Dynamics of the Lipopolysaccharide Assembly on the Surface of *Escherichia coli*

Lakshmi P. Kotra, Dasantila Golemi, Nabil A. Amro, Gang-Yu Liu, and Shahriar Mobashery*

Contribution from the Department of Chemistry, Wayne State University, Detroit, Michigan 48202

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Abstract: Lipopolychacharides (LPSs) cover the surface of Gram-negative bacteria. The LPS assembly provides a penetration barrier to molecules larger than 700−1000 Da, such as many antibiotics, and when LPS molecules are released from the surface of bacteria, they cause toxic shock in the infected patient. The surface of *Escherichia coli* JM109 was visualized by atomic force microscopy, providing the highest resolution images of any bacterium to date (50-Å lateral and 5-Å vertical resolutions). These images indicated that LPS molecules are assembled in bundles of 600−3500 molecules. The LPS molecule is comprised of lipid A, then an inner and outer core, and an outermost region of *O*-antigen units. Analyses indicated that the *O*-antigen repeat units vary between 1 and 26 for *E. coli* JM109, and the fraction of the LPS molecules with zero to three repeat units made up approximately 50% of the total LPS content. A matrix of 16 LPS molecules was constructed as a representative region of the surface of *E. coli*. The molecular dynamics simulations of this assembly indicated that the structural components closer to the milieu experienced more movement than those closer to the interior, and that the metal ions coordinated to the inner core were indispensable for the stability of the assembly. In the absence of metal coordination to the inner core, the assembly of the LPS molecules disaggregated such that simulations beyond 67 ps could not be attempted. Simulations also indicated that the metal ions allowed for assembly of the LPS molecules one next to another in a tight formation. Such a tight assembly dramatically decreases the surface accessibility to solvent, so that there is no access for even a water molecule beyond the inner core. This structural property accounts for the aforementioned penetration barrier that is characteristic of Gram-negative bacteria.

The Gram-negative bacterial envelope is a highly elaborate structure (Figure 1). The inner membrane encloses the cytoplasm. The space between the inner and the outer membrane is referred to as the periplasm, which contains the bacterial cell wall. The cell wall provides the structural rigidity that is essential for the survival of bacteria. Lipopolysaccharides (LPS) form the surface of Gram-negative bacteria as the major component of the outer leaflet of the outer membrane (Figure 1).[1] The outer membrane is absent in Gram-positive bacteria, hence it is a uniquely Gram-negative entity. Roughly 3.5 million LPS molecules cover the surface of Gram-negative bacteria,[2] but the nature of the assembly of LPS in bacteria is currently entirely unknown. There is considerable interest in understanding the properties of LPS, because its release in the course of infections is the cause of septic shock in patients with endotoxemia. This problem caused more than 21000 mortalities in 1996 in the US alone.[3]

Over the past several years, the chemical structure of LPS has been elucidated by several groups.[2] LPS is composed of the hydrophobic lipid A (the component that interacts with the inner leaflet of the outer membrane), an inner and an outer core, and the repeating units of *O*-antigen (Figure 1).[2] The number of *O*-antigen repeats is the characteristic of any given strain of bacterium and could range from 0 to 40 in *Escherichia coli*.[2] Attempts have been made to characterize the three-dimensional structure of LPS molecules by X-ray powder diffraction and computational techniques, with limited success.[4,5]

The outer membrane presents a major barrier to the entry of many small molecules, including antibiotics. In addition to LPS, there are certain proteins, such as porins[6] and gated channels,[7] within the outer membrane that form channels for entry of some small molecules into the cell (Figure 1). Individual LPS molecules on the surface of the bacterium are held together by various forces including electrostatic interactions, hydrophobic interactions, and hydrogen bonds. There are divalent metal ions such as Mg^{2+} and Ca^{2+} ions that bridge the anionic functionalities present in the inner and outer core regions of LPS. It has been known for some time that chelating agents such as ethylenediaminetetraacetate (EDTA) can remove these metal ions from the surface of the bacterium and increase the permeability of the membrane.[1] Hence, it is of interest to understand the structural details of the outer membrane of

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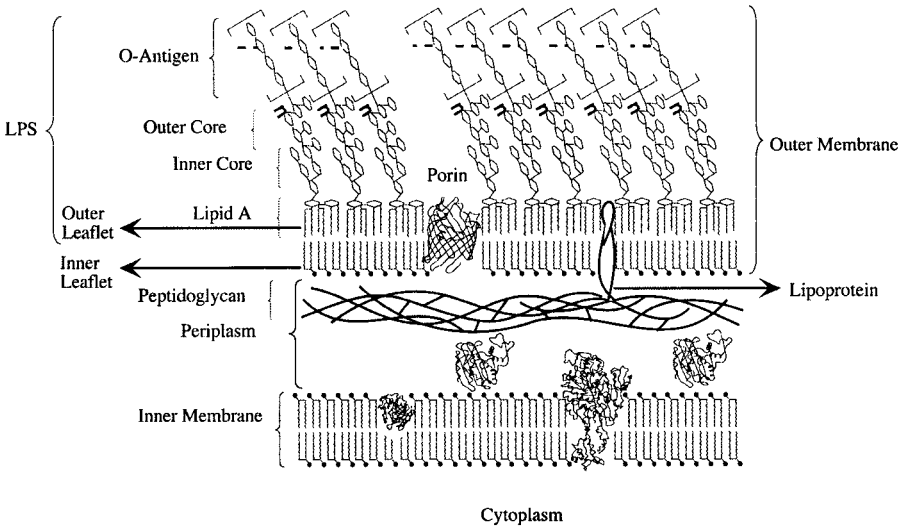

Figure 1. A schematic drawing of the envelope of Gram-negative bacteria.

bacterium and its components. We report herein the highest resolution image of the surface of the Gram-negative bacterium *E. coli*, determined by atomic force microscopy (AFM). The image revealed that the LPS molecules assemble on the bacterial surface in bundles. We have also performed the first dynamics simulations for the assembly of these LPS molecules. The details of these simulations shed light on the nature of the bundles and the basic properties of the surface of Gram-negative bacteria.

We chose *E. coli* JM109(pSF815A) for these studies. *E. coli* JM109 is a standard laboratory strain, which was transformed with pSF815A, a plasmid that encodes the bifunctional aminoglycoside resistance enzyme.[8] The resistance enzyme gave a selection handle to generate highly homogeneous cultures of the organism. A large scan area ($4 \times 4\ \mu m^2$) was first made for *E. coli* JM109(pSF815A) to select the desired cells for higher resolution imaging. The higher resolution images provide glimpses of the surface assembly in bacteria at unprecedented resolution (50-Å lateral and 5-Å vertical resolutions). As shown in Figure 2A (left panel), individual bacteria are readily distinguishable. The typical length of the bacterium is $2-6\ \mu m$, consistent with the previously reported values. The detailed structure of the outer membrane has been studied by systematically zooming in on the surfaces of the desired bacteria. An example is given in Figure 2A (right panel), which clearly reveals that the surface of *E. coli* is rough at the nanoscopic level. This high-resolution image shows that the outer membrane exhibits protrusions ("bumps") with the lateral dimensions of 250−600 Å and the vertical roughness of 22−90 Å. Certain "dark" features are also observed, representing the void spaces among the LPS patches. The widths of these dents range from 250 to 600 Å, with measurable depths of 25−85 Å. The actual depth is likely to be larger than the measured values here, because the AFM tip cannot always reach the bottom of the depressions. According to the lateral dimensions of the bumps measure by AFM and the models that we have generated (discussed below), each patch contains approximately 600 to 3500 LPS molecules. The differences in the heights of the bumps are most likely due to the different numbers of *O*-antigen units (1 to 26; vide infra) at the terminals of the LPS molecules.

As discussed above, these protrusions are the bundles of LPS molecules with different degrees of repeat of *O*-antigen. We determined the degrees of *O*-antigen repeats in *E. coli* JM109 by chemical analyses for colitose and heptose, two sugar constituents of the LPS structure, according to literature methods.[9] Our analyses indicated that the *O*-antigen repeat units vary between 1 and 26 for this bacterium. However, the fraction of LPS molecules with zero to three repeat units made up approximately 50% of the total LPS content. As revealed in the high-resolution images, the packing of the nearest neighbor LPS patches is tight. The bundles of LPS (Figure 2A) are known to provide a permeability barrier to many molecules, including various antibiotics. One also sees "holes" in the AFM images of the surface of the bacterium, such as the dark spots in Figure 2A. These spaces likely would lead to the outer membrane proteins such as porins, which are water-filled protein channels that span the outer membrane. Porins permit entry to molecules smaller than 700−1000 Da. It is a question of fundamental importance to understand how the LPS molecules function in these assemblies. We have performed molecular dynamics simulations to gain insight into the dynamic nature of LPS molecules in such assemblies.

Three different molecular dynamics simulations were performed on the LPS assembly: (1) The fatty acyl chains of lipid A were constrained, with the Mg^{2+} ions present in the assembly; (2) no constraints were imposed on the LPS molecular assembly with the Mg^{2+} ions present; and (3) all the metal ions and constraints were removed from the assembly (metals were removed and the system was equilibrated for 50 ps with constraints on the fatty acyl chains, and after 50 ps, constraints were removed). The first simulation was performed to understand the behavior of the *O*-antigen chains in the assembly. In essence, the lipid A moieties may be envisioned for this simulation as anchored in the bilayer environment by hydrophobic and van der Waals attractive forces. Molecular dynamics simulations were carried out for 255, 150, and 67 ps, respectively, for the above three assemblies, including the 50 ps of the equilibration period. The LPS molecules in the third assembly, which is devoid of the metal ions, showed dramatic motion of the various regions. This motion forced us to stop the simulations at 67 ps, because the individual LPS molecules were deviating out of the periodic box (not observed in the simulations of the other assemblies; vide infra). The results of the simulations were quantitated by measuring the deviation of

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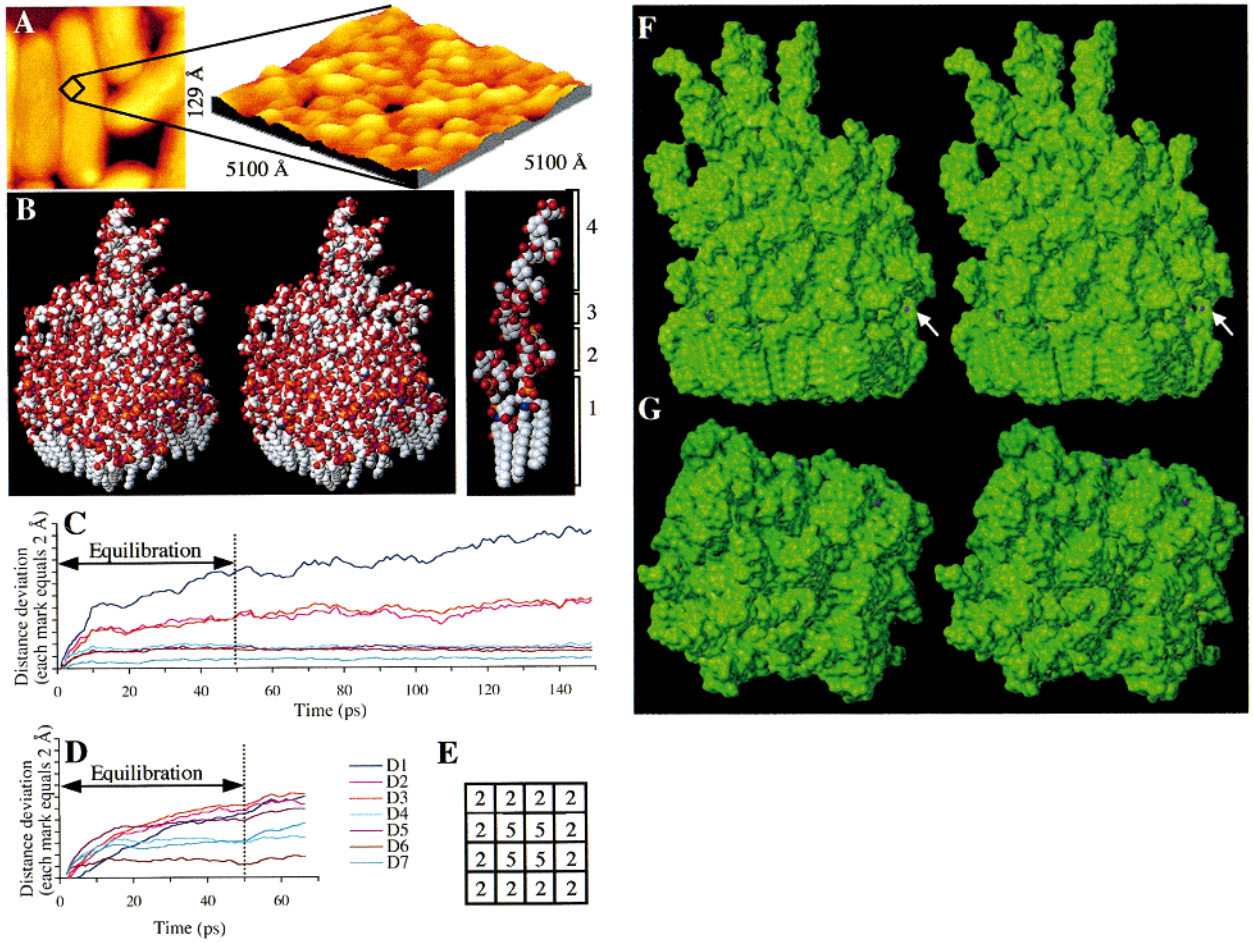

Figure 2. (A) Topographic image of *Escherichia coli* JM109 and an expanded view of the boxed portion of the surface. (B) The space-filled stereoview of an energy-minimized, time-averaged structure of the LPS assembly; the snapshot coordinates, collected over approximately 100 ps, were averaged and the resulting structure was energy minimized. The atoms are color coded according to the atom types (red for oxygen, white for carbon, blue for nitrogen, violet for magnesium; hydrogen atoms are not shown). The right panel shows different regions of the LPS molecule: 1 = lipid A, 2 = inner core, 3 = outer core, and 4 = two *O*-antigen repeat units. (C and D) The deviations from the starting conformations of the various regions of the LPS assembly during molecular dynamics simulations in the presence (C) and absence (D) of Mg^{2+} ions. Deviations of the centroid of a sugar in the fifth repeating unit of LPS5 (D1), in the second repeating unit of LPS2 (D2), in the second repeating unit of LPS5 (D3), in the outer core of LPS2 (D4), in the outer core of LPS5 (D5), in the inner core of LPS2 (D6), and in the inner core of LPS5 (D7) are plotted. (E) The grid shows the organization of LPS molecules in the assemblies that were used for the molecular dynamics simulations. Designations "2" and "5" refer to the LPS molecules with two and five *O*-antigen repeats, respectively. (F) The side stereoview of the Connolly water accessible surface of the energy-minimized, time-averaged structure of the LPS assembly (as described in panel B). (G) The top stereoview of the structure shown in part F. Metal ions interacting with the LPS molecules are shown as violet spheres, and one such metal ion is shown by an arrow in panel F.

the centroid of one sugar ring from each region (i.e., lipid A, inner core, outer core, and *O*-antigen) from its starting position and were averaged over either 12 LPS2 or 4 LPS5 molecules. The distances of deviations from the starting conformations are plotted as a function of time.

The results of the molecular dynamics simulations on the LPS assembly with and without Mg^{2+} ions (both without any constraints) are presented in Figures 2C and 2D, respectively. The results from the 255 ps simulations on the LPS assembly with Mg^{2+} ions and constrained fatty acyl chains (not shown) gave a similar profile as those without any constrains. Thus, in the presence of the metal ions in the inner core, the lipid A portions, inner core, and outer core regions (D4, D5, D6, and D7 in Figure 2C) did not show significant movements. These results demonstrate that the metal ions are critical in keeping the assembly of the LPS molecules together, an observation supported by experiments.[10,11] The *O*-antigen regions at the second repeating unit in the LPS2 molecules showed a stable profile (D2), similar to those at the same position in LPS5 (D3). In contrast, the terminal *O*-antigen repeats in LPS5 showed a dynamic nature throughout the simulation period (D1). Thus, from these profiles, it appears that the terminal *O*-antigen regions show greater movements in an assembly of LPS molecules and the longer chains show more flexibility. Figure 3 shows snapshots of two adjacent LPS molecules in the course of the 255 ps of simulation in the presence of the metal.

Removal of the metal ions using metal-chelating agents such as EDTA from the surface of the bacteria results in the loss of integrity of the LPS molecular assemblies and releases LPS molecules, producing damage to the outer membrane.[1,12] In our simulations in the absence of the Mg^{2+} ions in the LPS assembly, we observed a profile that led to disaggregation of the LPS molecules (Figure 2D). The deviations of the inner and outer core regions (D4, D5, D6, and D7) show that there is

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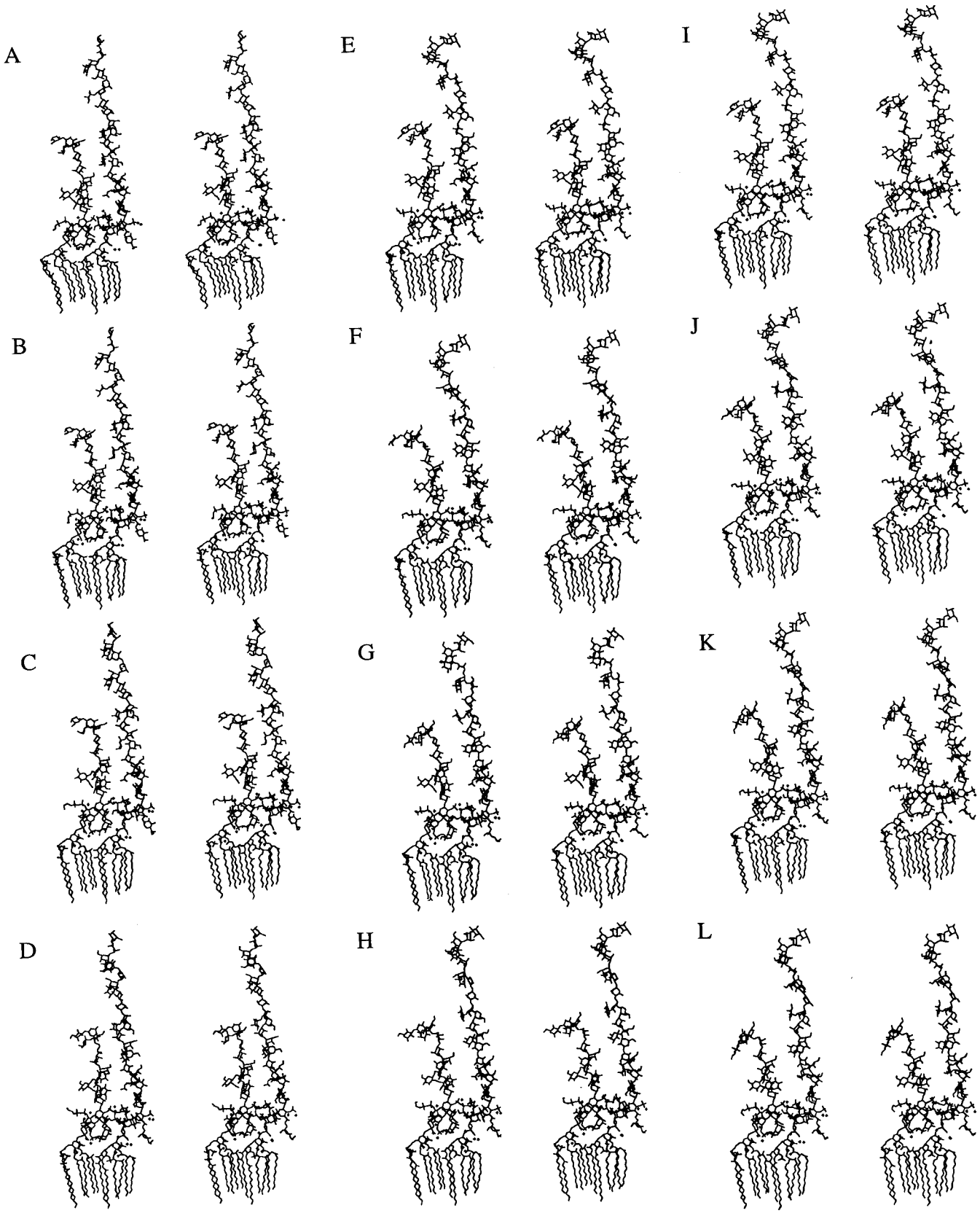

Figure 3. Snapshots from the trajectories of the molecular dynamics simulations on the LPS assembly in the presence of Mg^{2+} ions. Only one five-repeat LPS (LPS5) and its adjacent two-repeat LPS (LPS2) are shown for clarity of presentation of the dynamic nature of the assembly at various time points. The top extremities of the LPS molecules (the *O*-antigen regions) show significant movement compared to the inner portions. The corresponding time points for the snapshots are at 0 (A), 49 (B), 70 (C), 90 (D), 120 (E), 140 (F), 160 (G), 180 (H), 200 (I), 220 (J), 240 (K), and 255 ps (L).

more significant movement in these regions in the absence of metals (as discerned from the increasing slopes in Figure 2D after the equilibration period), in comparison to the profiles in the presence of the metal ions (Figure 2D). A similar profile

was also observed in the *O*-antigen regions (D1, D2, and D3, Figure 2D vs 2C). A comparison of the time-averaged structure from the simulations of the LPS assembly with metal ions (Figure 2B) to the snapshots of the trajectories of the LPS assembly in the absence of the metal ions clearly indicated that depletion of the metal ions from the inner core region facilitates the disaggregation of the assembly.

The simulations indicated that the individual molecules of LPS line up one against another, an arrangement that relies on the presence of the metals in the inner core for its stability. The molecules of LPS undergo motion in space, as would blades of grass in wind. Furthermore, the LPS assemblies present formidable penetration barriers for even small molecules into the bacterium. For example, the time-averaged structure of the LPS assembly (vide supra) does not permit the penetration of even a water molecule beyond the inner core (Figures 2F and 2G). These studies provide the first glimpses into the assemblies of LPS molecules in the Gram-negative bacteria. These arrays of LPS pose great difficulty at the present to pharmaceutical chemists in delivery of antibacterial drugs to their internal target sites. Availability of these computational models should stimulate further research in understanding the bacterial envelope in the immediate future.

Materials and Methods

Terrific Broth is a product of Difco Laboratories. Kanamycin A was purchased from Sigma Chemical Co**.** The *O*-antigen repeating units in *E. coli* JM109 were determined according to literature methods.[9]

Bacterial Preparation. *Escherichia coli* JM109(pSF815A) was grown to mid-log phase in Terrific Broth (10 mL) at 37 °C for 7 h in the presence of 50 μg/mL kanamycin A. The final bacterial concentration was approximately $(5-9) \times 10^9$ cells/mL. Bacterial concentrations were determined by measuring the absorbance of the culture at 600 nm ($0.1 \times OD_{600} = 10^8$ cells/mL) in a spectrophotometer (HP 8453 diode array UV−visible spectrophotometer). Stock cultures of *E. coli* JM109(pSF815A) were maintained at −80 °C.

Atomic Force Microscopy. The instrument is a state-of-the-art microscope constructed in house.[13] The samples may be imaged under ambient laboratory conditions, in a vacuum, or in solution. The cantilevers were standard Si_3N_4 microlevers from Digital Instrument with a force constant of 0.38 N/m. The scanning head incorporates a deflection type configuration with a quadrant photodiode detector that allows simultaneous acquisition of both topographic and frictional force images. The drop-and-dry method was used to transfer bacteria from solution to a solid support for AFM studies. To study structural features of individual cells, we used submonolayer coverage. For each specimen, 20 μL of the bacterial solution (total number of cells $\sim 9 \times 10^6$) was placed on a 1 cm^2 glass slide (thickness 0.5 mm). To enhance the adhesion, the glass slide was pretreated with the Piranha solution (1:3 ratio of 30% H_2O_2:concentrated H_2SO_4) and washed copiously with deionized water before introduction of the bacteria. The surface was then washed with distilled water (40 μL, 3×) and was allowed to dry in air, then the cells were immediately imaged in 2-butanol to preserve their original structures. 2-Butanol was the chosen imaging medium for the study. Tip−surface adhesion diminishes in this solvent, resulting in high spatial resolution. Water tends to liberate *E. coli* from the surface within minutes, which makes it difficult to study the surface structure in detail.

Molecular Modeling. An assembly of LPS molecules was constructed from multiple arrays of an individual energy-minimized structure for LPS.[2a] Two different types of LPS molecules were used in the assembly to mimic the bundles, one with 5 (LPS5) and another with 2 repeating units of *O*-antigen (LPS2). The three-dimensional structure of the LPS molecule was constructed using the Sybyl molecular modeling software,[14] and the structure was energy minimized using the AMBER 5.0 package.[15] The assembly contained 16 LPS molecules (13004 atoms; Figure 1E). The inner core of LPS is known to coordinate with calcium and magnesium ions.[1] A total of 64 Mg^{2+} ions were included, distributed uniformly within the assembly coordinated with the phosphate and carboxylate moieties. It is not known how many metal ions coordinate to the inner core of the LPS molecules. We chose the sites for coordination in a uniform fashion, and there are sites for coordination to additional metals beyond the 64 Mg^{2+} ions used in our LPS assembly. A typical assembly consisted of 85000−88000 atoms, including water molecules of solvation. The molecular dynamics simulations were performed on an Origin2000 supercomputer at the National Center for Supercomputing Applications or on an Octane dual processor computer. The calculations required in excess of 3 months of computer time. All the LPS molecules and the assembly structures were solvated and energy minimized using the AMBER 5.0 program. For the carbohydrate moieties of the LPS assembly, the GLYCAM force field developed by Woods et al. was used[16] and the atomic charges, when not available, were assigned based on the ESP charges obtained from the MOPAC package. Point charge on the magnesium ion was assigned to be +2 and the nonbonded parameters were assigned according to Aqvist.[17] All the energy minimizations and molecular dynamics simulations were performed on the completely solvated system in a box of water that was at least 8 Å thick from the surface of the LPS assembly. Periodic boundary conditions were used with the Ewald option as implemented in AMBER 5.0. The entire charge on the LPS system was not neutralized by adding Mg^{2+} ions and all the molecular assemblies contained residual charges that were smeared over all the atoms in the system by the Ewald approach to get back to neutrality. The LPS molecular assembly with 64 Mg^{2+} ions bears a residual charge of −32 and that without Mg^{2+} ions, −96. This will not have a large effect on the simulations due to the fact that the molecular systems contain a large number of atoms. The molecular assembly was heated to 300 K in 10 ps at constant volume and was equilibrated at 300 K for an additional 40 ps at constant pressure. The total energy of the system stabilized during this time. The total time of the simulations ranged from 100 to 255 ps, in the time step of 2 fs (except in the LPS assembly with fatty acyl chains constrained, where the time step was 1 fs from $t = 0-150$ ps). The simulations were carried out by immersing the complete assemblies of the LPS molecules in a box of water. One could argue that the hydrophobic fatty acyl chains need not be in a hydrophilic environment, such as the water in the box. But such a condition would simulate the fatty acids in a vacuum, with the oligosaccharide chains in the aqueous environment. We felt that the entire system should undergo dynamics simulations by immersing the complete assembly in water. Only the fatty acyl functions in the fringes of the LPS matrix experience solvation in the aqueous medium, and the remainder were in the interior and underwent dynamics as would be expected for the membrane to experience dynamics.

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