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Bacteria Survive Multiple Puncturings of Their Cell Walls

Zhiyong Suo,† Recep Avci,∗† Muhammed Deliorman,† Xinghong Yang,† and David W. Pascual†

†Imaging and Chemical Analysis Laboratory, Department of Physics and ‡Veterinary Molecular Biology, Montana State University, Bozeman, Montana 59717

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A bacterial cell wall is a highly dynamic multilayer structure interfacing the cytoplasm to the outside environment. It supports a multitude of chemical and biological processes necessary for life. It is therefore postulated that damage to the structure of bacterial cell wall would threaten cell integrity and result in cell death. We tested this hypothesis by repeatedly puncturing the cell wall of a live Gram-negative bacterium Salmonella typhimurium at different locations using a sharp atomic force microscope nanopip and conducting multiple viability tests. Our study demonstrated that a S. typhimurium survives repeated puncturings of its cell wall and retains its integrity, viability, and ability to divide. The results are explained on the basis of the concept of the self-repairing of lipid bilayers and the peptidoglycan layer.

Introduction

This manuscript focuses on addressing the issue of whether cell wall damage inflicted on a living Gram-negative bacterium using a sharp atomic force microscope (AFM) tip will kill the cell. The study has both fundamental and practical merit, in that the bacterial survival of a mechanical intrusion through piercing of a cell wall would pave the way to the efficient in vivo delivery of biomolecules, such as DNA, or nanoparticles, into living cells in their physiological environment. The cell walls of Gram-negative species are composed of three layers, with an overall thickness of ~20–30 nm: a cytoplasmic membrane, a peptidoglycan layer, and an outer membrane. The cytoplasmic membrane, made up of phospholipid bilayers and transmembrane proteins, establishes a highly controlled barrier between the cytoplasm and its surrounding environment. The peptidoglycan layer, interspersed with transport proteins, forms a continuous network of covalently linked polysaccharides and peptides with a uniform pore size of ~2 nm and a thickness of about 3–6 nm. It surrounds and supports the cell membrane. It is this layer that gives the bacterium its rigidity and maintains its shape. The outer membrane is composed of phospholipids and lipopolysaccharides interwoven with the outer membrane proteins. It interacts with the external environment and acts as a rough sieve between the cytoplasm and its surrounding environment. It supports a multitude of chemical and biological processes necessary for life. It is therefore postulated that damage to the structure of bacterial cell wall would threaten cell integrity and result in cell death. We tested this hypothesis by repeatedly puncturing the cell wall of a live Gram-negative bacterium Salmonella typhimurium at different locations using a sharp atomic force microscope nanopip and conducting multiple viability tests. Our study demonstrated that a S. typhimurium survives repeated puncturings of its cell wall and retains its integrity, viability, and ability to divide. The results are explained on the basis of the concept of the self-repairing of lipid bilayers and the peptidoglycan layer.

In contrast, electroporation is widely used for bacterial transformation. External plasmids are introduced into the periplasmic space through transient holes, comparable to the size of plasmids, formed on the bacterial outer membrane by high-voltage discharge. Although a number of investigations have demonstrated that puncturing a eukaryotic cell using a micropipette or a nanoneedle does not alter cell function, very little work has been reported on puncturing prokaryotic cells and testing its effect on cell viability. The effect of mechanical damage to a bacterial cell wall on cell integrity, activity, and viability remains poorly understood. This work focuses on studying the response to cell wall damage inflicted on a living Gram-negative bacterium Salmonella typhimurium using a sharp AFM tip. The results presented here demonstrate unambiguously that multiple puncturing of a cell wall neither kills S. typhimurium cells nor interferes with vital processes such as cell division. A hypothesis offering the mechanism by which the bacterium can survive multiple puncturing is also proposed.

Materials and Methods

Chemicals. Phosphate-buffered saline (PBS) and 3-aminopropyltriethoxysilane (APTES) were purchased from Sigma-Aldrich (St. Louis, MO). N-[β-Maleimidopropyl]succinimide ester (BMPS) was purchased from Pierce Biotechnology (Rockford, IL). Bacterial viability dyes (Live/Dead BacLight) were purchased from Invitrogen.

*Corresponding author: Address: EPS 264, Physics Department, Montana State University, Bozeman, MT 59717. Tel.: 406-994-6164. E-mail: avci@physics.montana.edu.

† Imaging and Chemical Analysis Laboratory, Department of Physics and Veterinary Molecular Biology, Montana State University, Bozeman, Montana 59717.

‡ Veterinary Molecular Biology, Montana State University, Bozeman, Montana 59717.

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of which corresponds to a colony forming units (CFU) value. Bacterial cells were harvested when the optical density of 70 min. Patterns composed of squares with a size of 5 μm without antibiotics and shaken at 125 rpm at 37 °C. The bacteria were then inoculated into an LB liquid medium in a solution of PEG-silane (2% in propanol) at 60 °C. Frozen bacteria stock at −80 °C was inoculated onto a Luria-Bertani (LB) plate and incubated at 37 °C overnight. The bacteria were then inoculated into an LB liquid medium without antibiotics and shaken at 125 rpm at 37 °C. The bacterial cells were harvested when the optical density of the medium at 600 nm (OD600) reached about 0.5–0.6, which corresponds to a colony forming units (CFU) value of ∼9.0 × 10^7/mL.

Immobilization of Bacteria on Silicon Substrates. Details of the immobilization were reported in our previous publication. In brief, a self-assembled monolayer of PEG-silane was formed on silicon substrates by heating the silicon chips in a solution of PEG-silane (2% in propanol) at 60 °C for 70 min. Patterns composed of squares with a size of 5 × 5 μm² or 10 × 10 μm² were etched onto PEG-silane-modified silicon chips using a focused Ga⁺ ion beam. The patterned silicon chips were treated with APTES (2% solution in methanol) and BMPS (10 mM solution in CH₃CN) to activate the patterned area for antibody linking. This was followed by soaking the chips in a 100-fold diluted solution of polyclonal rabbit antibody raised against CFA/I fimbriae for 1.5 h. Antibody-modified silicon chips were incubated in the bacterial culture described above for ∼30 min at room temperature and rinsed gently with sterile growth medium to remove the unattached cells. Such immobilization allows the formation of a bacterial monolayer within individual squares (5 × 5 or 10 × 10 μm²) on flat silicon chips. These samples were used immediately for puncturing experiments, as described below.

AFM Tips. Three types of AFM tips were used for puncturing experiments: MLCT-AUHW silicon nitride tips from VeecoProbes (Santa Barbara, CA), BioLever silicon nitride tips from Olympus (also carried by VeecoProbes, Santa Barbara, CA), and CSC-05 focused electron beam (FEB) tips from K-Tek Nanotech, LLC (Wilsonville, OR). Puncturing experiments were conducted using a MultiMode V AFM equipped with a PicoForce scanner (Veeco, Santa Barbara, CA). For each tip, the tip radius was measured by imaging the tips using field emission scanning electron microscopy (FESEM), and the spring constant was determined through the thermal tune function included in NanoScope software (Veeco, Santa Barbara, CA).

Puncture Experiments. S. typhimurium cells immobilized on a silicon chip remained imbedded in ∼200 μL growth medium or PBS buffer during the experiment. The cantilever deflection sensitivity was calibrated on the hard silicon surface (not covered with bacteria) before puncturing experiments were conducted. A tip was aligned at the center of a predetermined square (identified previously using a high-resolution optical image like those shown in Figures 3 and S2 (Supporting Information) covered with S. typhimurium cells. Either 1024 or 4096 pairs of force versus displacement curves were acquired from either a 7 × 7 μm² or 10 × 10 μm² area in force–volume mode, depending on whether the area was divided into 32 × 32 or 64 × 64 grids, respectively. Cantilever force constants varied between 0.01 N/m and 0.02 N/m, typical deflection set points were around 4 nN, and the experiment was run with a 3 μm ramp size at a frequency of 1 or 2 Hz.

Data Processing. Force versus displacement curves were analyzed with a Matlab code developed in our laboratory. The penetration depths in the puncturing curves were obtained by subtracting the cantilever deflection from the 2-piezo displacement. These values were designated as the x-axis of the indentation curves, and the zero point of the x-axis was defined as the point where the cantilever deflection started increasing from zero, which was interpreted as the tip making contact with the cell surface. The force at a given penetration depth was calculated by multiplying the measured spring constant of the cantilever by the cantilever deflection. The elastic modulus of the sample was obtained by fitting the indentation curve obtained from the approach curve with a Hertzian model. The origin of the indentation curve was chosen as the point where the approach curve makes contact with the bacterial surface. Because the indentation curve from 0 nm to the first maximum contains the initial elastic deformation of the whole cell, only the initial 20–30 nm was fitted to a Hertzian model, in order to avoid the interference from puncturing the cell wall.

Observation of Cell Division after Puncturing Experiments. After the puncturing experiment, the sample was transferred to a Petri dish filled with cell-free growth medium. Cell division was monitored in reflected bright field mode using an Olympus BX61 microscope (Leeds Precision Instruments, Salt Lake City, UT) equipped with a 60× water immersion objective. Time-lapse images were captured using a DP71 Olympus digital camera every 60 s for 100 min. The images taken at 0, 25, 50, 75, and 100 min were selected for Figure 4, and the complete time-lapse series was converted to a video using Microsoft Windows Movie Maker (Movie S1).

Cell Staining with Viability Dyes. Some samples were stained with viability dyes to check their viability after the puncturing experiment (Live/Dead BacLight, Invitrogen, Carlsbad, California). The sample was stained following the instructions from the manufacturer and rinsed with PBS buffer to remove the unbound excess dyes. The stained cells were observed using the optical microscope described above. Green and red fluorescence images were captured separately and combined using MicroSuites 5.0 (Olympus Soft Imaging Solutions GmbH, Johann-Krane-Weg, Germany).

References:

(14) Pascual, D. W.; Trunkle, T.; Sura, J. “ArticleDOI: 10.1021/la8033319 4589
Results and Discussion

Following the procedure described in the Materials and Methods section and in our previous publication, a layer of *S. typhimurium* cells in their exponential growth phase was immobilized on an atomically flat silicon surface in well-defined square patterns with about 20 cells within each square. Immobilized and patterned live bacteria lend themselves to continuous observation of the same group of cells under a light microscope before and after puncturing. After characterization of the immobilized cell patterns under a high-resolution light microscope, the substratum was transferred to the sample stage of a well-calibrated AFM system. The cells were kept in their physiological medium. A sharp AFM tip of variable aspect ratio was brought into contact with a live *S. typhimurium* cell and the loading force on the AFM tip was increased until the tip punctured the cell wall (Figure 1A). This was marked by a sharp decrease in the cantilever deflection value from \(~150\) nm to \(~35\) nm (inset, Figure 1B). The tip was pushed continuously until it stopped penetrating the cell, which was interpreted as the tip contacting the hard surface underneath the organism. When the loading force reached a preset value (i.e., \(~4\) nN), the tip was lifted to \(~2\) \(\mu\)m above the cell surface in order to start another puncture cycle at an adjacent location on the organism. This puncturing process was repeated at a rate of 1 Hz (sometimes 2 Hz) until each cell within a \(7 \times 7 \mu\text{m}^2\) or \(10 \times 10 \mu\text{m}^2\) area had undergone \(~20\) or \(~40\) puncturing events per square micrometer, depending on whether a pixilation matrix of \(32 \times 32\) per \(7 \times 7 \mu\text{m}^2\) or one of \(64 \times 64\) per \(10 \times 10 \mu\text{m}^2\) had been chosen. The substratum with the punctured bacteria was then placed under the light microscope for imaging and documentation of the subsequent behavior of the cells.

When a sharp tip punctures a cell wall of a Gram-negative bacterium, it tears the three-layer cell wall structure before entering the bacterial cytoplasm. The pressure required to tear, or puncture, the cell wall is determined from the “puncture curve.” The latter was obtained from the force versus displacement curve (inset in Figure 1B) by subtracting the cantilever deflection from the displacement of the AFM tip using a MatLab code developed in our laboratory. The resulting “puncture curve,” as the one depicted in Figure 1B, carries a wealth of information. For example, the distance \((\sim 800\) nm\) between where the tip makes contact with the bacterial surface and where it touches the substratum is the measure of the true height of the bacterium in its physiological medium. The maximum at approximately \(~100\) nm and the loading force of \(F \approx 2\) nN at the maximum suggest that the bacterium deformed under the pressure exerted by the sharp tip and that the bacterial surface was indented by \(~100\) nm before the tip broke through the cell wall, which is marked by a sharp reduction in the cantilever deflection of the approach curve in the raw data (inset of Figure 1B). The modulus of elasticity of the living cell at the initial contact and the turgor pressure of the organism can be determined from the early part of the loading versus tip penetration curve, as shown in Figure 1B, using simple models such as the Hertzian Model or more complicated ones. The pressure, \(P\), required to penetrate the bacterial cell wall can be determined from \(P = F/(\pi r^2) \approx 5.0 \pm 0.8\) atm, where \(r\) refers to the radius of the bacterium, it tears the three-layer cell wall structure before entering the bacterial cytoplasm. The pressure required to tear, or puncture, the cell wall is determined from the “puncture curve.” The latter was obtained from the force versus displacement curve (inset in Figure 1B) by subtracting the cantilever deflection from the displacement of the AFM tip using a MatLab code developed in our laboratory. The resulting “puncture curve,” as the one depicted in Figure 1B, carries a wealth of information. For example, the distance \((\sim 800\) nm\) between where the tip makes contact with the bacterial surface and where it touches the substratum is the measure of the true height of the bacterium in its physiological medium. The maximum at approximately \(~100\) nm and the loading force of \(F \approx 2\) nN at the maximum suggest that the bacterium deformed under the pressure exerted by the sharp tip and that the bacterial surface was indented by \(~100\) nm before the tip broke through the cell wall, which is marked by a sharp reduction in the cantilever deflection of the approach curve in the raw data (inset of Figure 1B). The modulus of elasticity of the living cell at the initial contact and the turgor pressure of the organism can be determined from the early part of the loading versus tip penetration curve, as shown in Figure 1B, using simple models such as the Hertzian Model or more complicated ones. The pressure, \(P\), required to penetrate the bacterial cell wall can be determined from \(P = F/(\pi r^2) \approx 5.0 \pm 0.8\) atm, where \(r\) refers to the radius of the bacterium.

Figure 1. (A) Schematics of puncturing experiments: An AFM tip was brought into contact with the surface of a live bacterium immobilized on a silicon substrate. The loading force was increased until the tip punctured the cell wall and subsequently made contact with the substrate. After the puncturing experiment, the substrate with punctured cells was observed under an optical microscope for cell division. (B) A typical puncturing curve obtained with a biolever tip. Puncturing curves reveal a variety of information, including the true height of a live bacterium, its initial elasticity under physiological conditions, the pressure required to puncture the cell wall and the cell indentation before the cell wall was punctured. The inset shows the raw cantilever deflection versus displacement curves from which the puncturing curves were obtained by subtracting the cantilever deflection from the z-piezo displacement using a MatLab code developed by our group.

AFM tip. In our experiments, the average modulus of elasticity was calculated to be \(0.4 \pm 0.2 \times 10^7\) Pa for living *S. typhimurium* cells in growth medium or in PBS buffer, comparable to the result reported for *Pseudomonas aeruginosa* and *Escherichia coli*.

The fine structures in the penetration range from approximately \(~250\) nm through approximately \(~600\) nm further suggest that the AFM tip experiences resistive forces even after it penetrates into the cytoplasm of the organism. Depending on their geometry and, more importantly, on their aspect ratio, tips experience varying resistance as they penetrate into the cytoplasm of the organism. The typical puncture curves associated with three different tip geometries are presented in Figure 2. The scanning electron microscope (SEM) image of the corresponding tip is shown as an inset in each panel. Figure 2A corresponds to a pyramidal tip. It had the lowest aspect ratio, \(~1:1\). Figure 2C shows an FEB tip. It had the highest aspect ratio, \(~10:1\). The aspect ratio is a measure of the height-to-width ratio of a conical or pyramidal construct. The tip used for Figure 2B (biolever tip) is an intermediate geometry corresponding to an aspect ratio of \(~1:7:1\). Its geometry can be represented by a plane folded along a diagonal. Clearly the largest resistance to the tip penetration occurred for the pyramidal tip (Figure 2A). The size of the puncture hole while the tip penetrates a bacterium
depends on the tip radius, the aspect ratio of the tip, and the depth of penetration. Compared with a high-aspect-ratio tip, one with a lower aspect ratio will tear the cell wall more as it pushes into the cytoplasm and will introduce more severe cell damage.

The puncture curves obtained by a pyramidal tip as in Figure 2A are shown in Figure 3, in which eight consecutive puncture curves taken across a bacterium surface at 1-s time intervals share many similarities: Each curve has an initial maximum at an indentation of ∼50 nm before the cell wall is punctured when the loading force reaches ∼1 nN. Even though the cell indentation and the force required to puncture the cell wall are smaller (because of the sharper AFM tip radius, r ≈ 25 nm) than those shown in Figure 1B, the critical pressure required to puncture the cell wall is the same (∼5 atm) as that found for the puncture curve shown in Figures 1B and 2B for r ≈ 35 nm. Fine structures in all the force profiles after the puncturing event suggest substantial resistance against penetration as the pyramidal tip is pushed into the bacterium. It should be noted that the profile of an individual curve is not affected by a previous puncturing event even though the curves are taken one after another at a frequency of 1 Hz. There is no evidence of damage or of a puncture hole left behind from the previous puncturing event, as though each curve were obtained from an unperturbed cell surface.

It appears that puncture holes self-repair so that bacterial integrity is maintained and bacterial mechanical properties are not altered. This becomes even more fascinating when we consider the aspect ratio of ∼1:1 associated with the pyramidal tip used in this experiment: by the time the tip reaches the underlying substratum, the cell wall is expected to be ripped open to a size comparable to the width of the bacterium!

The force the tip experiences when it is pushed into the cytoplasm can be traced to two sources: (1) vertical resistance from the cell membranes, particularly from the peptidoglycan layer, during the initial 50–200 nm indentation (depending on the tip radius), until the tip overcomes this resistance and punctures the cell wall; (2) lateral resistance from the peptidoglycan layer associated with the tip geometry. As the tip penetrates into the cytoplasm, it tears the covalently woven fabric of the peptidoglycan matrix. For tips with low aspect ratios, both interactions contribute to the resistive force, as suggested by the peak profiles observed in the puncture curves in Figure 2A,B. For a high-aspect-ratio tip the lateral resistance is expected to be minimal. This assumption is supported by the fact that Figure 2C suggests no resistance to the tip penetration after the puncturing event is completed, while Figure 2B shows intermediate resistance due to the tip’s intermediate aspect ratio and its folded-plane geometry. At the same time, all the tips, regardless of their aspect ratio or tip radius, exerts the same amount of initial pressure just before the cell wall penetration, which is about 5 atm.

The common feature among all the puncture curves is that there was very little or no resistance as the tip was pulled back from the cytoplasm of the bacteria (red lines in Figures 1, 2, and 3). This lack of resistance during the tip retraction implies that there are only weak tip/cell-wall interactions once the tip breaks the cell wall. The lipids, which are in continuous contact with the tip, offer no resistance to the motion of the tip as it is withdrawn from the cytoplasm. The small resistance during tip retraction observed for tips with large aspect ratios (Figure 2A,B) is interpreted as the recovery of the broken peptidoglycan layer, which possesses a Young’s modulus of ∼1.5 × 10⁷ to 6 × 10⁷ Pa, as measured for E. coli sacculi.21

Could other models besides puncturing explain our observations? For example, can the bacteria be pushed aside by the AFM tip so that the resultant force curves look similar to those shown in Figures 1, 2, and 3? There are a number of arguments against such a “pushing aside” model: (1) These bacteria are held against the substrate by a number of their pili, and it is not trivial to push them aside with a tip exerting a perpendicular, not shear, force. (2) The eight consecutive puncture curves taken at 1-s intervals shown in Figure 3 could not be so similar if these curves were due to a random “pushing aside” process. For example, the fact that the tip does not experience any force during the pulling process would suggest that the bacterium is out of the way of the tip for the subsequent puncturing event, and the next curve should not show another puncturing event: it should show a miss! This is not what is observed: instead we observe eight similar puncture curves. (3) As shown in Figure 2C, there is no force observed after the initial puncturing event if a high-aspect-ratio tip was used. If this were a “pushing aside” event, then the interaction between the cell and the tip should continue to exist after the initial interaction of the tip with the bacteria surface, and the tip should remain in contact with the cell surface.

Figure 2. Puncturing curves obtained using AFM tips with three different aspect ratios. An SEM image of the AFM tip is shown in the inset for each panel: (A) a pyramidal tip with an aspect ratio of ∼1:1, (B) a boleover tip with an aspect ratio of ∼1:7:1, and (C) an FEB tip with an aspect ratio of ∼10:1. For each tip the portion that pierces bacterial cells is marked with a red circle.

surface until the tip touches the hard substrate, regardless of tip geometry. This is not what is observed: instead we observe an initial resistance (peak) to the insertion of a sharp, high-aspect-ratio tip into the cell wall and no resistance thereafter. 

(4) For a given tip geometry, we consistently observe a puncturing event at ∼5 atm pressure following an initial indentation just before the puncturing event. The indentation length is consistent with the tip diameter: the larger the diameter, the deeper the indentations are. All the evidence lead to a single conclusion: our observations are consistent with a puncturing event and not with a pushing event.

It is not unusual for bacterial cells to retain their integrity for a short time after they die; therefore, the ultimate confirmation of bacterial viability is the observation of cell division. We have conducted a number of experiments to investigate whether *S. typhimurium* cells are capable of multiplication following puncturing. One example of such observations is shown in Figure 4 and in Movie S1 of the Supporting Information (SI). We conducted a puncturing experiment in force-volume mode on the colony of bacteria encircled by a blue dashed square in Figure 4B, and the corresponding force-volume image is shown in Figure 4A. Notice that there is a one-to-one correspondence between the bacteria inside the blue square and the bright features in the force-volume image. The light pixels in the dark background indicate the punctured bacteria with a density of 20 puncturing events per square micron. Figure 4B corresponds to the optical image acquired immediately after the puncturing experiment. We then kept the bacteria in their growth medium while the punctured bacteria divide after being punctured multiple times.

Figure 3. Puncturing curves associated with a force-volume image. The eight curves were obtained from eight consecutive locations on a live *S. typhimurium* cell using a pyramidal tip with a radius of about ∼25 nm. All the curves are similar, showing a peak at approximately ∼50 nm, corresponding to the resistance to the penetration of the cell wall when the loading force reaches ∼1 nN. The rest of the forces experienced by the tip are interpreted as tearing of the cell wall, particularly the peptidoglycan layer, as the tip penetrates into the cytoplasm. All retraction curves show small resistance. This is interpreted as the semielastic recovery of the broken peptidoglycan layer and the lack of friction between the tip and the multilayer cell wall structure.

Figure 4. Ultimate viability test: *S. typhimurium* cells divide after being punctured multiple times. (A) Force-volume image with bright spots showing the locations of the punctured bacteria. These spots have a one-to-one correlation with those shown in the blue dashed square of the optical images in panel B. Panels B–F show time-lapse images of the same group of bacteria taken 0, 25, 50, 75, and 100 min after the puncturing experiment, respectively. Cell division is highlighted by red, olive, and black ovals. Scale bar in B–F: 5 μm.
taking images of the same location at 1-min intervals for 100 min. These images were used to make the movie shown in Movie S1. The images shown in Figure 4B–F were taken at times 0, 25, 50, 75, and 100 min, respectively. The majority of cells that had been punctured multiple times are able to divide.

The effect of puncturing on the viability of S. typhimurium was also studied using viability dyes. After the puncturing experiment, the silicon substratum with immobilized bacteria was incubated in a solution of viability dyes. After being rinsed with PBS buffer to remove the unbound excess dyes, the immobilized bacteria were observed under an epifluorescence microscope. As shown in Figure S1, the washing process removed some of the immobilized bacteria (compare panels A and C of Figure S1), but the remaining bacteria had a very good one-to-one correlation with the force–volume image (Figure S1B). The green fluorescence (Figure S1D) suggests that the majority of the punctured bacteria remained alive. These experiments were repeated using tips with different aspect ratios, and in all cases we observed no statistical difference on the survival rate between the punctured and the unpunctured cells, regardless of tip geometry and puncturing density varying between 20 and 40 puncturing events per square micrometer. These observations further confirm that the puncturing of an S. typhimurium with a sharp tip does not interfere with its viability.

It was also noted that puncture curves for dead S. typhimurium cells were markedly different from those for living cells. Figure S2 presents puncture curves obtained from the same sample before and after it was treated with 5% glutaraldehyde to kill the cells. The puncture curves of the dead cells (Figure S2B) lack the maximum associated with the puncturing event observed for live bacteria (Figure S2A). We also observed that dead bacteria shrink by about 40% compared with the living organism, as revealed by the reduction of bacterial height. As discussed in connection with Figure 1B, the elastic modulus of a cell can be determined from its initial indentation curve, right after the tip makes contact with the bacterium but before the tip punctures the cell wall. Dead bacteria appear to be softer, with an elastic modulus of ∼0.13 ± 0.07 MPa in PBS, as opposed to an elastic modulus of ∼0.50 ± 0.10 MPa for a living bacterium, determined using a simple Hertzian model.17,18 Such a dramatic difference in elastic moduli between living and dead cells is in agreement with our claim that living S. typhimurium cells retain their viability after being punctured.

Our experiments suggest that damage inflicted on the bacterial cell wall, particularly on the peptidoglycan, is not a detriment to the viability of S. typhimurium. Part of the reason for the survival of S. typhimurium after extreme physical damage lies in the remarkable properties of the phospholipid bilayers that are the major building blocks of the cytoplasmic and outer membranes of bacteria. The phospholipid bilayers are highly dynamic, fluid-like constructs decorated with membrane proteins.22,23 Lipids themselves are highly mobile in the plane of the bilayer and can diffuse as much as 1 μm in 1 s above the phase transition temperature.22,24 According to the well-established fluid mosaic model and similar models,25,26 membrane proteins can easily diffuse in the plane of the surface of the membrane without much hindrance provided they are not connected to the subcellular structures of cell walls.27 The hydrophobic moieties on the intrinsic protein surface, which bisects the lipid bilayer, hold the proteins in their place and allow them to move with ease in concert with the phospholipids in the plane of the bilayer.

In an aqueous medium, phospholipid bilayers maintain their order and integrity because of the hydrophobic interactions of these amphiphilic molecules with their aqueous environment. We suggest that phospholipid molecules, including cross-membrane proteins with their hydrophobic regions intersecting the bilayers, undergo rapid reorientation in response to AFM tip penetration. The tip interacts with the bilayer structures (Figure 5) in a way similar to the interaction of integral membrane proteins with the cell membranes. The fact that the tip experiences little or no friction force during its retraction indicates either very weak or lack of interactions between the bilayers and the AFM tip. As the tip moves into or out of the cytoplasm, the fast-responding lipid membrane and membrane proteins are in close contact with the tip surface, which enables sealing against leakage into and out of the cytoplasmic and periplasmic regions regardless of the tip geometry. Lipids reorganize themselves faster than the AFM tip motion, hence, at every point in time that the AFM tip is plunging into or pulling out of the bacterial cytoplasm, the lipid and membrane proteins are in mechanical and thermodynamical equilibrium with the AFM tip body and adjust themselves in such a way as to seal any hole left behind by the AFM tip to prevent leakage of the cytoplasm, instantly. This is exactly what lipid bilayers and cross-membrane proteins do best: they seal cytoplasm against leakage.

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This model opens up the question of what happens to the peptidoglycan layer and to proteins that are attached to it. It was expected that not only the peptidoglycan, but also the proteins that are connected to the peptidoglycan layer, would be damaged as the tip tore the cell wall. However, there was no evidence of a broken or torn peptidoglycan layer in the force profiles of AFM puncture curves. Either bacteria are capable of repairing their peptidoglycan layer, or the peptidoglycan layer is self-sealed within a period of less than 1 s, or bacterial viability is not too sensitive to the integrity of this layer. The data presented in Figure 3 suggest that the peptidoglycan layer is repaired quickly and that the damage inflicted to this layer is negligible. Otherwise, the eight consecutive puncture curves (Figure 3) would have had very different profiles from one another. Our observations lead us to the conclusion that a damaged peptidoglycan layer has repairing capability, which maintains the turgor pressure exerted by the cytoplasm of the organism and prevents it from leaking into the environment. Although it is not clear how this repair is achieved, we hypothesize that covalent bonds that have been broken and resaturated as a result of AFM tip action regroup at random and reseal the broken fabric of the peptidoglycan layer through multitudes of interactions such as hydrogen bonds and van der Waals interactions within the restrictions of steric hindrance. The self-repair of the lipid bilayers and of the peptidoglycan layer are the key factors in the survival of *S. typhimurium* against multiple puncturing events exerted by an AFM tip.

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**Supporting Information Available:** Images depicting the results of the viability test conducted, puncture curves of live and dead *S. typhimurium* cells, and an explanation of Movie S1. This material is available free of charge via the Internet at http://pubs.acs.org.