Mechanical penetration of β-lactam–resistant Gram-negative bacteria by programmable nanowires

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β-Lactam–resistant (BLR) Gram-negative bacteria that are difficult or impossible to treat are causing a global health threat. However, the development of effective nanoantibiotics is limited by the poor understanding of changes in the physical nature of BLR Gram-negative bacteria. Here, we systematically explored the nanomechanical properties of a range of Gram-negative bacteria (Salmonella, Escherichia coli, Pseudomonas aeruginosa, and Klebsiella pneumoniae) with different degrees of β-lactam resistance. Our observations indicated that the BLR bacteria had cell stiffness values almost 10× lower than that of β-lactam–susceptible bacteria, caused by reduced peptidoglycan biosynthesis. With the aid of numerical modeling and experimental measurements, we demonstrated that these stiffness findings can be used to develop programmable, stiffness-mediated antimicrobial nanowires that mechanically penetrate the BLR bacterial cell envelope. We anticipate that these stiffness-related findings will aid in the discovery and development of novel treatment strategies for BLR Gram-negative bacterial infections.

INTRODUCTION

The β-lactam antibiotics have been among the most successful drugs with regard to reducing human morbidity and mortality for the past 60 years (1) because of their excellent safety profile, breadth of spectrum of activity, and low cost (2). However, as a result of the intensive use of β-lactam antibiotics in human therapy and animal agriculture, many bacterial pathogens have acquired β-lactam resistance and can cause infections that are essentially untreatable, representing an increasing threat to public health (3). This situation is especially troubling with respect to β-lactam–resistant (BLR) Gram-negative bacteria, as they are harder to kill than Gram-positive bacteria. The difficulty in eradicating BLR Gram-negative bacteria is largely due to the permeability barrier that is provided by their unique cell envelope, in which the outer membrane is very challenging for small molecules to cross (4).

To combat BLR Gram-negative bacteria, numerous conventional antibiotics replacements including chemical antibacterial nanomaterials such as metal nanoparticles (Au and Ag) (5, 6), metal oxide nanoparticles (ZnO and TiO2) (7, 8), photothermal agents (9), and graphene oxide nanosheets (10) and physical antibacterial materials such as antimicrobial peptides (4) and cationic polyelectrolytes (11) have been reported to decrease the possibility of antibiotic resistance. However, all of these antibacterial strategies may fail to achieve effective killing of BLR Gram-negative bacteria because they rely only on material characteristics such as toxicity (12), photothermal conversion efficiency (13), photocatalysis performance (14, 15), and charge characteristics (11), ignoring the acquired mechanical properties of BLR bacteria. Therefore, understanding the scientific basis of β-lactam resistance is essential to find an effective strategy against BLR Gram-negative bacteria. Recent studies have demonstrated that Gram-negative bacteria can acquire or develop resistance to β-lactam via several mechanisms such as genic mutations, resistance gene transfer, overexpression of efflux pumps, and production of β-lactamase (16), but the physical nature of BLR Gram-negative bacteria remains largely unexplored.

Here, we systematically studied the mechanical properties of 22 clinical isolates of the Gram-negative bacteria Salmonella, Escherichia coli, Pseudomonas aeruginosa, and Klebsiella pneumoniae to investigate the association between cell envelope mechanical behavior and acquisition of β-lactam resistance. We found that BLR Gram-negative bacteria exhibited a significant decrease in stiffness compared to β-lactam–susceptible (BLS) bacteria, indicating that changes in mechanical properties were correlated with acquired β-lactam resistance. To identify the mechanism of reduced stiffness, we used a fluorescent probe for in situ visualizing bacterial peptidoglycan (PG) biosynthesis, which revealed that this reduced stiffness could be induced by reduced PG biosynthesis resulting from long-term exposure to subinhibitory concentrations of β-lactam antibiotics. This change was further evidenced by a thickness analysis of bacterial cell envelope through cryo–transmission electron microscopy (cryo–TEM) and TEM. On the basis of these stiffness findings, we first developed a cell membrane penetration model to explore the possibility of mechanically killing bacteria and established criteria of cell membrane penetration. We then validated this prediction experimentally in Salmonella strains by varying the tip diameters of NiCo(OH)2:CO3 nanowires (NWs), showing that the soft BLR bacteria were more easily penetrated by sharp NWs. Our results provide insight into potential strategies for killing BLR Gram-negative bacteria with a combined focus on bacterial mechanical properties and performance of nanomaterials.
RESULTS

Nanomechanical properties of Gram-negative bacteria

We begin by asking whether the acquisition of β-lactam resistance is associated with changes in physical properties of the Gram-negative bacterial cell envelope. To systematically address this issue, we collected and characterized 22 clinical isolates of Salmonella, E. coli, P. aeruginosa, and K. pneumoniae (table S1), well-known pathogenic Gram-negative bacteria that usually cause a variety of life-threatening hospital-acquired infections (17). The selection and classification of BLS and BLR strains here are according to their degrees of β-lactam resistance. To this end, the antimicrobial resistance of these collected isolates was first identified using minimum inhibitory concentrations (MICs) to verify the selected bacterial populations including BLS and BLR bacteria (Fig. 1A and table S2). Several typical classifications of β-lactam antibiotics, the most important class of antibiotics currently in clinical use, have been tested, including penicillins, cephalosporins, carbapenems, and monobactams, which interfere with cell wall biosynthesis by covalent binding to the active site of penicillin-binding proteins (PBPs) (18). In addition, we used the acquired β-lactam resistance genes detected by using whole-genome sequencing and a spectrum of β-lactamase (ESBL) test to further evaluate the degree of β-lactam resistance (table S1). In combination of these results, the obtained Salmonella isolates (7 and 12), E. coli isolates (3, 17, and 15), P. aeruginosa isolates (84, 85, and 86), and K. pneumoniae isolates (3, 4, and 5) were deemed to be BLS bacteria, while the collected Salmonella isolates (44, 79, and 83), E. coli isolates (2 and 8), P. aeruginosa isolates (87, 88, and 89), and K. pneumoniae isolates (2, 12, and 25) were treated as BLR bacteria (see the details in Supplementary Discussion). Note that the collected P. aeruginosa isolates (87, 88, and 89) were resistant to all β-lactam antibiotics tested, indicating that such “superbugs” were very difficult to treat by classical β-lactam antibiotics.

To elucidate and correlate the respective nanomechanical profiles with bacterial resistance, we performed liquid atomic force microscope (AFM) analyses of Gram-negative bacteria in buffer corresponding with bacterial resistance, we performed liquid atomic force microscope (AFM) analyses of Gram-negative bacteria in buffer corresponding with bacterial resistance, we performed liquid atomic force microscope (AFM) analyses of Gram-negative bacteria in buffer corresponding with bacterial resistance, we performed liquid atomic force microscope (AFM) analyses of Gram-negative bacteria in buffer corresponding with bacterial resistance, we performed liquid atomic force microscope (AFM) analyses of Gram-negative bacteria in buffer corresponding with bacterial resistance, we performed liquid atomic force microscope (AFM) analyses of Gram-negative bacteria in buffer corresponding with bacterial resistance. This important role of β-lactam antibiotics in PG biosynthesis led us to hypothesize that the reduced bacterial stiffness resulted from exposing bacteria to subinhibitory concentrations of drugs during the selection of bacterial drug resistance, thus reducing PG biosynthesis. To test this, we performed an analysis of the mechanical properties of the typical BLS E. coli (ATCC25922) strain and New Delhi metallo-β-lactamase 1 (NDM-1)–producing wild-type E. coli strain after exposure to different concentrations of ceftazidime (CAZ) (Fig. 2D and fig. S5, A and B). First, it can be seen that the stiffness of NDM-1–producing wild-type strain was also nearly 10× lower than that of BLS strain before adding CAZ, which further confirmed that decrease in stiffness was associated with acquisition of β-lactam antibiotic resistance. Second, with increasing concentrations of CAZ, the stiffness of BLS E. coli gradually decreased while that of NDM-1–producing wild-type E. coli remained at approximately 0.7 MPa. This is because the BLS E. coli were susceptible to CAZ, and as a result, PG biosynthesis was inhibited. By contrast, NDM-1–producing wild-type E. coli isolate can produce β-lactamases, as confirmed by reaction with nitrocefin (fig. S5, C and D), which has the ability to hydrolyze β-lactam antibiotics (e.g., CAZ). These results revealed that the inhibition of PG biosynthesis by β-lactam antibiotics altered bacterial stiffness.

It is widely believed that the PG biosynthesis would be inhibited with the existence of β-lactam antibiotics (18). However, our mechanical results revealed that BLR Gram-negative bacteria exhibited a significant decrease in stiffness without adding β-lactam antibiotics, indicating that the alteration of stiffness was an intrinsic property of collected BLR isolates that can be expressed in the absence of β-lactam antibiotics. To examine this possibility, we used a published protocol for in situ labeling of PG of Salmonella cells with 7-hydroxyxoumarin-3-carboxylic acid 3-amino-α-alanine (HADA), a fluorescent α-amino acid (FDAA) (22). These small-molecule probes incorporate at the terminus (fifth position) of the stem peptide of nascent PG, wherein FDAAs act as a surrogate acceptor strand in the transpeptidation.

Analysis of the mechanism of reduced stiffness

The Gram-negative bacterial cell envelope structure includes a PG layer that lies in a periplasmic space located between the outer membrane and inner membrane, providing mechanical strength and rigidity for the bacterium (2A). The final stages of PG biosynthesis are catalyzed by PBPs, which have both transglycosylase and transpeptidase (Tpase) activities and are required to generate glycan strands and cross-linked adjacent glycan strands via stem peptides, respectively (21). β-Lactam antibiotics consisting of a four-member “β-lactam” ring act as a structural mimetic of the terminal d-Ala-d-Ala moiety of the donor stem peptide in transpeptidation. Therefore, β-lactam antibiotics inhibit PG biosynthesis by blocking the active site of Tpase (Fig. 2B). Gram-negative bacteria have developed different mechanisms to resist the action of β-lactams, including destroying the antibiotic molecule through the action of β-lactamases (Fig. 2C). This important role of β-lactam antibiotics in PG biosynthesis led us to hypothesize that the reduced bacterial stiffness resulted from exposing bacteria to subinhibitory concentrations of drugs during the selection of bacterial drug resistance, thus reducing PG biosynthesis. To test this, we performed an analysis of the mechanical properties of the typical BLS E. coli (ATCC25922) strain and New Delhi metallo-β-lactamase 1 (NDM-1)–producing wild-type E. coli strain after exposure to different concentrations of ceftazidime (CAZ) (Fig. 2D and fig. S5, A and B). First, it can be seen that the stiffness of NDM-1–producing wild-type strain was also nearly 10× lower than that of BLS strain before adding CAZ, which further confirmed that decrease in stiffness was associated with acquisition of β-lactam antibiotic resistance. Second, with increasing concentrations of CAZ, the stiffness of BLS E. coli gradually decreased while that of NDM-1–producing wild-type E. coli remained at approximately 0.7 MPa. This is because the BLS E. coli were susceptible to CAZ, and as a result, PG biosynthesis was inhibited. By contrast, NDM-1–producing wild-type E. coli isolate can produce β-lactamases, as confirmed by reaction with nitrocefin (fig. S5, C and D), which has the ability to hydrolyze β-lactam antibiotics (e.g., CAZ). These results revealed that the inhibition of PG biosynthesis by β-lactam antibiotics altered bacterial stiffness.

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Fig. 1. Nanomechanical properties of BLS and BLR Gram-negative bacteria. (A) Heat map representation of antimicrobial resistance of five Salmonella isolates, five E. coli isolates, six P. aeruginosa isolates, and six K. pneumoniae isolates on a logarithmic color scale. Red represents a high level of antibiotic resistance (MIC ≥ 64 µg/ml), while blue represents a low level of antibiotic resistance (MIC ≤ 0.125 µg/ml). PEN, penicillin; AMP, ampicillin; PIP, piperacillin; AMC, amoxicillin-clavulanic acid; OXA, oxacillin; CAZ, ceftazidime; FEP, cefepime; CTX, cefotaxime; CRO, ceftriaxone; CXM, cefuroxime; CZO, cefazolin; IPM, imipenem; MEM, meropenem; BIPM, biapenem; ATM, aztreonam. (B) Strategy for measuring stiffness of Gram-negative bacteria using liquid AFM with a bacterium immobilized on a poly-lysine–coated glass substratum. (C) AFM topographic image recorded in PBS showing S. 79 on a modified substratum. Scale bar, 500 nm. (D) The height profile along the white dotted line in the image (C). (E) Representative Derjaguin-Muller-Toporov Young’s modulus map of S. 79. Scale bar, 1 µm. (F) A high-magnification elasticity map is shown, and the enlarged section is outlined in white in (E). Scale bar, 200 nm. (G to J) Quantification of the average Young’s modulus of the five Salmonella isolates (G), five E. coli isolates (H), six P. aeruginosa isolates (I), and six K. pneumoniae isolates (J). The BLS isolates are indicated with a pink band, while the BLR isolates are indicated with a blue band in (G to J). Thick horizontal lines and error bars represent mean and SD, respectively. Student’s t test, **P < 0.01.
Fig. 2. Mechanism of reduction in stiffness for BLR Gram-negative bacteria. (A) The cell envelope of Gram-negative bacteria consists of lipopolysaccharide (LPS) molecules, an outer membrane (OM), a thin single layer of PG, and an inner membrane (IM). (B) β-Lactam antibiotics inhibit PG synthesis by inhibiting the peptide bond formation reaction that is catalyzed by Tpase. (C) Breaking of the β-lactam ring by β-lactamase. (D) Quantification of the average Young's modulus of the BLS and NDM-1–producing Escherichia coli treated with different concentrations of CAZ. Thick horizontal lines and error bars represent mean and SD, respectively. (E and F) Pulse labeling of BLS [S.7 and S.12; (E)] and BLR [S.44, S.79, and S.83; (F)] strains with HADA to determine PG synthesis. Scale bars, 2 μm. (G and H) Schematic representations showing the change of periplasmic gel in BLS bacteria (G) and BLR bacteria (H) following dehydration. (I and J) Cryo-TEM images of BLS Salmonella isolate (S.7) at low magnification (I) and high magnification (J). (K) TEM image of S.7. (L and M) Cryo-TEM images of BLR Salmonella isolate (S.79) at low magnification (L) and high magnification (M). (N) TEM image of S.79. Regions of interest are indicated by yellow arrows in (I to N). Scale bars, 200 nm (I and L), 50 nm (J and M), and 100 nm (K and N). (O and P) Quantification (n = 20) of the cell envelope thickness in five Salmonella isolates measured from cryo-TEM images (O) and TEM images (P). Results are represented as means ± SD. Student’s t test, **P < 0.01; ns, not significant.
reduced stiffness. cryo-TEM and TEM further indicated that a decrease in PG hydration with ethanol (figs. S12 to S15). The combined results of Gram-negative bacteria showed no marked differences after dehydration of the dehydrated cell envelope could be attributed to the decreased thick-ness of the cell envelope in two different states, frozen-hydrated and dehydrated, observed by cryo-TEM and TEM, respectively (Fig. 2, I to N, and fig. S8). There was no difference in the thickness of the cell envelope for BLS and BLR isolates in a frozen-hydrated state (Fig. 2O). Since these frozen-hydrated cells retained their structures in the native environment, these measured thicknesses of the cell envelope should be accurate for bacteria. When the bacterial cells were dehydrated, the cell envelope thicknesses of BLS bacteria (isolates 7 and 12) changed from ~40 to 28.83 ± 1.63 nm and 30.32 ± 1.91 nm, respectively. However, with regard to the BLR bacteria (isolates 44, 79, and 83), the thickness of the cell envelope decreased to 14.46 ± 0.97, 14.73 ± 0.93, and 16.99 ± 1.50 nm, respectively, which were half that of BLS isolates (fig. S9 to S11), suggesting that the decreased thicknesses of the dehydrated cell envelope could be attributed to the decrease in PG biosynthesis. Note that the size of BLS and BLR Gram-negative bacteria showed no marked differences after dehydration with ethanol (figs. S12 to S15). The combined results of cryo-TEM and TEM further indicated that a decrease in PG biosynthesis occurred in BLR Gram-negative bacteria, leading to reduced stiffness.

Computational modeling of cell membrane penetration

On the basis of the observation that BLR bacterial stiffness was altered from the native state and the knowledge that cell mechanics play a key role in cell membrane penetration events (25), we hypothesized that the stiff BLS and soft BLR bacteria may display different cell envelope penetration properties when prodded with an NW (Fig. 3, A and B). This penetration may provide the basis for an alternative antibacterial strategy in which NWs mechanically penetrate the BLR bacterial cell envelope, leading to cell death. Hence, gaining a deeper understanding of the bacteria penetration events is of great importance. However, the current criteria for cell penetration have been calculated from synthetic or membrane penetration experiments and are not suitable for the Gram-negative bacterial cell envelope because of its special structure. Therefore, to perform rigorous bacterial penetration studies, it is critical to establish consistent criteria to assess when (or whether) bacterial envelope penetration events occur.

Bacterial penetration occurs when the needle tip of an NW touches the surface of a bacterium, deforms it, penetrates the outer membrane, and then penetrates the entire bacterial envelope. To simplify this issue, we regard the penetration of the bacterial cell membrane as the penetration of the bacterial envelope. Cell membrane penetration can be described by the activation energy theory (26). On the basis of this theory, thermally activated molecular-scale defects arise and disappear spontaneously in membranes, with the steady-state hole formation rate affecting the probability of rupture. It has been reported that the free energy of such deformed cells plays a critical role in the rupture of the membrane (25). According to previous research, cell membrane penetration occurs when the free energy per unit area is larger than a threshold value [(2.9–8.3) × 10⁻³ J/m²].

From a mechanical perspective, the bacterial envelope can be considered as a composite elastic shell (27, 28). The mechanical equilibrium of the bacterial shell is determined by its surface tension and its elastic deformation and turgor pressure. Hence, the free energy per unit area of a deformed bacterial envelope can be expressed in the form (28)

\[ f = T_r + \Delta E_{\text{elastic}} \]

The tension (surface energy per unit area) is determined by \( T_r = T_{\text{in}} + T_d \), where \( T_{\text{in}} \) is the intrinsic tension at zero deformation caused by the bacterial turgor pressure and \( T_d \) represents the indentation tension that arises from the deformation of the bacterial envelope. \( \Delta E_{\text{elastic}} \) is the elastic energy induced by the deformation of the bacteria, which is often ignored in the bulge or vesicle indentation model (25) but plays a non-negligible role in the bacterial envelope model (27). Therefore, a useful method of quantifying membrane penetration is to simulate the free energy density distribution of deformed bacteria and compare its maximum value with threshold. On the basis of our experimental results, we chose the minimum value of threshold (2.9 × 10⁻³ J/m²) as the critical value in our following simulation to determine whether the bacteria were penetrated or not. To implement this approach, finite element method (FEM) analysis was performed to obtain the maximum free energy by using COMSOL Multiphysics version 5.2 on the basis of an inflated spherocylinder shell with isotropic elasticity model. Considering a realistic condition in which bacteria touch the NW surface from different directions, we started by analyzing the free energy of bacteria at different penetration angles (θ; fig. S16). The maximum value of the free energy reached a minimum when bacteria were perpendicular to NWs (θ = 0°). This suggests that if bacteria are penetrated from perpendicular angles, they are certain to be penetrated from other angles. Thus, we chose this case (θ = 0°) to determine whether the bacteria were penetrated for the subsequent FEM simulations.

Next, we analyzed the representative cases of NWs with tip diameters of 5-, 50-, and 100-nm indentations into a bacterium under a constant driving force of 100 pN (Fig. 3, C and D), which was calculated from our previously reported bendable NWs (29). As shown in Fig. 3, C and D, the free energy density is concentrated at the tip of the NW, and its maximum value decreases with increasing tip diameter. The soft BLR bacteria allow the generation of a maximum free energy density of 3.18 × 10⁻³ J/m² when being indented with an NW with a tip diameter of 5 nm, which is greater than the corresponding value for the stiff BLS bacteria (0.86 × 10⁻³ J/m²). Moreover, the stiff BLS bacteria exhibited no significant change in shape...
according to NW tip diameter, while the soft BLR bacteria were markedly more distorted by NWs with sharper tips. This difference in deformation was further evidenced by their corresponding indentation depth results (Fig. 3E), confirming that the extent of this swelling was strongly dependent on the NW tip diameter and bacterial stiffness. As expected, the soft BLR bacteria required a small critical tip diameter to induce membrane penetration and were triggered only when the NW tip diameter was 5 nm (Fig. 3F). By contrast, membrane penetration of stiff BLS bacteria did not occur in this range of NW tip diameter. These results suggested the possibility of directly penetrating only BLR bacteria using sharp NWs (\(d \leq 5\) nm). To determine the critical bacterial stiffness that can be penetrated by an NW with a diameter of 5 nm, bacteria with different stiffness were used for FEM analysis (Fig. 3G). Bacteria with low stiffness (\(E < 0.6\) MPa) could be readily penetrated according to this model, which also provides a basis for detecting bacterial stiffness based on cell membrane penetration events.

The driving force for bacterial capture is generally focused on molecular recognition processes such as carbohydrate-protein interaction (30) and antigen-antibody binding (31), which vary in the order of piconewton. To test the role of driving force in cell membrane penetration, we examined the amount of vertical force required to achieve free energy required for critical failure within the membrane. With increasing driving force, the deformation of soft BLR bacteria became more apparent, while the stiff BLS bacteria retained their original rod-like shapes (fig. S17, A and B). The maximum free energy density at the NW tip increased with driving force, with penetration occurring at driving force \(\geq 100\) pN for BLR bacteria and driving force \(\geq 300\) pN for BLS bacteria when the NW tip diameter was 5 nm (fig. S17C). Notably, the minimum driving
force required to induce membrane penetration increased with increasing tip diameter (fig. S17, D and E).

**Experimental testing of simulation predictions by programmable NWs**

To experimentally validate the predictions of our computational model, large-area high-density NiCo(OH)$_2$CO$_3$ NW arrays grown uniformly on fluorine-doped tin oxide (FTO) were used first as bacterial stiffness nanoprobes using a simple hydrothermal synthesis method (fig. S18, A and B). The obtained three-dimensional hierarchical NiCo(OH)$_2$CO$_3$ NWs were integrated and had sharp tips, which had a needle-like shape with a length of 5 ± 0.5 μm, root diameter of 160 ± 10 nm, and tip diameter of 20 ± 15 nm (fig. S18, C and D). The crystallographic structure of the NWs was investigated by x-ray diffraction, as shown in fig. S18E, and matched well with that of monoclinic binary NiCo(OH)$_2$CO$_3$ reported previously (32). The single-crystalline structure of NiCo(OH)$_2$CO$_3$ NWs was confirmed by high-resolution TEM (HRTEM), as well as the corresponding selected area electron diffraction pattern (fig. S18F), which had lattice fringes with interplanar spacing of 0.25 nm corresponding to the (040) plane of NiCo(OH)$_2$CO$_3$.

To test the prediction that the membrane penetration also depended on the NW tip diameter, we next aimed to alter the tip diameter of NWs through varying the aqua regia etching time to investigate the tip size effect (fig. S19A). The original NiCo(OH)$_2$CO$_3$ NWs with a diameter of 5 ± 1.8 nm were converted to a series of NWs with increasing tip diameter controlled by etching time (fig. S19, B to J). Eventually, these NWs reached an average tip diameter of 120 ± 7.8 nm after exposure in aqua regia solution for 8 min (fig. S19f). However, it was difficult to obtain larger tip diameters of NWs with this etching method because further increases in etch time resulted in exposed NWs ultimately falling off the FTO substrate (fig. S20).

Membrane penetration involves two steps, bacterial capture and indentation, both of which require a driving force. To this end, concanavalin A (Con A), as a bacterium-binding molecule that offers a driving force, was introduced to the surface of substrata by sequential chemical covalent coupling (fig. S21). The driving force was produced by specific lectin-carbohydrate recognition between Con A and mannose of the bacterial surface lipopolysaccharide (29), which enabled the bacteria to adhere to the substrata. To optimize the incubation time for optimal bacterial capture, a set of bacteria capture experiments with different incubation times were carried out using Con A–coated NiCo(OH)$_2$CO$_3$ NW/FTO and FTO as substrata and Salmonella isolate 79 (BLR) as the target (fig. S22A). The maximal bacterial capture efficiency was reached after 60 min of incubation on the NW/FTO substratum, while the bacterial capture efficiency of smooth FTO substrate remained intact despite contact with NWs (fig. S22B). The maximal bacterial capture efficiency was reached after 60 min of incubation on the NW/FTO substratum, while the bacterial capture efficiency of smooth FTO substrate remained intact despite contact with NWs (fig. S22B). The maximal bacterial capture efficiency was reached after 60 min of incubation on the NW/FTO substratum, while the bacterial capture efficiency of smooth FTO substrate remained intact despite contact with NWs (fig. S22B). The maximal bacterial capture efficiency was reached after 60 min of incubation on the NW/FTO substratum, while the bacterial capture efficiency of smooth FTO substrate remained intact despite contact with NWs (fig. S22B). 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penetration of bacteria by NWs, NWs could be applied to one or more inner surfaces of a medical device by growing the NWs directly on one or more surfaces. In contrast to conventional physical methods that require other resources, such as power, light, and heat sources, the energy efficiency and strong efficacy against BLR bacteria offer enormous and untapped potential for use of NWs in medical devices for preventing bacterial infection.

In summary, our findings are notable because they demonstrate that a decrease in BLR Gram-negative bacterial stiffness is associated with the acquisition of β-lactam antibiotic resistance, which is attributed to reduced PG synthesis. Theoretical work revealed that, with their decreased stiffness, BLR bacteria could be more efficiently penetrated by NWs, and that this penetrating effect can be controlled by the NW tip diameter and driving force. Experiments confirmed that ~95% of BLR bacteria with stiffness values below 0.5 MPa are penetrated by sharp NWs with a tip diameter of 5 nm compared to ~20% of stiff BLS bacteria with a Young’s modulus of 5 MPa, thus providing an alternative therapeutic strategy for attacking BLR Gram-negative bacteria. A fundamental understanding of BLR Gram-negative bacteria combined with our demonstration of the effectiveness of NWs open a viable route for addressing the emerging threat of BLR bacteria over the long term.
MATERIALS AND METHODS

Bacterial strains

The bacterial strains in this study are listed in table S1. A total of 10 *Salmonella* and *E. coli* isolates were obtained from the laboratory of B.Y. at Northwest A&F University (Yangling, Shaanxi, China). The clinically collected strains ( *P. aeruginosa* and *K. pneumoniae*) were obtained from the group of Y.Y. at Zhejiang University (Hangzhou, Zhejiang, China). To detect acquired β-lactam resistance genes, genomic DNA was extracted, fragmented, and tagged for multiplexing with Nextera XT DNA Sample Preparation Kits, followed by whole-genome sequencing on an Illumina HiSeq 2000 platform (Illumina, San Diego, CA, USA). Comparator strains consisted of two reference strains: *E. coli* (ATCC25922) and NDM-1–producing *E. coli* (07HAE27). Polymerase chain reaction–based analysis was used to screen for the presence of *bla*NDM-1 in *E. coli* isolate 07HAE27.

Bacterial culture conditions

Typically, the bacterial strains were stored at ~80°C in glycerol/ Difco nutrient (20% v/v) and reactivated by inoculation in 50 ml of sterile Luria-Bertani broth medium at 37°C. After being grown to exponential growth phase [optical density at 600 nm (OD600), 0.5 to 0.6], bacterial cells were harvested by centrifugation (5000 rpm, 5 min) and washed with sterile normal saline three times. Then, the cells were resuspended in sterile normal saline and diluted to predetermined volumes as stock solutions. The bacterial concentration could be monitored photometrically by measuring the OD600. Before performing the bacterial penetration experiments, the OD600 values of bacterial stock solutions were redetermined to 0.1, which corresponded to a concentration of ~10⁸ colony-forming units/ml.

Antimicrobial susceptibility test

All collected strains were tested for their susceptibility to antimicrobial agents. The MICs of the antimicrobial agents were determined by the agar dilution method using Mueller-Hinton agar according to the guidelines recommended by the Clinical and Laboratory Standards Institute (CLSI). The β-lactam antibiotics included penicillin, ampicillin, piperacillin, amoxicillin-clavulanic acid, oxacillin, ceftazidime, cefepime, cefotaxime, ceftriaxone, cefuroxime, cefazolin, imipenem, meropenem, biapenem, and aztreonam. *Enterococcus faecalis* ATCC29212 were used as quality control organisms in MIC determination. The breakpoints for the antibiotics were as described by Kuru and colleagues (23).

Sample preparations for AFM observation

An important requirement for liquid AFM investigations is that the sample must be immobilized on a surface. For this purpose, an aliquot of the bacterial suspension of ~10⁶ cells per ml was allowed to adhere through electrostatic interaction to a poly-L-lysine–coated glass substratum that was prepared as previously described (37). The excess liquid was then drained after 10 min to allow the bacteria to adhere; fresh, sterile PBS (200 μl) was refilled for the glass, and the sample was used for AFM observation.

**Nanomechanical measurements of the bacterial cell envelope with AFM**

AFM measurements in liquid were conducted using a Bruker Dimension FastScan AFM, equipped with the NanoScope V controller and a small scanner. For imaging live bacteria, cells were imaged in PBS at room temperature (22°C to 24°C) in PeakForce quantitative nanomechanical mapping mode using a silicon nitride tip (ScanAsyst-Fluid, Bruker). The spring constant of the cantilevers was 0.3 N/m, as determined by the thermal tune method for each experiment. The radius of the standard AFM probe was found to be 20 nm. Force-displacement curves were recorded at 0.8 Hz for determination of Young's modulus. Young's modulus was calculated by converting the force curves into force-indentation curves and fitting with the Derjaguin-Muller-Toporov model, which describes the indentation of an elastic sample using a stiff conical indenter, as described elsewhere. The half opening angle of the AFM tip was 18°, and the Poisson ratio of the cell was taken to be 0.5, as is typical for soft biological materials. To avoid the influence of the glass substrate on the measured bacterial stiffness, we used experimental conditions in which the cantilever indentation was 10 nm, far less than the thickness of the entire cell (~700 nm). Cantilever spring constants and sensitivity were calibrated before and after each experiment. Data processing was performed using the commercial NanoScope Analysis software (Bruker AXS Corporation).

**β-Lactamase assay**

β-Lactamase production was triggered for 2 hours (AMP, 100 μg/ml). Cells were then lysed on ice by sonication (2 × 2 min, 70%) and pelleted using a centrifuge. Then, 50 ml of nitrocefin (0.5 mg/ml) was added to 500 ml of the supernatant. Samples were observed after 30 min of incubation in the dark, and a red color indicated β-lactamase activity.

**Synthesis of HADA**

Preparation of HADA was carried out following the procedure described by Kuru and colleagues (23). The purity was determined using a Waters ACQUITY UPLC system (Waters Corp., Milford, MA, USA) with ACQUITY UPLC HSS T3 column (2.1 mm by 100 mm, 1.8 μm). Elution was performed with a gradient of water/acetonitrile from 95/5 to 5/95 for 10 min and maintained at 5/95 for another 10 min. The flow rate was 200 μl/min. Peaks were detected at 254 nm [high-performance liquid chromatography (HPLC), tR = 2.58 min]. The purification, as analyzed by HPLC, was 93% [¹H nuclear magnetic resonance (NMR) (400 MHz, DMSO-d6): δ = 3.41 (s, 1H), 3.48 (s, 1H), 3.69 to 3.77 (d, 2H), 3.82 to 3.96 (d, 2H), 6.87 (s, 1H), 6.89 (s, 1H), 7.82 (s, 1H), 8.76 (s, 1H), 9.12 (t, 1H), and 11.51 (br s, 1H); ¹³C NMR (250 MHz, DMSO-d6): δ = 39.11, 51.0, 101.4, 110.5, 112.5, 114.1, 131.3, 148.0, 155.9, 160.3, 162.5, 163.9, and 168.6; liquid chromatography–mass spectrometry mass/charge ratio calculated for C₁₁H₁₁O₇N₂ [(M-H)⁻]: 291.08, found 291.08].

**PG labeling**

We labeled the PG of *Salmonella* isolates with HADA as previously described (22). Cells were grown to exponential phase and incubated with HADA (final concentration, 0.5 mM) for 3 hours at 37°C. Cells were then fixed in 70% ethanol for 10 min to prevent potential cell stress resulting from the washing steps. Cells were collected by centrifugation (5000 rpm, 5 min) and washed three times with PBS (pH 7.4; 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM
KH₂PO₄) to remove excess dye. Approximately 20 µl of the samples was fixed on glass slides and dried at room temperature in the dark. The stained cells were observed using superresolution confocal microscopy (Leica TCS SP8 STED 3X, Leica Microsystems, Wetzlar, Germany). The samples were excited with a laser at 405 nm, and the emission was detected through a 419- to 465-nm emission filter.

**Structure analysis of PG**

ATR-FTIR was performed to clarify the structural differences of PG among the BLS and BLR strains. ATR-FTIR spectra were recorded using a spectrometer (VERTEX 70, Bruker) equipped with a KBr beam splitter and a deuterated triglycine sulfate detector. The sampling station was equipped with an overhead ATR accessory, which included transfer optics within the chamber through which infrared radiation was directed to a detachable ATR zinc selenide crystal mounted in a shallow trough for sample containment. Distilled water was used as the background spectra, and 256 scans were taken for each sample in the mid-infrared region of 4000 to 400 cm⁻¹ at a resolution of 4 cm⁻¹.

**Cryo-TEM imaging**

Salmonella cells were grown to exponential phase and harvested by centrifugation (5000 rpm, 5 min). The resulting pellet was washed with sterile normal saline three times and resuspended in sterile normal saline. All cell suspensions (5 µl) were placed on Quantifoil holey carbon film–coated 200-mesh copper grids (Quantifoil Micro Tools GmbH, Jena, Germany) in the chamber of a Vitrobot (FEI, Hillsboro, OR, USA). All the processes, blotting, and plunge-freezing into liquid ethane were performed by the Vitrobot. Images were acquired on a JEM-3200FSC transmission electron microscope (JEOL, Tokyo, Japan) incorporating a liquid helium stage and an omegatype energy filter operating at 300 kV. The stage was cooled with liquid nitrogen to 80 K during acquisition of all datasets.

**TEM analysis of bacteria**

The ultrastructure of the bacterial cells was evaluated using TEM. Bacterial suspensions were prepared in the same manner as described above for the cryo-TEM samples placed in a cold fixative solution composed of 2.5% glutaraldehyde in PBS (0.1 M, pH 7.2) at 4°C until use. After rinsing with PBS for 5, 10, 15, 20, and 30 min, the specimens were postfixed in 1% osmium tetroxide (in 0.2 M PBS, pH 7.2) at 4°C for 1 to 2 hours. The samples were then rinsed again with PBS for 5, 10, 15, 20, and 30 min. The samples were dehydrated for 15 min in a series of ethanol solutions (30, 50, 70, 80, 90, and 100%), and infiltrated overnight in a mixture of LR white resin (London Resin Company, Reading, UK) and alcohol (1:1, v/v), followed by infiltration with pure LR white resin twice (for 1 and 2 hours) at room temperature. Pure LR white resin was then used for embedding, and the samples were incubated at 60°C for 48 hours. Sections (50 nm) were obtained with a diamond knife on the Leica EM UC7 Ultramicrotome (Leica, Nussloch, Germany) and picked up on the coated grids. The ultrathin sections were floated on 3% aqueous solution of uranyl acetate for 10 to 15 min and refloated in 4% Pb solution for 8 to 10 min. The prepared bacterial samples were examined with TEM (JEOL, JEM-1011).

**Bacterial morphology**

Bacterial morphology was investigated by field emission SEM (FE-SEM; Hitachi, S-4800). After centrifugation, the bacteria were first fixed with 2.5% glutaraldehyde for 4 hours at room temperature. The bacteria were then washed with sterile normal saline followed by dehydration with increasing concentrations of ethanol (25, 50, 75, 90, 95, and 100%) for 10 min in each step and air-dried overnight. Before imaging, the bacteria on substrata were sputter-coated with platinum and imaged by FE-SEM.

**Computational methods**

An FEM environment (COMSOL Multiphysics version 5.2) was used to explore cell membrane penetration, of which the model of inflated spherocylinder shell with isotropic elasticity was carried out to simulate the scenario of a bacterium indented by an NW. In our simulations, the cell envelope was modeled as a single isotropic elastic sheet, where the Young’s modulus (E) encompasses the entire cell envelope. Therefore, the modulus of BLS bacteria is assumed to be 5 and 0.5 MPa for BLR bacteria. The bacterium body was assumed to initially be a spherocylindrical shape with a radius of 0.25 µm and a length of 0.9 µm, which were calculated from the SEM results. The net gravitational force on a cell or particle herein was determined from the difference between gravitational and buoyant forces and may be written as

\[ F_G = V_c g (p_p - p_w) \]

where \( V_c \) is the volume of the bacterium, \( g \) is the gravitational constant, \( p_w \) indicates the water density, and \( p_p \) indicates the particle density. Since most microorganisms are small and their density is similar to that of water, the net gravitational force is negligible (38). Therefore, the driving force for bacterial deformation is assumed to be the molecule force. Under a driving force of 100 pN, the bacterium is axisymmetrically indented by a truncated cone NW whose diameter varied from 100 to 5 nm and length remained constant (5 µm). The bacterial turgor pressure can be calculated as follows (39)

\[ \Delta P = E/100 \]

where \( \Delta P \) is the bacterial turgor pressure and \( E \) is the bacterial stiffness.

**Fabrication of programmable NWs**

In a typical synthesis process of NiCo(OH)₂CO₃ NWs, 2 mmol of CoCl₂·6H₂O, 1.25 mmol of NiCl₂·6H₂O, and 3 mmol of urea were dissolved in 15 ml of water to form a transparent pink solution. Following the addition of a piece of FTO (1.0 cm by 1.0 cm), the solution was transferred to a 25-ml Teflon-lined stainless steel autoclave and kept at 120°C for 6 hours. After hydrothermal growth, the NiCo(OH)₂CO₃/FTO was carefully washed with DI water and ethanol several times to remove the excess surfactant and dissociative ions and air-dried.

To obtain NWs with different tip diameters, the NiCo(OH)₂CO₃/FTO substratum was immersed into freshly prepared aqueous regia (HCl:HNO₃, 3:1 ratio by volume) at room temperature, and the etching time was varied to control the tip diameter. The substrata were then immersed in DI water to rinse their surfaces and dried by natural convection under ambient conditions.

**Materials characterization**

The morphology of the samples was characterized by FE-SEM (Hitachi, S-4800) and TEM (JEOL, JEM-1011). The HRTEM images were recorded using a JEM-2100F microscope. The crystalline structures
of the samples were characterized using a PANalytical x-ray diffraction instrument with Cu Kα radiation and ranging from 5° to 70° at room temperature.

**Surface modification with Con A**

To bind Con A onto substrata, the substrata of NiCo(OH)2CO3/FTO were first treated with oxygen plasma for 30 min (EXTRON25). The treated substrate surface was first incubated in 4% (v/v) 3-aminopropyltriethoxysilane in ethanol at room temperature for 60 min. After incubation, the surface was rinsed with ethanol and dried under argon. The substrate was then immersed into a 10 mM bis(N-succinimidyl) carbonate solution in acetonitrile at room temperature for 10 min. After the solution was removed, the surface was washed with acetonitrile and dried with argon. Last, the substrate was treated with bacteria binding molecule solutions (50 μg/ml) of Con A in PBS (0.02 M, pH 7.2) at room temperature for 60 min. After the solution was removed, the substrate was immersed in 5 ml of PBS buffer and shaken for 10 min on a shaker to remove noncovalently attached Con A. After the PBS buffer was removed, the substrate modified with Con A was obtained and stored at 4°C for later use.

**Analysis of bacterial penetration**

For bacterial capture and penetration tests, functionalized NiCo(OH)2CO3/FTO substrata with an exposed area of 1 cm2 was immersed in 1 ml of bacteria solution for 60 min for the bacteria to adhere to the substrate surface. After the test bacteria solution was removed, the substrata were washed three times with DI water to completely remove the nonattached bacteria. Then, the substrata were fixed, dehydrated, and dried before SEM observations.

CLSM measurement of bacterial viability was carried out using an Olympus FV1000-IX81 laser confocal microscope. The postwashed substrata were immediately stained with propidium iodide (PI; 5 μg/ml) for 15 min and then counterstained with 4′,6-diamidino-2-phenylindole (DAPI; 5 μg/ml) for 15 min in the dark. DAPI permeates all cells, binding to nucleic acids and fluorescing blue when excited by a 405-nm-wavelength laser. PI only enters cells with membrane damage, which were considered to be penetrated, and binds to nucleic acids with a higher affinity than DAPI. The postwashed substrata were then imaged by using CLSM. On the basis of LIVE/DEAD staining image analysis using ImageJ software (version 1.44), the penetration efficiency (P%) of the functionalized substrata can be quantified by the following equation

\[ P\% = \frac{F_r/F_{rs}}{F_b/F_{bs}} \]

where \( F_r \) and \( F_b \) represent the total bacteria fluorescence intensity in red (PI) and blue (DAPI) channels, respectively. \( F_{rs} \) and \( F_{bs} \) represent the fluorescence intensity per single bacterium in red and blue channels, respectively. The number \( N_c \) of bacteria captured on substrata can be calculated using the following equation: \( N_c = F_c/F_{bs} \).

**Statistical analysis**

In Figs. 1 (G to J) and 2D, a total of 20 bacteria (*Salmonella*, *n* = 72, 94, 106, 75, and 84; *E. coli*, *n* = 124, 183, 128, 176, and 155; *P. aeruginosa*, *n* = 102, 123, 147, 127, and 152; *K. pneumoniae*, *n* = 101, 107, 98, 123, 160, and 134; BLS *E. coli*, *n* = 130, 129, 148, and 141; ND-1–producing *E. coli*, *n* = 124, 100, 106, and 115; listed from left to right in each graph) were used to generate the dot plot. Each box chart in Fig. 2 (O and P) includes the minimum, lower quartile (lower horizontal line), median (middle horizontal line), mean (solid circle), upper quartile (upper horizontal line), and maximum. The number of captured bacteria and penetration efficiency for substrata were displayed as mean proportions ± SD from three independent experiments. Significance was calculated using a two-way analysis of variance (ANOVA) with a Tukey post hoc test using Minitab software. \( P < 0.05 \) was considered statistically significant (*\( P < 0.05 \), **\( P < 0.01 \), and ***\( P < 0.001 \)). Results with \( P > 0.05 \) were considered as not significant.

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/27/eabb9593/DC1

View/request a protocol for this paper from Bio-protocol.

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