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Physical properties of the bacterial outer membrane

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Abstract

The ability of the Gram-negative outer membrane (OM) to act as a permeability barrier has long been appreciated, but recent studies have uncovered a more expansive and versatile role for the OM in cellular physiology and viability. Due to recent developments in microfluidics and microscopy, the structural, rheological, and mechanical properties of the OM are becoming apparent across multiple scales. In this review, we discuss recent experimental and computational studies that have revealed key molecular factors and interactions that give rise to the spatial organization, limited diffusivity, and stress-bearing capacity of the OM. These physical properties suggest broad connections between cellular structure and physiology, and we explore future prospects for further elucidation of the implications of OM construction on cellular fitness and survival.

Introduction

The architecture and makeup of the bacterial cell envelope have long been topics of intense interest, in part due to the importance of the envelope for viability, virulence, and mechanical integrity. Unlike Gram-positive bacteria, Gram-negative bacteria have two highly distinct membranes that delimit an aqueous cellular compartment called the periplasm[1] (Figure 1A). The peptidoglycan (PG) cell wall, which determines cell shape[2], resides in this extra-cytoplasmic compartment, as does a variety of proteins. Surrounding the cell wall is the outer membrane (OM), a hallmark of Gram-negative species. The OM is an atypical biological membrane: while it is a lipid bilayer, it is asymmetric, with phospholipids (PLs) in the inner leaflet and a glycolipid known as lipopolysaccharide (LPS) in the outer leaflet[3]. The OM also contains two major classes of proteins. Nearly all of the transmembrane proteins assume a β-barrel fold and are commonly referred to as outer membrane proteins (OMPs)[1]. The OM additionally contains lipoproteins, which are mostly

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soluble but have a lipid moiety at the amino terminus that enables their embedding in the OM[4]. Unlike typical biological membranes, which are impermeable to protons, the OM is freely permeable to small, water-soluble molecules like sugars and amino acids[5]. These molecules diffuse through the OM via channels formed by a major class of OMPs called porins[5]. Also unlike typical membranes, the OM is resistant to detergents and other hydrophobic toxins. The strong lateral interactions between LPS molecules, together with their saturated acyl chains, stabilize the OM, greatly hindering the passage of hydrophobic molecules[5]. These biochemical properties hint at the mechanical importance of the OM, yet many of its structural properties remain mysterious. Only recently have studies started to probe the magnitude, molecular determinants, and tuneability of OM physical properties.

The OM is thought to be essential for viability in most Gram-negative bacteria[1]. Due to its adjacency with the extracellular milieu, the OM serves as the location of a substantial fraction of environmental sensors[6-9] and as an anchor point for adhesive organelles[10]. The OM acts as a platform for interacting with host immune systems[11-13], as well as with neighboring bacterial cells through surface contact[14] and vesicles[15]. Moreover, the OM is an important permeability barrier providing Gram-negative bacteria resistance to large and hydrophobic antibiotics that are effective against Gram-positive bacteria with conserved targets[5,16-18]. Indeed, multi-drug resistant Gram-negative bacteria currently pose a serious threat to human health[19,20], and the inability to overcome OM barrier function has, in part, hampered antibiotic discovery efforts[21,22] aimed at avoiding a possible return to the time when infectious diseases were more feared than cancer[23]. The OM is also a barrier to large molecules, for example by sterically hindering antibodies and phages from binding to surface targets[24-28]. Although clearly useful, this barrier function does not explain why the OM is essential. In *Escherichia coli* for example, there are only a few enzymes present in the OM, and none of these enzymes performs an essential function. Indeed, the only essential proteins in the OM are ones necessary for building the OM[1].

Besides the presence or absence of an OM, another major difference between Gram-negative and Gram-positive bacteria is the thickness of the PG cell wall, which is a monolayer in at least several Gram-negative model organisms[29] and multi-layered (often tens of nanometers thick[30,31]) in Gram-positive organisms. The classical picture of the Gram-negative bacterial envelope has assigned responsibilities for stress-bearing to the cell wall and barrier functions to the OM. However, the two structures are physically connected: across the entire surface by proteins such as OmpA[32,33], at the division site by the Tol/Pal complex[34,35], and during cell wall synthesis by the LpoA/B proteins that bind to the wall synthesis enzymes PBP1A/B[36,37]. In certain enteric bacteria such as *E. coli*, the cell wall and the OM are also coupled covalently by Braun's lipoprotein[38]. Moreover, in recent years, accumulating evidence has suggested that the essential function of the OM may be mechanical[39-42], contributing physical strength to compensate for the thin cell wall. Indeed, the crosslinking of LPS molecules by divalent cations[43] has been suggested as a mechanism of mechanical stabilization[44]. Moreover, although antibiotics targeting cell-wall biogenesis are effective against Gram-negative bacteria, they can often survive at least temporarily without a cell wall[45,46], with certain mutations enabling stable, wall-less proliferation[47]. In this review, we will focus on the emerging physical and mechanical properties of the OM and the ways this unique membrane contributes strength to improve bacterial fitness.

Molecular components of the OM

The OM is made up of PLs, LPS, and proteins[3,48-50], whose localization patterns and interactions determine the basic chemical and physical properties of this structure. The abundances of OMPs and lipoproteins[51] as well as LPS offer insights into OM function. For decades, OM research has focused on bilayer asymmetry and the biochemical functions of the molecular components[16,17,48,49,52-58]. Now, advances in imaging and force spectroscopy are improving our understanding of the molecular organization and physical properties of the OM at higher spatial and temporal resolution (Figure 1B).

The LPS that occupies the outer leaflet[3] consists of three moieties: the hydrophobic lipid A, a conserved oligosaccharide core, and a variable polysaccharide called the O-antigen[59]. The negatively charged LPS molecules are neutralized and bridged (often referred to as "cross-linked") by divalent cations such as Mg^{2+}. At roughly 1 million molecules per cell[53], LPS makes up a substantial fraction of the OM and hence has the potential to be a major contributor to maintaining structural integrity of the OM. LPS localization to the OM outer leaflet was first determined in *Salmonella*[3] and is now thought to generally apply to Gram-negative bacteria. Some antibacterial agents specifically target LPS: LPS molecules can be stripped out of the OM by the divalent cation chelator EDTA[52], and are bound stoichiometrically by the cationic cyclic lipopeptide polymyxin B[55,60]. By examining the ability of uncharged, hydrophilic molecules to penetrate the OM[16], it was found that disruption of the negative charge of LPS substantially increased permeability, suggesting a central role for LPS in OM barrier function[55,61].

OMPs are transmembrane β-barrels that also have important structural and functional implications for the OM. AFM revealed that OMPs cover ~70% of the cell surface[62]. Many studies have revealed diverse and critical roles played by OMPs, including signaling, nutrient import, virulence, and OM biogenesis[34,37,63,64]. In addition to these biological functions, the high abundance of OMPs, at roughly 500,000 per *E. coli* cell[51], suggests a role in maintaining the physical properties of the OM. Indeed, AFM measurements revealed OMPs packed into islands rather than existing as single proteins that diffuse freely in the membrane, a sign of strong interactions that restrict mobility[62,65,66] (Figure 2A,B). Coarse-grained molecular dynamics simulations suggested that specific interaction surfaces between OMPs are key to island formation[67].

Lipoproteins, which are anchored in the membrane with a lipid moiety, are another important class of protein in the OM with over 1 million per *E. coli* cell[51]. As noted above, in certain enteric bacteria such as *E. coli,* the most abundant is Braun's lipoprotein (Lpp), whose C-terminus is covalently bound to the PG layer, providing coupling between the OM and the cell wall[38] (Figure 1A). Lipoproteins are involved in various pathways including capsular synthesis regulation[7], colonization, immune system evasion, peptidoglycan synthesis[36,37], OMP biogenesis, and LPS transport[68,69]. The extent to which lipoproteins affect OM organization is largely unknown, although evidence of their transfer between *Myxococcus xanthus* cells suggests that they are highly mobile[14]. Taken together, every component of the OM has the potential to impact its physical properties.

Molecular interactions between LPS and OM proteins

In the OM, proteins and LPS interact in multiple ways. Insertion of LPS into the outer leaflet requires the Lpt transport proteins[63,69]. Recent evidence increasingly supports a physical picture of the outer leaflet made up of LPS and porins as a partially ordered structure with strong lateral interactions at the molecular scale (Figure 2A). In X-ray reflectivity and grazing incidence X-ray diffraction measurements, LPS monolayers at water-air interfaces showed lateral ordering highly distinct from phospholipid membranes, and this organization could be perturbed by the ionic strength of the aqueous phase; Ca^{2+} ions increased the rigidity of the monolayer by cross-linking LPS molecules[43]. Conversely, treatment with EDTA, which chelates divalent cations, induces release of LPS from the OM[52]. Atomic force microscopy (AFM) measurements on living cells showed that OM porins distributed among LPS molecules formed a densely packed, net-like structure that diffused slowly[62,66]. Mutagenesis and structural studies (X-ray, neutron scattering) together revealed specific interactions between LPS and porins that stabilize the ordered network of LPS molecules and maintain permeability[64]. Molecular dynamics simulations have provided further support for strong interactions among LPS molecules and between LPS and proteins[70-73], and have suggested that the LPS environment can affect the accessibility of certain small molecules to the passive transport porin OmpF[74], as well as access to surface epitopes by antibodies[28] and phage[26,75,76].

Physical structure and properties of the OM

The dynamics of and interactions between molecules in the OM lead to a variety of structural characteristics and physical properties, including limiting the diffusion of molecules within the OM and producing spatial heterogeneity. Thus, molecular composition, structure, and dynamics can generate physical behaviors at the cellular scale. Recent studies integrating experimental and computational approaches are beginning to reveal the mechanisms relating these properties to molecular-scale organization and envelope mechanics, and to connect them to cellular fitness and survival.

The lipids and proteins within a bilayer such as the bacterial inner membrane typically diffuse quickly, with fluorescence recovery after photobleaching (FRAP) occurring within seconds[77]. However, the unusual asymmetric bilayer structure of the OM can give rise to distinct diffusive properties, altering the motion of constituent molecules and hence their spatial distribution. FRAP experiments and pulse-chase labeling with a general OM label, fluorescent succinimidyl ester (flSE), suggested that LPS and some proteins in the OM are largely immobile while other molecules can diffuse relatively quickly[78,79], suggesting that the OM behaves more like a gel (a semi-solid with dilute crosslinks) than a fluid (Figure 2A). Proteins with limited mobility that are inserted locally must rely on the heterogeneity of OM growth and on cell division to cover the entire OM (Figure 2B). In addition to proteins, the limited motility of LPS molecules may impact the diffusivity of the OM as a whole given their high abundance and lateral interactions[79].

By contrast to the homogenizing tendency of rapid diffusion, low diffusivity has the potential to produce spatial heterogeneity at the cellular scale by limiting exploration of individual molecules. Indeed, pulse-chase labeling of the abundant OM protein LamB in

E. coli cells revealed the insertion of large clusters along the cylindrical region, not at the poles, that were essentially immobile[80]. Local interactions of LPS and proteins, together with expansion of the OM during cell growth are predicted to result in a spatial pattern wherein newly inserted clusters near mid-cell push older clusters toward the poles[80] (Figure 2C). Pulse-chase flSE labeling showed that localization of OM components at the poles is more stable than along the cylindrical region[78]. A separate study demonstrated that synthesis of clusters of OMPs that include the β-barrel assembly machine (BAM) is biased away from the poles, with clusters migrating to the poles due to growth[81] (Figure 2C). Heterogeneity of the OM is accompanied by heterogeneous growth of the PG[82,83], with bursts of similar sizes[84]. While *E. coli* has been the predominant model for studying OM synthesis and properties, evidence from many other species such as *Agrobacterium tumefaciens*[85], *Shigella flexneri*[86,87], and *Salmonella* Typhimurium[17] indicates that the OM may be spatially heterogeneous across Gram-negative bacteria.

Given the asymmetric structure of the OM, its molecular organization may play a critical role in its biochemical functions. Interestingly, Lpp was found to occupy distinct subcellular compartments in which molecules were either surface-exposed or periplasmic and PG-bound[88]. This transition between bound and unbound states is reversible, and detachment from the PG has been proposed to be beneficial under certain stress conditions[89,90]. It remains unclear why free-form Lpp is spatially distinct from its PG-bound counterparts, and whether alteration of the balance between the two states alters OM organization and/or mechanics.

Despite continuing knowledge gaps, recent studies have highlighted the impact of OM physical organization and properties on cellular physiology and interactions with the environment. For instance, binding of phages to the OM and subsequent motion before internalization is impacted by the heterogeneous distribution of its receptors[91]. Moreover, the physical organization of molecules within the OM can contribute to its structural integrity and mechanical strength. Characterization of the rheological properties of an *in vitro* monolayer showed that LPS undergoes a viscous-to-elastic transition upon compression[44]. This finding suggests that the molecular interaction between LPS molecules provides an important basis for the viscosity, stiffness, and/or strength of the OM. Similarly, it has been speculated that protein interactions can contribute to OM mechanical integrity[92] and biosynthesis[93]. In addition, Lpp defines the periplasmic width[94], and AFM measurements showed that mutants with disrupted Lpp crosslinking to the PG or increased Lpp length have weaker cell envelopes[95]. In some species lacking Lpp, β-barrel proteins were found to take on the role of covalently linking the OM to the cell wall, potentially also providing mechanical coupling[96,97]. Taken together, OM integrity and mechanics are likely intrinsically tied to the assembly and movement of its molecular components.

Evidence for the OM as a mechanical structure

Historically, the cell wall has been assumed to be the sole agent responsible for the task of maintaining mechanical integrity. However, over the past few decades, a growing appreciation for the mechanical role of the OM has emerged, driven in large part by advances in experimental methodologies. Studies of perturbations that lead to cell lysis

revealed that Gram-negative bacterial cells can survive temporarily when the cell wall is disrupted or degraded, suggesting a role of the OM in maintaining cell integrity. For example, when phages induce breakdown of the wall, cells round up rapidly but persist in a viable state for up to an hour before lysis[98]. Upon vancomycin treatment of *E. coli* mutants with a permeable OM, the cytoplasm bulges through holes in the cell wall, but nevertheless lysis does not occur for tens of minutes[99]. In a similar study of bulging, cells treated with both β-lactam antibiotics and EDTA to disrupt OM integrity lysed more rapidly than during β-lactam treatment alone, implicating the OM in mechanical integrity[41]. Under certain conditions, spheroplasts (cell wall-deficient cells) can even survive indefinitely without a wall[45,47,100-102]. Importantly, Mg^{2+} is often required for generation of spheroplasts, suggesting that in the absence of the cell wall the OM must be stabilized by LPS crosslinking in order for cells to survive[100].

However, direct evidence for a mechanical role of the OM had been lacking until recently. Spurred by the advent of microfluidics, which allows for the tracking of changes to single-cell morphology during perturbations, a recent study subjected *E. coli* cells to hyperosmotic shock (Figure 3A). The shock caused cells to decrease in length, consistent with the removal of turgor-mediated stress on the cell envelope. Under the hypothesis that the cell wall bears most (if not all) stress, in the plasmolyzed state after hyperosmotic shock the cell wall would be stress-free. However, after cells were subsequently treated with detergent to remove the IM and OM, a further reduction in length of up to 40% occurred[40]. This second contraction indicated that the cell wall was still under stretching forces after hyperosmotic shock, and that removal of the membranes allowed the cell wall to relax to its true rest length. To test whether the OM was the element contributing to cell-wall stretching, cells were treated before and during hyperosmotic shock with EDTA to cause rapid removal of LPS from the OM[52]. Now, there was significantly larger contraction after hyperosmotic shock, while the rest length of the cell wall after detergent treatment remained the same as without EDTA treatment. These two contractions (post-shock and post-detergent) provided key input to a biophysical model predicting that the relative stiffness of the OM is comparable to that of the PG cell wall[40]. This conclusion is supported by molecular dynamics simulations of a model of the OM as an asymmetric bilayer with a mixture of *E. coli* PLs in the inner leaflet and LPS in the outer leaflet[103].

Several other complementary experimental approaches support the prediction of high stiffness of the OM. In a microfluidic device in which cells are placed in narrow channels to grow filamentously into a region subjected to tunable flow pressure, cells deflect under fixed hydrodynamic force[104], and deflection was increased by treatment with EDTA (Figure 3B); fitting the data to a biophysical model of thin-shell bending enabled an estimate that Young's modulus (Box 1) decreased ~3-fold[40]. AFM measurements of EDTA-treated cells, in which cells are subjected to local indentation in contrast to the longitudinal stretching induced by turgor pressure (Figure 3C), further supported the mechanical contribution of the OM[40].

In addition to EDTA treatment, a number of factors can tune OM stiffness (Figure 3D). Underscoring the importance of LPS properties, deleting the LPS O-antigen from an *E. coli* gut isolate dramatically reduced OM stiffness, and the OM of *V. cholerae* O139 was comparatively less stiff, consistent with a lack of LPS multivalency in this species[40].

Deletion of several abundant OM proteins, including OmpA, Pal, and Lpp, also reduced stiffness[40]. Interestingly, as mentioned above, all three of these proteins have roles in linking the PG and OM[32-35,38,88]. In the case of Lpp, which is the only protein covalently linked to the PG, its deletion, disruption of its crosslinking to PG, or modification of its length reduced cell stiffness as measured by AFM[95]. It remains to be seen whether the role of these proteins in OM mechanics is direct, or a consequence of modified OM composition, particularly PL and LPS levels, which can be highly responsive to the deletion of OMPs[54]. Consistent with this idea, the mutant allele *lptD4213*, which suppresses transport of LPS to the OM, increases PL content at the expense of LPS[105] and decreases OM stiffness[40]. However, the inner membrane protein YhdP, which modulates anterograde PL flow and affects biosynthesis of the enterobacterial common antigen[106], decreases OM stiffness without affecting LPS levels[107].

The physiological importance of OM stiffness

The structural importance of the cell wall is made clear by the deleterious effects of chemical inhibition of its biogenesis. Treatment with β-lactam antibiotics, which inhibit penicillin binding proteins involved in PG synthesis, leads to swelling (increase in cell width)[108] or bulging[99,109], and eventually cell lysis at high concentrations. Nonetheless, cells can be propagated in a wall-less state, as so-called “L-forms”[45], which survive via mechanical stabilization by the OM[41] (Figure 3E). As with spheroplast generation, successful L-form propagation relies on the addition of Mg^{2+} to the medium[100], likely due to the importance of LPS cross-linking in the absence of the wall, and proliferation has been postulated to rely on membrane blebbing and tubulation[47], implicating membrane composition as an important factor in mechanical stability. Mutants with a mechanically weakened OM have dramatically lower spheroplast yields[40,107]. In fact, during the process of spheroplast generation, lysis of *lptD4213* cells occurred as the cytoplasm started to escape from the cell wall[40], the time at which the OM would be required to take up the stress. Similarly, after degradation of the cell wall during phage infection, disruption of the OM was necessary to enable virus release[110,111]. Thus, it is likely that the burden of mechanical integrity falls on the OM when the cell wall is compromised or removed.

Nonetheless, during steady-state growth, the *E. coli* OM is synthesized in a state such that the area of the OM does not change after plasmolysis and cell wall digestion[40]. Thus, OM area in a turgid state is the same as in the absence of turgor, indicating that the OM does not bear stress in this state. However, in environments such as the gastrointestinal tract and soil, cells are constantly subject to fluctuating osmolality[108,112,113], in which the OM is likely to be relied upon for stress-bearing upon perturbation to avoid excessive compression or extension of the cell wall and IM (Figure 3F).

Modification of LPS, for example through addition of amino-arabinose or phosphoethanolamine[114], can provide protection against AMPs and antibiotics[115,116]; cells likely have to balance the cost of these modifications on mechanical stability with the need to protect from molecules that can kill them. Osmotic oscillations led to increased lysis and greater amplitude of length change in situations with a compromised OM[40], indicating that OM stiffness may be critical for survival in fluctuating environments.

In addition, bacterial membrane composition can be modulated by environmental cues such as culture conditions and temperature[117-120], which will likely induce changes in physical properties[121]. For instance, the relative abundance of unsaturated or branched lipids in the IM can cause drastic changes in membrane viscosity[122], and similar effects may occur in the OM[123], given that the lipid A component of LPS can contain unsaturated and branched acyl chains under certain conditions[124-127]. Thus, it is likely that cells can tune the physical properties of the OM by adjusting its molecular composition, and thereby adapt to different growth stages, conditions, and environmental assaults.

Addressing knowledge gaps

From its structural and mechanical properties, it is clear that the OM can act as a versatile layer that provides both protection against chemicals and also resistance to turgor pressure and other mechanical loads. How general is a stiff OM across Gram-negative species, and across growth conditions? OM mechanical properties were similar across *E. coli* strains and in *Pseudomonas aeruginosa* as well as in minimal medium[40], hinting at conservation across species and environments. Nonetheless, the decreased potential for crosslinking in *V. cholerae* was predictive of the comparatively low stiffness of its OM, suggesting that LPS may be the most informative signature of OM mechanics. Regardless, it will be important to understand the feedback mechanisms that regulate the fractions of LPS and OMPs in the OM, which may impact whether the OM behaves as a linear elastic material or one that shows strain stiffening like peptidoglycan[39,103] (Box 1). It may be the case that cells can more easily tune the mechanical stiffness of the OM compared with that of the cell wall, since the OM harbors more components and is partially fluid. Certain environmental conditions, such as growth in a biofilm, may emphasize the importance of stiffness tunability. To pin down the structural role of the OM in other (particularly non-enteric) Gram-negative bacteria, key experiments such as determining the level of asymmetry, degree of PLs in the outer leaflet, and the chemical nature of the LPS will be highly informative.

How are OM mechanical properties related to other physical parameters, such as growth rate, cell shape, OM fluidity, and OM transport? For instance, in stationary phase, when cells are depleted of nutrients and as a result activate stress responses, the OM is more resistant to SDS than in log phase[128] and it might be advantageous to construct a stiffer OM. Another possibility is a trade-off between the fluidity of the OM and its stiffness, based on the assumption that solid-like structures will be more rigid. One intriguing possibility is that perturbations known to disrupt physical properties such as fluidity also change OM mechanics; exploring such a potential coupling will be critical for determining the extent to which each property can be independently tuned. While cells with a less stiff OM are more sensitive to osmotic fluctuations, it remains to be determined what environmental mechanical forces have shaped the selective environment for OM properties. It is also possible that tradeoffs exists between the parallel functions of the OM as a load-bearing structure and a chemical barrier, in which modifications that enhance protection against AMPs or antibiotics[114] may compromise OM stiffness.

Many aspects of OM physical properties remain unknown. From the compositional perspective, a large mystery is how PLs are transported from the IM to the OM. A recent study showed that the inner membrane protein YhdP modulates the rate of a high-flux diffusive anterograde PL flow pathway in *E. coli*[107], but YhdP is non-essential so other mechanisms must also exist for PL transport. Although potentially an artifact of sample fixation for electron microscopy[129], Bayer's junctions are hypothesized connections between the IM and OM[130] that, if they do exist, would allow for lipid transport via hemifusions. There may also be protein bridges for PLs like there are for LPS[69,131-133]. How are PLs linked to the physical properties of the OM? Although unproven, the natural hypothesis is that increasing PL concentration would make the OM less stiff based on the flexibility of PL vesicles, consistent with the finding that the FM4-64 OM-specific lipophilic stain disrupts OM stiffness[40] (Figure 3D) as does *lptD4213*, which increases PL concentration by decreasing LPS concentration[105]. PLs may also be connected to the rheology/fluidity of the membrane, thereby affecting the localization of proteins within the OM. The cytoplasmic factors that dictate the pattern of OM protein insertion[134] and localization have yet to be fully uncovered, but it is possible that the elongation machinery responsible for cell wall insertion also dictates OM insertion. Regardless of insertion, the relative lack of fluidity in the OM means that memory of the insertion pattern is maintained for long intervals.

From a mechanical perspective, a major knowledge gap is the magnitude of turgor pressure across organisms. There have not been any direct measurements of turgor in bacteria; in *E. coli*, the magnitude of turgor pressure was estimated indirectly from blebbing cells[39]. In the green algae *Chara corallina*, which are extremely large compared to a bacterium, turgor has been measured and controlled by piercing the cytoplasm with a needle[135]; there is some hope of a similar strategy being successful with bacteria using open AFM tips[136]. Without information about turgor, particularly regulation of turgor under conditions in which the cell wall is compromised, the distribution of stresses across the cell envelope is hard to discern. Moreover, it remains unclear whether turgor pressure is predominantly acting on the IM or the OM. One study provided evidence for the latter[137], consistent with the idea that the IM would rupture at high turgor, although Gram-positive bacteria can maintain high turgor in the absence of an OM. Curiously, the cell wall can itself bear turgor after detergent treatment removes the membranes[40]; this ability may be due to the wall being clogged with cytoplasmic components, and hence behaving as a semipermeable membrane. The molecular mechanisms of OM construction itself (i.e., OMP and LPS insertion) are still not completely resolved, but will have to account for building this structure under mechanical pressure.

While the OM is specific to Gram-negative species, it remains possible (perhaps even likely) that elements beyond the PG provide mechanical/structural support in Gram-positive bacteria. One possibility is the teichoic acids, which are essential for shape determination in *B. subtilis*[138] and may serve in a crosslinking capacity by binding divalent cations[139]. Consistent with this notion, sudden removal of Mg^{2+} from the extracellular medium caused *B. subtilis* cells to suddenly expand, suggesting that the cell envelope rapidly weakened[140]. Moreover, there are many other candidate structures that may contribute to cell mechanics. In response to cell-wall damage, *E. coli* cells produce a capsule that could be structurally important when the cell is mechanically compromised[6,7]. Similarly, gut commensals such

as *Bacteroides* species produce capsular polysaccharide layers in response to different environmental cues. These layers can be extremely thick (>1 μm)[141], and hence have potential for altering the physical properties of cells. Other Gram-negative bacteria, such as *Caulobacter crescentus*, have highly ordered S-layers surrounding the OM[142-144]. In fact, it seems likely that many structural layers contribute mechanically (it is even possible that the IM also plays a mechanical role). If so, then it is likely that they all have similar stiffnesses as the OM and cell wall because otherwise the stiffest layer would dominate mechanical responses.

It remains unresolved whether the OM is truly essential. No mutants of Gram-negative species have been identified that completely lack the OM, although the composition can be changed quite dramatically (e.g., removal of LPS) and still support growth. Removing the OM would likely also remove many periplasmic proteins, which could have serious consequences. Does perturbing the OM impact survival in different environments, for instance the transition between rich and poor nutrients? Ultimately, it may be that the role of the OM becomes most clear during survival of cell wall-deficient states.

Future prospects

Future studies will benefit from the recent expansion in experimental methods to probe cell mechanics, including direct methods such as AFM and indirect methods such as the degree of growth in a gel of known stiffness[145,146], neutron reflectometry[147], and microfluidic perturbations[104]. In addition, manipulation methods such as optical traps that can be used to pull tethers from the OM could provide information on OM viscoelasticity, particularly whether such tethers retract. Helping to interpret such experiments will be an enhanced understanding of cellular and single-molecule properties, such as the mechanics and folding of β-barrels.

Recent discoveries that highlight the physical properties of the OM and its mechanical importance complement the rich history of genetic and biochemical investigation into this versatile and complex structure, stimulating reflection on the evolution of cellular membranes in general. Physical stress clearly shapes bacterial physiology in profound ways, and mechanical factors were likely strong selective forces early on in the evolution of single-cell organisms. Thus, identifying the key determinants of OM physical properties and their association with other aspects of cellular physiology will provide a deeper understanding of microbial growth and fitness.

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Box 1:

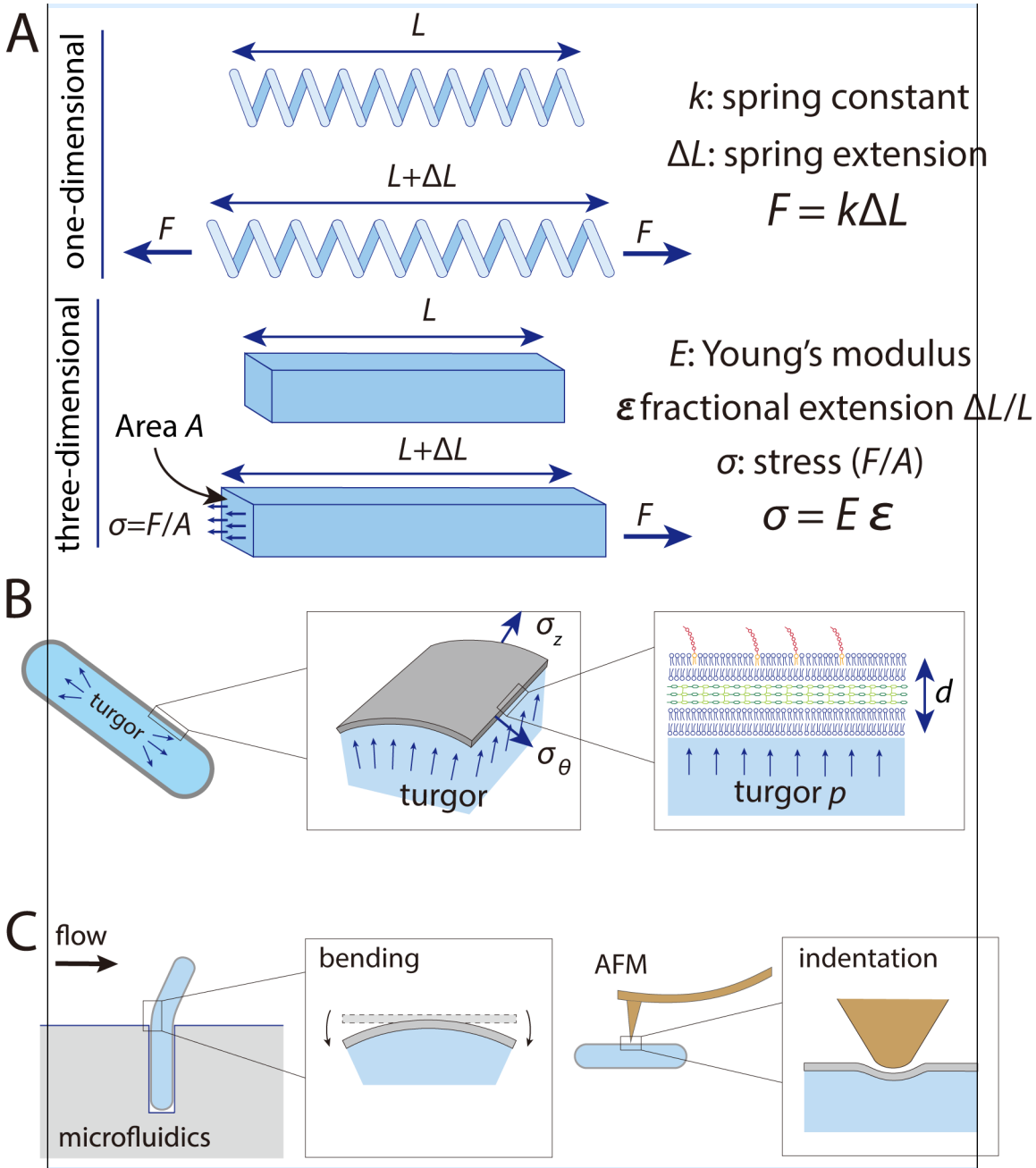
Key concepts relevant to the mechanics of a bacterial cell

The envelope of a bacterium is constantly subjected to mechanical perturbations such as turgor pressure. Understanding the magnitudes of these forces and the deformations that they cause provides insights into physical factors that dictate cellular function and survival. For solid objects, the simplest relationship between forces and deformation is linear elasticity, wherein the ratio between stress (force per unit area, σ) and strain (fractional change in size, ε) is a constant. This ratio characterizes the material's stiffness and is known as Young's modulus (E), such that $\sigma = E\varepsilon$. A one-dimensional analog of Young's modulus is the spring constant k in the well-known Hooke's law $F = kx$ (Box Figure A).

To account for the three-dimensional nature of the stress-bearing cell envelope and the enclosed cytoplasm, a bacterial cell can be modeled as a thin shell under internal fluid pressure. For *E. coli*, the shell is rod-shaped, with length L several times larger than the cross-sectional radius r, and both are at least an order of magnitude larger than the thickness of the envelope d. In this case, along the cylindrical portion the stresses in the axial and circumferential directions are approximately $\sigma_z = \frac{pr}{2d}$ and $\sigma_\theta = \frac{pr}{d}$, respectively, where p is turgor pressure (Box Figure B). If we assume linear elasticity, the axial strain, which is the length extension ΔL relative to the rest length L_0 when no forces are acting on the shell, is $\frac{pr}{2dE}$.

In addition to axial stress, rod-shaped objects can be subjected to other modes of deformation such as bending and indentation (Box Figure C). Bending of *E. coli* cells has been studied in microfluidic devices to reveal the coupling between the envelope strain and cell growth[104]. Indentation is typically studied via AFM using a nanoscale tip, through which Young's modulus and other mechanical parameters of the cell surface can be inferred in combination with mechanical modeling[39].

Although linear elasticity serves as a reasonable approximation for static loads and small strains, the true mechanical nature of cellular components can be more complicated and largely remains an open question. Generally, materials deviate from linear elasticity as the size of the deformation increases. Molecular dynamics simulations[103] and AFM measurements[39] have suggested that the cell wall stiffens under high tension, whereas the OM becomes softer[103]. Moreover, due to the fluid contents of the cytoplasm and specific aspects of membrane rheology, cells can exhibit viscoelasticity in which the strain is time-dependent[148]. As a result, much work remains to fully elucidate the mechanical properties of the bacterial cell envelope.



Box Figure: The mechanics of cylindrical thin shells.

A) For a one-dimensional spring with spring constant k, the force required to extend the spring by an amount ΔL is $F = k\Delta L$. For a three-dimensional material, Young's modulus E is the analog of the spring constant, and is the ratio of the stress σ to the fractional extension ε.

B) The cell envelope of a rod-shaped bacterial cell with cross-sectional radius r can be modeled as a thin shell of thickness d (which is much smaller than r) under turgor pressure p. Along the cylindrical portion the stresses in the axial and circumferential directions are approximately $\sigma_z = \frac{pr}{2d}$ and $\sigma_\theta = \frac{pr}{d}$.

C) Bending in a microfluidic chamber (Figure 3B) and AFM indentation (Figure 3C) explore other modes of deformation such as bending and indentation, respectively.

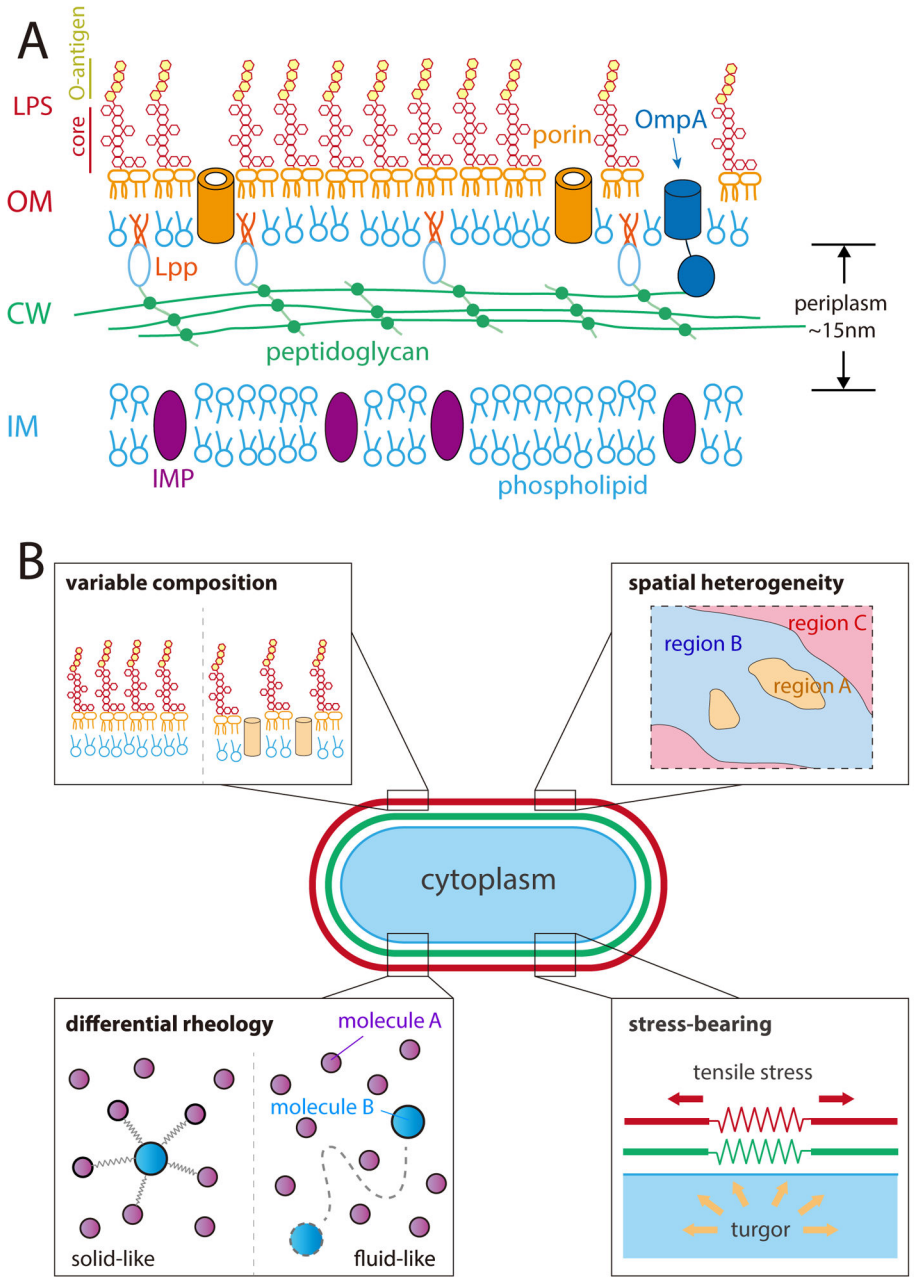


Figure 1: The Gram-negative outer membrane has a diverse makeup and a wide variety of physical and mechanical properties.

A) The cytoplasm of all bacterial cells is surrounded by an inner membrane (IM) composed of phospholipids and inner membrane proteins (IMPs), which is enclosed by the cell wall (CW), a macromolecular network of peptidoglycan (PG) that single-layered in *E. coli*[29]. In Gram-negative bacteria, the cell wall resides in the periplasm, a ~15-nm thick aqueous compartment enclosed by the inner and outer membranes. The outer membrane (OM) is asymmetric, composed of phospholipids in the inner leaflet and lipopolysaccharides (LPS), along with outer membrane proteins (OMPs) such as porins and lipoproteins such as Lpp, which links the OM and PG.

B) The OM (red) can exhibit variable composition, spatial heterogeneity, solid- or fluid-like rheology (diffusive dynamics), and the ability to bear mechanical stresses of similar magnitude to the cell wall (green). As a result, the chemical, physical, and mechanical properties of the OM can have broad impacts on bacterial physiology.

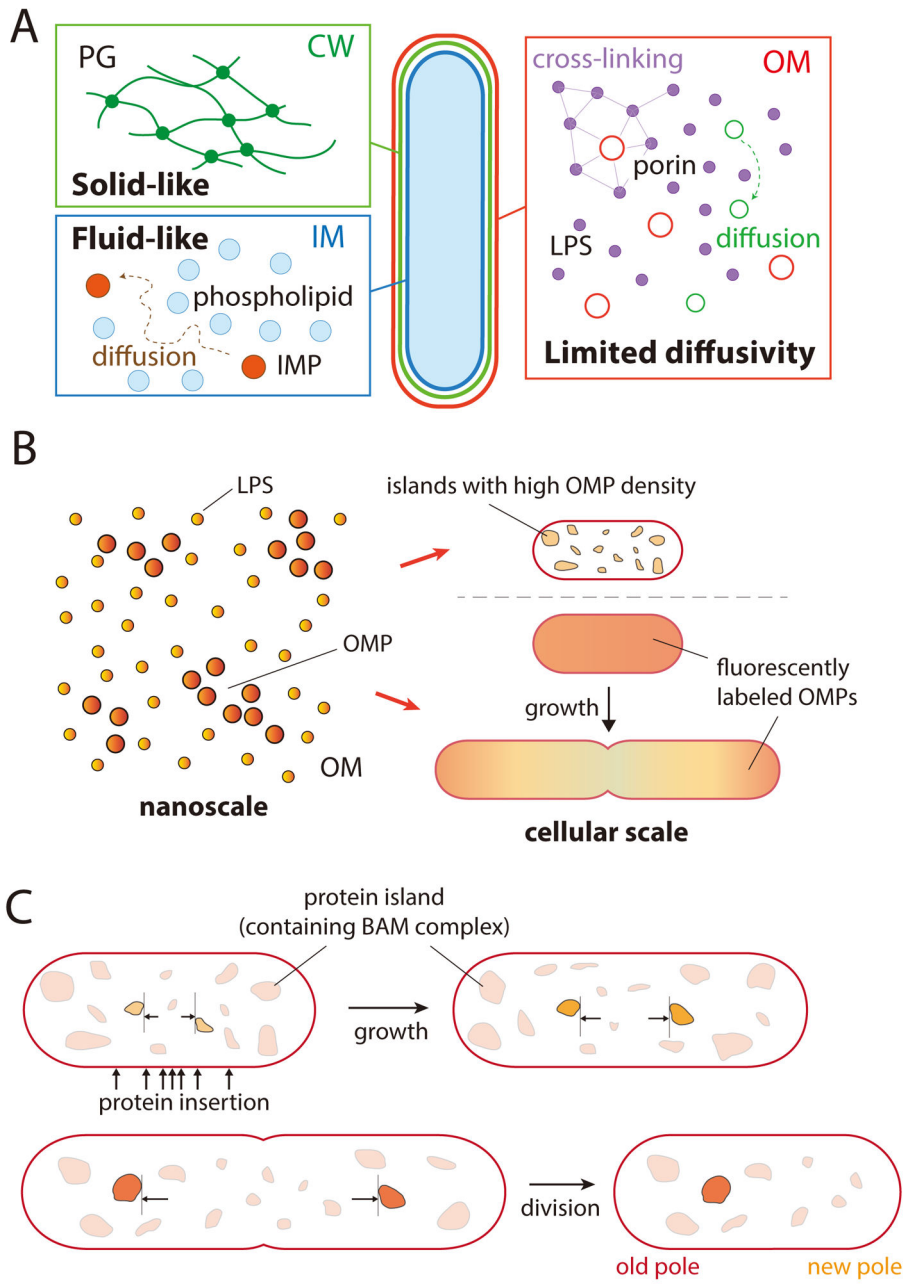


Figure 2: Outer membrane rheology and spatial organization are highly distinct from that of the PG or IM.

A) While the PG behaves as a relatively immobile solid due to its highly crosslinked nature, and the IM behaves as a liquid with rapid diffusion of lipids, the OM exhibits limited diffusivity. LPS molecules (purple) and OMPs (red) appear largely immobile, although there is some evidence of diffusive motion in the OM[79]. Green circles represent proteins with relatively high diffusivity.

B) At the nanoscale, LPS molecules and OMPs exist in small clusters. At the cellular scale, the density of fluorescently labeled OMPs along the cylindrical portion of the cell is diluted by growth, while the density at the poles remains high.

C) At the mesoscale, islands (represented by irregular shapes) including proteins such as the BAM complex expand and spread apart due to insertion of new materials along the cylindrical portion of the cell. After division, clusters at the old pole remain in one daughter cell, while clusters at the new pole have been trapped there by cytokinesis.

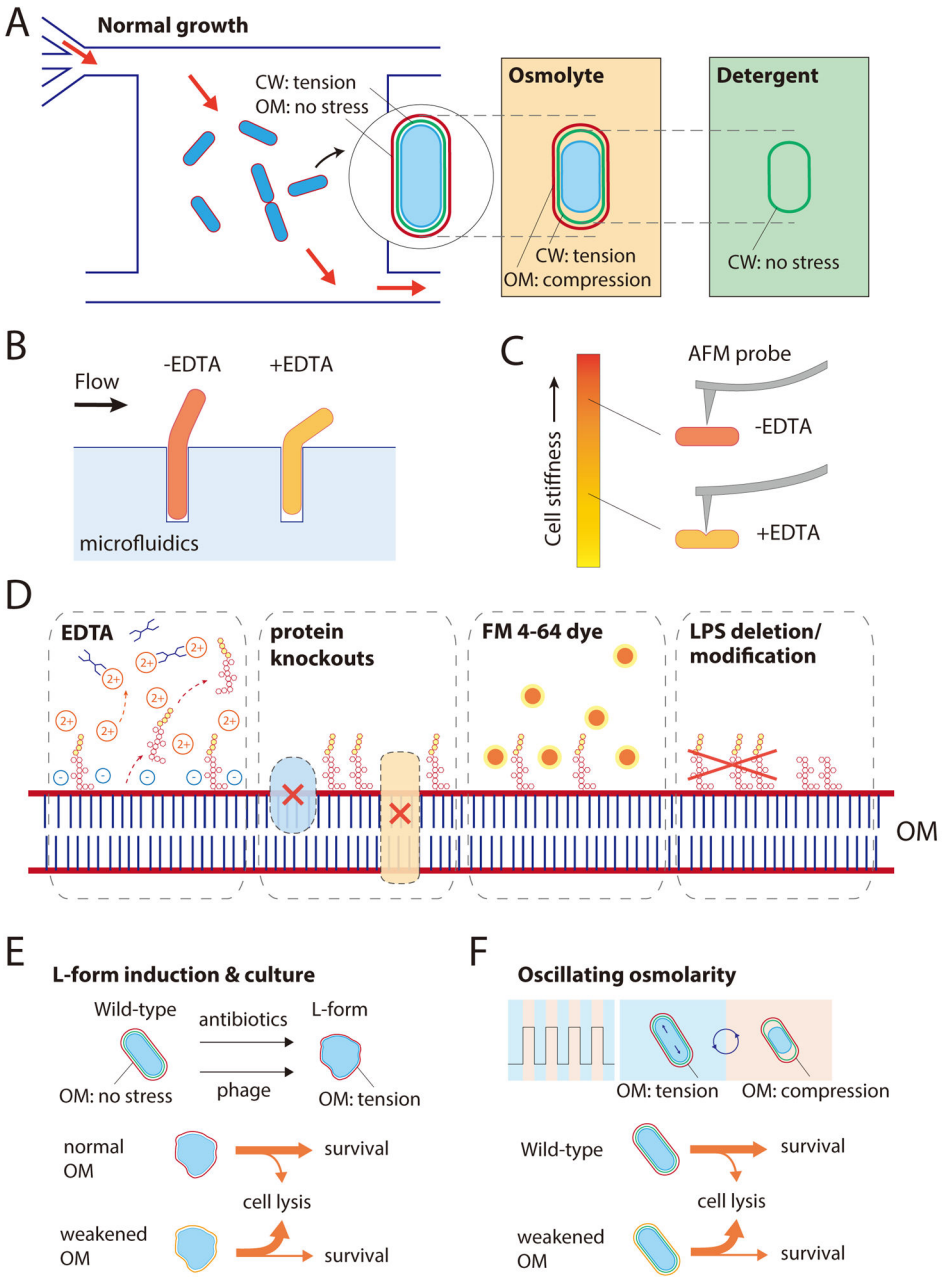


Figure 3: Probing the magnitude and impact of OM stiffness.

A) In a microfluidic flow cell, exponentially growing *E. coli* cells are under mechanical stress due to turgor pressure, which is born predominantly by the PG rather than the OM. Cells shrink suddenly when exposed to a hyperosmotic shock due to water efflux, decreasing the overall stress on the cell envelope. In this shrunken state, the cell wall remains partially extended as the OM experiences compression. Removal of the OM by detergent or EDTA treatment allows the cell wall to fully relax to its rest length.

B) Growth of *E. coli* cells initially embedded within a narrow channel leads to exposure of part of the cell body to fluid flow, whose hydrodynamic force can be tuned. EDTA-treated

cells deflect more than untreated cells for a given flow strength[40], highlighting the strength of the OM.

C) AFM measurements directly confirm the loss of cell stiffness by EDTA treatment. EDTA-treated cells indented more than untreated cells for the same amount of applied force; several outer membrane mutants also exhibited more indentation[40].

D) In addition to EDTA treatment, OM stiffness can be compromised by deletion of various OM proteins, intercalation of the OM-specific dye FM4-64, or LPS modification such as deletion of the O-antigen.

E) E. coli cells adopt a wall-less L-form or wall-deficient spheroplast state when exposed to phage or cell wall-targeting antibiotics, in which the OM bears stress to avoid envelope rupture.

F) Cells with compromised OM stiffness are more susceptible to death during osmotic-shock oscillations.