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Seeing is believing: Illuminating the Gram-negative outer membrane with molecular dynamics simulations

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Abstract

Recent advances in molecular dynamics (MD) simulations have led to rapid improvement in our understanding of the molecular details of the outer membranes (OMs) of Gram-negative bacteria. In this review, we highlight the latest discoveries from MD simulations of OMs, shedding light on the dynamic nature of these bacteria's first line of defense. With the focus on cutting-edge approaches, we explore the OM's sensitivity to structural features, including divalent cations and membrane composition, which have emerged as crucial determinants of antimicrobial passage. Additionally, studies have provided novel insights into outer-membrane proteins (OMPs), revealing their intricate roles in substrate translocation and their distinct interactions with lipopolysaccharides (LPS) in the OM. Finally, we explore the challenging process of β-barrel membrane protein insertion, showcasing recent findings that have enhanced our grasp of this fundamental biological phenomenon.

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Introduction

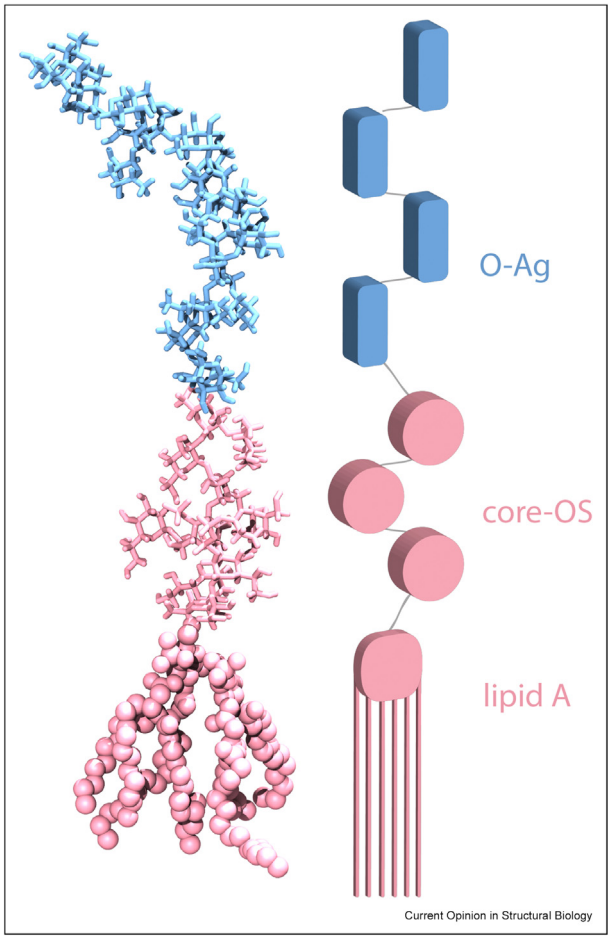
The OM of Gram-negative bacteria constitutes a dynamic and multifaceted interface instrumental in various processes, including nutrient transport, protein insertion and secretion, and defense against antibiotics. In recent years, we have seen review papers on these topics separately [1–3]. Here, we offer a concise and comprehensive perspective on bacterial OM dynamics as observed through MD simulations over approximately the last few years, distinguishing it from recent reviews covering a longer period [4,5] or focused on methodological aspects [6].

The asymmetric OM contains LPS in its outer leaflet and phospholipids in its inner leaflet. In addition to a structural role, LPS can trigger the innate immune response through its interaction with Toll-like receptors such as TLR4, highlighting its significance beyond the bacterial OM [7]. LPS is itself composed of lipid A, core sugars, and, in many cases, O-antigen polysaccharides (Figure 1). MD simulations have emerged as a potent tool for enabling a detailed exploration of the molecular intricacies governing bacterial OM behavior. In this review, we have structured our coverage into four key sections, each delving into a specific facet of OM functionality. The first section explores the OM's sensitivity to structural features, the second section reviews recent studies of OM-OMP interactions, the third section covers the function of pore-forming porins, and the fourth section delves into the intricate process of folding and insertion of β-barrel membrane proteins into the OM.

OM sensitivity to structural features

The composition of the OM, including its asymmetry, the unique structure of LPS molecules, and the high net negative charge of LPS, make modeling it especially complex. To address these challenges, several tools have been developed, with CHARMM-GUI's Membrane Builder emerging as the most prominent and effective among them [8]. MD simulations of bacterial OMs have demonstrated the selection of factors such as phosphate group charges, cation type, lipid composition, and ion parameterization impact membrane properties such as the area per lipid (ApL) and inter-lipid hydrogen bonding [9,10]. Protonating the phosphate groups and adjusting the non-bonded-interaction parameters for monovalent cations with LPS produced the best agreement with experimental results [9]. Notably, a lower LPS charge resulted in a reduced ApL and a thicker hydrophobic region with a less hydrated LPS core, highlighting the importance of phosphate protonation states [9]. Even the way the two leaflets of an asymmetric OM are combined can affect its properties. The most common approach is to match the surface areas of the two leaflets, but this can induce a differential stress between them when combined, which can alter, e.g., the free energies of small molecules in the

Figure 1



Structural representation of LPS. The three primary components of LPS are indicated in both atomistic (left) and schematic (right) depictions: lipid A (pink spheres on left) forming the membrane anchor, core oligosaccharides (core-OS, pink sticks), and O-antigen (O-Ag, blue sticks) extending outward from the cell surface. The molecular detail on the left exemplifies the intricate chemical structure, while the schematic on the right highlights the three distinct regions of LPS, emphasizing its modular architecture.

membrane [10]. Nonetheless, a zero-differential-stress approach can also introduce inaccuracies in mechanical properties of the membrane, and thus the appropriate choice of method needs to be considered carefully [11].

LPS is the predominant component of the outer leaflets of OMs and, when present, the O-antigen polysaccharide chains can extend tens of nm above the surface, forming a mesh-like barrier. In a combined immunology-simulation study, it was found that the accessibility of epitopes on OMPs is modulated by O-antigen length, with longer chains preventing antibody binding [12]. In addition to LPS, other molecules may be present as well. These include the glycolipids Enterobacterial common antigens (ECA), which are found in members of the *Enterobacteriaceae* family, and the much longer capsular polysaccharides (CPS), which are more commonly found across bacteria. Atomistic simulations of symmetric bilayers composed of LPS and ECA showed that a higher percentage of ECA makes the LPS more flexible, increasing its molecular area [13]. In contrast, LPS-linked CPS makes the membrane more rigid, decreasing the molecular area of LPS, while PG-lipid-linked CPS has the opposite effect [14].

Coarse-grained (CG) simulations, investigating both smooth (with O-antigens) and rough (without O-antigens) LPS, revealed that smooth LPS is packed more tightly, resulting in stronger intermolecular interactions and lower lipid mobility. Tighter packing also affects the mechanical strength of the OM, meaning that rough-LPS-containing OMs rupture at lower surface tensions than smooth-LPS-containing ones [15]. In order to study more varied LPS-containing systems with CG simulations, new models and parameters for additional sugars have been developed and validated through comparison to atomistic simulations [16].

In order to breach the OM, antibiotics must enter through a porin or other OMPs or disrupt the LPS to permeabilize it. Polymyxins, for example, take the latter route, in part through displacement of the divalent cations that bridge LPS molecules [17]. Free-energy analysis of the route shows a significant free-energy barrier at the interface between the LPS inner core and lipid A [18]. MD simulations illustrate how membrane composition influences polymyxin's interaction with the OM and its effects on membrane dynamics; for example, it increases diffusion of LPS in the OM, while it decreases diffusion of lipids in the outer leaflet of the inner membrane (IM) [19]. Additionally, polymyxin's effects on structural properties such as the ApL and membrane thickness depend significantly on membrane composition [17,19]. Another approach to breaching the OM is to combine antibiotics with adjuvants, which enhance their potency. MD simulations of novel adjuvants with a diamidine core structure demonstrated that the large separation between the positively charged ends is likely a key factor in their activity [20].

The roles of outer-membrane proteins

While MD simulations of membranes have yielded a number of new insights, incorporating proteins into the simulations provides a more comprehensive and detailed perspective. OMPs play a pivotal role in transport across the OM, as demonstrated by MD simulations showing that the gap in LPS created by OmpF could be beneficial for the translocation of short antimicrobial peptides [21]. MD simulations also highlighted the profound importance of electrostatic interactions in ligand-protein binding [22]. CG simulations of multiple OMPs demonstrated that each has a

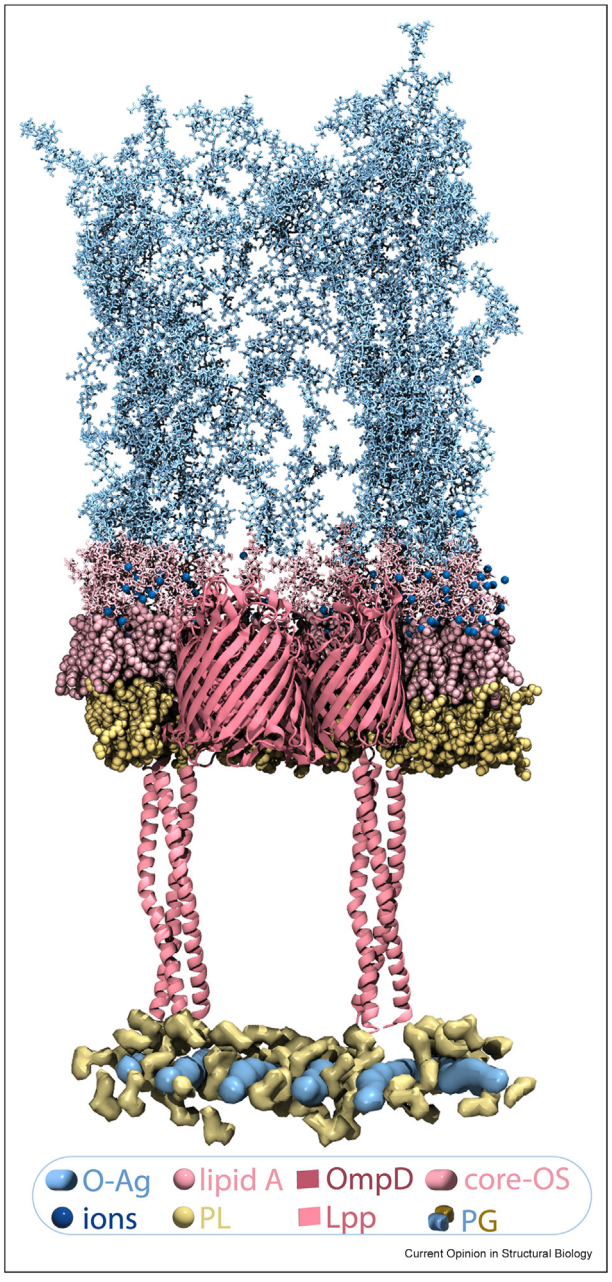
"fingerprint", i.e., a distinct orientation and pattern of interactions with the OM, which is influenced by both the OMP and the membrane composition, including LPS length [23]. Additionally, the tight binding of divalent cations has been shown to prevent convergence of CG potential of mean force (PMF) calculations during LPS extraction, a problem that was mitigated by restraining the ions to remain in the OM [24].

OMPs, such as Ail, can induce rigidity and thickening of the OM, improving its resistance to antibiotics [25]. On the other hand, the stability of OMPs is influenced by the position along the membrane normal of nonpolar side chains, as revealed by calculations of the interface-to-bilayer transfer free energy [26]. OMPs often form clusters, shown by cross-linking and CG simulations, typically mediated by a single lipid/LPS between them [27]. The presence of these interfacial LPS and lipids not only enhances the impermeability of the OM but also contributes to the stabilization of OMP assemblies, ensuring structural integrity [27]. OMP–LPS interactions are also mediated by divalent cations. Because of the resulting low rate of diffusion of LPS in the OM, MD simulations can be heavily biased by initial conditions, necessitating the development of novel approaches for construction of OMP-membrane systems [28]. Atomistic and CG simulations of the OM of *Pseudomonas aeruginosa*, with and without embedded OMPs, have further illustrated how these ions stiffen the OM, making it an effective permeability barrier [29].

The OM in Gram-negative bacteria is coupled to the IM through large protein complexes and also to the intervening peptidoglycan (PG) cell wall through various linkages (Figure 2). MD simulations have been used to investigate both of these couplings, revealing, for example, how crowding affects diffusion of polymyxins from the OM to the IM [30] and how Braun's lipoprotein (Lpp) [31] as well as OmpA [32] regulate the spacing between the OM and PG in *Escherichia coli*. OM-PG linkages in species that lack Lpp have been identified and studied as well [33]. Simulations were also used to resolve interactions between PG and the periplasm-spanning multi-drug efflux pump AcrAB-TolC, which may influence its assembly and stability [34]. Another periplasm-spanning complex, the Tol-Pal system, has been investigated with steered MD simulations, which helped to elucidate the fundamental mechanisms by which the Tol assembly orchestrates the localization of Pal in the OM at the septum for cell division [35].

LPS molecules are inserted into the OM by LptD/E, part of the Lpt system. LptD forms a large, 26-stranded β-barrel in the OM. Both MD simulations and wet-lab experiments suggest that not only does LptD have a lateral gate for egress of LPS into the OM, its opening can be primed by the presence of an LPS molecule in LptD's "β-taco" domain near the gate [36,37].

Figure 2



OM and peptidoglycan (PG) components of the Gram-negative cell envelope. O-antigen is displayed in light blue (sticks), core sugars in pink (sticks), OmpD trimer in dark pink, lipid A in pink (spheres), phospholipids (PL) in dark yellow, Lpp in rose, ions in dark blue, and PG in light blue and dark yellow (surface).

Conversely, this β-taco domain can be stabilized by binding of the antimicrobial peptide thanatin, which blocks transport of LPS [37].

Insights into transport across the OM

Pore-forming OMPs, also known as porins, facilitate the diffusion of essential nutrients into the Gram-negative

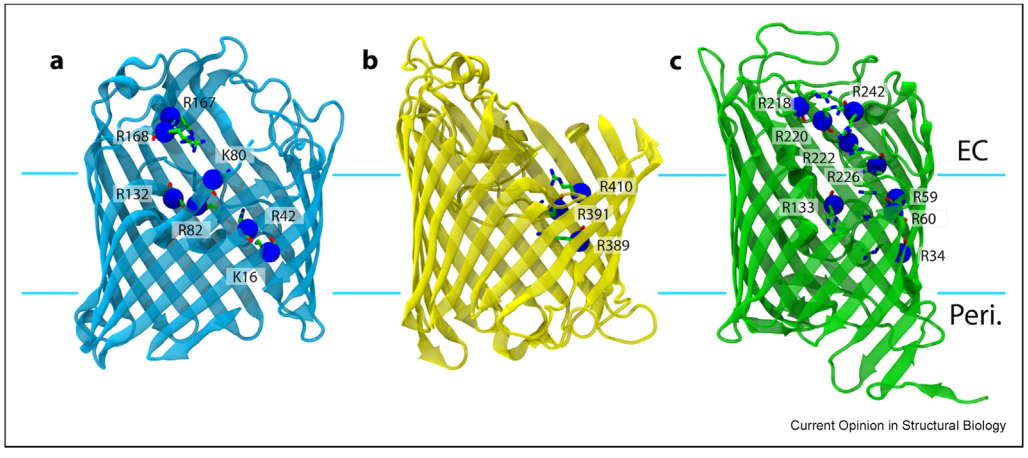
bacterial cell. Additionally, they serve as a conduit for the influx of many antibiotics. MD simulations have provided valuable insights into these processes. For instance, research on OmpF has demonstrated its L3 loop functions as a gate for antibiotic translocation, with the open-closed state transition influenced by negatively charged residues on the loop [38]. Furthermore, the charge distribution of bulky antibiotics can disrupt the protein's hydrogen-bond network to open the gate and, consequently, create a low-energy pathway for permeation [39]. Interestingly, L3 of OmpF became more dynamic with increased temperature than L3 of OmpC in MD simulations, which could be connected to the different conditions under which the two porins are expressed [40]. Other aspects of translocation through OMPs, such as the presence of Mg^{2+} ions [41], the arrangement of basic residues on the β-barrel wall (Figure 3a) [42], and the electric field in the constriction region [43], have also been studied using MD simulations and shown to play significant roles in influencing antibiotic permeability. Moreover, OmpM of *Veillonella parvula*, which possesses a flexible stalk that interacts with PG, has been studied (through experiments and MD simulations) for its dual functionality in stabilizing the OM and facilitating nutrient absorption, indicating its potential evolutionary importance in bacteria and the development of the OM [44].

The OMs of certain bacteria, such as *P. aeruginosa*, feature poor permeability due to the intrinsic rigidity of the OM [29] as well as to the lack of highly permeable, large-channel porins such as OmpC and OmpF. Instead, they utilize various substrate-specific channels, like the Occ family, for nutrient uptake. For example, OprD, a member of this family, is responsible for transporting basic amino acids as well as some carbapenem antibiotics. MD simulations have shown that OprD possesses a distinct "basic ladder" on one side of its β-barrel (Figure 3b), serving as an electrostatic guide for substrate permeation [45]. Similarly, phosphate-selective channels like OprP (Figure 3c) and OprO display an arginine ladder on the extracellular side and a lysine cluster on the periplasmic side. This feature enables them to interact with the phosphonic acid group of substrates; MD simulations showed that it also effectively guides antibiotic molecules through each porin monomer [46].

Even though large molecules are generally not transported across the OM, there are exceptions. A notable instance is the passive permeation of cationic antimicrobial peptides through the channel CymA. This process is facilitated by the negatively charged residues within the channel as observed in MD simulations [47]. Another OMP, BtuB employs an induced-fit mechanism during transport of vitamin B12. MD simulations have suggested that the conformational changes of the extracellular loops of BtuB, necessary for its opening and closing movements, are governed by non-specific interactions with substrates [48]. This mechanism has been explored for potential applications in delivering peptide nucleic acids to *E. coli* cells [49], demonstrating the feasibility of using oligonucleotides as programmable agents to inhibit bacterial growth. Some species, such as *Bacteroides thetaiotaomicron*, encode multiple copies of BtuB. MD simulations of one example, named BtuB2, illuminated its interactions with BtuG2, an accessory extracellular protein, and helped to resolve how BtuG2 binds B12 and hands it off to BtuB2 [50].

As discussed above, MD simulations have become an important tool for evaluating the permeability of

Figure 3



Key basic residues of some porins. Blue spheres indicate the position of C_α atoms, while entire residues are displayed in stick representation. **(a)** Side view of an OmpF monomer (PDB: 2OMF) [42]. **(b)** Side view of OprD (PDB: 3SY7) highlighting the "basic ladder" [45]. **(c)** Side view of an OprP monomer (PDB: 2O4V) [46]. The light blue lines indicate the approximate position of the hydrophobic region of the OM. The extracellular side is indicated by "EC" and the periplasmic side by "Peri".

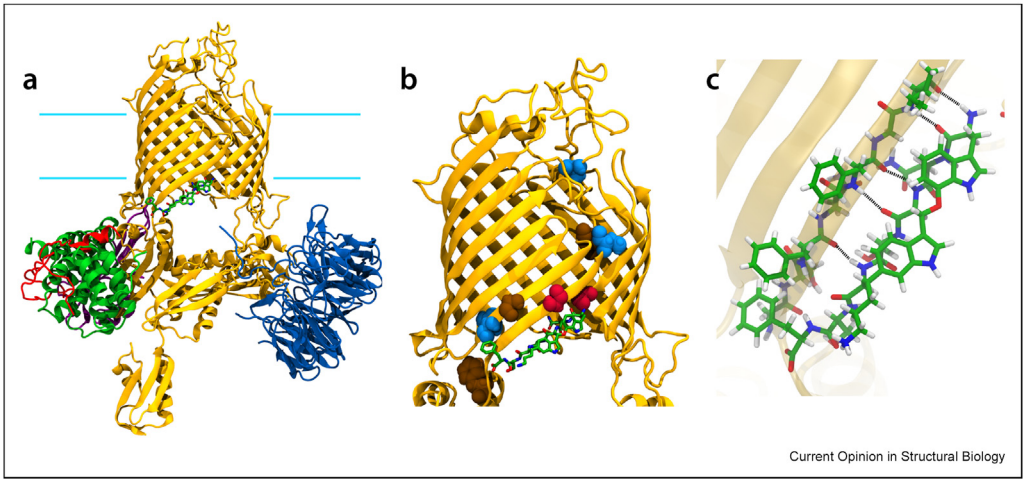
bacterial porins to antibiotics, with a particular focus on calculating the free energy along the permeation pathway. For example, through a comparative analysis of various umbrella sampling (US) methods, replica-exchange US, in particular, has proven effective at achieving converged PMF calculations with an accuracy reaching sub-kcal/mol [51]. Additionally, employing temperature acceleration in conjunction with slowly evolving collective variables has helped to mitigate sampling issues, thus enhancing convergence of free-energy estimates [52]. The integration of Monte Carlo simulations and graph theory has also been shown to be beneficial in determining pathways for enhanced sampling [43], further advancing the field.

Folding and insertion of OMPs into the OM

The folding and insertion of β-barrel membrane proteins into the OM is a challenge for bacteria, exacerbated by the lack of chemical energy at the OM. Certain physicochemical properties of the OM and the proteins themselves contribute to resolving this challenge. For example, the asymmetric nature of the OM, most notably the higher amount of negative charges in the outer leaflet due to LPS compared to the inner leaflet, aids the proper insertion of OMPs via specific interactions with positive charges on their extracellular side identified via MD simulations [53]. Other protein features, such as the length and composition of extracellular loops [54] and the presence of inward-facing glycines within the β-barrel [55], also modulate the insertion process. Critically, folding and insertion is catalyzed by the OM-associated β-barrel assembly machinery (BAM) complex [56]. Unfolded OMPs, which are maintained in distinct folding-competent ensembles in the periplasm [57], are delivered to BAM by chaperones to initiate folding and insertion.

The BAM complex, the core component of which is the 16-stranded β-barrel OMP BamA, catalyzes insertion in multiple ways. MD simulations of BamA in its native OM [59] and in a nanodisc [60] have revealed significant membrane distortions near the seam between the N- and C-terminal β-strands, which could lower the energetic barrier to OMP insertion by thinning and destabilizing the OM locally. Additionally, the seam, also called the lateral gate, adopts multiple conformations, including an open one that may be its predominant form in the OM [60]. The open lateral gate was proposed to act as a template for folding of OMPs, which could insert one or more β-strands at a time, forming a so-called "hybrid barrel" with BamA. MD simulations provided support for the hybrid barrel model by demonstrating the large degree of plasticity of the β-barrel domain of BamA [61], which must contort to accommodate the growing β-barrels of numerous substrates [62]. High-resolution cryo-EM structures of intermediate states, first of a four-β-strand substrate inserted into BamA's lateral gate [61] and then of a 12-β-strand substrate [63], validated predictions from computational modeling. For example, hydrogen bonds between BamA's β1 strand and the substrate's C-terminal β strand are more numerous and uniform than those at BamA's β16 strand [62], supporting a more detailed "asymmetric hybrid barrel" model of folding and insertion [64]. Insights into OMP development have also carried over to the mitochondrial homolog Sam50, which was revealed to have a similar hybrid-barrel mechanism;

Figure 4



BAM complex with darobactin (PDB 7NRI) [58]. **(a)** BAM complex (BamA in gold, BamB in blue, resolved portion of BamC in red, BamD in green, BamE in purple) with darobactin (sticks representation) bound to the β1 strand of BamA. Light blue lines indicate the approximate location of the hydrophobic core of the OM. **(b)** Focus on darobactin bound to BamA. Residues involved in resistant mutants are shown in a space-filling representation (tan – F394, E435, G443; blue – T434, Q445, A705; red – G429, G807). **(c)** Close-up view of darobactin (right) bound to β1 (left). Hydrogen bonds are indicated by dotted lines.

MD simulations were used to fill in three β-strands that were unresolved in the electron density map [65].

Unsurprisingly given its essential function in Gram-negative bacteria, BamA has recently been a focus of the development of novel antibiotics. Perhaps most notable, the natural product darobactin has been discovered to mimic a BamA substrate, allowing it to bind to the lateral gate and block access of native substrates to it (Figure 4) [58]. MD simulations further showed that darobactin displaces lipid molecules from the lateral gate region, forming stronger contacts with BamA [58]. In addition to evaluating the bound-state of darobactin, MD simulations have also revealed how darobactin-resistant mutants function by enhancing lateral-gate dynamics (Figure 4b) [66].

Concluding remarks

In conclusion, the application of MD simulations has provided unprecedented insights into the behavior and functionality of bacterial OMs. From their sensitivity to specific constituents to the pivotal roles of OMPs, MD simulations have illuminated the intricate structure and dynamics of the OM. Furthermore, these simulations have deepened our understanding of the folding and insertion of β-barrel membrane proteins, shedding light on processes crucial for both bacterial biology and antimicrobial strategies. Looking ahead, MD simulations are poised to continue making an impact, including in the development of antibiotics as well as other innovative therapies to combat bacterial infections. The dynamic and adaptable nature of bacterial OMs is still a subject of intense exploration, and MD simulations will remain an invaluable tool in elucidating their properties.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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