crystal structures of SLH domains from monoderm Firmicutes and in the extended conformation of the VpOmpM1 stalk predicted by AlphaFold2 (Figure 3).

Rank	Chain	Z	rmsd	lali	nres	%id	Description
1	5f7l-C	5.7	2.5	57	375	11	Blood group antigen binding adhesin BabA
2	6bml-B	5.2	3.3	67	294	12	Human palmitoyltransferase DHHC20
3	6fws-B	5.2	3.3	68	683	4	Helicase DinG
4	6gmm-A	5.2	2.9	61	433	11	Adhesin LabA
5	6fws-A	5.0	2.7	65	686	8	Helicase DinG
6	7khn-A	4.8	3.3	68	290	12	Human palmitoyltransferase DHHC20
7	5f7w-A	4.8	2.7	60	419	10	Blood group antigen binding adhesin BabA
8	5f7y-A	4.8	3.2	62	417	10	Blood group antigen binding adhesin BabA
9	5has-A	4.7	3.2	64	381	5	DCB-HUS domain of Sec7
10	5f8q-A	4.7	2.4	56	419	11	Blood group antigen binding adhesin BabA

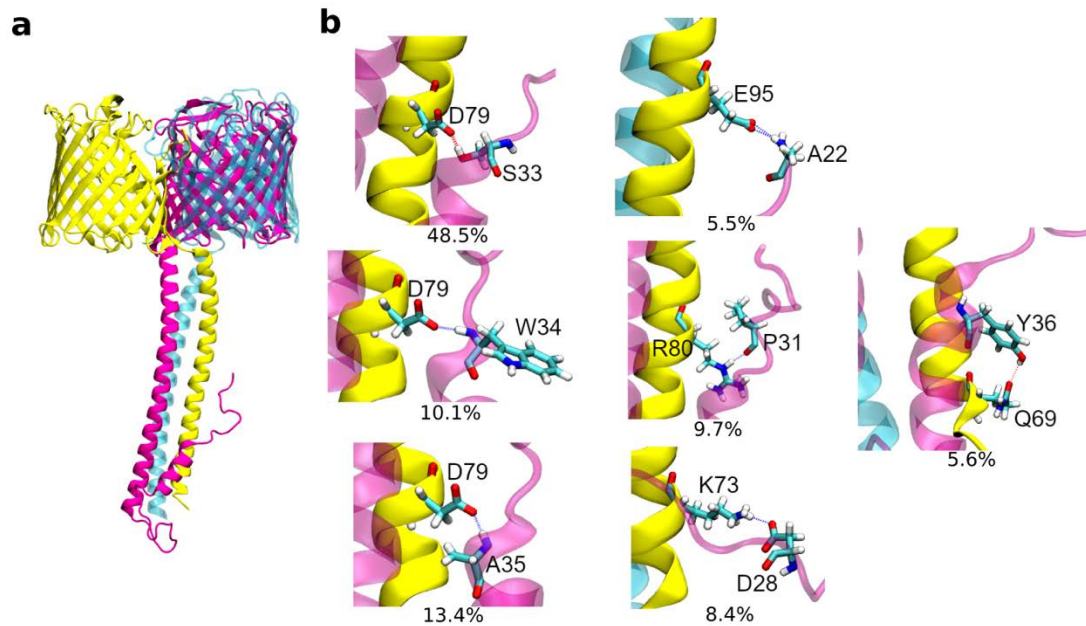
Supplementary Figure 7. SLH domain crystal structure DALI search results. DALI¹ analysis shows that the VpOmpM1 SLH domain crystal structure (PDB 8BZ2) has low similarity to other proteins from the PDB.



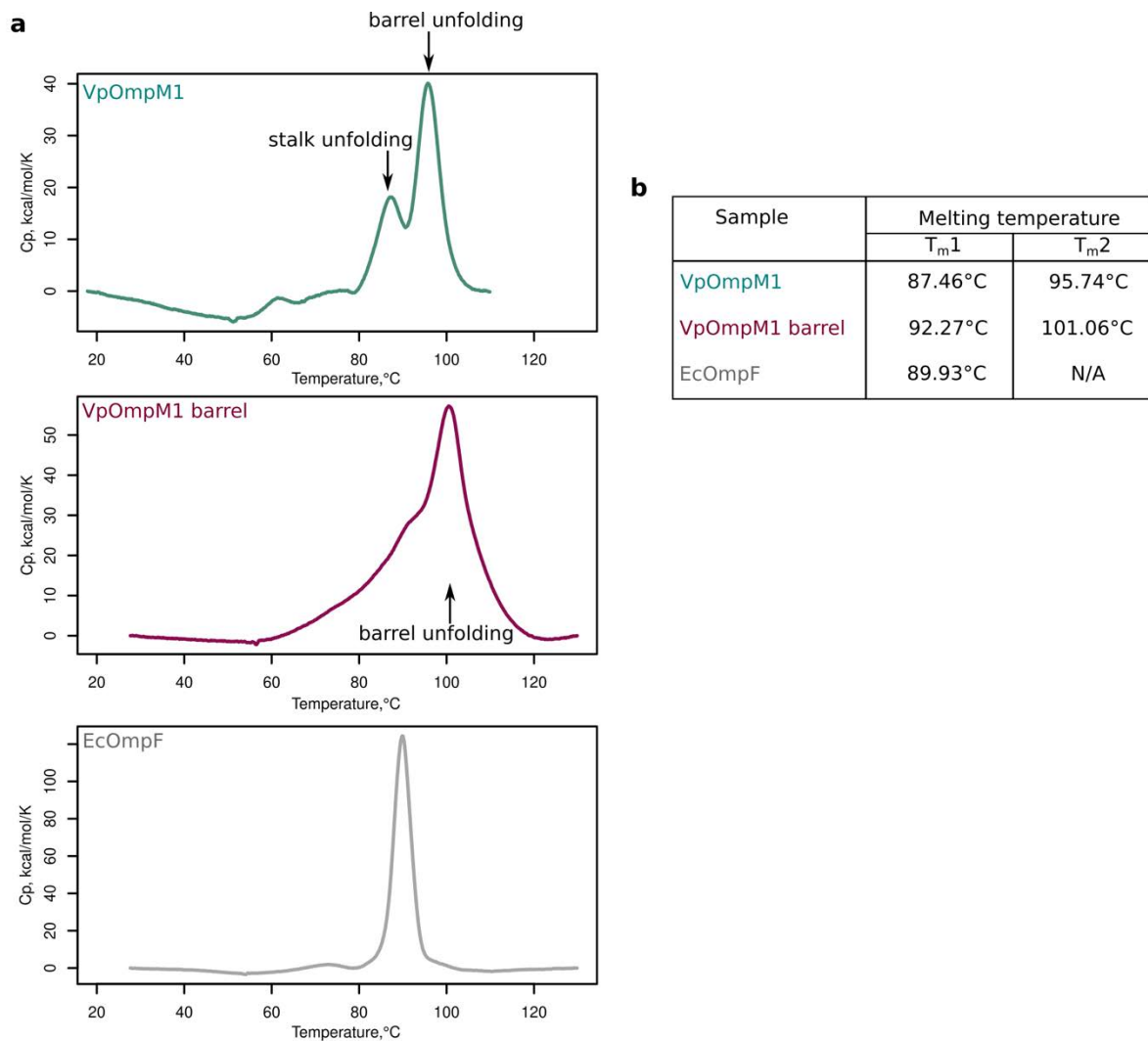
Supplementary Figure 8. VpOmpM1 trimer AlphaFold2² predictions. Cartoons coloured by per residue confidence score (colour key): pLDDT>90 – very high; 90>pLDDT>70 confident; 70>pLDDT>50 low; pLDDT<50 – very low.



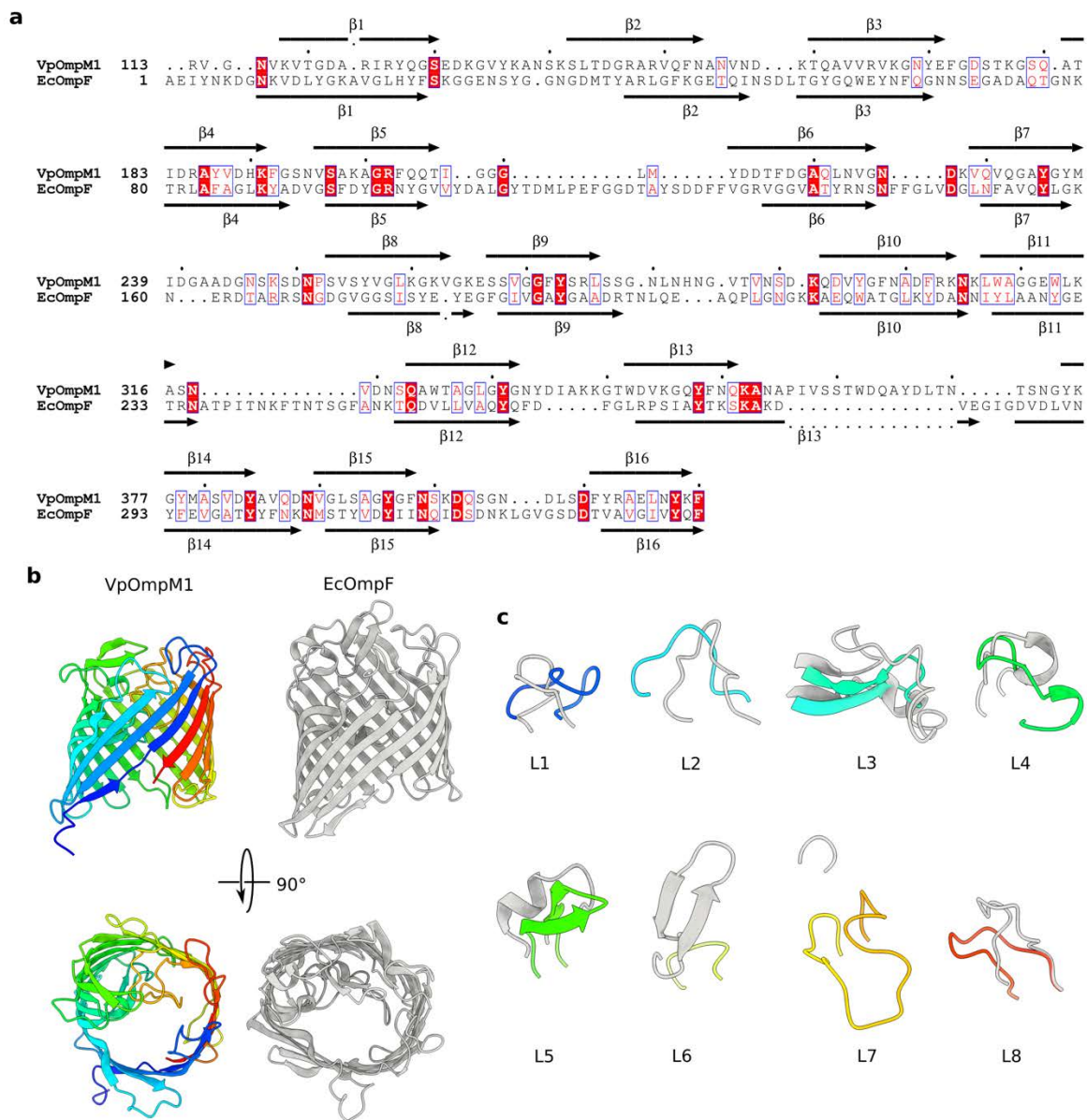
Supplementary Figure 9. VpOmpM1 compact stalk simulation. **a** The stalk α -helices in each protomer are kinked at residues 97-102 (arrows). This kink is the hinge that allows movement of the stalk towards the OM, as seen for the pink protomer in the simulation. **b** The conformation of the pink protomer is stabilised by salt bridge formation with the stalk of the yellow protomer. Salt bridge occupancy throughout the simulation (top to bottom): 14.33%, 3.21% and 1.27%.



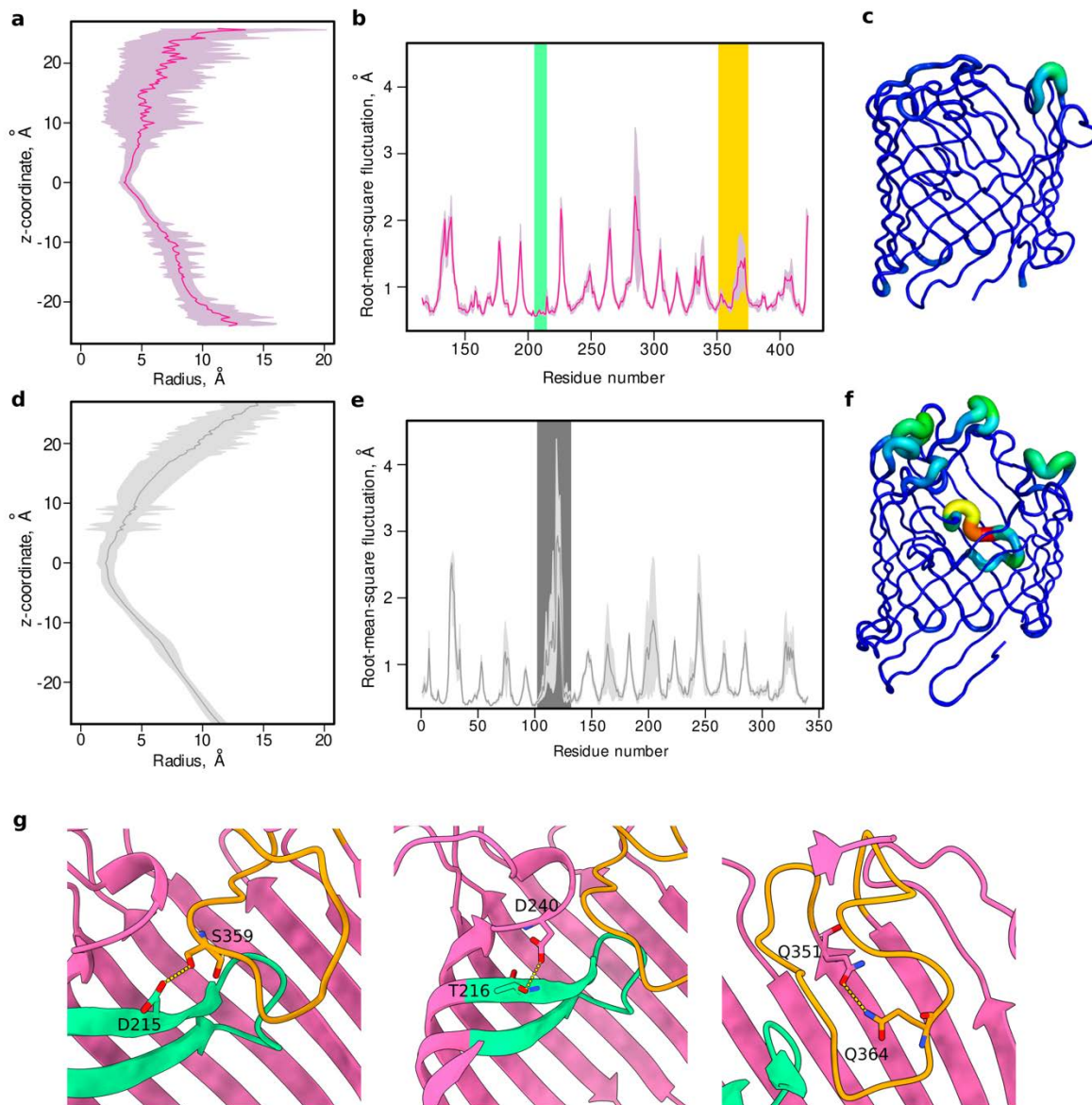
Supplementary Figure 10. Interaction of SLH domain with coiled-coil during extended stalk MD simulations. **a** During one all-atom simulation of VpOmpM1 with the grafted stalk, the SLH domain of one protomer (pink) unfolded and formed contacts with the coiled-coil region of another protomer (yellow). The SLH domains of the yellow and cyan protomers are not shown for clarity. **b** Close-up views of the SLH-coiled coil interactions. The percentage occupancy of each interaction throughout the 1 μ s simulation is shown under each panel.



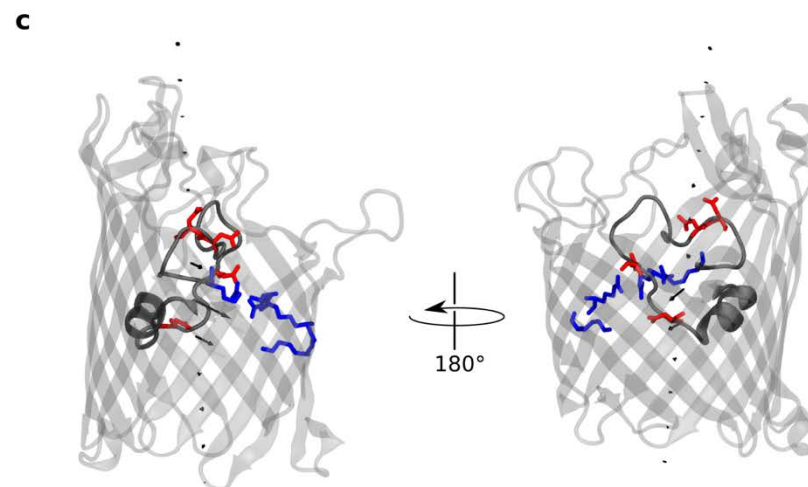
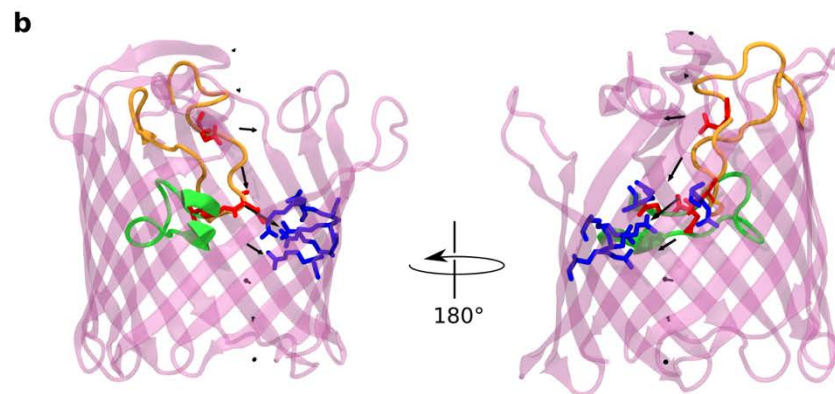
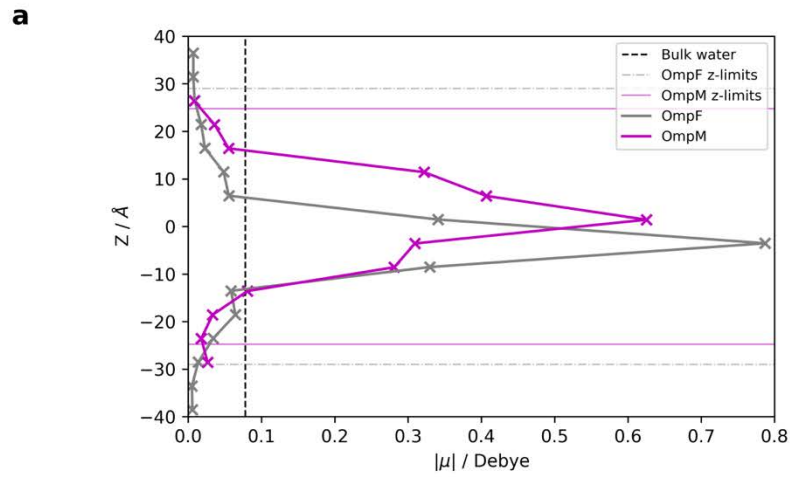
Supplementary Figure 11. Protein melting temperature analysis. **a** Dynamic scanning calorimetry thermograms for full-length VpOmpM1, VpOmpM1 barrel domain, and EcOmpF. Measurements were performed on a Malvern VP Capillary DSC instrument, using 18.7 μ M protein in 10 mM HEPES-NaOH pH 7.0, 100 mM NaCl and 0.12% DM in each experiment. **b** Data were fitted and the melting temperatures (T_m) were extracted via the instrument manufacturer's software.



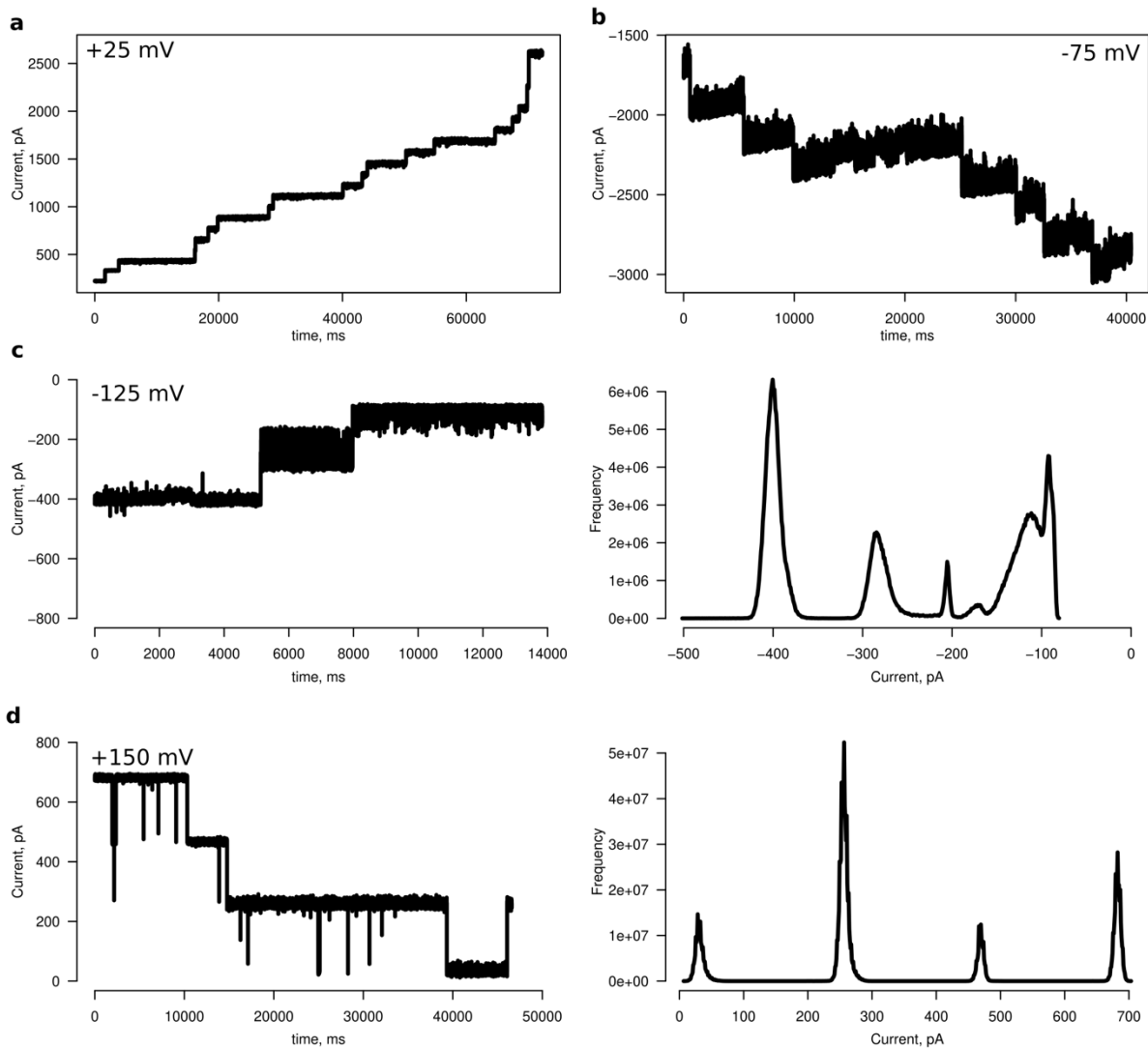
Supplementary Figure 12. Comparison of VpOmpM1 and EcOmpF. **a** Alignment of VpOmpM1 β -barrel and EcOmpF amino acid sequences. The alignment was generated by sequence-independent pairwise structure alignment of VpOmpM1 and EcOmpF (PDB 2ZFG) using TM-align³ (RMSD 3.84 Å). Residues forming the barrel β -strands are annotated above and below the alignment for VpOmpM1 and EcOmpF, respectively. The sequences share 8% identity and 25% similarity. **b** Comparison of the VpOmpM1 β -barrel from C3 reconstruction (rainbow, N-terminus blue, C-terminus red) and EcOmpF (2ZFG; grey) structures. Views generated from a superposition. **c** Comparison of the extracellular loops (L1-8) of the two β -barrels as viewed from inside the β -barrel.



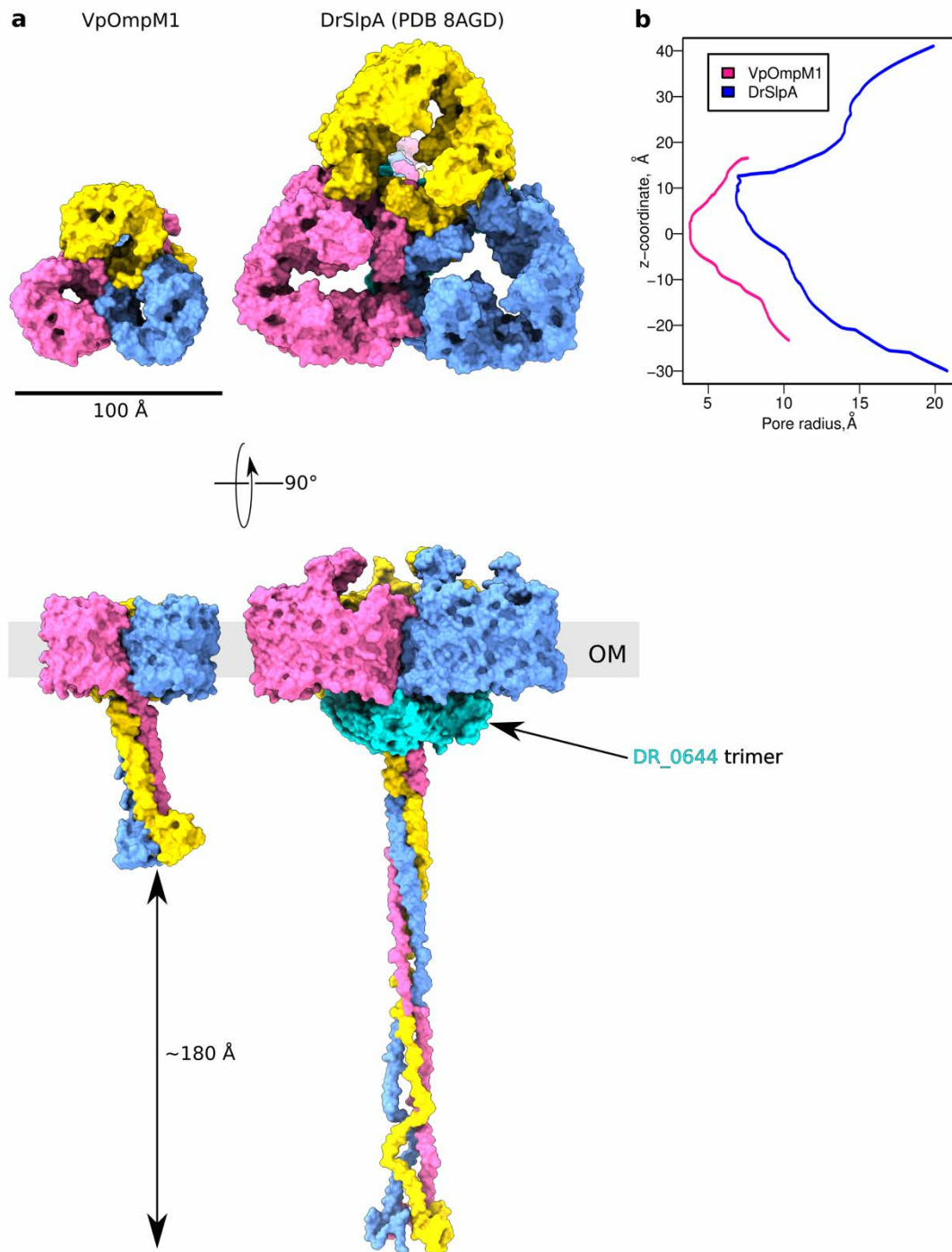
Supplementary Figure 13. Fluctuations in the channel regions of VpOmpM1 and EcOmpF during MD simulations. **a-f** Analysis of residue fluctuations in VpOmpM1 and EcOmpF during MD simulations. HOLE⁴ analyses of VpOmpM1 (**a**) and EcOmpF (**d**) calculated averaged from all-atom MD simulation frames: 1 frame every 10 ns over the final 100 ns from 3 simulations, averaged over the 3 protomers (VpOmpM1, n=90 traces); 1 frame every 10 ns over the final 300 ns of a single all-atom simulation, averaged over the three protomers (EcOmpF, n=90 traces). Lines show the average values \pm S.D. depicted by the shaded area. **b, e** Per-residue root-mean-square fluctuation (RMSF) plots for VpOmpM1 (**b**) and EcOmpF (**e**) during MD simulations. Same sampling as in **a** and **d**. Lines show the average values \pm S.D. depicted by the shaded area. Residues forming the constriction loops are highlighted: VpOmpM1 L3, green; VpOmpM1 L7, orange; EcOmpF L3, grey. **c, f** Per-residue RMSF values converted to B-factors and displayed on VpOmpM1 (**c**) and EcOmpF (**f**) structures on the same scale. Cartoon thickness and colour (blue to red) are proportional to B-factor value. **g** Examples of interactions observed during simulations that likely restrict movement of the eyelet-forming loops of VpOmpM1: between the L3 and L7 loops (left), between L3 and L4 (middle), and between L7 and the internal barrel wall (right).



Supplementary Figure 14. Transverse electric field properties in VpOmpM1 and EcOmpF channels. **a** Total water dipole moment by z-coordinate, normalised to the number of water molecules in each z-slice. **b**, **c** Arrows indicate the direction of the dipole moment averaged across the waters in a particular z-slice over the final 100 ns of equilibrium simulations for VpOmpM1 (**b**) and EcOmpF (**c**). The length of the arrow is proportional to the magnitude of the dipole moment. VpOmpM1 loop 3 is green and loop 7 is orange; EcOmpF loop 3 is black. Basic loop residues are in blue and acidic residues are in red.



Supplementary Figure 15. Representative electrophysiology recordings. **a, b** Representative ion-current traces of full-length VpOmpM1 (**a**) and the barrel-only construct (**b**) showing multiple channel insertion events observed as 'steps' or sudden increases in current. **c, d** Ion-current traces for full-length VpOmpM1 (**c**) and the barrel-only construct (**d**) showing channel gating activity at high potential (left), with the corresponding all-current point histograms shown on the right. A 2.5 kHz low-pass eight-pole Bessel filter was applied to all traces. The applied voltage is indicated on each trace.



Supplementary Figure 16. Comparison of VpOmpM1 and DrSlpA structures. **a** To-scale surface representations of VpOmpM1 and *Deinococcus radiodurans* SlpA (DrSlpA) (PDB 8AGD⁵) from outside the cell (top) and from the OM plane (bottom). **b** HOLE⁴ profiles of VpOmpM1 and DrSlpA. Note that the profiles show the path of a ball-shaped probe via the narrowest part of the pore and, therefore, this analysis grossly underestimates the size of the SlpA pore, since the constriction is not roughly circular as in VpOmpM1.

Supplementary Table 1. Cryo-EM data collection, processing and refinement statistics.

	VpOmpM1 from <i>E. coli</i>		VpOmpM1 from <i>V. parvula</i>
Data collection			
Electron microscope	FEI Glacios		FEI Glacios
Voltage (kV)	200		200
Spherical aberration (μm)	2.7		2.7
Camera	Falcon 4 (counting)		Falcon 4 (counting)
Energy filter	none		none
Magnification	240,000		240,000
Pixel size (\AA)	0.574		0.574
Total dose ($\text{e}^-/\text{\AA}^2$)	50.1		50
Defocus minimum maximum (μm)	-1.0 to -2.0		-0.8 to -2.0
Number of movies collected	4,284		6,505
Image Processing			
Imposed symmetry	C1	C3	C1
Initial number of particles	838,051	838,051	1,501,693
Final number of particles	96,280	119,001	144,245
Global resolution (FSC = 0.143)	3.15	2.78	3.28
Map sharpening B-factor (\AA^2)	-70.2	-85.5	-97.5
Refinement			
Model composition			
Non-hydrogen atoms	7,386	7,077	7,406
Protein residues	963	924	968
R.m.s. deviations			
Bonds lengths (\AA)	0.003	0.002	0.004
Bond angles ($^\circ$)	0.493	0.420	0.563
Validation			
MolProbity score	1.56	1.39	1.76
Clash score	4.52	3.57	6.45
Rotamer outliers (%)	0	0	0
Ramachandran plot			
Favoured (%)	95.30	96.41	93.97
Outliers (%)	0	0	0
PDB	8BYM	8BYT	8BYS
EMDB	EMD-16328	EMD-16333	EMD-16332

Supplementary Table 2. Crystallography data collection, processing and refinement parameters.

	VparOmpM1 stalk*
Data collection	
DLS beamline	I03
Wavelength	0.89842
Space Group	H3
Unit cell parameters	
a, b, c (Å)	118.71, 118.71, 47.24
α, β, γ (°)	90, 90, 120
Molecules in AU	3
Resolution range (Å)	34.78-1.57 (1.57-1.6)
I/ σ I	17.1 (0.7)
Completeness (%)	99.5 (92.9)
Multiplicity	9.0 (4.7)
Rpim (%)	1.7 (88.4)
CC _{1/2} (%)	100 (23)
Phasing	
Ab initio	Arcimboldo ⁶
Refinement	
Resolution (Å)	34.78-1.7
Rwork/Rfree (%)	21.9 /24.8
Reflections	
Non-hydrogen atoms	2,015
Protein only	1,918
Average B-factor (Å ²)	39.68
Rmsd	
Bond lengths (Å)	0.011
Bond angles (°)	1.21
MolProbity clashscore	3.75
Ramachandran plot	
Favoured (%)	99.59
Outliers (%)	0
PDB	8BZ2

*Statistics for the highest-resolution shell are shown in parentheses.

Supplementary Table 3. Bacterial strains used in this study.

Strain	Genotype	Reference or Source
<i>E. coli</i> DH5 α	<i>F-</i> <i>endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20</i> ϕ 80 <i>dlacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169, <i>hsdR17</i> (<i>r_K⁻m_K⁺</i>), λ^-	Promega
<i>E. coli</i> TOP10	<i>F-</i> <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 <i>lacZ</i> Δ M15 Δ <i>lacX74 nupG recA1 araD139 Δ(<i>ara-leu</i>)7697 <i>galE15 galK16 rpsL</i>(<i>StrR</i>) <i>endA1</i> λ^-</i>	Invitrogen
<i>E. coli</i> BL21(DE3)	<i>F-</i> <i>ompT lon hsdSB</i> (<i>rB- mB-</i>) <i>gal dcm</i> (DE3)	Invitrogen
<i>E. coli</i> C43(DE3) Δ <i>cyoABCD</i>	<i>F-</i> <i>ompT lon hsdSB</i> (<i>rB- mB-</i>) <i>gal dcm</i> (DE3) <i>lacUV5</i> ** Δ <i>cyoABCD</i>	⁷ (modified)
<i>E. coli</i> MFDpir	MG1655 <i>RP4-2-Tc</i> ::[Δ <i>mu1</i> :: <i>aac</i> (3)IV- Δ <i>aphA</i> - Δ <i>nic35</i> - Δ <i>mu2</i> :: <i>zeo</i>] Δ <i>dapA</i> : <i>erm-pir</i>) Δ <i>recA</i>	⁸
<i>V. parvula</i> SKV38	Wild type strain	⁹
<i>V. parvula</i> SKV38 Δ <i>ompM1-3</i>	Δ FNLLGLLA_01389-7:: <i>tetM</i>	¹⁰

Supplementary Table 4. Plasmids used in this study.

Plasmid	Description	Reference or Source
pB22	pBAD22 vector with <i>E. coli</i> TamA signal sequence, N-terminal His ₇ -tag	^{11,12}
pJW45	pB22 with beta barrel of <i>ompM1</i> cloned between XhoI and XbaI restriction sites	This study
pJW46	pB22 with <i>ompM1</i> (without signal peptide) CDS cloned between XhoI and XbaI restriction sites	This study
pJW48	pRPF185 with His-tagged <i>ompM1</i> (native RBS) cloned into the SacI restriction site	This study
pRPF185	<i>Escherichia/Clostridium</i> shuttle conjugative expression vector with <i>tet</i> promoter	¹³
pBAD24	Vector for expressing proteins with their native signal sequence in <i>E. coli</i>	¹¹
pBAD24-EcOmpF	pBAD24 vector with the full-length <i>E. coli</i> OmpF coding sequence cloned between EcoRI and XbaI restriction sites	This study
pET28b	<i>E. coli</i> expression vector	Novagen
pET28b-SLH_22-107	pET28b with <i>ompM1</i> SLH domain and stalk residues 22-107 coding region cloned between NcoI and XhoI sites (C-terminal His ₆ -tag)	This study
pB22-00518_21-200	pB22 with beta barrel (residues 21-200) of <i>FNLLGLLA_00528</i> cloned between XhoI and XbaI restriction sites	This study
pB22-00833	pB22 with beta barrel of <i>FNLLGLLA_00833</i> cloned between XhoI and XbaI restriction sites	This study

Supplementary Table 5. Primers* used in this study.

Primer	Sequence	Description
JW172	agcgttaacagatctgagct <u>taagaaagaaggaattcattatgaa</u> <u>aaaac</u>	Forward primer for amplifying full length <i>ompM1</i> with its native RBS with homology arm to the pRPF185 digested with SacI
JW201	ccatcaccatcaccatcacctcgagc <u>gcttggagaccgtgtag</u>	Forward primer for amplifying OmpM1 beta barrel with homology arm to the pB22 digested with XhoI
JW202	ctgaagcgggtgtaatactctagattaga <u>attgtaatttaattctgc</u> <u>acg</u>	Reverse primer for amplifying <i>ompM1</i> with homology arm to the pB22 digested with XbaI
JW203	ccatcaccatcaccatcacctcgaggctgcaaatccattctcc	Forward primer for amplifying <i>ompM1</i> without signal peptide with homology arm to the pB22 digested with XhoI
JW206	tctccttactgcaggagct taatgatgatgatgatgaccac <u>cgaattgtaatttaattctgcacggtag</u>	Reverse primer for amplifying <i>ompM1</i> , adding C-terminal 6His tag (indicated in bold) with homology arm to the pRPF185 digested with SacI.
00833_F	atactcgaggcatttgctgcagctcc	Forward primer for amplifying <i>FNLLGLLA_00833</i> without signal peptide with XhoI site
00833_R	atatctagattagaaggagtagcctacacc	Reverse primer for amplifying <i>FNLLGLLA_00833</i> without signal peptide with XbaI site
00518_F	atactcgagactcctcaactcaattcaataaag	Forward primer for amplifying <i>FNLLGLLA_00518</i> barrel with XhoI site
00518_R	atatctagattaaccaccgaaacggttaagataatc	Reverse primer for amplifying <i>FNLLGLLA_00518</i> barrel with XbaI site
stalk_F	ataccatggctgcaaatccattctccg	Forward primer for amplifying <i>ompM1</i> stalk region with NcoI site
stalk_R	atactcgagctttacattacctacacggtc	Reverse primer for amplifying <i>ompM1</i> stalk region with XhoI site

* The part of the primer hybridizing to the template is indicated by an underscore.

Supplementary Table 6. Position restraints, timesteps (dt), and durations of the equilibration phases in the all-atom MD simulations.

Equilibration phase	Position restraint / kJ mol ⁻¹ nm ⁻²				dt/ fs	Duration / ns
	Protein Backbone	Protein Sidechains	Lipid Headgroups	Dihedrals		
NVT1	4000	2000	1000	1000	1	0.125
NVT2	2000	1000	400	400	1	0.125
NPT1	1000	500	400	200	2	0.5
NPT2	500	200	200	200	2	0.5
NPT3	200	50	40	100	2	0.5
NPT4	50	-	-	-	2	0.5

Supplementary Table 7. VpOmpM1 simulation composition.

System	Contents									Total atoms	Box dimensions, nm
	OmpM protomers	POPE	POPG	Cardiolipin	LPS	Potassium	Chloride	Calcium	TIP3P Water		
Truncated stalk (residue 100 onwards)	3	180	10	10	62	258	178	279	69,146	317,563	12.74 x 12.74 x 21.56
Full length extended stalk	3	162	9	9	55	324	241	247	91,443	377,626	12.22 x 12.22 x 27.40
Full length compacted stalk	3	162	9	9	56	268	185	252	71,804	319,677	12.25 x 12.25 x 23.18

Supplementary Table 8. EcOmpF simulation composition.

System	Contents									Total atoms	Box dimensions, nm
	OmpF protomers	POPE	POPG	Cardiolipin	LPS	Potassium	Chloride	Calcium	TIP3P Water		
EcOmpF	3	180	10	10	63	273	143	283	56,781	282,355	12.71 x 12.71 x 19.61

Supplementary Table 9. Substrate concentrations used in liposome swelling assays.

Substrate	Concentration (mM)
Figure 6 – VpOmpM1, VpOmpM1 barrel and EcOmpF	
Lactate	8
Acetate	8
Putrescine	8
Arginine	8
Lysine	8
Glutamate	10
Aspartate	8
Glycine	15
Alanine	15
Leucine	12
Methionine	15
Arabinose	12
Glucose	12
Fructose	12
Lactose	15
Maltose	15
Sucrose	15
Kanamycin	8
Ampicillin	8
Gentamicin	8
Figure 7 – FNLLGLLA_00518 barrel, VpOmpM1 barrel and EcOmpF	
Lactate	10
Putrescine	8
Glycine	15
Arabinose	10

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