A novel fold for acyltransferase-3 (AT3) proteins provides a framework for transmembrane acyl-group transfer

Kahlan E. Newman[1*], Sarah N. Tindall[2*], Sophie L. Mader[3], Syma Khalid[3**], Gavin H. Thomas[2**], Marjan W. van der Woude[2**]

[1]School of Chemistry, University of Southampton, SO17 1BJ, UK
[2]Department of Biology and the York Biomedical Research Institute, University of York, UK
[3]Department of Biochemistry, University of Oxford, OX1 3QU, UK

* These authors contributed equally to this study
**Correspondence:
Syma Khalid: syma.khalid@bioch.ox.ac.uk
Gavin H. Thomas: gavin.thomas@york.ac.uk
Marjan W. van der Woude: marjan.vanderwoude@york.ac.uk

Abstract

Acylation of diverse carbohydrates occurs across all domains of life and can be catalysed by proteins with a membrane bound acyltransferase-3 (AT3) domain (PF01757). In bacteria, these proteins are essential in processes including symbiosis, resistance to viruses and antimicrobials, and biosynthesis of antibiotics, yet their structure and mechanism is largely unknown. In this study, evolutionary co-variance analysis was used to build a computational model of the structure of a bacterial O-antigen modifying acetyltransferase, OafB. The resulting structure exhibited a novel fold for the AT3 domain, which molecular dynamics simulations demonstrated is stable in the membrane. The AT3 domain contains 10 transmembrane helices arranged to form a large cytoplasmic cavity lined by residues known to be essential for function. Further molecular dynamics simulations support a model where the acyl-coA donor spans the membrane through accessing a pore created by movement of an important loop capping the inner cavity, enabling OafB to present the acetyl group close to the likely catalytic resides on the extracytoplasmic surface. Limited but important interactions with the fused SGNH domain in OafB are identified and modelling suggests this domain is mobile and can both accept acyl-groups from the AT3 and then reach beyond the membrane to reach acceptor substrates. Together this new general model of AT3 function provides a

framework for the development of inhibitors that could abrogate critical functions of bacterial pathogens.

Introduction

Acyltransferase family 3 (*Acyl_transf_3,* AT3) domain (Interpro: IPR002656, PFAM: PF01757) containing proteins are found in all domains of life. These membrane-bound AT3 proteins are involved in acylation of a wide range of extracytoplasmic and surface bacterial polysaccharides (1), but are also important in Eukarya, for example in the regulation of lifespan in *Caenorhabditis elegans* (2) and in *Drosophila* development (3). In bacteria, where these proteins have been primarily studied, the resulting acylations have been shown to be involved in root nodulation (4), increase the efficacy of macrolide antibiotics (5), conferring resistance to lysozyme (6), influencing bacteriophage sensitivity (7), and altering antibody recognition (8–10). While AT3 domains most commonly exist as standalone proteins, there are many examples of AT3 domains fused with SGNH or alanine racemase domains, and fusions to other domains also exist in Eukarya (see Pfam architectures for PF10757). AT3 domains are predicted to have ten transmembrane helices (11–29); however, despite the wide-ranging functions of this family of proteins, there are currently no models for their overall structure and only limited information on mechanism.

The overall understanding of AT3 proteins is mainly derived from studies of these proteins in the context of bacterial virulence through changes in the cell surface (2,3). One example of this is the acetylation of lipopolysaccharide (LPS) and lipooligosaccharide (LOS) by AT3 domain containing proteins present in the inner (cytoplasmic) membrane of Gram-negative bacteria, including species of *Neisseria* (30), *Salmonella* (31,32), *Shigella* (33–38), *Haemophilus* (39,40), *Burkholderia* (41–44) and *Legionella* (27,45,46), that modify the O-antigen of the LPS during its synthesis and before LPS export to the outer membrane. Acetylation performed by these AT3 family proteins increases heterogeneity of the O-antigen, which can lead to an altered bacterial serotype and resistance to bacteriophage attack (31–36). Some of these reactions are carried out by AT3 only proteins, others by proteins with fused AT3 and SGNH domains. SGNH proteins are a family of proteins with hydrolase/transferase activity (47).

A second important process in bacteria that involves an AT3 protein is the modification of peptidoglycan that leads to resistance to both lysozyme and β-lactam antibiotics (29,48). One such protein, OatA, is an AT3 protein with attached SGNH domain (AT3-SGNH), that acetylates the MurNAc residue in peptidoglycan (29,48). Initially discovered in

70 *Staphylococcus aureus* (29), OatA homologues have since been identified in many Gram-
71 positive bacteria including *Listeria monocytogenes* (48), *Lactococcus lactis* (49,50) and
72 *Streptococcus pneumoniae* (14,51,52). Furthermore, standalone AT3 proteins contribute an
73 acyl group in the biosynthesis of macrolide antibiotics in *Streptomyces* species which
74 increases antibiotic efficacy (5,53–55). These selected examples (see (1) for a recent
75 review) illustrate that AT3 domain-containing proteins are involved in a range of highly
76 relevant processes for bacterial pathogens and may be relevant for biotechnological
77 applications.
78
79 Found in the cytoplasmic membrane, AT3 domains are highly hydrophobic integral
80 membrane proteins predicted to contain 10 transmembrane helices (11–29). While the
81 majority of AT3 domains consists of only an AT3 domain (standalone AT3), many are AT3
82 domains with an additional transmembrane helix (TMH) and C-terminal, periplasmic SGNH
83 domain attached via a periplasmic linking region (AT3-SGNH) (56). Thanweer *et al.* (26) and
84 Jones *et al*. (57) have performed PhoA-LacZα fusion analysis of the O-antigen
85 acetyltransferase Oac from *Shigella flexneri* (a standalone AT3) and OatA from
86 *Staphylococcus aureus* (an AT3-SGNH), respectively. Analysis by Thanweer *et al.*
87 suggested that Oac has 10 TMH, with the N- and C- termini in the cytoplasm (26). This is
88 consistent with fusion analysis of the O-antigen acetyltransferase OafB from *Salmonella* ser.
89 Typhimurium (20). The *Salmonella* O-antigen acetyltransferases OafB and OafA are both
90 AT3-SGNH fusion proteins and predicted to have an 11th TMH to allow the fused SGNH
91 domain to be located in the periplasm (20,58). In contrast, Jones *et al.* proposed that OatA
92 contain only 9 TMH with one large re-entrant loop (57); as an AT3-SGNH protein, OatA was
93 also previously predicted to contain 11 TMH, orientating the SGNH domain in the periplasm
94 (12,29). In addition, a large cytoplasmic loop was described between TMH 8-9 of OatA,
95 where Oac has only short cytoplasmic loops but a longer periplasmic loop between TMH3
96 and 4 (26,59). Currently, this is the extent of the knowledge of the architecture of AT3
97 domains and there are no published experimentally determined protein structures.
98
99 AT3 proteins are predicted to transport acyl groups, including acetyl groups, from the
100 cytoplasm across the cytoplasmic membrane to be transferred onto the receptor molecule
101 (6). However, the mechanism is currently largely unknown. The acetyl group has been
102 proposed to be donated by acetyl coenzyme A (acetyl-CoA)(24). Three arginine residues on
103 the cytoplasmic side of TMH1 and 3 have been identified as highly conserved in the PFAM
104 HMM logo, and in both standalone AT3 and AT3-SGNH proteins these residues are
105 essential for function (26,58). Arginine residues have been shown to be involved in binding
106 of the 3'-phosphate group of acetyl-CoA (60) suggesting that these conserved residues may

interact with the proposed acetyl group donor. Previous *in vitro* studies suggest that standalone AT3 proteins CmmA, MdmB and Asm19 are able to utilise acetyl-CoA as an acetyl donor (5,61,62). Importantly, Jones et al. (57) found OatA from *Staphylococcus aureus* (an AT3-SGNH protein) is able to hydrolyse the acetyl group from acetyl-CoA and transfer it to the a peptidoglycan-like acceptor substrate, effectively observing the entire catalytic cycle *in vitro* (57). Thus, the available data indicate that acetyl-CoA is the most likely acetyl group donor is acetyl-CoA, but how this interacts with the AT3 domain or how the acetyl group can cross the membrane, is as yet unknown.

In addition, site-directed mutagenesis identified three conserved tyrosine residues which are required for function (57). One of these Tyr residues is predicted to be located in the periplasm and is proposed to be involved in transfer of the acyl group from the AT3 domain to the SGNH domain in AT3-SGNH proteins (57). The catalytic triad of the SGNH domain of OafB from *Salmonella enterica* subsp. *enterica* serovar Typhimurium is known to be required for acetylation of the rhamnose moiety of the O-antigen of LPS (58), suggesting that in standalone AT3 proteins an equivalent, as yet unknown, partner protein may exist. If this were the case, it would suggest that the system may be similar to that of the PatA and PatB peptidoglycan acetyltransferase system. In this system it is hypothesised that the MBOAT (membrane-bound O-acyltransferase) protein PatA transports the acetyl group across the membrane where it is transferred to the peptidoglycan substrate by PatB (an SGNH domain containing protein) (63).

To further study the AT3-SGNH family of proteins, OafB from *Salmonella enterica* subsp. *enterica* was used as a model system. OafB acetylates the rhamnose moiety of the O-antigen repeating unit of LPS. Modification of the O-antigen has repeatedly been identified as important for virulence and persistence of bacteria (27,30,39,45). Similarly, O-acetylation by OafB has been acknowledged in the development of vaccines against *Salmonella* ser. Paratyphi A, where acetyl groups were shown to be required to elicit bactericidal antibodies (64,65). Furthermore, OafB-mediated acetylation in invasive non-typhoidal Salmonella (iNTS) alters susceptibility to bacteriophage (20). OafB consists of an AT3 domain linked to an SGNH domain via an 11th TMH and periplasmic linking region (AT3-SGNH) (58) (**Fig. 1A**). The structure of the SGNH domain and periplasmic linking region was previously solved using x-ray crystallography (58). The structure was found to be similar to that of other SGNH domains, but the periplasmic linking region formed a structured extension suggesting the AT3 and SGNH domains are likely to exist in close proximity and may even interact (58).

Herein we use RaptorX to determine a computationally derived structure for the transmembrane region, including the 10-TMH AT3 domain, of OafB. We show this novel structure to be stable under physiological conditions *via* molecular dynamics simulations. This model is integrated with the existing x-ray structure of the SGNH domain and published mutational data to allow additional structure-function analysis. Utilising both classical molecular dynamics simulations and quantum mechanical calculations, we consider the acetyl donor and acceptor substrates to generate a refined functional model for this important family of membrane proteins.

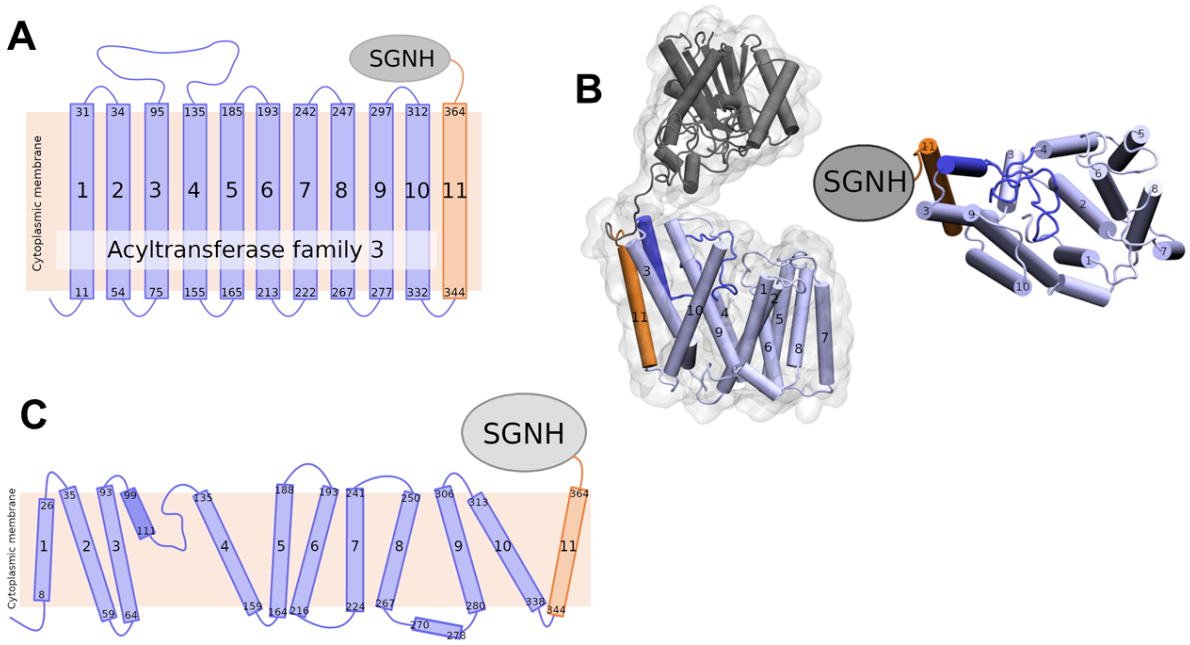


Figure 1: Topology and predicted structure of OafB. **(A)** TOPCONS topology prediction of OafB with N- and C-termini of each TMH indicated. Consistent with the RaptorX structure, TOPCONS predicts short cytoplasmic loops and a long periplasmic loop between TMH 3 and 4. **(B)** RaptorX predicted structure of OafB with AT3 domain and TMH 11 coloured light blue, periplasmic loop 3-4 coloured blue, SGNH domain coloured grey with the periplasmic linking region in orange and additional helix in teal. **(C)** Topology schematic of OafB based on the RaptorX predicted structure. The RaptorX structure has 11 TMH, with a long periplasmic loop between TMH 3 and 4 consisting of a short helix followed by an unstructured region. TMH1-10 form the AT3 domain and are coloured blue, TMH11 forms the linking region between the AT3 and SGNH domains and is coloured orange.

Results & Discussion

RaptorX model of OafB supports specific topology predictions and identifies new features

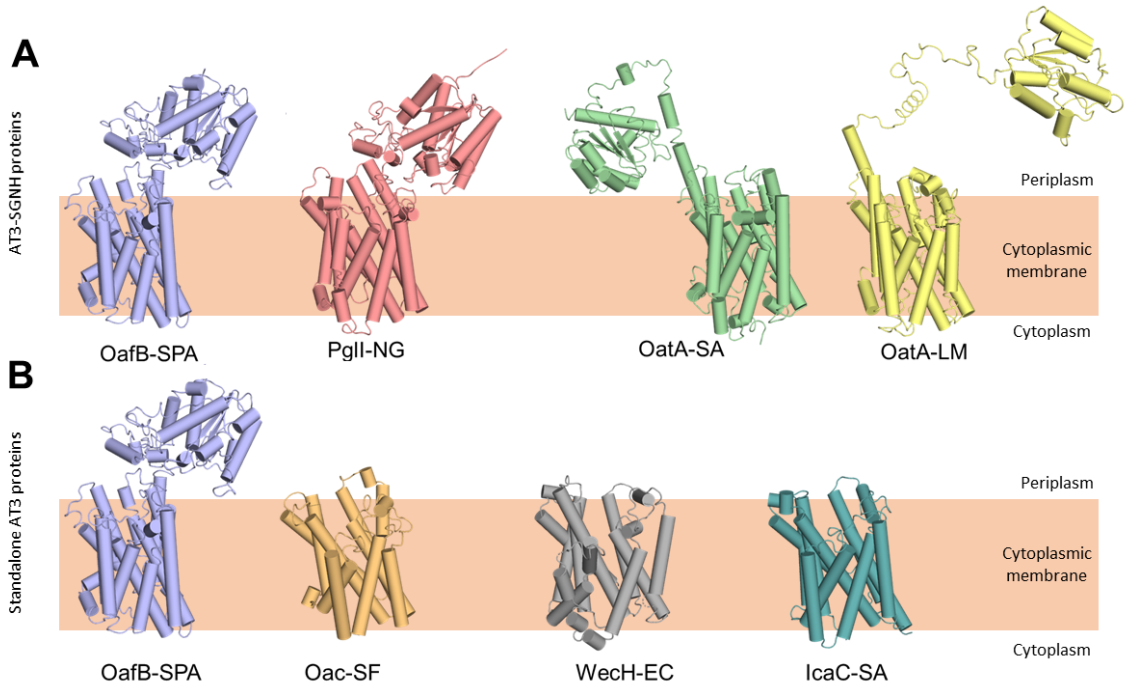
The structure of OafB was predicted by RaptorX (66–68) using the protein sequence of a *Salmonella* rhamose O-acetyltransferase (OafB). This method has been successfully validated with numerous proteins (69–73). The structure of OafB consists of two key

168 domains: the AT3 domain and SGNH domain (**Fig. 1B**). The transmembrane region has 13
169 helices, of which 11 completely span the membrane (**Fig. 1B, C**). The AT3 domain (residues
170 1-338) has 10 TMH and an additional 11th TMH (residues 339-376) fused to the periplasmic
171 linking region (containing SGNH-ext, residues 377-421), which facilitates a periplasmic
172 location of the fused SGNH domain (residues 422-640) (58), as predicted from simple
173 TOPCONS topological analysis (74,75). The topological analysis also predicted a loop
174 region between helices 3 and 4 (**Fig. 1A**); in the RaptorX model this region is clearly
175 suggested to be a short re-entrant loop consisting of a short, well-structured helix (residue
176 99-111) followed by a ~24 residue-long unstructured region **(Fig. 1B,C**). This means that
177 there are no long hydrophilic loops in the structure on either side of the membrane (58).
178
179 The structure of the SGNH domain and periplasmic linking region of OafB was previously
180 solved using x-ray crystallography (58). The RaptorX structure of the same region showed
181 remarkable similarity, with a root-mean-square deviation (RMSD) of 2.8 Å. As seen in the
182 crystal structure, the SGNH domain from OafB resembles a typical SGNH domain but with
183 an additional helix and structured extension. Neither of these regions are seen in the
184 structures of other SGNH domains in the RCSB PDB; despite this, RaptorX predicts these
185 regions with high accuracy. There are very few residues between the C-terminus of TMH11
186 (residue 367) and the N-terminus of the structured extension (residue 377), suggesting that
187 the AT3 and SGNH domains are likely in close proximity and may interact (58). However, in
188 the RaptorX structure of OafB, the two domains show limited interaction and are not in close
189 proximity (**Fig. 1B**).
190
191 Similar to RaptorX, AlphaFold (76) predicted 11 transmembrane helices, with the SGNH
192 domain in the periplasm. The structures predicted for residues 1-338 (TMH1-10 of the
193 transmembrane domain, the AT3 domain) showed 97% coverage (RMSD of 3.04 Å) and a
194 template matching score of 0.85, indicating the same structure across the two models
195 (**Supp. Fig. S1**).
196

197 Diverse bacterial AT3 domain-containing proteins share a common 10 TMH 198 structure

199 Having built a model for our primary experimentally characterised protein, we expanded the
200 analysis to include other important AT3 domain-containing proteins, including both additional
201 AT3-SGNH fusion proteins and standalone AT3 proteins. The RaptorX generated structures
202 of the AT3-SGNH proteins OatA from *Staphylococcus aureus* (OatA-SA), OatA from *Listeria*
203 *monocytogenes* (OatA-LM) and PglI from *Neisseria gonorrhoeae* (PglI-NG) all closely

204 resembled the OafB (**Fig. 2A**), with an RMSD of less than 3 Å for the AT3-domain over the
205 10 TMHs. Importantly, as they all contain a C-terminal SGNH domain that functions extra-
206 cytoplasmically, they all contain an 11th TMH to enable correct localisation of the fused
207 domain (**Fig 2A**) (23,58,77,78). In addition, the core of the SGNH domains are also similar
208 (79) with variation occurring in the length of the linking regions that connect the AT3 to the
209 SGNH. While the PglI-NG protein closely resembles OafB with a short, structured linker (1),
210 OatA proteins show a more extended, and potentially more mobile and flexible, structure,
211 locating the SGNH domain further from the AT3 domain (**Fig. 2A**).
212
213 Expanding the analysis to standalone AT3 proteins, the RaptorX outputs from Oac from
214 *Shigella flexneri* (Oac-SF), WecH from *Escherichia coli* (WecH-EC) and IcaC from
215 *Staphylococcus aureus* (IcaC-SA) were analysed (**Fig. 2B**). While the RMSD of these
216 compared to the AT3-domain of OafB was more variable (RMSD of 4.5 - 6.7 Å when
217 compared to residues 1-376 of OafB) the overall 10 TMH features were conserved and are
218 consistent with the experimentally-determined topology of Oac-SF (10,26). However, a
219 recent analysis of *Staphylococcus aureus* OatA combining LacZ-PhoA fusion data with *in*
220 *silico* predictions (56), led to an alternative model for the topology of the AT3 domain with 9
221 TMH, a further re-entrant helix and a long cytoplasmic loop preceding the final TMH that
222 leads to the SGNH domain in the periplasm. This topology is not seen in any of our models,
223 including similar models generated in AlphaFold (data not shown) and these conflicts will be
224 discussed later.



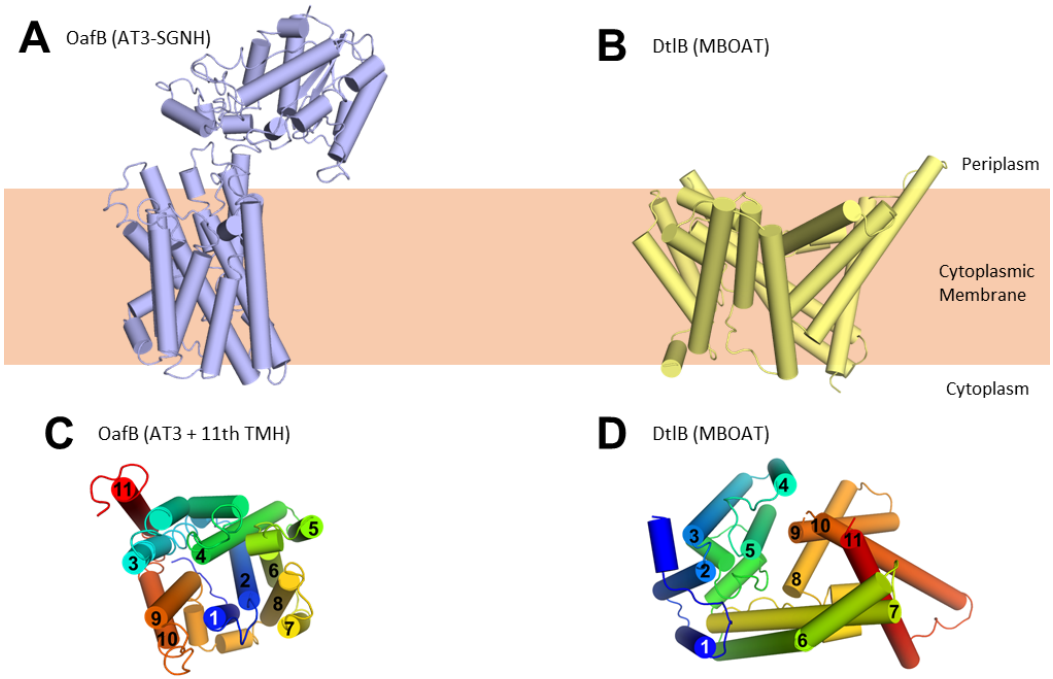
225
226 **Figure 2:** RaptorX predicted structures of AT3 domain containing proteins. **(A)** Fused AT3-SGNH proteins with
227 OafB for comparison. Left to right: OafB from *Salmonella enterica* subsp. *enterica* ser. Paratyphi A (OafB); PglI

228 from *Neisseria gonorrhoeae* (PglI-NG); OatA from *Staphylococcus aureus* (OatA-SA); OatA from *Listeria*
229 *monocytogenes* (OatA-LM). **(B)** Standalone AT3 proteins with OafB for comparison. Left to right: OafB-SPA; Oac
230 from *Shigella flexneri* (Oac-SF); WecH from *Escherichia coli* (WecH-EC); IcaC from *Staphylococcus aureus*
231 (IcaC-SA). The structure of the AT3 domain is largely conserved between these proteins.
232

233 **The AT3 domain defines a novel and stable membrane protein fold**

234 Using our new structural models for AT3 proteins, we assessed their similarity to known
235 structures using the DALI server (80). The closest structural homologues consisted of small
236 helical bundles that were fragments of larger proteins and no significant matches to full
237 length proteins were identified. Looking specifically at other membrane proteins with 10-11
238 TMH (3M73, 4J72, 4KJR, 1RH5) we could not see similarities with the AT3 structure
239 (RMSDs between 12-24 Å) and the arrangement of TMH is very different. Finally, we
240 compared our AT3 structure to that of the other known family of membrane-bound
241 acyltransferases, the MBOAT proteins. These also function in the acylation of complex
242 extracytoplasmic carbohydrates (81) and previous analysis has shown an MBOAT protein to
243 be functionally interchangeable with a standalone AT3 protein (19,82) (**Fig. 3B**), again
244 suggesting that these proteins have closely related functions. The structure of DltB (an
245 MBOAT protein from *Streptococcus thermophilus*) (81) similarly contains 11 TMH, however,
246 the helical arrangement is entirely different (**Fig. 3C,D**). Consistent with a core 10 TMH fold
247 in the AT3-SGNH protein, the 11th TMH present sits on the outside of the bundle as would
248 be expected for a non-essential feature of the AT3 domain, in contrast to the 11th TMH in
249 DltB (**Fig. 3C**).
250

251 To investigate the biological plausibility of the proposed AT3 fold, we applied Molecular
252 Dynamics (MD) techniques to emulate the behaviour of the protein in a physiological
253 environment, and to extract meaningful data on the dynamics and structure-function
254 relationships of the system. The RaptorX model of the transmembrane domain (TMH1-11 of
255 OafB, residues 1-376) was embedded in a model *Escherichia coli* inner membrane in 150
256 mM KCl solution. When subjected to MD simulations of 50 and 100 ns at 320 and 303 K,
257 respectively, the protein model remained stable within the membrane. There was no
258 significant unfolding observed; secondary structure analysis indicates that the alpha-helical
259 segments of the transmembrane domain are maintained throughout and the RMSD of the
260 protein backbone remained below 0.5 nm at both temperatures (**Supp. Fig. S2A**). The root-
261 mean-square fluctuation (RMSF) by residue, as expected, is highest for the termini and for
262 residues in unstructured loops, and lower for the transmembrane helices (**Supp. Fig. S2B).**
263 Together these data suggest that the computational approach described a novel, stable
264 membrane protein fold for AT3 proteins.



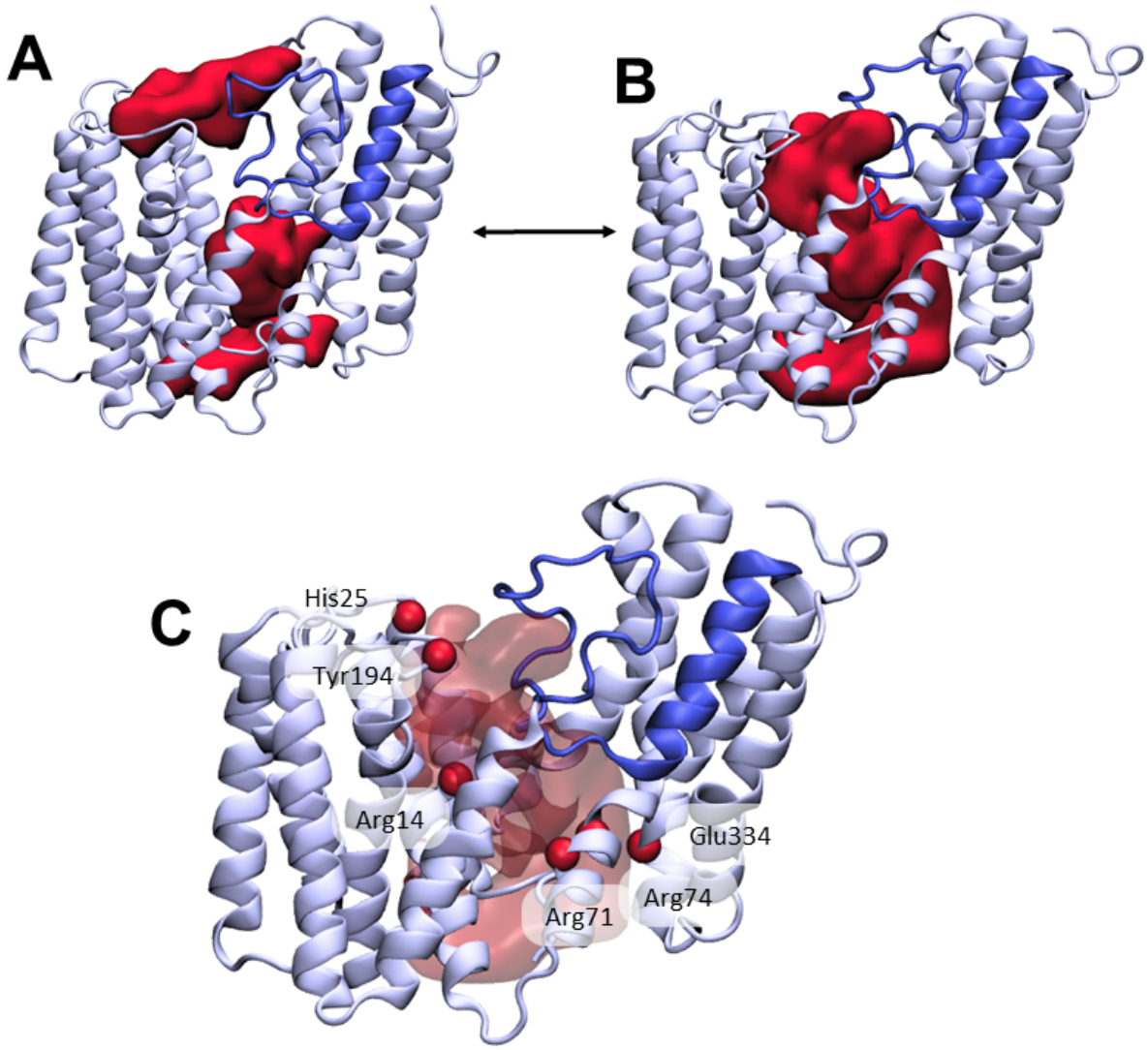
265
266 **Figure 3:** Structure of OafB, an AT3-SGNH protein (left, panels **A** and **C**), compared to DltB from *Streptococcus*
267 *thermophilus*, an MBOAT (Membrane Bound O-Acyltransferase) protein (right, panels **B** and **D**). Both OafB and
268 DltB have 11 TMH **(C, D)** however the arrangements differ considerably. **(C)** OafB and **(D)** DltB viewed from
269 periplasm, coloured blue to red, N- to C- termini with transmembrane helices numbered.
270

271 **The AT3 domain from OafB has a cavity lined with essential residues forming a**
272 **putative acetyl-CoA binding site**

273 Having established that the AT3 domain constitutes a novel stable fold in the membrane we
274 then wished to use this structural model to try and understand the mechanism of this
275 membrane-bound enzyme. A large cavity with a volume of ~3,620 $Å^3$ can be observed in
276 the AT3 protein on the cytoplasmic side, which is enclosed by enclosed by TMH 1, 2, 9, and
277 the unstructured loop between TMH 5 and 6 (**Fig. 4A**). The top end of this cavity is blocked
278 by the novel re-entrant loop between THM 3-4, which in contrast to TOPCONS predictions
279 (**Fig. 1A**) is not in the periplasm but resides within the transmembrane domain (**Fig. 4A**).
280 Examination of the positioning of core amino acids known to be important and/or essential
281 for function of different AT3 proteins onto the predicted structure reveals that many of these
282 in fact line this cytoplasmic cavity in the protein (**Fig. 4C**).
283

284 Two conserved motifs have been identified in AT3 domains: an R-X_{10}-H motif located in
285 TMH1 (Arg14 and His25 in OafB, **Fig. 4C**) (58); and an RXXR motif located on the
286 cytoplasmic face of TMH3 (Arg71 and Arg74) (17,20,24,83). The four residues found in
287 these motifs have all been identified in experimental studies as being essential for function

288 (20,25,57,58,84). Both arginine and histidine residues have previously been implicated in
289 other acetyltransferase proteins as important for binding of acetyl-CoA (60,81,85). Perhaps
290 significantly, all three Arg residues are in close proximity in positions approximately in the
291 middle of the membrane, a usually unfavourable location, suggesting a key function in the
292 mechanism of the enzyme.
293



294
295 **Figure 4:** The loop between TMH3 and 4 controls the formation of a transmembrane channel lined with essential
296 residues. **(A)** Initial equilibrated structure of the transmembrane domain (residues 1-376) of OafB. Residues 1-94
297 and 136-376 in pale blue, residues 95-135 in blue. The two largest cavities identified by the ProPores2 server are
298 shown as red surfaces. Initially, the loop between TMH3 and 4 occludes the central pore in the AT3 domain. **(B)**
299 Structure of the transmembrane domain after 50 ns equilibrium MD at 320 K. The loop between TMH3 and 4 is
300 dynamic – this snapshot from the end of the simulation shows the loop to have moved away from the center of
301 the cavity to allow a channel to form. This channel could be occupied by the acetyl group donor. **(C)** Same
302 snapshot as (B), but pore shown as a transparent red surface. Important conserved residues shown as red
303 spheres: these residues line the pore in the center of the AT3 domain.

304 In addition, Jones et al. performed site-directed mutagenesis on a number of residues in
305 OatA from *Staphylococcus aureus* (57). These identified, amongst others, three conserved
306 tyrosine residues which are required for function (57). It is thought that Tyr206, located in the
307 periplasm (equivalent residue in OafB Tyr194, **Fig. 4A**), is involved in transfer of the acetyl
308 group from the AT3 domain to the SGNH domain (57). Similarly, a catalytic triad consisting
309 of this aforesaid tyrosine, plus a glutamic acid and a histidine in the AT3 domain was
310 proposed to remove the acetyl group from acetyl-CoA (57), allowing transport across the
311 membrane (the three equivalent residues are highlighted in **Figure 4A** – Tyr194, His25 and
312 Glu334). In the RaptorX structure of OafB, Tyr194 and His25 are in close proximity (C_α
313 separated by 8 Å) and both sit on the periplasmic face of the membrane. However, Glu334 is
314 located on the cytoplasmic side very distant from His25 and Tyr194, suggesting that it is not
315 part of a catalytic triad.
316
317 Together this mapping suggests two important sites in the protein, a mid-membrane region
318 of positive charge created by 3 conserved Arg residues and a potential catalytic site on the
319 periplasmic surface comprising at least His25 and Tyr194. In the RaptorX model these two
320 features of the protein are physically separated by the loop 3-4 region (**Fig. 4A**). During
321 equilibrium MD simulations this loop is in fact dynamic (**Fig. 4B**). Structures taken from later
322 in the simulations indicate that the loop can move outwards (displacement of residues on the
323 order of 3-4 Å) from the centre of the cavity towards TMH2, allowing the potential formation
324 of a pore between the cytoplasmic and periplasmic surfaces of the transmembrane domain,
325 which could be a critical stage in the mechanism of the acyltransferase in the trans-
326 membrane movement of acyl-groups (**Fig. 4B**).
327

328 The AT3 domain facilitates presentation of acyl-CoA molecules to the 329 periplasm

330 Any model for an acyl-coA dependent acylation of an extracytoplasmic acceptor sugar
331 requires the transfer of the cytoplasmically-located acyl group across the membrane. The
332 inner cavity and dynamic conversion of this to a pore, could provide the route for acyl-CoA
333 molecules to enter the protein in a way to present the acyl group for use in the catalytic
334 process (**Fig. 5A, left**). OafB would use acetyl-CoA as the substrate, which is an extended
335 molecule with a volume of ~630 $Å^3$. To test this hypothesis, one molecule of acetyl-CoA was
336 pulled into this channel (entrance delimited by the N-terminus and TMH9 and 10) from the
337 cytoplasmic entrance of the AT3 domain of a hybrid full-length OafB model (assembly
338 discussed in the following section) using steered MD (SMD). It was found that this channel
339 could indeed accommodate acetyl-CoA (**Fig. 5A, right**) and, significantly, the thioester bond

340 was positioned close to the essential His25 residue within the membrane. The conserved
341 Arg14 was also positioned to help coordinate the 3'-phosphate of acetyl-CoA, as previously
342 predicted (58) (**Fig. 5A**).
343
344 The acetyl-CoA molecule was then allowed to equilibrate within the transmembrane domain
345 using equilibrium MD. Three replicates of this system were generated and simulated with
346 altered starting conformations for the acetyl-CoA within the protein. In the unrestrained final
347 20 ns of these simulations, acetyl-CoA was observed to hydrogen bond to several basic
348 residues within the pore (**Fig. 5B**). In addition to the Arg14 coordinating the phosphate of 3'-
349 phosphate acetyl-CoA, we noted that the conserved Arg74 (of the RXXR motif of TMH3)
350 along with Lys279 and Arg338 formed a small pocket capable of orienting such that they can
351 all simultaneously hydrogen bond to the 3'-phosphate group (**Fig. 5B**).
352
353 Further to this, energy decomposition analysis using ONETEP (86,87), indicates strong,
354 attractive interactions between the AT3 domain and acetyl-CoA. The transmembrane
355 domain-acetyl-CoA complex was optimised, and a single point energy calculated. This
356 energy decomposition analysis approach was employed to calculate the quantum
357 mechanical interaction energy and decompose it into its contributing electronic components
358 accounting for electrostatic, exchange, correlation, Pauli repulsion, polarisation, and charge
359 transfer contributions. Adding the dispersion component, the overall interaction energy was
360 calculated to be -1,035.943 kcal mol-1. Strong charge transfer interactions were identified
361 between the protein and small molecule. The most prominent of these were between the 3'-
362 phosphate of acetyl-CoA and nearby basic residues in the AT3 domain (Arg14, Arg74,
363 Arg338, Lys279); these interactions are shown in **Figure 5C** and are in strong agreement
364 with the above MD simulations.
365
366 We calculated the electrostatic potential of the transmembrane domain and acetyl-CoA, and
367 found them to be complementary: the transmembrane domain displayed positive potentials,
368 while the acetyl-CoA displayed negative potentials, of similar magnitudes (**Fig. 5D,E**). The
369 greatest positive potentials are found towards the cytoplasmic side of the transmembrane
370 domain (in agreement with the positive inside rule (88)), and the greatest negative potentials
371 are found at the phosphate groups of acetyl-CoA (which are positioned towards the
372 cytoplasmic side of the AT3 domain). Together these data are strongly supportive of a model
373 whereby acetyl-CoA from the cytoplasmic side is able to penetrate the enzyme to bring the
374 acetyl group into range for use in catalytic transfer to the receptor sugar on the periplasmic
375 face of the membrane.
376

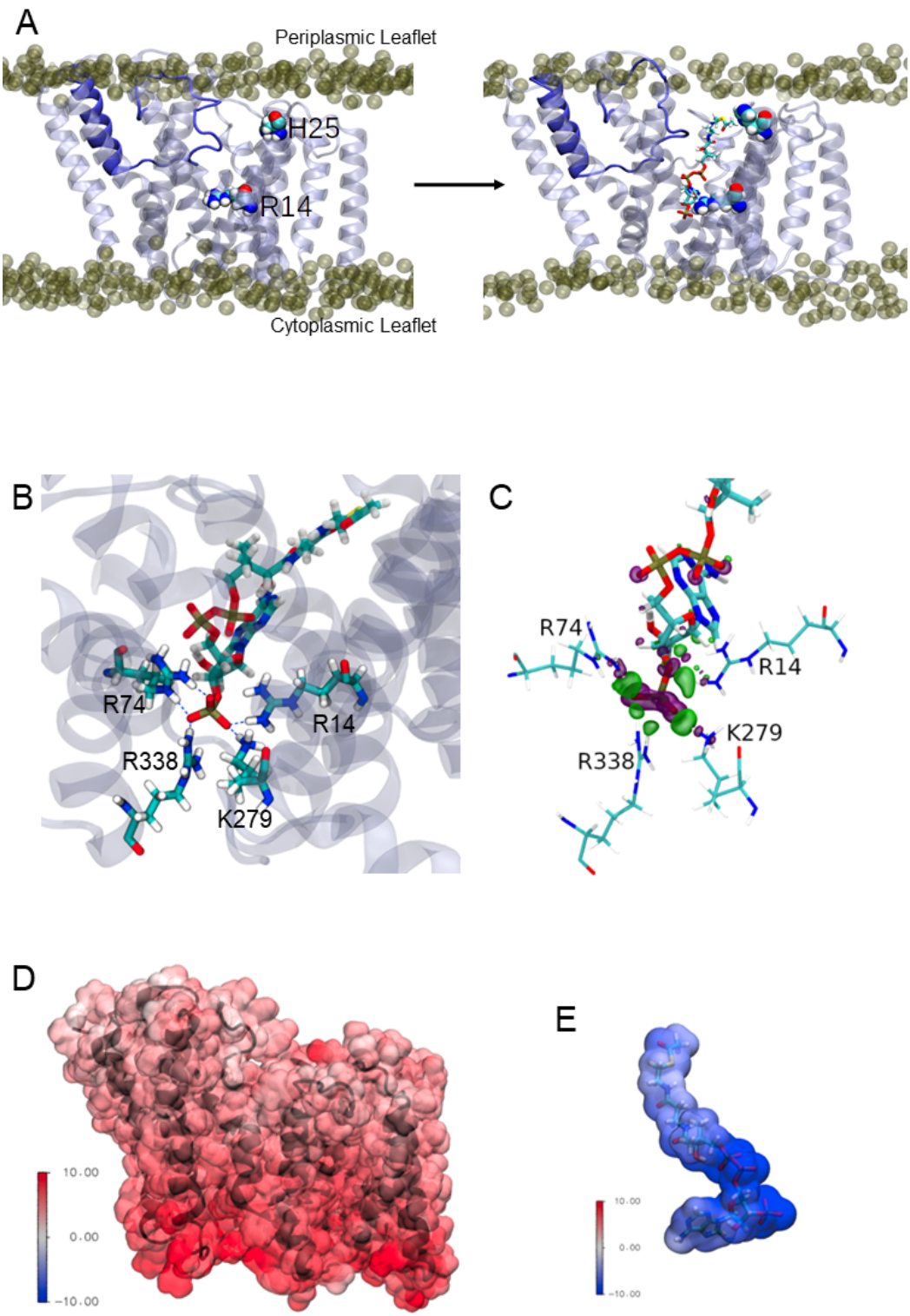


Figure 5: Interactions between the Transmembrane domain of OafB and the putative acetyl donor molecule, acetyl coezyme-A. Note these structures are taken from simulations in which we use the full OafB protein, but only the transmembrane domain (residues 1-377) is shown here for clarity. **(A)** Left: Initial structure of the OafB transmembrane domain. Essential residues H25 and R14 shown as spheres, the loop between helices 3 and 4 is

shown in dark blue. Phosphate headgroups of the phospholipids shown as tan spheres. The loop initially occludes the pore in the AT3 domain. Right: Structure from the end of the steered MD simulation in which acetyl Coenzyme-A was pulled into the central channel within the AT3 domain. The loop between helices 3 and 4 has moved away from the center of the pore towards TMH2 (to the left) sufficiently to allow passage of acetyl-coa into the transmembrane domain. **(B)** A pocket of basic residues is observed in the AT3 domain, complementary to the 3'-phosphate of acetyl coenzyme-A. Several high occupancy hydrogen bonds are observed between the 3'-phosphate and transmembrane domain residues R14, R74, R338, and K279. **(C)** Charge transfer interactions between acetyl coenzyme-A and the transmembrane domain of OafB, identified via ONETEP Energy Decomposition Analysis. Loss of electron density is depicted as a purple surface and gain of electron density in a green surface. Significant charge transfer interactions were identified between the 3'-phosphate of acetyl Coenzyme-A (losing electron density) and surrounding basic residues R14, R74, R338, and K279 (gaining electron density) **(D)** Electrostatic potential of the OafB transmembrane domain. Protein in black New Cartoon representation; calculated electrostatic potential overlayed in Surface representation. Scale from -10 V (blue) to +10 V (red). **(E)** Electrostatic potential of acetyl Coenzyme-A. Molecule in Licorice representation; calculated electrostatic potential overlayed in Surface representation. Using the same scale as the AT3 domain, it is clear that acetyl coenzyme-A and the AT3 domain are complementary.

Modelling suggests the two domains in OafB could function in a pedal bin mechanism

To learn more about the function of the model OafB proteins, which requires both the AT3 domain and its linked SGNH domain to function, we first constructed a hybrid structure that combined the RaptorX predicted structure of the transmembrane domain (residues 1-376) with the x-ray crystal structure (380-640) of the SGNH-ext (380-421) and the SGNH domain (422-640). Residues 377-379 were added to the N-terminus of the SGNH domain using MODELLER 10.0 (89), and a peptide bond was generated between residues 376 and 377 in ChimeraX (90) with varying C-N-C_α-C dihedral angles (80, 100, 120, 140°) to generate proteins with differing relative conformations of the AT3 and SGNH domains (henceforth referred to as $OafB_{80}$, $OafB_{100}$, $OafB_{120}$, and $OafB_{140}$). To investigate the flexibility of the hybrid structure we used equilibrium MD simulations (see Methods).

The x-ray crystal structure of the SGNH-ext region of OafB (PDB ID 6SE; residues 380-421) (58) shows it to be structured and interact substantially with the SGNH domain. The biological relevance of this close interaction is supported by the equilibrium MD simulations of OafB: the SGNH-ext remains structured and packed close to the SGNH domain throughout the 4 x 250 ns simulations, indicating this fold is stable under physiological conditions. Unfolding of the SGNH-ext to generate a longer flexible linker - such as that seen in the RaptorX predicted structures of OatA-LM and OatA-SA - is not observed.

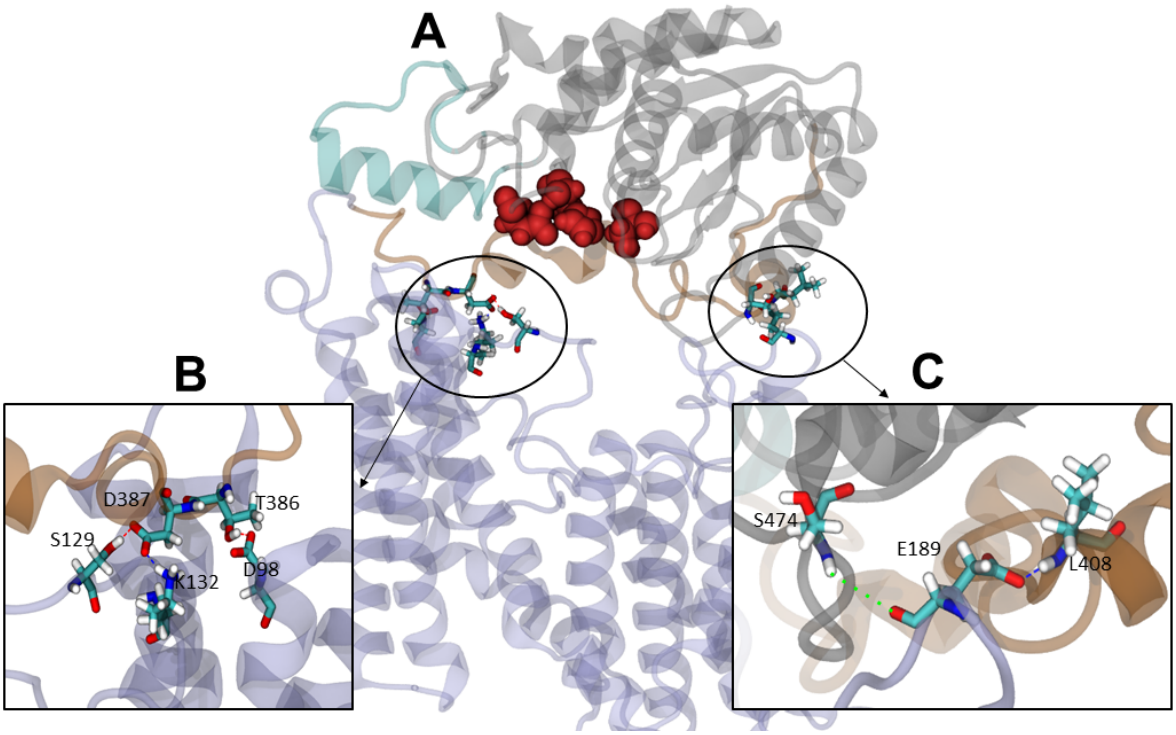
A short stretch of only around 10 residues (~370-380) remains that could form a flexible linker between the transmembrane and periplasmic domains. Indeed, in the MD simulations, the periplasmic domain exhibits a wide range of orientations relative to the transmembrane domain. Principal component analysis (PCA) of the backbone atoms was used to extract the first 2 major motions (accounting for 77% or more of the variance) of the protein in the four equilibrium replicates. The first of these motions was a pedal bin-like action, where the periplasmic domain 'lid' opens and closes relative to the transmembrane domain, hinged at the flexible linker. The second motion was the rotation of the SGNH domain within the periplasmic space about the linker. These two motions can be seen in **Videos 1 and 2**.

Despite these motions and the range of relative orientations, the SGNH and AT3 domains were not observed to spontaneously interact in the equilibrium simulations. This does not necessarily mean that the domains do not interact *in vivo*; given the short timescales of MD simulations and the vast conformational space available to the protein, sampling all possible conformations is an intractable problem. To direct computational efforts to the conformational space of interest, elastic network bonds were applied to pull the transmembrane and periplasmic domains together.

Elastic networks are a set of artificial harmonic 'bonds' added to a molecular model. These are commonly used in coarse-grained models to maintain the secondary structure of molecules (91); here, we use them to induce and then maintain a change in the tertiary structure of $OafB_x$ by pulling the periplasmic and transmembrane domains towards each other. The 'bonds' were added between residues likely to be proximal to one another as identified through coevolution analysis (see Methods). Around 75% of co-evolving residues have heavy atoms (carbon, oxygen, nitrogen, sulphur) within 5 Å of one another (92). The side chains of these residues were generally 3-5 Å in length; to avoid artificial re-orientation of the side chains but still bring the residues close enough to interact, elastic bonds of length 10 Å were added between the alpha carbons of the residue pairs. Applying elastic network bonds between the transmembrane and periplasmic domains of the $OafB_x$ proteins resulted in the rapid and irreversible 'closing' of the protein (the closed state), for the duration of the simulation time that the elastic network bonds were applied.

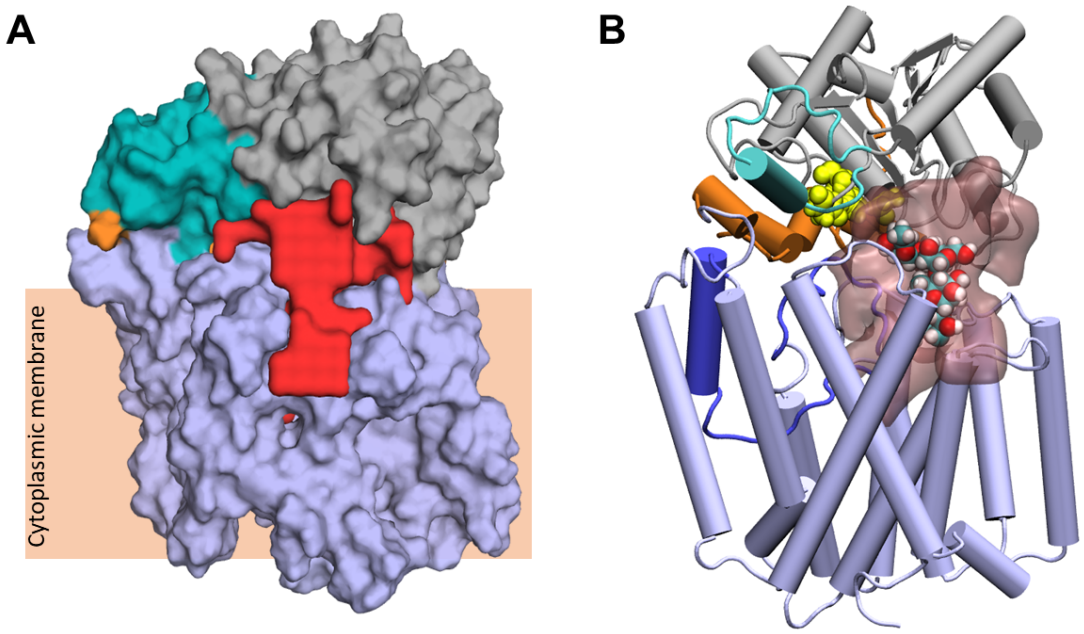
The elastic network bonds were then removed from these closed state structures, and over the subsequent 100 ns equilibrium MD simulations, all four replicates remained closed throughout. In this configuration, inter-domain interactions, sufficiently stable to maintain the closed state without the elastic network, are identified. Hydrogen bond analysis (distance and angle cutoffs of 3 Å and 20°, respectively) of the subsequent equilibrium simulations

459 revealed a minimum of 35 unique hydrogen bonds between the periplasmic and
460 transmembrane domains across each simulation. When averaged across all four
461 simulations, the number of hydrogen bonds between the two domains at any given time was
462 3.16 ± 1.67 (**Fig. 6A**). Three of the higher occupancy hydrogen bonds found in all four
463 replicates reside close to the linker region, as shown in **Figure 6B**. Specifically, these are
464 Lys132-Asp387 (all >51% occupancy), Thr386-Asp98 (>25% occupancy), and Ser129-
465 Asp387 (>70% occupancy in 3 of the 4 simulations, 23% in the fourth). Additional hydrogen
466 bonds were observed further from the linker in each system, on the other side of the catalytic
467 triad: for example, Leu408-Glu189 (34% occupancy) and Ser474-Glu189 (21%) are
468 observed in the $OafB_{100}$ system (**Fig. 6C**), but do not form simultaneously (**Supp. Fig. S4**).
469 Taken together, these findings from equilibrium MD simulations identify interactions between
470 the residues in the AT3 membrane domain and the periplasmic domains, supporting the
471 hypothesis that the two domains may cooperate.
472



473
474 **Figure 6:** Interactions between the transmembrane and periplasmic domains in the closed state of OafB. **(A)**
475 Closed OafB structure in New Cartoon representation (TM domain in lilac, SGNH-ext in orange, α8 in teal, SGNH
476 domain in grey). Catalytic triad (D618, H621, S430) as red spheres. Residues identified in high-occupancy or
477 important hydrogen bonding interactions between the SGNH and AT3 domains in Licorice representation. **(B)**
478 Hydrogen bonding observed between SGNH-ext and AT3 domains in all equilibrium simulations of the closed
479 OafB structure. High-occupancy hydrogen bonding between side chain: carboxylate of E387 and hydroxyl of
480 S129, amine of K132; carboxylate of E98 and hydroxyl of T386. **(C)** Hydrogen bonding between the SGNH and
481 AT3 domains observed in the $OafB_{100}$ simulations. E189 can hydrogen bond to S474 via its backbone carbonyl

482 (green dashed line), or to L408 via its carboxylate side chain (blue dashed line) but cannot form these
483 interactions simultaneously. Timeseries of the separation of these residues shown in Supplementary Figure S4.
484
485 These fully closed structures were submitted to the ProPores2 webserver (93) to identify and
486 parameterise cavities within the protein. The closing of the pedal bin converts the outer
487 cavity from the transmembrane domain into a larger enclosed cavity at the interface of the
488 AT3 and SNGH domains (**Figure 7A**) This pore has a volume of ~4,780 Å^3 and includes
489 both the SGNH catalytic triad comprising residues Asp618, His621 and Ser430, and at the
490 membrane surface the proposed catalytic residues of the AT3 domain, namely, His25 and
491 Tyr194. This raises the possibility that the transfer of the acyl-group to the acceptor molecule
492 could occur in this region of the protein.



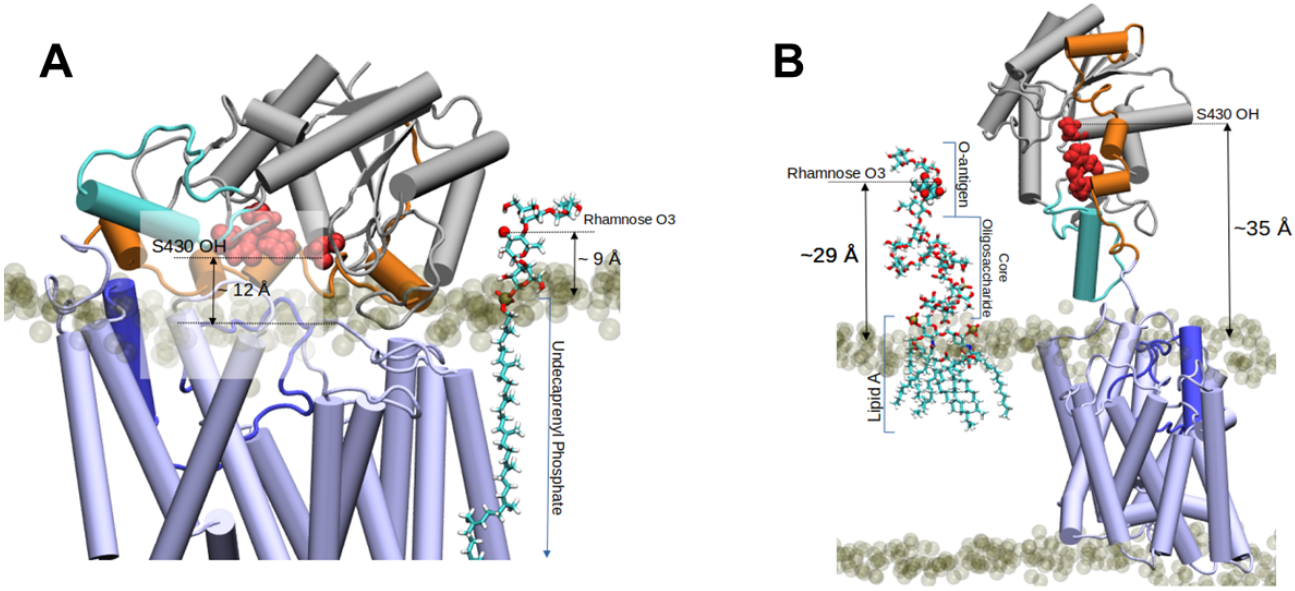
493 **Figure 7:** Largest cavity in the closed state of OafB. **(A)** Largest cavity identified by the ProPores2 webserver in
494 the closed OafB structure shown as a red surface. The cavity extends down into the transmembrane domain. **(B).**
495 Closed OafB in Cartoon representation: transmembrane domain coloured light blue, periplasmic loop between
496 TMH 3-4 coloured dark blue, SGNH domain coloured grey with the periplasmic linking region in orange, and
497 additional helix in the SGNH domain in teal. The SGNH catalytic triad (Ser430, Asp618, His621) is shown as
498 yellow spheres. Largest pore identified by the ProPores2 Webserver shown as a transparent pink surface. A
499 single O-antigen unit is shown inside the pore in van der Waals representation, coloured by atom name (cyan for
500 carbon; red for oxygen; white for hydrogen). The single O-antigen unit can be comfortably accommodated in this
501 space in several orientations.
502

503 **Fully closed structure exhibits cavity able to accommodate the LPS O-antigen**

504 Finally, we considered the potential mechanisms by which the acceptor molecule(s) would
505 interact with open and closed 'pedal bin' model of OafB, working with two alternative

506 hypotheses that the acceptor is presented either as a single or multiple O-antigen repeat
507 unit(s) anchored to a lipid carrier or later after transfer to the lipidA core molecule.
508
509 The closed form of the pedal bin model presents a possible enclosed catalytic site for OafB,
510 which is known to acetylate the rhamnose residue in the O-antigen repeating unit of the LPS
511 (10). This cavity resides directly below the catalytic triad (Ser430, His621, Asp618), and
512 extends down into the AT3 domain (**Fig. 7A**). The measured volume of ~4,780 $Å^3$ is an order
513 of magnitude greater than the volume of the LPS O-antigen unit (volume of ~502 $Å^3$), hence
514 one or more units could be manually placed within the cavity (**Fig. 7B**). Importantly the
515 enclosed cavity on the periplasmic surface of the membrane does extend into the
516 membrane, the lower portion residing below the plane of the upper leaflet headgroups and
517 hence is exposed to the membrane core (**Fig. 7A**), consistent with a ligand delivered on a
518 lipid anchor.
519
520 *S. enterica* serovars Typhimurium and Paratyphi use the prevalent Wzx/Wzy-dependent
521 pathway for O-antigen assembly, in which a single O-antigen unit is assembled in the
522 cytoplasm on undecaprenyl phosphate (Und-PP) in the membrane, which is then flipped
523 placing the O-antigen unit in the periplasm. Lipid linked O-antigen units are assembled to
524 the final length in the periplasm before ligation to lipid A-core. Subsequently, the completed
525 LPS molecule is transported to the outer membrane (94). Thus, the question remains, does
526 OafB acetylate the rhamnose moiety of a single O-antigen unit bound to Und-PP or after O-
527 antigen polymerisation? If the latter, does acetylation occur with the polymerised OAg
528 unit(s) attached to the Und-PP or after transfer to the lipidA-core molecule?
529
530 To contextualise the OafB models with biologically relevant molecules, a system containing
531 one molecule of the lipid carrier undecaprenyl phosphate (Und-PP) attached to an O-antigen
532 unit was placed next to the OafB model (**Fig 8A**). A distance of ~9 Å separates O3 of the
533 rhamnose from the periplasmic surface of the membrane, which is similar to the distance of
534 the SGNH active site (Ser430) from the membrane (which was ~12 Å calculated with
535 $OafB_{100}$ after 100 ns with elastic network bonds applied), meaning it is possible that the
536 substrate diffuses laterally into the cavity, either with the pedal bin open or closed.
537
538 The analysis above suggests that acetylation could occur using single O-antigen units,
539 before O-antigen polymerisation. Alternatively, acetylation occurs during or after
540 polymerisation on the Und-PP carrier before transfer to the lipidA-core (not shown). The
541 distance of multimers of 9 Å (single O-antigen unit) stretching away from the membrane

542 could possibly be accommodated by the open state of the pedal bin with the SGNH domain
543 extending into the periplasm.
544
545



546 **Figure 8:** Two proposed models for the O-antigen rhamnose moiety acetylation by OafB. In both panels, OafB is
547 shown in a cartoon representation with the AT3 domain coloured light blue, periplasmic loop between TMH3-4
548 coloured blue, SGNH domain coloured grey, the periplasmic linking region in orange, and additional helix in the
549 SGNH domain in teal. Catalytic triad of the SGNH domain (Ser430, Asp618, His621) shown as red spheres.
550 POPE and POPG phosphate headgroups shown as transparent tan spheres. **(A)** Undecaprenyl phosphate
551 carrier lipid with a single O-antigen unit in Licorice representation. O3 of the rhamnose moiety (marked by a red
552 sphere) has a vertical separation from the periplasmic leaflet surface of ~9 Å. The OH of S430 has a vertical
553 separation from the bulk membrane of ~12 Å. **(B)** OafB-LPS system after equilibration steps - one LPS molecule
554 with a single O-antigen unit shown in Licorice representation, and its rhamnose moiety shown in van der Waals
555 representation. OafB remains in an open conformation. After relaxation, the oligosaccharide of LPS has kinked,
556 with the distance between the lipid headgroups of the periplasmic leaflet surface and the acetylation site reduced
557 to ~29 Å (from ~42 Å, as shown in Figure S5). This is comparable to the ~35 Å separation between the OH of
558 S430 and the periplasmic leaflet surface
559

560 To investigate the final model of acetylation after assembly onto the lipidA core, we built a
561 model with LPS (with one O-antigen unit) and OafB-SPA embedded in the inner membrane
562 to assess the distances defined between the periplasmic leaflet surface and various groups
563 within the system relevant to the acetylation (**Fig. 8B**). One molecule of *Salmonella* LPS was
564 generated in CHARMM-GUI's LPS Modeler and inserted into the periplasmic leaflet of the
565 $OafB_{100}$ system (replacing 2 POPE and 2 POPG molecules). Initially the modelled LPS
566 adopts a quasi-linear conformation; the z-separation between the rhamnose moiety and the
567 periplasmic membrane surface was found to be ~42 Å, as shown in **Supplementary Figure**
568 **S5**. During minimisation and equilibration, the oligosaccharide was allowed to relax; this

569 resulted in a kinked structure, where the distance between the acetylation site and the
570 periplasmic leaflet surface was reduced to around 29 Å (**Fig. 8B**). By comparison, after
571 equilibration, the z-separation of the hydroxyl of Ser430 in the catalytic triad of $OafB_{100}$ and
572 the periplasmic leaflet is ~35 Å, hence it is not inconceivable that the open pedal bin could
573 function to deliver acetyl groups at a distance from the membrane. However, we note that in
574 reality the full-length O-antigen will be attached to the lipidA core, which would require
575 significant, entropically unfavourable conformational rearrangement of the O-antigen for
576 OafB to catalyse significant overall levels of O-acetylation across the whole polymer.

577 Steered molecular dynamics (SMD) was used to pull the rhamnose moiety of LPS and Ser
578 430 of OafB towards one another, and subsequent restrained MD simulations held the
579 protein and LPS close to one another to assess possible interactions. Many hydrogen bonds
580 were observed between the O-antigen and the periplasmic domain of OafB over the two
581 replicates. Several of these were with residues from the SGNH-ext (galactose-Asp387;
582 galactose-Tyr389; rhamnose-Tyr394). Tyr389 and Tyr394 are both residues which were
583 previously identified by FTMap *in silico* docking analysis as involved in binding to rhamnose
584 sugars. Hydrogen bonding was also observed between the 3' and 2' hydroxy groups of the
585 rhamnose moiety and Ser430. Even when all position restraints were removed in the MD
586 simulations, this interaction persisted for a further 10 ns.

587 Together these analyses indicate that O-acetylation of the O-antigen may occur either when
588 a single O-antigen unit attached to a lipid carrier is presented in the periplasm, or when the
589 O-antigen is polymerized, either on the lipid carrier, or when attached to the lipid A-core.
590

591 Ideas and Speculation

592 The realisation that diverse bacteria use AT3 proteins in a plethora of different biological
593 processes for the modification of extracytoplasmically localised glycans, directed our efforts
594 into describing the fundamental mechanism of these proteins (1). While the role of AT3
595 proteins in these diverse processes is often implicated from genetics only, the OafB protein
596 has been studied in much more detail, along with other selected AT3 proteins including the
597 OatA protein from *S. aureus* (10,57,58). These systems are examples of AT3 proteins
598 working with SGNH domains to acylate LPS and peptidoglycan, respectively, which are key
599 components of bacterial envelopes.
600

601 Integration of experimental and computational data has provided detailed insight into the
602 structure and potential mechanism of action of AT3-SGNH proteins. Our structural model,
603 combined with molecular dynamics simulations, suggests first that the transmembrane

604 domain of OafB has a novel fold. Importantly this is distinct from the MBOAT family of
605 membrane-bound acyltransferases, suggesting that nature has evolved two independent
606 routes to solve this problem. The discovery of a large cytoplasmic cavity, which can open
607 dynamically to accommodate an acyl-CoA molecule that spans the membrane, provides a
608 key mechanistic breakthrough in our understanding of the protein function, which in OafB is
609 mediated by a loop region which would need to block the pore in the absence of
610 donor/acceptor to prevent leakage of protons.
611
612 The putative catalytic residues His25 and Tyr194 are close enough to the acetyl-group (~ 5
613 Å) to conceivably be involved in catalysis, although the MD simulations cannot capture bond
614 making or breaking (nor proton transfer). The loop between TMH 5 and 6, in which Tyr194
615 resides, shows significant mobility and the side chain of Tyr194 is observed to point inwards,
616 towards the centre of the pore (and therefore towards acetyl-CoA). Given the conformational
617 mobility of the 4'-phosphopantetheine segment of the coenzyme, and of the loop between
618 TMH 5 and 6, it is not unreasonable to suggest that the thioester bond is accessible to both
619 His25 and Tyr194 to aid the acetyl transfer in a mechanism such as that suggested by Jones
620 et al (57). Addressing the role of Glu334, which Jones et al (56) proposed was the third
621 residue in the catalytic site (Glu357 in OatA), our model places this distantly from the His25
622 and Tyr194, but yet in OafB we also know this residue is important for function (79). The
623 residue sits close to the cytoplasmic side and the internal cavity and perhaps has a role in
624 the initial recognition of the acyl-CoA. Other conserved residues, including Phe42, Tyr124,
625 Trp138 and Glu143 in OafB, which were identified by mutagenesis as being important for
626 both the OafB-related protein OafA and OatA, cluster on the extracytoplasmic surface and
627 could have related roles in acyl-CoA binding, catalysis or acceptor recognition.
628
629 While the function of the AT3 to bind and deliver acyl-groups to a periplasmic catalytic site is
630 well supported by our model, and must be a conserved feature of all AT3 proteins, the
631 further discrete role of the SGNH domain is still not fully understood, although is essential for
632 transferase function in both OafB and OatA (57,58). The linker region, while short and
633 structured in OafB suggests that the domain can function in a 'pedal bin' like model, opening
634 and closing to define a large cavity on the extracytoplasmic surface, which we demonstrate
635 could then capture a lipid-anchored O-antigen repeat structure. For OatA and other related
636 proteins, this linker can be much longer (**Fig. 2**) and whether this then allows the pedal bin
637 lid to close and accept the acyl-group and then open and extend away from the membrane
638 to reach the acceptor substrate needs further investigation, but the MD simulations suggest
639 it is possible. However, for the OafB system we favour the model of lid opening and closing
640 to help trap the substrate in a membrane-surface-located activity site, similar to the function

641 of the Sus protein in capturing ligand in the outer membrane of *Bacteroidetes* bacteria (95).
642 For LPS O-acetylation by OafB and similar proteins we favour the model where the substrate
643 is the single O-antigen repeat bound by the Und-PP lipid anchor before later polymerisation.
644 Given that overall levels of OafB-dependent O-acetylation vary between 40-70% (10,96), the
645 stochastic interaction of OafB with the Und-PP anchored O-antigen repeat appears a more
646 parsimonious mechanism than relying on the enzyme trying to reach rhamnose sugars in a
647 fully assembled O-antigen.
648
649 Beyond LPS and peptidoglycan, AT3 domain-containing proteins acylate a diverse range of
650 complex carbohydrates in bacteria (1). Each receptor substrate may present its own unique
651 challenges for the AT3 domain. In both the LPS and peptidoglycan examples, additional
652 SGNH domains are required for function, however, AT3 domains can function as standalone
653 proteins. One example of this is IcaC, which is involved in the O-succinylation of poly N-
654 acetylglucosamine (PNAG) biofilm component of *Staphylococci.* Here, the acceptor is not
655 delivered on a lipid anchor, but rather through a continuous synthesis and extrusion
656 mechanism catalysed by IcaAD, a 'synthase' mechanism (94). Perhaps a close physical
657 association of IcaC with IcaAD removes the need for the additional SGNH domain and
658 allows direct transfer of the acyl group onto the emerging PNAG polymer giving stochastic
659 levels of O-succinylation of about 40% (97,98). Hence, the differences between AT3 and
660 AT3 with additional domains such as SGNH, perhaps could relate to the route of
661 presentation of the acceptor molecule within the context of different biosynthetic pathways
662 for extracytoplasmic glycans.
663

664 Conclusions

665 Here we provide strong evidence that the integral membrane AT3 domain (PF01757),
666 mediating acylation of a diverse range of complex carbohydrates, has a novel fold. The
667 stability of this structure was confirmed using molecular dynamics simulations, which also
668 enabled insights into the mechanism of action of AT3 domains. Importantly, for the first time
669 our model provides a solution to the problem of acetylation occurring in the periplasm using
670 the cytoplasmic acetyl donor acetyl-CoA. We show a membrane-spanning pore occurs
671 transiently in the AT3 domain which can accommodate acetyl-CoA, presenting the acyl
672 group to the periplasmic side. This part of our model is supported by quantum-level
673 calculations to probe the hypothesised protein-acetyl-CoA interactions, and the presence of
674 key, conserved and essential residues that line the pore. The second cavity at the
675 periplasmic side in OafB is able to accommodate the receptor substrate O-antigen.
676 Together, our data give important new insights into the family of AT3 domain containing

677 proteins. Our well-supported model can support and drive targeted research to gain a full
678 understanding of the mechanism of action of this family or membrane bound acyltransferase
679 proteins that mediate acylation of complex carbohydrates across the domains of life.
680

681 Methods

682 Co-evolution and protein structure predictions

683 OafB Protein sequence (Uniprot accession A0A0H2WM30) was submitted to either the
684 RaptorX contact prediction server (66–68) or AlphaFold server (76) and analysis was run
685 with default parameters. Predicted structures using RCSB PDB Pairwise Structure Alignment
686 webserver, employing the flexible jFATCAT alignment algorithm (99).
687

688 Molecular dynamics simulations

689 All simulations used the GROMACS simulation package (version 2020.3) (100). Results
690 were analysed using GROMACS tools and in-house python scripts utilising MDAnalysis
691 (101–104). Molecular graphics were generated using VMD 1.9.4 (105).
692

693 Atomistic simulations

694 Atomistic simulations used the CHARMM36m forcefield (106) with the TIP3P water model
695 (107). A cut-off of 1.2 nm was applied to Lennard-Jones interactions and short-range
696 electrostatics using the potential shift Verlet scheme. Long-range electrostatics were treated
697 using the particle mesh-Ewald (PME) method (108). Atoms were constrained using the
698 LINCS algorithm to allow the use of a 2 fs timestep (109). For production simulations,
699 temperatures were maintained using the Nosé-Hoover thermostat (110,111) (1.0 ps coupling
700 constant), and pressure was maintained at 1 bar using the Parrinello-Rahman semi-isotropic
701 barostat (112) (5.0 ps coupling constant).
702

703 Transmembrane Domain Model

704 A model of the transmembrane domain (residues 1-376, i.e. the 10 TMH of the AT3 domain
705 and the 11th TMH) was generated using the RaptorX webserver (66,67). Using the
706 CHARMM-GUI membrane builder module (113), this structure was embedded within a
707 model *Escherichia coli* inner membrane: a symmetric bilayer of 18:1:1 1-palmitoyl-2-oleoyl-
708 sn-glycero-3-phosphoethanolamine (POPE), 1-Palmitoyl-2-oleoyl-sn-glycero-3-
709 phosphoglycerol (POPG), and 1’,3’-bis[1-palmitoyl-2-oleoyl-sn-glycero-3-phospho]glycerol
710 (cardiolipin). The bilayer system was subsequently solvated in 0.15 M KCl. The system was
711 energy minimised in 5000 steps using the steepest descent method (114). The subsequent
712 structure was equilibrated in six phases in which the protein and lipid head groups were

713 subjected to position restraints with varying force constants. The full equilibration protocol is
714 detailed below. Two short, independent equilibrium molecular dynamics simulations were
715 carried out on the equilibrated system: one simulation of 100 ns at 303.15 K, and one of 50
716 ns at 320 K.

717 **Equilibration Protocol**

718 Each system was equilibrated using two NVT phases followed by four NPT phases. NVT
719 phases used a timestep of 1 fs and each lasted 0.125 ns, and the NPT stages used a
720 timestep of 2 fs and each lasted 0.5 ns. The velocity-rescaling thermostat (115) was applied
721 at all stages to bring the system to 303.15 K with a coupling constant of 1.0 ps. Semi-
722 isotropic Berendsen pressure coupling (116) was applied to the NPT phases to equilibrate
723 with a pressure bath of 1 bar (τ_P = 5.0 ps, compressibility of 4.5×10^{-5} bar^{-1}).
724
725 **Table 1:** Position restraints used in the equilibration steps for all atomistic systems

Equilibration Phase	Position restraint / kJ mol^{-1} nm^{-2}			
	Protein backbone	**Protein Sidechains**	**Lipid head groups**	**Dihedrals**
NVT1	4000	2000	1000	1000
NVT2	2000	1000	400	400
NPT1	1000	500	400	200
NPT2	500	200	200	200
NPT3	200	50	40	100
NPT4	50	-	-	-

726

727 **Full OafB model**

728 The RaptorX transmembrane domain model and periplasmic domain crystal structure (PDB
729 ID: 6SE1) were used to build a full OafB model. Missing linker residues (residues 377-379)
730 were modelled into the N-terminus of the SGNH domain using MODELLER 10.0 (89). A
731 peptide bond was generated between the N-terminus of the resulting periplasmic domain
732 (K377) and the C-terminus of the transmembrane domain (N376) using UCSF ChimeraX
733 (90). Four structures were generated, varying the C-N-C_α-C dihedral angle; values of 80,
734 100, 120, and 140° were used to give structures with different relative orientations of the two
735 domains. The C_α-C-N-C_α dihedral was maintained at 170°. These proteins will henceforth be
736 referred to as $OafB_x$, where x is the C-N-C_α-C dihedral angle (80, 100, 120, or 140°).
737
738 The transmembrane domain of each model was embedded in an *Escherichia coli* inner
739 membrane model and solvated in the CHARMM-GUI membrane builder as described for the
740 RaptorX model. Systems were energy minimised in 5000 steps using steepest descent
741 algorithm, and subsequently equilibrated using the protocol described above.

742

743 Duplicates of each equilibrated system were generated. The first replicate of each system
744 was simulated for 250 ns at 303.15 K. The topologies of the second replicates were modified
745 to add elastic network bonds that would bring the periplasmic and transmembrane domains
746 together. Elastic bands (of length 1 nm and strength 100 kJ mol^{-1} nm^{-2}) were generated
747 between the alpha carbons of 10 residue pairs, identified through coevolution analysis, to be
748 more than 50% likely to be proximal to one another.

749

750 **Table 2:** Residues identified through co-evolution as likely to be proximal to one another

Residue 1 (TM domain)	Residue 2 (periplasmic domain)	% Probability
94	545	56.1
95	546	80.5
96	546	57.2
97	546	70.5
98	546	58.4
123	458	53.3
125	403	59.2
126	404	55.9
194	627	50.9
242	503	51.3

751

752 These four replicates were simulated for 100 ns with the elastic network bonds applied. A
753 subsequent 100 ns simulation was undertaken on each system with the network removed.

754

755 **Full OafB Model: AlphaFold**

756 For comparison to the hybrid models built above, the OafB amino acid sequence was
757 submitted to AlphaFold (76) to generate a structure prediction for the full protein. The RCSB
758 PDB Pairwise Structure Alignment web service (99) was used to compare our structures to
759 the AlphaFold prediction. Residues 1-327 of the RaptorX model, residues 328-406 of
760 OafB80, and residues 407-640 of the periplasmic domain crystal structure (PDB ID: 6SE1)
761 were aligned to the equivalent residues in the AlphaFold model using the flexible jFATCAT
762 algorithm.

763

764 **Acetyl Coenzyme A**

765 One molecule of acetyl coenzyme A (acetyl-coA) was generated in CHARMM-GUI's Ligand
766 Reader & Modeler (117). Using VMD as a visual tool to guide positioning, the acetyl group of
767 acetyl-coA was placed beneath the gap between transmembrane helices 9 and 10, and the
768 N-terminus of the $OafB_{100}$ protein. The protein was embedded in the *Escherichia coli* inner
769 membrane model in CHARMM-GUI and solvated in 0.15 M KCl. The system was energy
770 minimised using the steepest descent algorithm, and subsequently equilibrated using the
771 protocol described above.
772
773 A short steered molecular dynamics (SMD) simulation was used to insert acetyl-coA into the
774 pore (delimited by transmembrane helices 1, 2, and 9, and the periplasmic loops between
775 helices 5 and 6, and 3 and 4). Pulling at a rate of 0.5 nm ns^{-1} and with a harmonic force of
776 1000 kJ mol^{-1} nm^{-2}, the sulphur of the acetyl-coA molecule was pulled upwards, towards the
777 centre of mass of the alpha carbons of residues E243, D126, E189 (all in loops at the
778 periplasmic surface of the transmembrane domain, surrounding the pore) until the z-
779 separation of the two groups was 0 (7.5 ns).
780
781 Three variations on the final frame of the SMD were generated in VMD by rotating and
782 translating acetyl-coA within this pore to use as starting conformations for equilibrium MD.
783 These systems were energy minimised using the steepest descent algorithm to resolve
784 steric clashes, and equilibrated as described above, with additional position restraints (**Table**
785 **3**) on the acetyl-coA molecule. Each system was subjected to three consecutive MD
786 simulations of 20 ns at 303.15 K, with decreasing position restraint strength on acetyl-coA to
787 allow the protein to relax around the substrate.
788
789 **Table 3:** Position restraints used in the equilibration and simulation of the OafB-acetyl CoA
790 systems.

Equilibration Phase	**Position restraint / kJ mol^{-1} nm^{-2}**				
	Protein backbone	**Protein Sidechains**	**Lipid head groups**	**Dihedrals**	**Acetyl-coA**
NVT1	4000	2000	1000	1000	4000
NVT2	2000	1000	400	400	4000
NPT1	1000	500	400	200	4000
NPT2	500	200	200	200	3000
NPT3	200	50	40	100	3000
NPT4	50	-	-	-	2000
MD1	-	-	-	-	1000
MD2	-	-	-	-	500
MD3	-	-	-	-	-

791
792
793
794 **LPS in the Periplasmic Leaflet of the Inner Membrane**
795 One molecule of *Salmonella spp.* LPS was generated using the CHARMM-GUI LPS
796 modeller (O9-4 core with one O-antigen unit). Using VMD as a tool to guide positioning, 2
797 POPE and 2 POPG molecules were removed from the periplasmic leaflet of the $OafB_{100}$
798 system and the LPS lipid A moiety inserted in their place. 8 water molecules were replaced
799 by potassium ions to neutralise the additional negative charge of the LPS. The system was
800 energy minimised in 5000 steps via the steepest descent algorithm to resolve steric clashes.
801 The system was equilibrated in 6 phases, as described for the other atomistic systems.
802
803 Steered MD was used to guide the rhamnose moiety of the LPS towards the Serine 430
804 residue (2 replicates, one with a pull rate of 0.5 nm ns^{-1}, the other with a pull rate of 1 nm ns^{-}
805 ^{1}; both with a pull force of 500 kJ mol^{-1}). The final frame of each of these trajectories (13.1 ns
806 and 6.5 ns, respectively) was used as the starting conformation for 3 consecutive MD
807 simulations at 303 K of 20 ns each, with varying position restraints on the protein backbone
808 (250, 0, 0 kJ mol^{-1}) and LPS molecule (1000, 500, 0 kJ mol^{-1}). Hydrogen bond analysis was
809 undertaken in VMD (distance cutoff = 3 Å, angle cutoff = 20°) to assess the interactions
810 between the LPS and the SGNH domain.
811
812 **DFT Calculations**
813 The electrostatic potentials of the transmembrane domain of OafB (residues 1-370) and the
814 acetyl-CoA molecule were calculated in a vacuum using the linear-scaling density functional
815 theory package, ONETEP (86,87), using the PBE exchange-correlation functional (118),
816 augmented with Grimme's D2 dispersion correction (119). Open boundary conditions *via*
817 real-space solution of the electrostatics were used in a simulation cell with dimensions 9 nm
818 × 7 nm × 8 nm. Norm-conserving pseudopotentials were used for the core electrons, and the
819 psinc basis set, equivalent to a plane wave basis set with a kinetic energy cut-off of 800 eV,
820 was employed. 8.0 Bohr localisation radii were used for the nonorthogonal generalised
821 Wannier functions (NGWFs). The energy decomposition analysis (EDA) implemented in
822 ONETEP (120,121) was used to calculate the intermolecular interaction energy between
823 Acetyl-CoA and the AT3 domain and to extract its contributing components and to visualise
824 charge transfer interactions.
825
826 **Data availability**

The trajectories generated and the run input files necessary to repeat the simulations presented here are openly available in Zenodo at https://doi.org/10.5281/zenodo.6834637.

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