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Oritavancin Binds to Isolated Protoplast Membranes but not Intact Protoplasts of *Staphylococcus aureus*

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Abstract

Solid-state NMR has been used to examine the binding of [^{19}F]oritavancin, a fluorinated analogue of oritavancin, to isolated protoplast membranes and whole-cell sucrose-stabilized protoplasts of *Staphylococcus aureus*, grown in media containing [$1\text{-}^{13}\text{C}$]glycine and L-[$\epsilon\text{-}^{15}\text{N}$]lysine. Rotational-echo double-resonance NMR was used to characterize the binding by estimating internuclear distances from ^{19}F of the oritavancin to ^{13}C and ^{15}N labels of the membrane-associated peptidoglycan, and to the ^{31}P of the phospholipid bilayer of the membrane. In isolated protoplast membranes, both with and without 1M sucrose added to the buffer, the nascent peptidoglycan was extended away from the membrane surface and the oritavancin hydrophobic sidechain was buried deep in the exposed lipid bilayer. However, there was no [^{19}F]oritavancin binding to intact sucrose-stabilized protoplasts, even though the drug bound normally to the cell walls of whole cells of *S. aureus* in the presence of 1M sucrose. As shown by the proximity of peptidoglycan-bridge ^{13}C labels to phosphate ^{31}P , the nascent peptidoglycan of the intact protoplasts was confined to the membrane surface.

Keywords

Bacterial cell wall; glycopeptide antibiotic; rotational-echo double resonance; solid-state NMR; transglycosylase

Introduction

Peptidoglycan is common to all eubacteria, with the exception of mycoplasmas and bacterial L-forms.¹ Peptidoglycan is synthesized from a building block consisting of a disaccharide, a stem, and a bridge. The disaccharide pair, *N*-acetylglucosamine and *N*-acetylmuramic acid, is conserved in most bacteria, but the stem and bridge structures are varied. In *Staphylococcus aureus* a pentapeptide (L-Ala-D-iso-Gln-L-Lys-D-Ala-D-Ala) is the stem, and a pentaglycyl segment, the bridge. Mature peptidoglycan is assembled at the exoface of the bacterial cytoplasmic membrane by the action of transglycosylase and transpeptidase on lipid II and nascent peptidoglycan. Lipid II refers to a single peptidoglycan repeat unit carried by the C₅₅-lipid transporter, pyrophosphoryl-undecaprenol, and nascent peptidoglycan to multiple repeat units carried by the C₅₅-lipid transporter (Figure 1).

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Transglycosylase catalyzes the transfer of nascent peptidoglycan to lipid II by formation of a $\beta(1\rightarrow4)$ glycosidic bond between the *N*-acetylglucosamine of lipid II, and the *N*-acetylmuramic acid of nascent peptidoglycan.¹ The result is an elongation of the nascent peptidoglycan by one repeat unit and the release of C₅₅. The growing nascent peptidoglycan is subsequently incorporated into the surrounding mature peptidoglycan by transpeptidase, which catalyzes the formation of a cross-link, a peptide bond between the terminal amine of the pentaglycyl bridging segment of one stem, and the carbonyl of the 4th amino acid (D-Ala-1) of an adjacent stem (Figure 1). Multiple layers of cross-linked peptidoglycan embedded within the cell walls of *S. aureus*, which are typically 20-30 nm thick, provide protection against fluctuating osmotic pressure but still allow a cellular volume expansion of as much as 400%.²

Numerous antibiotics target peptidoglycan biosynthesis. Vancomycin (Figure 2, left), a glycopeptide antibiotic, is thought to inhibit the transglycosylation step of peptidoglycan biosynthesis by binding to the D-Ala-D-Ala terminus of lipid II and preventing the recycling of C₅₅.³ Vancomycin therapy was once reserved for serious infections caused by multiple-antibiotic resistant pathogens, including methicillin-resistant *S. aureus*. As such infections became wide spread, vancomycin usage became routine, which eventually led to the emergence of vancomycin-resistant enterococcal and staphylococcal strains. Currently there are only a few antimicrobial agents that are effective against such strains, one of which is oritavancin (The Medicines Company, New York), a semi-synthetic disaccharide-modified glycopeptide (Figure 2, right) developed by Eli Lilly.⁴ The tendency of oritavancin to dimerize *in vitro* and to bind to lipid vesicles,^{5,6,7,8} has led to the suggestion that *in vivo*, weak drug binding to the modified lipid II of vancomycin-resistant pathogens is compensated by the formation of drug dimers with hydrophobic-tail membrane anchors.^{5,9} However, such structures have not been observed *in situ* for oritavancin and oritavancin-like glycopeptides complexed to intact whole-cells of *S. aureus*. Instead, the drugs were found in the cell wall bound to mature peptidoglycan as monomers.^{10,11}

We therefore decided to search for oritavancin binding directly at the cytoplasmic membrane of *S. aureus*. Lysostaphin was used to digest the cell wall transforming a *S. aureus* whole cell to a protoplast. Lysostaphin is an endopeptidase secreted by *Staphylococcus simulans* that selectively cleaves the cross-linked pentaglycyl bridge in the cell wall of *S. aureus*.¹² The preparation of *S. aureus* (ATCC 6538P) protoplasts using lysostaphin, and the isolation of protoplast membranes, have been well documented.^{13,14,15,16} The lysostaphin digestion does not affect *S. aureus* metabolic activity as monitored by O₂ uptake and protoplast growth,¹⁷ and the protoplast membrane retains all of the membrane proteins and membrane-associated precursors required for peptidoglycan biosynthesis.¹⁸

In this report, we present the results of solid-state NMR investigations of [¹⁹F]oritavancin binding to whole-cell protoplasts and isolated protoplast membranes of *S. aureus* grown in defined media containing ¹³C and ¹⁵N-labeled amino acids. [¹⁹F]oritavancin has fluorine in place of chlorine as a biphenyl substituent but has the same antimicrobial properties as oritavancin.¹⁹ Rotational-echo double resonance (REDOR) NMR²⁰ was used to determine approximate distances from the fluorine of [¹⁹F]oritavancin to the ¹³C and ¹⁵N labels in the membrane-associated peptidoglycan, and to the ³¹P of phospholipids of intact protoplasts and isolated protoplast membranes. These results suggest an explanation for the discrepancy between *in vitro* model studies and *in situ* whole-cell studies. They also reveal the organization of glycan chains of nascent peptidoglycan at the cytoplasmic membrane, and help to refine the proposed^{11,21,22,23} oritavancin mode of action in *S. aureus*.

Results

Transmission Electron Micrographs

Transmission electron micrograph (TEM) images of *S. aureus* cross-section at various stages of lysostaphin digestion are shown in Figure 3. In the parent form of *S. aureus* (Figure 3A) the cell wall, cross-wall, and cytoplasmic membrane are clearly visible. The intermediate and final stages of the protoplast conversion were prepared by the treatment of *S. aureus* in 5 $\mu\text{g/mL}$ of lysostaphin. The intermediate stage (Figure 3B) shows the partially digested cell-wall fragments exfoliating from the bacterial surface, exposing patches of cytoplasmic membrane. In the final stage of protoplast conversion (Figure 3C), the cell wall is absent from the cytoplasmic membrane.

To ensure the lysostaphin digestion was complete and uniform throughout the sample, protoplasts and protoplast membranes were prepared from *S. aureus* using lysostaphin at a concentration of 75 $\mu\text{g/mL}$. Typically, *S. aureus* conversion to protoplast occurred after 15 minutes of treatment, as monitored by turbidity measurements, but the treatment was allowed to progress for 1 hr. The conversion was uniform as confirmed by the TEM image shown in Figure 4 (left). Occasionally, a hollow membrane of lysed protoplast, marked by an asterisk in Figure 4 (left), and its cellular debris were visible, but these constituted minor components. The protoplast conversion was irreversible; that is, the protoplast did not divide or revert to the parent form when grown in the absence of lysostaphin. The TEM image of protoplast membranes prepared by osmotic rupturing of the cell membrane is shown in Figure 4 (right). Only membrane vesicles with sizes varying from 0.05 μm to 0.8 μm in diameters were visible. The small vesicles are classified as mesosomal. The thickness of a protoplast membrane (plus associated nascent peptidoglycan and bound protein) was approximately 80 $\text{\AA}$.

Drug Binding-Site Occupancy

For whole cells of *S. aureus* grown in 1 L of media and harvested at $\text{OD}_{660 \text{ nm}} = 0.7$, there are approximately 13 μmol of glycopeptide binding sites, based on the D-Ala-D-Ala primary vancomycin-binding capacity determined for whole-cells.¹⁰ Of approximately 20 layers of peptidoglycan in the cell wall of *S. aureus*,²⁴ there is at least one layer anchored to the cytoplasmic membrane by the C₅₅-lipid transporter. Hence, the estimated glycopeptide binding capacity of protoplasts and protoplast membranes after lysostaphin digestion is 0.65 μmol (13/20 μmol). Therefore, the addition of 0.56 μmol of [¹⁹F]oritavancin corresponds to a binding-site occupancy of 86%, less than the total primary glycopeptide-binding capacity.

¹³C{¹⁹F} REDOR of [¹⁹F]oritavancin Complexed to Whole Cells, Protoplasts, and Protoplast Membranes of *S. aureus*

The full-echo S_0 ¹³C-NMR spectrum of whole cells of *S. aureus* grown in media containing [1-¹³C]glycine is compared to that of its various components in Figure 5. The individual spectra have been scaled by the fraction of whole-cell mass (excluding buffer) represented by each component. Based on the 171-ppm peak intensities, which are due primarily to incorporation of [1-¹³C]glycine, about 50% of the whole-cell glycine label appears in the cell walls.²⁵ The sum of the cell-wall and protoplast 171-ppm peak intensities equals the whole-cell peak intensity. The isolated protoplast membrane 171-ppm peak intensity arises from nascent peptidoglycan and attached proteins. Some of the differences between protoplast and protoplast membrane S_0 spectra are due to the buffers. In the protoplast spectrum, peaks from 1M sucrose in the hypertonic buffer appear at 71 and 91 ppm, and in the membrane spectrum, peaks from the 0.05 M TRIS in the hypotonic buffer appear at 58 and 68 ppm.

Despite the similarities in chemical, lipid, and nascent peptidoglycan composition of intact protoplasts and isolated protoplast membranes, [¹⁹F]oritavancin binding is completely

different. There is no $^{13}\text{C}\{^{19}\text{F}\}$ REDOR difference peak for the protoplasts, whereas numerous ^{13}C - ^{19}F contacts are observed for the isolated protoplast membranes (Figure 6). Chemical shifts of the REDOR difference peaks give clues to the nature of some of these C-F contacts. The relatively weak dephasing (Figure 7, top right) shows that $[^{19}\text{F}]$ oritavancin is not proximate to nascent peptidoglycan bridges. The 174-ppm REDOR difference is assigned to $[1\text{-}^{13}\text{C}]$ glycine labels in peptidoglycan stem debris on the membrane surface (see Figure 4, right). This assignment is consistent with a small fraction of $[^{19}\text{F}]$ oritavancin in contact with and perhaps bound to peptidoglycan fragments. The 160-ppm ΔS is assigned to natural-abundance carbonyl carbons of TRIS amine nitrogens carbamylated by atmospheric CO_2 .²⁶

The natural-abundance lipid-carbon REDOR difference peaks at 34, 15, and 10 ppm (Figure 8, top right) show proximity of a significant fraction of the $[^{19}\text{F}]$ oritavancin hydrophobic tails to the middle of the isolated protoplast membrane bilayer. The lipid CH_2 carbon signal at 30 ppm is not prominent in either the full-echo or REDOR-difference spectra, probably because of a short echo-train lifetime.²⁷ The fatty-acid compositional analysis of *S. aureus* protoplast membranes by capillary column gas chromatography¹⁶ has shown that *iso* and *anteiso* branched methyl C_{15} , C_{17} , and C_{19} fatty acids constitute over 85% of the lipids. We assign the 34-ppm peak to the CH carbon of the *anteiso*-branched lipids, the 15-ppm peak to the methyl carbons of the *normal*-branched lipids, and the 10-ppm peak to the methyl carbons of the *anteiso*-branched lipids. These assignments are consistent with the ^{13}C -chemical shifts of fatty-acid alkyl carbons observed in solution-state NMR.²⁸

The high concentration of sucrose was not directly responsible for exclusion of $[^{19}\text{F}]$ oritavancin from the surface of the intact protoplasts. Normal drug binding²⁰ was observed for whole cells of *S. aureus* (labeled by $[1\text{-}^{13}\text{C}]$ glycine) in the presence of 1M sucrose (Figure 9).

$^{15}\text{N}\{^{19}\text{F}\}$ and $^{15}\text{N}\{^{31}\text{P}\}$ REDOR of $[^{19}\text{F}]$ oritavancin Complexed to Isolated Protoplast Membranes

Figure 10 (left) shows the $^{15}\text{N}\{^{19}\text{F}\}$ REDOR spectra of $[^{19}\text{F}]$ oritavancin complexed to protoplast membranes of *S. aureus* labeled with $[1\text{-}^{13}\text{C}]$ glycine and L- $[\epsilon\text{-}^{15}\text{N}]$ lysine. The Gly-5-Lys bridge-link of membrane-attached peptidoglycan appears as an $\epsilon\text{-}^{15}\text{N}$ amide peak at 93 ppm. Park's Nucleotide is presumably removed during membrane isolation so that only peptidoglycan stems with no bridges and the L- $[\epsilon\text{-}^{15}\text{N}]$ lysine in membrane proteins contribute to the $\epsilon\text{-}^{15}\text{N}$ amine peak at 10 ppm. This conclusion is consistent with the loss of cytoplasmic content for the protoplast membranes shown in Figure 4, and the absence of ^{13}C sugar peaks shown in Figure 6 (right). The intensity of the amine peak is approximately 3-4 times greater than that of the $\epsilon\text{-}^{15}\text{N}$ amide peak. In mature cell walls of *S. aureus*, stems without bridges constitute only 15% of all stems.¹⁰ Thus, the observed $\epsilon\text{-}^{15}\text{N}$ amine-nitrogen peak at 10 ppm is mainly from lysine label in membrane proteins. There is no observable $^{15}\text{N}\{^{19}\text{F}\}$ dephasing of either $\epsilon\text{-}^{15}\text{N}$ lysyl amide or amine peaks (Figure 10, top left), consistent with fluorine near the middle of the bilayer. The $^{15}\text{N}\{^{31}\text{P}\}$ REDOR dephasing of the $\epsilon\text{-}^{15}\text{N}$ amine peak at 10 ppm (Figure 10, right) is about 10%, which shows spatial proximity of membrane protein lysyl sidechains and the phosphates of headgroups and possibly lipoteichoic acid at the membrane surface.

$^{31}\text{P}\{^{19}\text{F}\}$ REDOR of $[^{19}\text{F}]$ oritavancin Complexed to Isolated Intact Protoplasts

The $^{31}\text{P}\{^{19}\text{F}\}$ REDOR spectra of $[^{19}\text{F}]$ oritavancin complexed with protoplasts labeled with $[1\text{-}^{13}\text{C}]$ glycine and L- $[\epsilon\text{-}^{15}\text{N}]$ lysine are shown in Figure 11 on the left, and the sum¹⁰ of the centerband and all the spinning sidebands on the right. The weak dephasing (0.27%) is attributed to fluorine in adventitious contact with ^{31}P of polyphosphate groups in lipoteichoic

acids. Cell-wall teichoic acids are presumed to have been removed along with the mature cell walls in proportion to the extent of mature cell-wall digestion by lysostaphin.

$^{13}\text{C}\{^{31}\text{P}\}$ REDOR of Intact Protoplasts and Protoplast Membranes

The $^{13}\text{C}\{^{31}\text{P}\}$ REDOR dephasing ($\Delta S/S_0$) of the 171-ppm glycyl carbonyl-carbon peak is 12% for protoplasts (Figure 12, left) labeled by $[1-^{13}\text{C}]$ glycine, but only 4% for the similarly labeled protoplast membranes (Figure 12, center). The dephasing of the natural-abundance carbon peaks (71 and 91 ppm) of sucrose in the protoplast hypertonic buffer is about 30%. This level of dephasing for the intact protoplasts is consistent with nascent peptidoglycan, lipid II, and sucrose in proximity to the lipid head groups of the membrane bilayer. Estimated ^{13}C - ^{31}P distances for the glycyl-carbonyl carbons and sucrose to the ^{31}P of the headgroups are on the order of 6 Å. By contrast, most of the nascent peptidoglycan and lipid II of the isolated protoplast membranes are necessarily more distant from the membrane surface. This situation is not changed by the presence of a high sucrose concentration (Figure 12, right) although the signal-to-noise ratio is substantially reduced by displacement of peptidoglycan in the rotor by sucrose.

Discussion

$[^{19}\text{F}]$ oritavancin Binding

Addition of $[^{19}\text{F}]$ oritavancin to protoplasts did not result in the formation of complexes to nascent peptidoglycan or lipid II (Figure 6, top left). A similar observation has been made by Perkins and Nieto, who reported that 98 to 99% of vancomycin added to *M. lysodeikticus* protoplasts was found unbound in the sucrose-rich supernatant.²⁹ However, the high concentration of sucrose that was essential for protecting the protoplasts did not directly interfere with complex formation because normal oritavancin binding to the cell walls of whole cells was observed in the presence of 1M sucrose (Figure 9). In fact, we believe that the sucrose likely served as a lyoprotectant,³⁰ preserving the local structure of the protoplast surface.

In isolated protoplast membranes, $[^{19}\text{F}]$ oritavancin was found primarily partitioned to the cytoplasmic membrane, resulting in the $^{13}\text{C}\{^{19}\text{F}\}$ REDOR dephasing of the natural-abundance lipid peaks at 34, 15, and 10 ppm (Figure 8, top right). The fluorine of $[^{19}\text{F}]$ oritavancin is therefore in contact with the membrane lipid tails. If the fluorine is near the middle of lipid bilayer, then the hydrophobic sidechain and portions of the $[^{19}\text{F}]$ oritavancin aglycon are deeply embedded within the lipid bilayer. A schematic representation of the $[^{19}\text{F}]$ oritavancin-membrane complex in isolated protoplast membranes consistent with the REDOR results is shown in Figure 13 (bottom right). The hydrophobic tail can be described as anchoring the aglycon D-Ala-D-Ala binding cleft at the membrane surface, just as in model systems in synthetic bilayers.⁵⁻⁸

Nascent Peptidoglycan Organization at the Cytoplasmic Membrane

The $^{13}\text{C}\{^{31}\text{P}\}$ dephasing of the 171-ppm peak is 12% for protoplasts and only 4% for isolated protoplast membranes (Figure 12). This indicates a compact nascent-peptidoglycan organization in protoplasts at the bacterial cytoplasmic membrane (Figure 13, bottom left). Figure 13 (top) illustrates possible nascent-peptidoglycan organization in protoplasts (left) and isolated protoplast membranes (right) consistent with the REDOR results. The gray circle represents the lipid bilayer of the cytoplasmic membrane. The black line represents the disaccharide of a repeating peptidoglycan unit; the green line shows the stem structure; and the red line represents the bridge structure. Equal numbers of lipid II and nascent peptidoglycan are bound to the membranes of both intact protoplasts and isolated protoplast membranes, but not enough for full surface coverage in either case. For protoplasts, the growing ends of nascent peptidoglycan are shown in a layer parallel to the membrane.³¹ In this arrangement, all the

[1-¹³C]glycines in the bridges contribute to the ¹³C{³¹P} REDOR dephasing. For isolated membranes on the other hand, the ends of the nascent peptidoglycan are shown as strands extended away from the membrane surface, and therefore away from the phosphates of lipid headgroups and lipoteichoic acid. In this situation, the ¹³C{³¹P} REDOR dephasing contribution arises from only a few [1-¹³C]glycines in peptidoglycan bridges.

If nascent peptidoglycan in whole cells were extended perpendicular to the membrane surface³² as depicted in the strand model, collapse (without aggregation) on protoplast formation to a uniform coverage of the membrane surface so that all ¹³C labels were proximate to phosphate ³¹P seems implausible. In fact, high concentrations of sucrose and lyophilization do not cause collapse of nascent peptidoglycan to the surface of isolated protoplast membranes (Figure 12, right). A parallel-layer shell model for the nascent peptidoglycan of *S. aureus* therefore seems likely, a conclusion consistent with recent electron cryotomographic images of the peptidoglycan of *E. coli*³³ which also support a shell model.

Mode of Action of [¹⁹F]oritavancin

The addition of [¹⁹F]oritavancin to whole-cells of *S. aureus* does not result in the detection of a membrane anchor,¹⁰ which suggests more favorable [¹⁹F]oritavancin binding to mature peptidoglycan than to the membrane. We have attributed the selective binding of [¹⁹F]oritavancin to mature peptidoglycan in part to the presence of a secondary binding site, the cleft formed between the aglycon structure and the hydrophobic sidechain.¹¹ Recently, such a secondary binding site has been confirmed for the Edman degradation products, des-*N*-methylleucyl-[¹⁹F]oritavancin²³ and des-*N*-methylleucyl-4-fluorophenylbenzyl-vancomycin.²² Each of these disaccharide-modified glycopeptides has a damaged D-Ala-D-Ala binding pocket. Nevertheless, they not only bind to mature peptidoglycan in whole-cells of *S. aureus*^{22,23} but also retain their antimicrobial activities. In contrast, the Edman degradation product of vancomycin does not bind and has no antimicrobial activity.

We believe that the bridge in mature peptidoglycan of *S. aureus*, which has a specific conformation and orientation with respect to the peptidoglycan stem due to lattice constraints, is the preferred substrate for the secondary binding site in [¹⁹F]oritavancin. Nascent peptidoglycan is only partially cross-linked and therefore only partially constrained and ordered for binding. We believe that the nearest mature peptidoglycan layer serves as a guide or template for the alignment and orientation of stems and bridges. Contrary to the prevalent view that glycopeptide binding to mature peptidoglycan is unproductive,³⁴ we propose that [¹⁹F]oritavancin binding to mature peptidoglycan is in fact essential for its mode of action. By targeting the template peptidoglycan, [¹⁹F]oritavancin interferes with transpeptidase activity in *S. aureus*²³ and the accurate copying of the template.²¹ Once the template is destroyed, a bacterium cannot resume normal biosynthesis of peptidoglycan any more than a protoplast can revert and divide.³⁵

Materials and Methods

Growth and Labeling of *S. aureus* Protoplasts

A starter culture of *S. aureus* (ATCC 6538P) was prepared by inoculating colonies to test tubes containing 5 ml of trypticase soy broth and grown overnight at 37 °C shaken at 200 rpm in a Environ-Shaker (Lab-Lines Instruments, Inc., Melrose Park, IL). The overnight starter culture was added (1% final volume) to two one-liter flasks each containing 500 mL of defined media containing ¹³C and ¹⁵N isotope-labeled amino acids, [1-¹³C]Gly and L-[ε-¹⁵N]Lys.²⁵ The cells were harvested at mid-exponential phase (OD_{660 nm} = 0.66) by centrifugation at 10,000 g for 5 min at 4 °C in a Sorvall GS-3 rotor. Cells were washed once in hypotonic buffer (0.05 M TRIS pH 7.5), resuspended in 40 ml of hypertonic buffer (1 M sucrose in 0.05 M TRIS pH

7.5) and were equilibrated on ice for 20 min with gentle stirring. The conversion to protoplasts was initiated by the addition of lysostaphin (75 $\mu\text{g}/\text{ml}$) and bovine pancreatic DNase (25 $\mu\text{g}/\text{ml}$). The conversion progress was monitored by detection of the turbidity of lysostaphin-charged *S. aureus* suspension (10 μL) added to a 1 ml of hypotonic buffer. The protoplasts were harvested after an hour of digestion by centrifugation at 10,000 g for 20 mins, washed, and suspension in 10 ml of hypertonic buffer containing 1 mg of [^{19}F]oritavancin (LY329332). [^{19}F]oritavancin was a gift from Eli Lilly. [^{19}F]oritavancin-protoplast mixture was incubated on ice for 10 min followed by centrifugation at 10,000 g for 20 min. Excess sucrose in hypertonic buffer was removed and the pellet containing [^{19}F]oritavancin-protoplast complexes was lyophilized.

Protoplast Membrane Isolation

Protoplasts were osmotically lysed by suspending in 60 ml of hypotonic buffer and stirring on ice for 20 mins. DNase and RNase were added to the suspension to final concentration of 17 $\mu\text{g}/\text{ml}$ each, and the mixture was incubated in 37 $^{\circ}\text{C}$ water bath with slight agitation for 20 mins. The cellular debris was removed by centrifugation at 2,000 g for 10 mins at 4 $^{\circ}\text{C}$, and the supernatant containing isolated protoplast membrane was centrifuged at 35,000 g (Sorvall SS34) for 25 mins at 4 $^{\circ}\text{C}$ yielding a soft pellet. Following five wash in hypotonic buffer, the protoplast membrane was suspended in 10 ml of hypotonic buffer containing 1 mg of [^{19}F]oritavancin. The mixture was incubated on ice for 10 min followed by lyophilization.

Transmission Electron Micrographs

The WCP and IPM cultures were fixed by adding 25% glutaraldehyde to the media to yield a glutaraldehyde concentration of 4%, fixed for 1.5 hr at room temperature. After several water washes, samples were postfixed with aqueous 1% OsO_4 for 2 hr, water washed, and treated with aqueous 1% uranyl acetate for 1 hr. Following several water washes, the samples were dehydrated in ascending concentrations of ethyl alcohol to three changes of 100%, then three changes of propylene oxide, and infiltrated with Polybed 812 epoxy embedding resin. Specimen blocks were polymerized at 60 $^{\circ}\text{C}$ in a vacuum oven. Thin sections were cut using a diamond knife, poststained with 2.5% uranyl acetate and lead citrate, and examined in a Hitachi H-600 TEM operated at 75 kV accelerating voltage. Images were recorded on Kodak 4489 electron microscope film.

Rotational-Echo Double Resonance

Rotational-echo double resonance (REDOR) is a solid-state NMR method used to recouple dipolar interactions under magic-angle spinning (MAS) to determine heteronuclear dipolar couplings and hence internuclear distances.³⁶ The REDOR experiment consists of two parts. In the first part of a $^{13}\text{C}\{^{19}\text{F}\}$ REDOR experiment, a polarization transfer from the protons prepares the ^{13}C magnetization and the spectrum is collected after N rotor periods. Rotor-synchronized π pulses on the ^{13}C channel refocus the isotropic chemical shift. There is no ^{13}C - ^{19}F dipolar evolution over the rotor period due to MAS and thus no net dipolar phase is accumulated. The observed ^{13}C spectrum is referred to as the full echo (S_0) spectrum and is used as an intensity reference. In the second part of the REDOR experiment, the dipolar coupling is reintroduced by applying rotor-synchronized dephasing π pulses to the ^{19}F nuclei in addition to the ^{13}C pulses. The ^{19}F pulses invert the sign of the dipolar coupling and interfere with the MAS spatial averaging, resulting in a net dipolar phase accumulation. The ^{13}C spectrum with the dephasing pulses is referred to as the dephased (S) spectrum. Then intensity of the difference spectrum ($\Delta S = S_0 - S$) arises only from the ^{13}C that are dipolar coupled to ^{19}F . The difference spectra are typically collected as a function of N rotor periods to map out the ^{13}C - ^{19}F dipolar evolution.

REDOR was performed at a static magnetic field of 12 T (proton radio frequency of 500 MHz) provided by 89-mm bore Magnex (Oxfordshire, England) superconducting solenoid. A six-frequency transmission-line probe was used with a 12-mm long, 6-mm inside diameter analytical coil and a Chemagnetic/Varian ceramic spinning module. A lyophilized whole-cell sample was spun in a thin wall Chemagnetics/Varian (Fort Collins, CO/Palo Alto, CA) 5-mm outside diameter zirconia rotor. Magic-angle spinning was at 7143 Hz with the speed under active control within ± 2 Hz. A Tecmag Libra pulse programmer (Houston, TX) controlled the spectrometer.

Radio-frequency pulses for ^{31}P (202 MHz), ^{13}C (125 MHz), and ^{15}N (50.7 MHz) were produced by 1 and 2-kW American Microwave Technology power amplifiers. ^1H (500 MHz) and ^{19}F (470 MHz) radio-frequency pulses were generated by 2-kW Creative Electronics tube amplifiers driven by 50-W American Microwave Technology power amplifiers. The π -pulse lengths were 8 μs for ^{31}P , 10 μs for ^{13}C and ^{15}N , and 5 μs for ^{19}F . Proton-carbon cross-polarization transfers were made in 2 ms at 62.5 kHz. Proton dipolar decoupling was 100 kHz during data acquisition, and TPPM³⁷ of the ^1H radio frequency was used throughout both dipolar evolution and decoupling periods. Standard XY-8 phase cycling³⁸ was used for all refocusing and dephasing pulses. The re-cycle delay period was 2 seconds during which each amplifier produced a 300- μsec test pulse. The resulting diode-detected voltages were compared to a reference voltage previously calibrated. The resulting differences were used to correct the drives of the power amplifiers for the next repetition of the REDOR pulse sequence. Combination of the active control of the amplifiers³⁹ and the alternate-scan data acquisition for each pair of REDOR spectra (S and S_0) eliminated long-term drifts in the performance of the spectrometer.

Calculated REDOR Dephasing

The normalized REDOR difference ($\Delta S/S_0$) is a direct measure of dipolar coupling. This quantity was calculated using the modified Bessel function expressions given by Mueller et al.⁴⁰ and de la Caillerie and Fretigny⁴¹ for an IS spin- $1/2$ pair.

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Abbreviations

C₅₅

pyrophosphoryl-undecaprenol

[^{19}F]oritavancin

N'-4-[(4-fluorophenyl)benzyl]chloroeremomycin

lipid II

N-acetylglucosamine-*N*-acetyl-muramyl-pentapeptide-pyrophosphoryl-undecaprenol

MAS	magic-angle spinning
REDOR	rotational-echo double resonance
TEM	transmission electron microscopy
TRIS	tris(hydroxymethyl)aminomethane

Figure 1. Chemical structure of *Staphylococcus aureus* peptidoglycan at the cytoplasmic membrane. Lipid transporter C55 is highlighted in yellow, and a single peptidoglycan precursor repeat unit, in gray. Each repeat unit consists of a disaccharide, a stem (L-Ala-D-iso-Gln-L-Lys-D-Ala-D-Ala), and a bridge. Nascent peptidoglycan has multiple repeat units and is connected to the C55 transporter.

Figure 2.
Chemical structures of vancomycin and oritavancin.

Figure 3.

Transmission electron micrographs of cross-sections of *S. aureus* at various stages of lysostaphin digestion. (A) Parent form. (B) Intermediate stage of protoplast conversion prepared by treatment of *S. aureus* with 5 µg/mL lysostaphin. (C) Protoplast prepared by the treatment of *S. aureus* with 75 µg/mL lysostaphin.

Figure 4.

Transmission electron micrographs of whole-cell protoplasts and isolated protoplast membranes prepared from *S. aureus*. (Left) protoplasts prepared by incubating *S. aureus* in 75 $\mu\text{g/mL}$ of lysostaphin for one hour. The protoplast conversion was uniform with no visible parent forms. A lysed protoplast with a hollow membrane shell is marked by an asterisk. (Right) isolated protoplast membranes prepared from whole-cell protoplasts. Only membrane vesicles varying in size with diameters ranging from 0.05-0.8 μm are visible. Mesosomal vesicles have diameters less than 0.1 μm .

Figure 5. 125-MHz full-echo ^{13}C NMR spectra of whole cells and various components of whole cells of *S. aureus* labeled by $[1-^{13}\text{C}]$ glycine. The individual spectra have been scaled by the fraction of whole-cell sample mass (excluding estimated buffer) represented by each component. Magic-angle spinning for the spectra at the top of the figure was at 6250 Hz, and at the bottom of the figure, 7143 Hz.

Figure 6.

125-MHz $^{13}C\{^{19}F\}$ REDOR spectra after 8.96 msec of dipolar evolution of [^{19}F]oritavancin complexed to intact protoplasts (left) and isolated protoplast membranes (right) prepared from *S. aureus* labeled by [$1-^{13}C$]glycine. The full-echo spectra (S_0) are at the bottom of the figure, and the REDOR-difference spectra ($\Delta S = S_0 - S$, where S is the dephased-echo spectrum), are shown at the top. The S_0 spectra were normalized for equal intensity of the 171-ppm peak. The spectra on the left were the result of the accumulation of 38,948 scans, and the spectra on the right, 21,112 scans. Magic-angle spinning was at 7143 Hz.

Figure 7.
An enlargement of the carbonyl-carbon regions shown in Figure 6.

Figure 8.
An enlargement of the lipid-carbon regions shown in Figure 6.

Figure 9.

125-MHz $^{13}\text{C}\{^{19}\text{F}\}$ REDOR spectra after 8.96 msec of dipolar evolution of $[^{19}\text{F}]$ oritavancin complexed to whole cells of *S. aureus* in the presence of 1M sucrose and labeled by $[1-^{13}\text{C}]$ glycine. Full-echo spectra (S_0) are at the bottom and middle of the figure, and the REDOR-difference spectrum ($\Delta S = S_0 - S$, where S is the dephased-echo spectrum), is shown at the top. The S_0 spectra were normalized for equal intensity of the 171-ppm peak. After about three weeks, some of the sucrose glass (bottom) crystallized, resulting in sharper sucrose peaks (middle). The 2.2% REDOR difference was unaffected. The spectra were each the result of the accumulation of 37,664 scans. Magic-angle spinning was at 7143 Hz.

Figure 10.

50.7-MHz $^{15}\text{N}\{^{19}\text{F}\}$ REDOR spectra (left) and $^{15}\text{N}\{^{31}\text{P}\}$ REDOR spectra (right) spectra after dipolar evolution times of 8.96 msec of [^{19}F]oritavancin complexed to isolated protoplast membranes of *S. aureus* labeled by [$1\text{-}^{13}\text{C}$]glycine and L- $[\epsilon\text{-}^{15}\text{N}]$ lysine. The full-echo spectra (S_0) are at the bottom of the figure, and the REDOR-difference spectra (ΔS) are at the top. The $\epsilon\text{-}^{15}\text{N}$ amide peak of the pentaglycyl-lysine bridge-link appears at 93 ppm, and the $\epsilon\text{-}^{15}\text{N}$ amine peak at 10 ppm. The $^{15}\text{N}\{^{19}\text{F}\}$ REDOR spectra are the result of the accumulation of 29,836 scans, and the $^{15}\text{N}\{^{31}\text{P}\}$ REDOR spectra of 18,456 scans. Magic-angle spinning was at 7143 Hz.

Figure 11.

(Left) 202-MHz $^{31}\text{P}\{^{19}\text{F}\}$ REDOR spectra after 17.9 msec of dipolar evolution of $[^{19}\text{F}]$ oritavancin complexed to intact protoplasts prepared from *S. aureus* labeled by $[1\text{-}^{13}\text{C}]\text{glycine}$. The full-echo spectrum is at the bottom of the figure, and the REDOR-difference spectrum at the top. (Right) Fourier transforms of the rotor-echo maxima of the time-domain signals associated with the spectra on the left. The resulting spectra are the quantitative sums of the centerband and all spinning sidebands of the spectra shown on the left of the figure. The observed total dephasing ($\Delta S/S_0$) is 0.27%. The spectra are the result of the accumulation of 309,704 scans. Magic-angle spinning was at 7143 Hz.

Figure 12.

125-MHz $^{13}\text{C}\{^{31}\text{P}\}$ REDOR spectra after a dipolar evolution time of 8.96 msec of intact protoplasts (left), and isolated protoplast membranes either without (middle) or with (right) 1 M sucrose added to the buffer, of *S. aureus* labeled by $[1-^{13}\text{C}]\text{glycine}$. The S_0 spectra are at the bottom of the figure, and the ΔS spectra are at the top. The S_0 spectra were normalized for equal intensity of the 171-ppm peak. The spectra on the left (WCP) were the result of the accumulation of 15,200 scans, those in the middle, of 16,228 scans, and those on the right of 62,368 scans. Magic-angle spinning was at 7143 Hz.

Figure 13.

Illustrations of possible nascent peptidoglycan organization in intact protoplasts (left) and isolated protoplast membranes (right) consistent with the REDOR results. (Top) The grey circle represents the lipid bilayer of the cytoplasmic membrane. The black line represents the disaccharide of a peptidoglycan repeat unit; the green line, the stem structure; the red line, the bridge structure. Equal quantities of lipid II and nascent peptidoglycan are shown in both intact protoplasts and isolated protoplast membranes. The nascent peptidoglycan strands are parallel to the membrane surface in intact protoplasts and perpendicular in isolated protoplast membranes. Close-up views of the nascent peptidoglycan organization are shown in the bottom panels of the figure. Color coding of the peptidoglycan units are the same in all panels. In the

isolated protoplast membranes, [^{19}F]oritavancin (blue) is shown with its hydrophobic sidechain embedded within the membrane and the fluorine positioned near the middle of the lipid bilayer.