Molecular Simulations of Gram-Negative Bacterial Membranes Come of Age

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Abstract (150 words)

Gram-negative bacteria are protected by a multicompartment, molecular architecture known as the cell envelope that contains two membranes and a thin cell wall. As the cell envelope controls influx and efflux of molecular species, in recent years both experimental and computational studies of these architectures have seen a resurgence due to the implications for antibiotic development. We review recent progress in molecular simulations of bacterial membranes. We show that enormous progress has been made in terms of the lipidic and protein compositions of bacterial systems. The simulations have moved away from the traditional setup of one protein surrounded by a large patch of the same lipid type, towards a more biologically representative viewpoint. Simulations with multiple cell envelope components are also emerging. We review some of the key method developments that have facilitated recent progress, discuss some current limitations, and offer a perspective on future directions.

Introduction.

Antimicrobial resistance is a phrase that is now familiar to us all due to the recent mainstream media attention on this topic, prompted by the huge threat posed by antimicrobial resistance to human, animal, and plant life. If the antibiotic drug development is in the current pace, recent projection has estimated that by the year 2050 more patients will die from bacterial infections than cancer with costs approaching $100 trillion dollars(1). Bacteria are relatively simple organisms, certainly compared to eukaryotes. Yet, the membranes that protect them are anything but simple, and indeed the complexity and adaptability of these membranes are key contributing factors to the development of bacterial resistance to antibiotics. In order to develop new more potent antibiotics, we must develop a thorough molecular-level understanding of the mechanisms by which bacteria protect themselves. To this end, in recent years there has been a resurgence in experimental and simulation studies aiming to understand the structure-function relationships of bacterial cell envelopes. Here we review progress in simulations of these bacterial cell envelopes over the last 5 years or so. To facilitate the reader, we begin with a reminder of the structural and chemical details of molecular architectures of bacterial cell envelopes.

Gram-negative and Gram-positive bacteria can be differentiated by the compositions of their cell envelopes. The former are characterised by a thin peptidoglycan cell wall, which lies in the periplasm between the two (inner and outer) membranes(2). In contrast, Gram-positive bacteria have a much thicker cell wall (e.g., 30-100 nm)(3) which faces the external environment, and there is only one cell membrane below the cell wall. While the fine details of the compositions of the membranes of the two types

of bacteria are species-dependent, some generalities do exist. The compositions of the two membranes of Gram-negative bacteria differ; the inner membrane is essentially a symmetric bilayer composed of a mixture of phospholipids, whereas the outer membrane (OM) is asymmetric in that the outer leaflet contains lipopolysaccharide (LPS) molecules exclusively, whereas the inner leaflet contains phospholipids similar in composition to the inner membrane. In Gram-positive bacteria, the membranes contain phospholipids in both leaflets, but often some of these lipids have larger, more complex headgroups than simple phospholipids, e.g., lysyl PG lipids in *S. aureus*. Below we provide a summary of major progress in the molecular modeling and simulation field that have seen models of complex Gram-negative bacterial membranes beyond simple homogenous lipid bilayers that incorporate one or two proteins.

The outer membrane.

Perhaps the most significant impediment to studies of even minimal OMs of viable Gram-negative bacteria was the parameterisation of LPS. An LPS molecule is a complex amphiphatic compound consisting of a phylogenetically conserved lipid A, an (inner and outer) core oligosaccharide, and highly diverse O-antigen polysaccharides with various lengths of repeating units (up to ~100) that determine bacteria's antigenic diversity(4; 5). **Figure 1** shows some LPS chemical structures of *Pseudomonas aeruginosa* (*P. aeruginosa*) with 3 different core and O-antigen structures. Clearly, the heterogeneity of core structures, as well as O-antigen sequence diversity and repeating unit length can create dynamic protein-LPS and LPS-LPS interactions.

Simulation studies of the OM that incorporate detailed models have tended to focus on *Escherichia coli* (*E. coli*) and *P. aeruginosa*, although more recently studies of the OMs of other species are appearing in the literature (**Table 1**). The first LPS model used in a membrane simulation study was from *P. aeruginosa* by Straatsma and co-workers in 2001(6). This model employed the Amber force-field and incorporated Ra-LPS (with a full core only; it is also called the rough LPS). Later models of LPS from *E. coli* using the GROMOS and CHARMM force-fields were reported by the Khalid group(7) and the Im group(8; 9), respectively. Notably, the latter also incorporated the O-antigen component (i.e., smooth LPS) for the first time. Both *E. coli* models incorporated a realistic mixture of phospholipids in the inner leaflet.

A major practical breakthrough in constructing bacterial OM models has been advent of automated membrane construction tools, of which the most popular and versatile is *LPS Modeler(10)* and its incorporation into *Membrane Builder(11)* in CHARMM GUI(12) from the Im group, featuring LPS from 15 bacterial species and incorporates 37 lipid A, 52 core, and 305 O-antigen types. Prior to the release of LPS-containing *Membrane Builder*, few laboratories had the expertise to build complex Gram-negative bacterial OM systems containing LPS reliably and systematically. Indeed, it took approximately 6 months to correctly construct the first smooth LPS model from *E. coli* (personal note from Im). In contrast, it takes on the order of 5-10 minutes to build an LPS structure from various bacteria in *LPS Modeler*, and within minutes to a few hours, depending on the system size, to construct a membrane incorporating this structure in *Membrane Builder*. In addition, one can use *Martini Maker* (13; 14) in CHARMM-GUI to build various OMs using coarse-grained LPS models based on the Martini force-field.

The number of atomistic and coarse-grained models of LPS has now grown. **Table 1** provides a summary of these models that have been reported to date (to the best of our knowledge). Rather than discussing the historical aspects of the development of these models, which can be found elsewhere(15), here we focus on the biophysical insights that have resulted from molecular simulations with these models.

Table 1. A summary of the literature of LPS models and outer membrane simulations.

Year	Bacterial Species	Membrane Type	AA[1] or CG[2]	Force-Field	Reference
2019	*E. coli*	Asymmetric/ Symmetric	CG/AA	Martini/CHARMM	(16)
2019	*C. jejuni*	Asymmetric	AA	CHARMM36	(10)
2019	*E. coli*	Asymmetric	AA	CHARMM36	(17)
2018	*P. aeruginosa*	Asymmetric	AA	GLYCAM06&Slipids	(18)
2017	*Various Bacteria*	Asymmetric	CG	Martini	(19)
2017	*E. coli*	Asymmetric	CG	Martini	(14)
2017	*E. coli*	Asymmetric	AA	CHARMM36	(20)

2016	*Various Bacteria*	Symmetric	AA	CHARMM36	(21)
2015	*P. aeruginosa*	Asymmetric	CG	Martini	(22)
2015	*P. aeruginosa*	Asymmetric	CG/AA	Martini/GROMOS	(23)
2013	*E. coli*	Asymmetric	AA	CHARMM36	(8)
2012	*P. aeruginosa*	Asymmetric	AA	GLYCAM06	(24)
2011	*E. coli*	Asymmetric	AA	GROMOS53A6	(7)
2008	*P. aeruginosa*	Asymmetric	AA	AMBER96&GLYCAM93	(25)
2008	*P. aeruginosa*	Asymmetric	AA	AMBER96&GLYCAM93	(26)
2008	*P. aeruginosa*	Asymmetric	AA	NWChem	(27)
2007	*P. aeruginosa*	Asymmetric	AA	AMBER96&GLYCAM93	(28)
1999	*E. coli*	One Layer	AA	GLYCAM	(29)

An intriguing aspect of the stability of LPS-containing membranes is the role played by divalent cations. The importance of divalent cations has been highlighted by studies using all major force-fields and has also been shown experimentally. The first mention of lipid A headgroups cross-linked by divalent cations was from simulations by Lins and Straatsma(6), which are rather short by current standards. Nevertheless, Khalid(7) and Im(8; 9) in their studies using different force-fields and longer simulations reported the same behaviour. Divalent cations form long-lived electrostatic interactions with phosphate moieties of LPS **(Figure 1)**, and indeed do so simultaneously with phosphate groups from multiple LPS molecules, thereby forming an extended network of interactions across the headgroup region of the outer leaflet of the OM. This contributes to slow diffusion rates of LPS. The simulation studies, in agreement with experimental data, showed that LPS diffuses an order of magnitude slower than phospholipids(7; 21). This is also likely a consequence, in part, of the greater number of lipid tails in LPS and the sheer bulk of the sugars. Thus, LPS diffuses slowly due to having headgroups that are tightly cross-linked by divalent cations, between 4-8 hydrocarbon tails, and multiple sugar groups. Recently, Hughes et al combined neutron reflectometry and molecular simulation to explore physical properties of OM mimetics(17). They found excellent agreement between experiment and simulation, allowing experimental testing of the conclusions from simulations studies, and also atomistic interpretation of the behaviour of experimental model systems, such as the degree of lipid asymmetry, the lipid component (tail, head, and sugar) profiles along the bilayer normal, and lateral packing (i.e., average surface area per lipid). Therefore, the combination of both experimental and simulation approaches continues to provide a powerful new means to explore the biological and biophysical behaviour of the bacterial OM.

The importance of protein-membrane interactions in modulating and regulating the behaviour of membrane proteins has been known for some time now. Development of the LPS models discussed above rendered it possible to study such interactions in the OM of Gram-negative bacteria. Here we discuss only a few selected case studies due to space limitations, but **Table 2** provides a summary of all simulation studies of outer membrane proteins (OMPs) in OM models (to the best of our knowledge).

Table 2. A summary of the literature of simulation studies of outer membrane proteins in OM environments.

Year	Protein name(s)	Bacterial Species	AA[1] or CG[2]	Reference
2019	OmpF	*E. coli*	AA	(16)
2019	OprD	*E. coli*	AA	(30)
2019	OmpE36	*E. coli*	AA	(31)
2019	OmpA/OmpX/OmpF/FhuA/EstA/BtuB	*E. coli*	CG	(32)
2019	BtuB	*E. coli*	AA	(10)
2018	OmpF	*E. coli*	CG	(33)
2018	Aquaporin Z, OmpF	*E. coli*	AA	(34)
2018	Occk5	*P. aeruginosa*	AA	(35)

2018	OmpA/OmpF	*E. coli*	CG	(36)
2017	OmpF	*E. coli*	AA	(15)
2017	PorB	*N. gonorrhoeae*	AA	(37)
2017	OprH	*P. aeruginosa*	AA	(38)
2017	OmpA/OmpF	*E. coli*	CG	(14)
2017	AcrABZ-TolC/OmpA	*E. coli*	CG	(39)
2017	Polymyxin B1	*E. coli*	CG	(40)
2016	OmpA	*E. coli*	AA	(41)
2016	OmpA	*E. coli*	AA	(42)
2016	OmpF	*E. coli*	AA	(43)
2016	BtuB	*E. coli*	AA	(44)
2016	BamA	*E. coli*	AA	(45)
2015	OccD1	*P. aeruginosa*	CG	(23)
2015	Polymyxin B1	*E. coli*	AA	(46)
2014	OmpLA	*E. coli*	AA	(47)
2013	Hia	*H. influenzae*	AA	(48)
2013	FecA	*E. coli*	AA	(49)
2009	OprF	*P. aeruginosa*	AA	(50)

Rather frustratingly, there are only three structures of OMPs in complex with LPS in the PDB: BtuB (PDB ID 1UJW; *E. coli* vitamin B12 (cyanocobalamin) transporter), FhuA (PDB ID 1QJQ; *E. coli* ferric hydroxamate receptor), and OmpE36 (PDB ID 5FVN; a

major porin from *Enterobacter cloacae*). This paucity of experimental structural data requires molecular simulations to provide an in-depth and unprecedented understanding on interactions between the OMPs and OMs. One of the first studies of LPS interactions with an OMP from *E. coli* was reported by Khalid and co-workers(49). They showed that the large loops of FecA, which is a TonB-dependent transporter, interacts with various moieties of LPS, providing evidence that considering these OMPs in simple phospholipid bilayers is not likely to provide accurate, realistic description of the conformational dynamics of the loops of the OMPs. BtuB, another TonB-dependent transporter from *E. coli* was studied by Gumbart and co-workers. In agreement with the aforementioned simulations of FecA, the extracellular loops of BtuB were also found to be stabilised to some extent through interactions with LPS. It was suggested that these interactions may eliminate the need for Ca^{2+} binding. However, it was also noted that Ca^{2+} was observed to play a role in stabilising the substrate-binding region, and also in the apo protein, facilitating conformational rearrangement of the substrate binding residues to closely resemble the conformation of the substrate-bound protein. Thus, while these simulations of TonB dependent transporters were rather short by today's standards, they did emphasise the importance of including LPS in models of the OM if the *in vivo* behaviour of these proteins is being explored.

Lee et al explored the influence of various *P. aeruginosa* and *E. coli* LPS environments on the physical properties of OprH (outer membrane protein H of *P. aeruginosa*) using all-atom molecular simulations(38). Although the *P. aeruginosa* OMs are thinner hydrophobic bilayers than the *E. coli* OMs, which is expected from the difference in the acyl chain length of their lipid A, the simulations revealed that this effect is almost imperceptible around OprH due to a dynamically adjusted hydrophobic match between

OprH and the OM **(Figure 2)**. Consistent with previous experimental findings, calculated interaction patterns identified key residues for interactions between OprH and LPS. As it is difficult to determine well-defined orientations of the OprH loops by solution NMR experiments in detergent micelles, this study also illustrated that OprH-OM simulations could provide a general approach to refine functionally important loop conformations of OMPs. Recently, Kleinekathofer and co-workers investigated the geometrical properties of the first LPS shell and the role of Ca^{2+} ions in LPS binding to OmpE36(31). The simulations reproduced LPS binding to OmpE36 observed in a recently determined crystal structure, but not as compact as in the crystal structure. Their findings highlight the role of divalent cations in stabilizing the binding between proteins and LPS molecules in the OM of Gram-negative bacteria.

Kleinekathofer and co-workers have reported a number of studies of OMPs in phospholipid bilayers to complement the experimental work of Winterhalter and colleagues, for example see refs (51; 52). More recently, they teamed up with the Im group to study the Occk5 channel from *P. aeruginosa* in various LPS-containing model OMs(35). The simulations revealed that OccK5 has a remarkable anion selectivity independent of both the OM composition and effective cation concentration. The entrance of Occk5 was found to be occluded by the outer core and O-antigens of LPS, which resulted in lowered diffusion constants of ions approaching the channel and served further to reinforce that LPS-containing membranes cannot be neglected when considering the functioning of OMPs in their native membrane environments.

Free-energy calculations are notoriously expensive, and this is even more so when slow-moving LPS is considered. Nevertheless, characterising energetics is a crucial

aspect of the physical chemistry of these systems. The free energies of permeation of a range of small molecules across the OM were calculated by Carpenter et al(53). The profiles were found to be distinctly asymmetric. For example, hexane was found to experience a free energy barrier of ~6 kcal/mol upon entering the head group region of LPS, but no appreciable barrier to entry into the phospholipid head group region; the latter is due to local lipid reorientation to minimise the energetic penalty for hexane entry. This was followed up with a study of the protein OprD that allows passage of basic amino acids across the OM of *P. aeruginosa(30)*. Again, umbrella sampling simulations were performed to calculate the free energy barriers encountered by arginine as it moves through the protein. Location of arginine within the polar interior of the protein was found to be energetically favorable, whereas for permeation directly across the OM, it would have to overcome an energetic barrier of around 30 kcal/mol. While it had previously been posited that the correct orientation of arginine is key for translocation through OprD(54), these later simulations also showed that LPS likely plays a role in orientating arginine as it enters the vestibule of the protein.

The slow diffusion rates of LPS results in limited sampling of the phase space, and thus while the models are now very accurate, there is a concern about diffusional / conformational sampling achievable with standard all-atom molecular dynamics. For example, simulations of the *P. aeruginosa* OM reported by Soares and co-workers required on the order of 500 ns for equilibration of deuterium order parameters and area per lipid headgroup. This incomplete/slow sampling issue has necessitated the search for routes to achieve enhanced sampling. One approach has been to employ coarse-grained (CG) models. Such models have largely been based on the popular MARTINI CG force-field. A number of different membrane phenomena have been studied using

CG models of LPS-containing membranes. Hsu et al reported a study of the pathways taken by pristine fullerenes to permeate into *E. coli* OM. They showed that under 'normal' conditions, on the timescale of the simulations, the fullerenes were unable to penetrate beyond the LPS headgroups. However, they are easily able to enter the core of the membranes from the outer leaflet side at elevated temperatures, when the LPS headgroups are cross-linked by monovalent cations, or if there are some phospholipids present in the outer leaflet. Interestingly, once inside the core of the membrane, the fullerenes were not observed to form aggregates, and indeed any aggregates that had formed on the surface of the membrane, dissociated once inside the low dielectric region.

A recent study by the Khalid group included five OMPs (BtuB, FhuA, OmpA, OmpX, and EstA) in differing levels of LPS(32). The simulations showed that each protein has a unique LPS fingerprint and furthermore, the patterns of interactions differed for the different levels of LPS considered. For example, for BtuB, if the interactions of charged protein residues with LPS are considered, the greatest number of interactions was with lysines in Re-LPS (including only the first two Kdo residues in the core), aspartates in Ra-LPS, and comparable between lysines and aspartates when LPS with O-antigen was considered. Another key feature of this study was that even with CG simulations, convergence of properties such as protein tilt angle within the OM can require simulations of the order of 5 μs. Thus, for equilibrium molecular dynamics simulations, the slow diffusion of LPS requires far longer simulations to achieve converged behaviour compared to membranes composed of simpler phospholipids. Simulation studies of larger, more crowded outer membrane systems have also been reported. Thus far, these have employed simplified OM models (both planar and spherical

vesicles), but they have provided novel insights into patterns of molecular interactions within these systems(55-57). For example, the Sansom group simulated ~100 copies of *E. coli* OMPs in OM mimetic lipid bilayers (~100 nm length scale) composed of PE and PG lipids(56). During the course of the simulations, the OMPs (the TonB-dependent transporter, BtuB and the trimeric porin, OmpF) were observed to form clusters. The CG simulations were used to parameterise a mesoscale model with which systems of thousands of proteins were simulated for timescales of up to 24 μs. These simulations throw up interesting questions about the membrane localisation of OMPs and the role of protein-protein and protein-lipid interactions in regulating the choreography within the OM. For example, it was found that clusters formed only by BtuB tend to be linear in their configuration, whereas those of OmpF were less linear, both proteins engaged in patterns of interactions that are quite different to those observed for multiple copies of GPCRs or potassium channels in comparable simulations **(Figure 3)**. Interestingly, a coarse-grained study by the Khalid group predicted distinct patterns of behaviour to also extend to lipids in the OM(36). Specifically, cardiolipin clustering was observed in the inner leaflet, directly below regions of low LPS density in the outer leaflet. Thus, it seems that the dynamics of lipids and proteins in the OM are rather more complex than perhaps anticipated. In the future, it will be of interest to extend such studies of crowded membranes to OM models that incorporate LPS (of various levels) and multiple copy numbers of OMPs of a range of different sizes and oligomeric states to ascertain whether any further patterns of interaction emerge in such complex, crowded systems.

The inner membrane

The inner membrane (IM) of Gram-negative bacteria is composed of a mixture of phospholipids including POPE (palmitoyl-oleoyl-phosphatidyl-ethanolamine), POPG (palmitoyl-oleoyl-phosphatidyl-glycerol), cardiolipin in various ratios; the precise details of the ratios and headgroup and tail combinations varies from species to species (ref). There has been a plethora of simulation studies of bacterial IM systems over the years. As the parameters of phospholipids have been available for far longer than those for LPS, IM models have more closely resembled the *in vivo* environment for a greater period of time compared to OM models. Here we focus our attention on a few notable examples of IM simulation studies.

Components of resistance-nodulation-cell division (RND) efflux pumps have been the focus of a number of simulation studies, many of which are reviewed in (58) **(Figure 4)**. These pumps are drug/H+ antiporters that are fuelled by the proton gradient across the IM. They are involved in the recognition and extrusion of a broad range of compounds including many antibiotics, and thus they are of interest from a biomedical perspective as well as basic biophysics. Ruggerone and Vargiu have led the field in terms of relating the structural dynamics to the function of these protein pumps for many years. Here we highlight a few notable examples from their work. A recent simulation study of the *E.coli* antiporter, AcrB, predicted conformational changes, which lead to formation of a layer of structured waters on the inner surface of the channel(59). The water layer ensures hydration of the solute moving through the channel and screen the protein-solute interactions to enable transport of the former through the channel. This role of water in the transport process had not previously been considered, thus the simulations added to the mechanistic understanding of this protein. In a later study, Ruggerone and Vargiu along with colleagues investigated the molecular origins of the substrate specificity of

the transporters AcrB and AcrD *via* a series of comparative, μs-timescale simulations(60). The substrate binding pockets of both proteins were assessed in terms of range of metrics including volume, shape, lipophilicity, electrostatic potential, hydration, and distribution of multi-functional sites. Importantly, they were able to characterise the conformational flexibilities of loops that may also play a role in substrate recognition. Such studies provide invaluable data for drug design that target specific sites. This is important for the RND pumps as inhibition of their activity is a key goal in combating pathogenic bacteria. In this context, an elegant multidisciplinary study, in which X-ray crystallography, atomistic MD simulations, and cell biology assays were employed to characterise the inhibition of AcrB by pyranopyridine derivates reported in 2016, by a team of researchers once again including Ruggerone and Vargiu(61). The structural data and simulations were able to provide molecular-level rationalisation of the cellular assays. The different studies together showed that pyranopyridines are stabilised within a phenylalanine-rich cage in AcrB, largely through hydrophobic interactions. The molecules studied showed inhibition of the efflux activity of AcrB at lower concentrations than many other molecules that have been proposed as potential inhibitors of RND pumps. Of key importance is that free-energy calculations enabled the identification of the contributions from the protein, solvent, and ligand molecules to the binding energetics. Thus, each different technique provided a piece of the jigsaw, which has helped to build up a picture of the mechanism of inhibition of AcrB.

Simulation studies of bacterial membrane systems such as the study of AcrB described above, which are performed alongside experimental work, are now becoming *de regeur*, such that new membrane protein structures are now rarely reported without accompanying simulation studies, for example see references (62-64). Furthermore,

studies in which other types of computational methods are combined with simulations and experiments are also becoming more prevalent. For example, sequence co-evolution, simulations, cross-linking experiments, and imaging methods were combined to study the twin-arginine protein translocation (Tat) system of *E. coli(65)*. The results enabled identification of protein-protein interactions and led to a structural model for assembly of the active Tat translocase in which substrate binding triggers replacement of TatB by TatA. Another example of combining simulations with experiments is work on YidC insertase from *E. coli(66).* In this study, *in vivo* cysteine alkylation scanning and MD simulations were used to assess the water accessibility of the protein and its impact on the local membrane environment. Interestingly, the simulations revealed the protein structure to be more compact when in the model IM compared to the X-ray structure. This observation once again highlights the importance of considering the local environment when interpreting structural data as well as simulation trajectories.

Recently, Kim et al used all-atom simulations to investigate the conformational ensemble of lipid II and its elongated forms (lipid VI and lipid XII) in an IM model and their interactions with penicillin-binding protein 1b (PBP1b) from *E. coli(67)*. As the precursor in the peptidoglycan biosynthetic pathway, lipid II carries a nascent peptidoglycan unit that is processed by glycosyltransferases and thus is a target of several classes of antibiotics. Simulations revealed that as the glycan chain grows, the non-reducing end of the nascent peptidoglycan displays much greater fluctuation along the membrane normal and minimally interacts with the membrane surface. When a nascent peptidoglycan is bound to PBP1b, the stem peptide remains in close contact with PBP1b by structural rearrangement of the glycan chain. Most importantly, this study characterised the number of nascent peptidoglycan units required to reach the

transpeptidase domain to be 7 or 8. These findings complement experimental results to further understand how the structure of nascent peptidoglycan can dictate the assembly of the peptidoglycan scaffold for bacterial cell wall formation.

The periplasm.

The periplasm is an aqueous region sandwiched between the two membranes of Gram-negative bacteria. A number of proteins reside either wholly within this region or have some soluble domain within the periplasm while being anchored to either one of the membranes through a transmembrane domain or a membrane anchoring moiety. The cell wall, which is composed of peptidoglycan layers, is also contained within the periplasm. Simulation studies of some of periplasmic proteins have recently been reported. For example, Boags et al, showed the molecular basis for inhibition of LolA by hydrophobic molecules(68). LolA carries Braun's lipoprotein from the IM across the periplasm and delivers to the LolB which is anchored to the inner leaflet of the OM. Experimental work had earlier shown that the LolA function is inhibited by hydrophobic molecules. Atomistic simulations and free energy calculations showed that Braun's lipoprotein and the inhibitor form a complex within the cavity of LolA. Free-energy calculations revealed complex to have weaker binding within the cavity of LolA compared to uncomplexed Braun's lipoprotein. This was proposed to be the basis of the inhibition, in other words the inhibitor does not physically block access to the binding site, rather it reduces the strength of the binding between the protein and its natural ligand by forming a complex with the latter.

Modeling and simulation of peptidoglycan provide a rather different challenge due to its structural uncertainty. While the overall composition of long glycan strands cross-linked by short peptides is largely conserved, some details are still debated, e.g., the arrangement of the strands, as well as the size and shape of the 'pores' within the mesh. High resolution microscopy methods, such as work by the Hobbs and Foster groups, are now beginning to provide data to clear up some of these uncertainties(69). Some of the first atomistic level simulations of the cell wall to give results comparable to experimental data were those reported by Gumbart and Roux in 2014(70). A single layer of peptidoglycan was constructed, starting from individual residues and building up into increasingly larger patches by validating against a range of experimental data (including pore size, elasticity, and thickness) at each stage of size expansion. Thus, the authors were able to test various possibilities regarding the arrangement of the strands. The agreement between simulations and experiments converged on a model for *E. coli* in which the cell wall is composed predominantly of a single layer of peptidoglycan with glycan strands aligned in a disordered fashion, circumferentially around the cell. Gumbart then followed up this work with simulations combined with experimental work from the Lakey group, on the inner and outer membranes and the cell wall(34). The combined study showed the different responses of these cell envelope components to surface tension. They produced a model of the distribution of mechanical stress in the *E. coli* cell envelope in which the OM and cell wall share the tension at low turgor pressure (0.3 atm), but the tension in the cell wall dominates at high values (>1 atm).

A series of simulations focussing on the cell interactions of OmpA were reported by the Khalid group(42; 71). Having first characterised the conformational dynamics of a model of the dimer formed by the full-length *E. coli* protein, OmpA, simulations of this protein

bound to small portions of the peptidoglycan were reported(42). The protein-peptidoglycan complexes were set up using the X-ray structure of the C-terminal domain of OmpA from *A. baumannii* (which is bound to a pentapeptide from peptidoglycan, PDB: 3TD5)(72). The simulations of the full-length *E. coli* OmpA revealed long-lived electrostatic interactions between the soluble C-terminal domain of the protein and the cell wall when the protein is in its monomeric and dimeric forms. It was noted that dimerization reduced the mobility of the C-terminal domains somewhat and thus the dynamics of the protein in the two oligomeric forms differed. These interactions with the cell wall were further explored in simulations that also included the inner membrane; these are discussed in the following section(73).

Progress in simulations incorporating multiple components of the bacterial cell envelope.

While thus far we have considered simulations of one component of the cell envelope, it is important to consider the interplay between the different components to fully understand their dynamic behaviour. Recently, such simulation studies and indeed tools to construct these biochemical complex architectures are beginning to emerge. Earlier this year, the first study to incorporate all three components was reported(73). OmpA in the OM, Braun's lipoprotein anchored in the OM and covalently bound to the cell wall in periplasm, and TolR in the IM (as well as variations of this combination) were simulated at the atomistic level of detail **(Figure 5)**. The results showed that OmpA and TolR both form stable interactions with the cell wall and that Braun's lipoprotein plays a role in facilitating these interactions by tilting and kinking to adjust the width of the periplasmic space. Furthermore, these simulations also showed that the morphology of the cell wall

is impacted by the local protein interacting with it. For example, balanced protein-cell interactions from the IM and OM sides result in a flat cell wall, whereas if protein binding is from only one side, the cell wall becomes buckled. These observations are from simulations of OmpA and Tol only, and necessitate further studies, in which other proteins are considered and in which the cell envelope is more crowded, in order to provide additional insights.

The current *Membrane Builder* and *Martini Maker* modules in CHARMM-GUI do allow users to build a membrane system with multiple proteins once these proteins are prearranged in a PDB file. Recently, the *Multicomponent Assembly* module in CHARMM-GUI is released to make it easy to build a simulation system with multiple components with and without phospholipid membranes. When LPS molecules are supported, *Multicomponent Assembly* can be used to model and simulate crowded environments in the OM, thereby moving ever closer to more biologically representative models.

Known issues in molecular simulation of bacterial membranes.

In order to identify avenues for further work, it is important to consider caveats and limitations of the current state-of-the-art molecular simulations of bacterial membranes. Unsurprisingly, for bacterial membranes these are largely associated with LPS(74). Firstly, there is little structural data on LPS available. As mentioned previously, there are very few structures of OMPs in complex with LPS, thus there can be an element of educated guess-work in terms of positioning OMPs with respect to LPS within model OMs. Furthermore, when constructing models, it is important to ask if it is realistic to

assume that the outer leaflet is essentially all LPS. Can this be generalised across different bacterial species? The vast majority of experiments that provide estimates of how much LPS there is in the outer leaflet were done over a decade ago, so likely need to be revisited with modern experimental setups. While LPS is the major component, the OM outer leaflet does contain other complex glycolipids such as the enterobacterial common antigens (ECAs) and capsular polysaccharides (CPS) whose occurrence is frequently overlooked(75; 76). Therefore, it will be interesting to see the efforts of modeling and simulation of OMs with LPS, ECAs, and CPS together. As a separate note, encouragingly, experimental work by Lakey, Clifton, and others has led to the development of *in vitro* asymmetrical, LPS-containing membranes which enable detailed characterisation of the physical properties of OM-like membranes, while having tight control over their lipidic compositions(77). It will be interesting to see more direct comparison between simulations and these experiments in the future.

From the perspective of performing MD simulations of the OM, by far the biggest obstacle remains achieving convergence due to the slow diffusion rate of LPS. It was hoped that the smoother energetic landscapes of CG models would enable significantly dynamics, alas this has not been the case with models that use an ~ 4 to 1 heavy atom to CG particle mapping. However, development of newer CG parameter sets such as MARTINI3, in which sugars are more accurately represented does offer more hope for the future. Higher LPS mobility has been achieved at all-atom and CG level when phospholipids are incorporated into the outer leaflet, which brings us back to the need to quantify the phospholipid content of the outer leaflet of the OM(78). Sampling of protein-LPS interactions may be enhanced through methods such as replica exchange. Recently, some tests performed by the Khalid group with Hamiltonian replica exchange

using CG (MARTINI 2) models in which the LPS interactions are scaled have shown that sufficient exchange can be achieved after ~5 μs of simulation, in systems without proteins (Khalid. Personal note). More complex systems are currently being tested. Thus, it seems that a combination of CG models and enhanced sampling methods may be a route to overcoming the issue of slow LPS diffusion. However, for specific details of protein-LPS interactions, it would then still be desirable to convert the system back to atomistic detail, given the slightly lower resolution of the CG models. So, there is still work to be done in terms of establishing protocols for efficient simulations of the OM at multiple levels of resolution. There is good news however, when it comes to setting up simulations of these system, as the modules within the CHARMM-GUI server provide automated procedures for setting up systems for this purpose (www.charmm-gui.org). Furthermore, analyses for biomolecular simulations in general are now far less laborious that in the past due to analyses packages that are code/force-field agnostic such as MDAnalysis(79) as well as more advanced tools being available with the popular MD codes.

Conclusions.

In conclusion, the literature discussed here as well as studies to which we have provided references but were unable to elaborate upon due to space limitations all demonstrate that the huge strides have been taken in the last 5 or 6 years in simulations of bacterial membrane systems that are increasingly representative of the *in vivo* environment. Some of the more recent simulation studies that incorporate the cell wall within the periplasm are now enabling the study of movement across bacterial cell envelope compartments, which represents a step change in simulations of these systems. A

particularly gratifying aspect of the state of the bacterial membrane simulation community, and in some ways also a sign of its maturity, are that the models of system components are available in various force-fields, and also all-atom and CG levels of detail. The recent progress in large, complex simulation systems has been facilitated in a large part by automation of simulation set up, largely led by the Im group, and sharing of parameters through various web servers and repositories. We feel strongly that for future progress, not only is the sharing of parameters/models continued, but so too are frank and open discussions regarding the limitations of current models and methods. In summary, much progress has been made in advancing molecular simulations of not only bacterial membranes, but the whole cell envelope. While there are still issues to be resolved, the progress in enhanced sampling methods, newer CG models, and the availability of greater computing power mean that the future for this field is undoubtedly exciting.

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Figures

Figure 1: Chemical structures of *P. aeruginosa* LPS. Note that the cores 1a and 1b cannot have any O-antigen attached to them, but the G2 core can. Three O-antigen structures (O10, O11, and O5a) are shown as examples. Kdo: 2-keto-3-deoxyoctulosonate; Hep: L-glycero-D-manno heptose; Gal: D-galactose; Glc: D-glucose; Rha: L-rhamnose; QuiNAc: *N*-acetyl- D-quinovosamine; GalNAc: *N*-acetyl-L-galactosamine; FucNAc: *N*-acetyl-L-fucosamine; ManNAc: *N*-acetylation- D-mannosamine; ManNAc: *N*-acetamidine-D-mannosamine.

Figure 2: Representative snapshots of OprH embedded in various OM environments with LPS containing (A) only two Kdo residues, (B) G2 core, and (C) G2 core plus two repeating units of O10-antigen (see Figure 1 for their chemical structures). Lipid A is represented as pink spheres, core sugars as grey sticks, O10-antigen polysaccharides as orange sticks, PPPE (1-palmitoyl(16:0)-2-palmitoleoyl(16:1 cis-9)-phosphatidylethanolamine) as blue spheres, PVPG (1-palmitoyl(16:0)-2-vacenoyl(18:1 cis-11)-phosphatidylglycerol) as orange spheres, PVCL2 (1,1'-palmitoyl-2,2'-vacenoyl

cardiolipin with a net charge of -2*e*) as magenta spheres, Ca^{2+} ions as small cyan spheres, K^{+} ions as small magenta spheres, and Cl^{-} ions as small green spheres. For clarity, some portion of each system is truncated and water molecules are not shown.

Figure 3: Coarse-grain simulations of a model outer membrane containing multiple copies of the TonB-dependent vitamin B12 transporter, BtuB. Distinct clustering behaviour is seen. The snapshot is taken after 20 μs of simulation. The proteins are orange and yellow, and the lipids are cyan and blue

Figure 4: Asymmetric structure of the AcrB protein from *E. coli* embedded in a phospholipid bilayer. Loose and Open monomers are represented as orange and lime ribbons respectively, while the tight monomer, also shown as ribbons, with pore (periplasmic) and transmembrane domains colored grey and purple respectively. The main binding pockets (Access and Distal) are represented as molecular surfaces colored green and red, while the exit gate is shown in blue. The switch-loop separating the two binding pockets is highlighted in yellow.

Figure 5: A multicomponent *E. coli* cell envelope system composed of the OM, IM and a single-layered cell wall(73). Proteins and cell wall are represented as surfaces; OmpA is cyan, TolR is lime green and peptidoglycan is purple. The LPS molecules are represented as yellow spheres and phospholipids as blue spheres.