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ABSTRACT

The clinically important vancomycin antibiotic inhibits the growth of pathogens such as Staphylococcus aureus by blocking cell wall synthesis through specific recognition of nascent peptidoglycan terminating in D-Ala-D-Ala. Here, we demonstrate the ability of single-molecule atomic force microscopy with antibiotic-modified tips to measure the specific binding forces of vancomycin and to map individual ligands on living bacteria. The single-molecule approach presented here provides new opportunities for understanding the binding mechanisms of antibiotics and for exploring the architecture of bacterial cell walls.

Glycopeptide antibiotics of the vancomycin group are of crucial clinical importance in the treatment of methicillin resistant Staphylococcus aureus. Vancomycin binds with high affinity and specificity to the terminal D-Ala-D-Ala of peptidoglycan precursors, thereby preventing their incorporation into the bacterial cell wall and leading eventually to cell lysis.1–5 Besides being used in the treatment of disease, vancomycin molecules represent an important research tool. In particular, they can be used as a highly sensitive molecular probe to label peptidoglycan, a major component of bacterial cell walls which maintains cell shape and integrity, thereby providing novel insights into the growth and assembly of the cell walls.6 During the past decades, key insights into the structural biology of molecular recognition by vancomycin have been gained using NMR, X-ray crystallography, computational analysis, kinetic and thermodynamic studies, and new semisynthetic derivatives.5 Yet, progress in understanding the molecular forces that control the vancomycin/D-Ala-D-Ala interaction has been hampered by the lack of ultrasensitive force probes.

In the past years, the atomic force microscope (AFM) has emerged has a powerful tool to measure the minute forces within or between single biomolecules.7–14 Besides its piconewton force sensitivity, AFM also offers nanometer positional accuracy, thereby allowing researchers to map the distribution of single recognition sites on cell surfaces. These single-molecule force spectroscopy analyses imply modifying the AFM tip with specific molecules, bringing the tip in contact with the sample and then pulling it away in order to measure the receptor–ligand interaction forces.14 So far, the use of antibiotic-modified tips in molecular recognition studies has never been reported. Here, we have demonstrated the ability of AFM with vancomycin tips to measure the forces and the dynamics of the vancomycin/D-Ala-D-Ala interaction and to image individual D-Ala-D-Ala sites on the division septum of living Lactococcus lactis bacteria. The AFM results were supported by the use of a genetically engineered mutant lacking D-Ala-D-Ala sites and by imaging peptidoglycan insertion sites with fluorescent vancomycin.

We first used single-molecule force spectroscopy to explore the vancomycin/D-Ala-D-Ala binding forces (Figure

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To this end, we coupled vancomycin molecules to gold-coated AFM tips via strong Au—S bonds. Gold supports treated in parallel with the tips were analyzed using both X-ray photoelectron spectroscopy (surface chemical composition) and AFM imaging (surface morphology) to validate the quality of the surface modification. The data (not shown) confirmed the presence of a ∼1 nm thick layer of vancomycin molecules strongly bond to the gold surface. d-Ala-d-Ala peptides were covalently immobilized onto flat supports modified with mixed self-assembled monolayers (SAMs) terminated with oligo(ethylene glycol) (OEG) and OEG propionic acid (Figure 1A). This surface chemistry allows peptides to be uniformly oriented on the surface at low density, thus ensuring single-molecule recognition.

Using this strategy, we recorded force—distance curves between vancomycin tips and d-Ala-d-Ala supports (Figure 1B). A significant fraction of the retraction curves displayed single adhesion forces, the remaining measurements exhibiting no adhesion. The corresponding adhesion force histogram displayed a single maximum with a mean magnitude of 98 ± 33 pN (n = 978). We suggest that the measured binding forces reflect the rupture of single vancomycin/d-Ala-d-Ala complexes for the following reasons. First, supports were modified with a mixed SAM containing only 10% of
propionic acid groups to make sure that a low surface density of peptides is exposed, thus favoring the detection of single monomeric interactions. At lower peptide density, we noted a dramatic reduction of adhesion frequency without any modification of the mean adhesion force, while at larger density much stronger adhesion forces were often detected suggesting multiple interactions were measured (data not shown). Second, when force curves were recorded in a 0.4 mM solution of D-Ala-D-Ala-D-Ala (Figure 1C), a dramatic reduction of adhesion frequency was observed (from 90% to 15%), demonstrating that the measured adhesion forces originate from specific vancomycin/D-Ala-D-Ala interactions.

Theoretical and experimental studies have shown that receptor–ligand binding forces depend on the loading rate, that is, the rate at which the force is applied to the diffusion-controlled association mechanism. Such a slow interaction time while keeping the loading rate (or pulling speed) constant (Figure 1E). Interestingly, we found that the adhesion frequency increased exponentially with contact time to reach a constant value. This time dependency agrees well with earlier kinetic studies, which revealed that the association rate constant of dissociation at zero force: 

\[ k_{\text{off}} = \frac{r_{\text{on}} N_{\text{eff}} T}{k_{\text{B}} T} = 2 \times 10^{-3} \text{s}^{-1}. \]

Besides changing the loading rate, we also varied the interaction time while keeping the loading rate (or pulling speed) constant (Figure 1E). This allowed us to estimate the association rate constant, where \( V_{\text{eff}} \) is the effective volume explored by the tip-tethered vancomycin, approximated here to a half-sphere of 1 nm radius. Considering the above rate constant values, we then estimated the equilibrium dissociation constant: 

\[ K_D = k_{\text{off}} / k_{\text{on}} = 0.4 \text{mM} \]

This is much larger than the value determined for the vancomycin/D-Ala-D-Ala pair in solution, a finding that may reflect the influence of steric restrictions on the recognition process. Indeed, it is likely that the short spacer used here did not provide maximal motional freedom to the attached vancomycin molecules. Nevertheless, the above data indicate that AFM is a valuable tool to assess the rate constants of vancomycin on solid surfaces, which was not possible using SPR owing to mass transport limitations.

Next we used vancomycin tips to map the distribution of single D-Ala-D-Ala ligands on living bacteria, with a lateral resolution of ~20 nm (Figure 2). We chose Lactococcus lactis as a model for Gram-positive cocci in view of its great biotechnological and biomedical relevance and because its cell wall peptidoglycan was recently shown to contain D-Ala-D-Ala termini. Bacteria were immobilized in porous polymer membranes, a method allowing AFM analysis of living cells while preserving their native macromolecular architecture. Topographic images obtained for dividing cells revealed a smooth and elongated cell morphology as well as a well-defined division septum (Figure 2A). Furthermore, we observed ringlike structures at a certain distance from the septum. These annular structures, presumably formed by an outgrowth of the cell wall, are reminiscent of so-called “equatorial rings” in streptococci, which mark the position of the division site, duplicate, and then separate to form the edges of the newly synthesized peptidoglycan zone during cell elongation.

Force curves recorded in the septum region with vancomycin tips showed single unbinding events in 12% of the cases (Figure 2B, 2C). As opposed to D-Ala-D-Ala supports, cells showed elongation forces with rupture lengths of about 10–20 nm reflecting stretching of flexible-cell surface macromolecules (Figure 2C). The adhesion force histogram (n = 1536) revealed a bimodal distribution of adhesion forces with maxima at 83 ± 22 pN and 150 ± 16 pN (Figure 2C). The ~83 pN peak was close to the ~98 pN value obtained for the D-Ala-D-Ala supports, suggesting this peak reflects the detection of single D-Ala-D-Ala sites on the bacterial surface. The slightly smaller value observed on cells may result from a reduction of the actual loading rate ascribed to the higher flexibility of the ligands. The ~150 pN peak may reflect cooperative binding which is well-documented for vancomycin. Clearly, it would be most interesting in future research to explore the dimerization and cooperative interaction of vancomycin, for example, by using different tip surface chemistries, since these phenomena are known to enhance the drug affinity for bacterial cell walls.

Notably, affinity maps demonstrated that binding sites were essentially located in the septum region, and more specifically on the equatorial rings (Figure 2B), suggesting that newly formed peptidoglycan was inserted in these regions. This finding is consistent with recent immunofluorescence microscopy observations on Streptococcus pneumoniae, showing that penicillin-binding proteins that synthesize peptidoglycan are localized at duplicated equatorial rings. We confirmed the detection of D-Ala-D-Ala sites on cell surfaces by constructing a mutant of L. lactis that produces peptidoglycan precursors ending by D-Ala-D-Lac instead of D-Ala-D-Ala. We found that both the affinity maps and force histograms recorded in the septum region showed a dramatic reduction of adhesion frequency (Figures 2D–F), supporting the notion that D-Ala-D-Ala ligands were probed on the wild-type strain.

As a complementary approach to our single-molecule study, we used fluorescence microscopy with a fluorescent vancomycin probe to visualize the sites of peptidoglycan insertion into the entire cell wall of L. lactis (Figure 3). In agreement with AFM, fluorescence staining of the wild-type strain was found around the septum and at the poles of the daughter cells at a later stage of the cell cycle, while no fluorescent labeling was detected for the D-Ala-D-Lac mutant.
strain. Accordingly, the above data show that AFM with vancomycin tips is a complementary approach to fluorescent vancomycin to explore the architecture and assembly process of peptidoglycan during the cell cycle of Gram-positive bacteria. While fluorescence microscopy generates micrometer images allowing the localization of peptidoglycan in the entire cell wall, AFM provides nanoscale affinity maps revealing the distribution of single peptidoglycan molecules on the outermost cell surface.

In summary, our data demonstrate that AFM with antibiotic-modified tips is a valuable tool for exploring the forces and the dynamics of antibiotic-ligand interactions. Particularly,
this single-molecule approach allows researchers to assess association and dissociation rate constants that are not accessible using SPR because of mass transport limitations. In addition, affinity imaging with antibiotic tips is shown to be a complementary approach to fluorescence microscopy for studying the architecture and assembly process of peptidoglycan in Gram-positive bacteria.

**Materials and Methods.** The *L. lactis* wild-type strain used in this study was NZ3900. The D-Ala-D-Lac-producing mutant MD003 was an isogenic derivative of NZ3900 that was obtained by mutating the endogenous D-Ala-D-Ala ligase and expressing the D-Ala-D-Lac-ended peptidoglycan precursor biosynthesis genes from *Lactobacillus plantarum*, an intrinsically resistant Gram-positive bacterium (unpublished data). The *L. lactis* wild type and MD003 mutant strains were cultured at 28 °C in M17 medium (DIFCO) added with glucose 0.5%. For MD003, growth medium was supplemented with 15 mM of D-lactate as a substrate for D-Ala-D-glucose 0.5%. For MD003, growth medium was supplemented with 15 mM of D-lactate as a substrate for D-Ala-D-Ala ligase and expressing the D-Ala-D-Lac-ended peptidoglycan precursor biosynthesis genes from *Lactobacillus plantarum*, an intrinsically resistant Gram-positive bacterium (unpublished data). The *L. lactis* wild type and MD003 mutant strains were cultured at 28 °C in M17 medium (DIFCO) added with glucose 0.5%. For MD003, growth medium was supplemented with 15 mM of D-lactate as a substrate for D-Ala-D-Lac ended peptidoglycan precursor synthesis.

AFM tips and supports were functionalized with vancomycin molecules and D-Ala-D-Ala-D-Ala peptides as follows. AFM cantilevers and silicon wafers (Siltronix, France) were coated using electron beam thermal evaporation with a 5-nm thick Cr layer followed by a 30-nm thick Au layer. Before use, the gold-coated surfaces were cleaned for 5 min by UV/ozone treatment (Jelight Co., Irvine, CA), rinsed with ethanol, and dried with a gentle nitrogen flow. Gold tips were immersed overnight in an aqueous solution containing 0.05 mM bis(vancomycin) cystamide (for details about the synthesis of this compound, see ref 21), rinsed three times with deionized water, and then immediately used. Gold supports were immersed for 36 h in an ethanol solution containing a 20 μM mixture (90:10 mol/mol) of alkanethiols terminated with OEG and OEG propionic acid, respectively (Figure 1A). For synthesis of the former, azido-OEG (Polypure, Oslo, Norway) was reduced with triphenylphosphine and the resulting amine was acylated with 16-(acetylsulfanyl)-hexadecanoic acid11 and the thioester was cleaved with K₂-butoxide in methanol. For synthesis of the latter, azido-OEG was alkylated with r-buty1 acrylate in toluene/ K₂-butoxide, the butyl ester was cleaved with trifluoroacetic acid/dichloromethane, the azide was reduced with zink/acetic acid, the resulting amino group was reacted with the N-succinimidyl ester of 16-(acetylsulfanyl)-hexadecanoic acid, and the thioester was cleaved as above. All compounds were purified on Sephadex LH20 in methanol. After the supports were rinsed with ethanol, sonication was briefly applied to remove alkanethiol aggregates that may be adsorbed. The supports were immersed for 30 min into a PBS solution containing 20 g/l N-hydroxyessuccinimide (NHS) (Aldrich) and 50 g/l 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) (Sigma), rinsed with PBS, incubated with 0.01 mg/l D-Ala-D-Ala-D-Ala (Sigma) in PBS for 3 h, further rinsed, and then immediately used.

AFM contact mode images and force-distance curves were obtained using a Nanoscope IV Multimode AFM (Veeco Metrology Group, Santa Barbara, CA). For cell experiments, bacteria were harvested from exponentially growing cultures (OD₆₀₀nm of 0.3), resuspended in Tris-maleate, and mechanically immobilized onto porous poly-carbonate membranes (Millipore). Measurements were performed either in PBS for D-Ala-D-Ala supports or in Tris-maleate for cells, at room temperature, using triangular-shaped silicon nitride cantilevers (Microlevers, Veeco Metrology Group, Santa Barbara, CA). Blocking experiments were performed with a D-Ala-D-Ala-D-Ala solution (0.1 mg/l in PBS). For force measurements, the maximal applied force was kept at 250 pN to minimize indentation. For D-Ala-D-Ala supports, the loading rate was obtained by multiplying the retraction speed by the cantilever spring constant, since rupture forces did not show significant elongation events. Affinity images were obtained by recording 16 × 16 force-distance curves on areas of given size and calculating the adhesion force for each force curve. To estimate the spring constants of the cantilevers, we measured their geometrical dimensions using scanning electron microscopy as well as their free resonance frequency. Then, the cantilever mechanical properties were adjusted in order to match the calculated frequencies to the measured ones. The determined mechanical properties and the measured geometrical dimensions were then used to calculate the spring constants, which were found to be ~0.011 N/m.

Van-FL staining was carried out on cells from exponentially growing cultures using vancomycin BODIPY FL conjugate (Molecular Probes) mixed with an equal amount of unlabeled vancomycin (SIGMA) and added to the cultures giving a final concentration of 3 μg/mL. After 30 min of incubation at 28 °C, cells were fixed with formaldehyde 1.6% (in PBS), mounted on polylysine coated slides and then visualized by fluorescence microscopy (484 nm set filter). Images were taken and analyzed as previously described.

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