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REDOR constraints on the peptidoglycan lattice architecture of *Staphylococcus aureus* and its FemA mutant ☆

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ABSTRACT

The peptidoglycan of Gram-positive bacteria consists of glycan chains with attached short peptide stems cross-linked to one another by glycyl bridges. The bridge of *Staphylococcus aureus* has five glycyl units and that of its FemA mutant has one. These long- and short-bridge cross-links create totally different cell-wall architectures. *S. aureus* and its FemA mutant grown in the presence of an alanine-racemase inhibitor were labeled with D-[1-^{13}C]alanine, L-[3-^{13}C]alanine, [2-^{13}C]glycine, and L-[5-^{19}F]lysine to characterize some details of the peptidoglycan tertiary structure. Rotational-echo double-resonance (REDOR) NMR of isolated cell walls was used to measure internuclear distances between ^{13}C-labeled alanines and ^{19}F-labeled lysine incorporated in the peptidoglycan. The alanyl ^{13}C labels in the parent strain were preselected for C{F} and C{P} REDOR measurement by their proximity to the glycine label using ^{13}C–^{13}C spin diffusion. The observed ^{13}C–^{13}C and ^{13}C–^{31}P distances are consistent with a tightly packed architecture containing only parallel stems in a repeating structural motif within the peptidoglycan. Dante selection of D-alanine and L-alanine frequencies followed by ^{13}C–^{13}C spin diffusion rules out scrambling of carbon labels. Cell walls of FemA were also labeled by a combination of D-[1-^{13}C]alanine and L-[^{15}N]alanine. Proximity of chains was measured by C{N} and N{C} REDOR distances and asymptotic plateaus, and both were consistent with a mixed-geometry model. Binding of an ^{19}F-labeled eremomycin analog in the FemA cell wall matches that of binding to the parent-strain cell wall and reveals the proximity of parallel stems in the alternating parallel–perpendicular mixed-geometry model for the FemA peptidoglycan lattice. This article is part of a Special Issue entitled: NMR Spectroscopy for Atomistic Views of Biomembranes and Cell Surfaces. Guest Editors: Lynette Cegelski and David P. Weliky.

1. Introduction

We recently described the rotational-echo double-resonance analysis of the peptidoglycan of the cell walls of the FemA mutant of *Staphylococcus aureus* into which four specific labels were incorporated (Fig. 1). Distances from the ^{19}F label to the ^{13}C labels (preselected by carbon-carbon spin diffusion) were interpreted by a model in which a peptidoglycan structural motif was a hybrid of tightly packed alternating parallel and perpendicular stems [1]. The preselection was restricted to proximity to the glycyl-carbon label (see Fig. 1). In this report we describe measurements in which the preselection site is moved to the D-alanyl and L-alanyl carbon-label sites. In addition, we contrast C{F} and C{P} REDOR results for short-bridge FemA with those for its long-bridge parent strain (which has an all-parallel-stem lattice) with respect to inter-stem proximities, location of wall teichoic acid, and preferred binding sites for a fluorine-labeled eremomycin glycopeptide antibiotic [2]. These comparisons add detail to the lattice architecture of *S. aureus* which should be useful for future modeling.

2. Materials and methods

2.1. Growth and labeling of FemA whole cells

Growth conditions were described before [3]. The same method was used for both the parent strain of *S. aureus* (BB255) and its FemA mutant (UK17). To prevent the scrambling of L-[3-^{13}C]alanine and D-[1-^{13}C]alanine through alanine racemase, alaphosphin (L-alanyl-L-1-aminoethylphosphonic acid), an alanine-racemase inhibitor was added

Abbreviations: ^{13}C{^{19}F} or C{F}, carbon-channel observation with fluorine dephasing; ^{13}C{^{31}P} or C{P}, carbon-channel observation with phosphorus dephasing; ^{13}C{^{15}N} or C{N}, carbon-channel observation with nitrogen dephasing; ^{15}N{^{13}C} or N{C}, nitrogen-channel observation with carbon dephasing; NAG, *N*-acetylglucosamine; NAM, *N*-acetylmuramic acid; REDOR, rotational-echo double resonance

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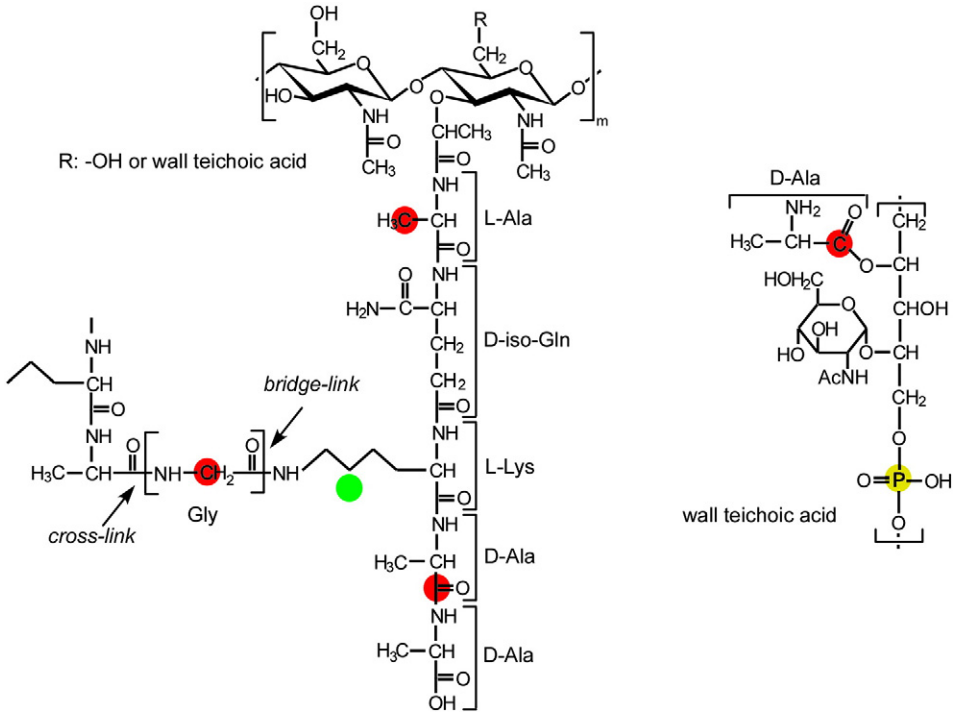

Fig. 1. Location of the labels (red, ^{13}C; green, ^{19}F) of FemA peptidoglycan, and (red, ^{13}C; yellow, ^{31}P) of the repeating unit of wall teichoic acid.

to a final concentration of 10 μg/ml in two steps [3]. The FemA and parent-strain whole cells were harvested after 6 h of growth by centrifugation at 8000 *g* for 10 min at 4 °C in a Sorvall GS-3 rotor. The cells were washed twice in 50 mL of ice-cold 40 mM triethanolamine buffer, pH 7.0, and then resuspended in 10 mL of 40 mM triethanolamine buffer and lyophilized. Cell walls were isolated as described in detail previously [3].

2.2. Dipolar recoupling

REDOR is a solid-state NMR method that recouples heteronuclear dipolar interactions under magic-angle spinning [4] and so can be used to determine inter-nuclear distances. REDOR is a difference experiment in which two spectra are collected, one in the absence of heteronuclear dipolar coupling (full echo, S_0 spectrum), and the other in the presence of the coupling (dephased echo, S spectrum). In the S_0 spectrum, dipolar dephasing is refocused due to spatial averaging resulting from motion of the rotor in magic-angle spinning. In the S spectrum, the spin part of the dipolar interaction is manipulated by the application of rotor-synchronized dephasing π-pulses to prevent full refocusing. The extent of the dephasing is related to the spin-pair dipolar coupling and hence the internuclear separation [4,5].

2.3. Solid-state NMR spectrometer and REDOR Pulse Sequence

Experiments were performed at 12 T with a six-frequency transmission-line probe having a 12-mm long, 6-mm inner-diameter analytical coil, and a Chemagnetics/Varian ceramic spinning module. Samples were spun using a thin-wall Chemagnetics/Varian (Fort Collins, CO/Palo Alto, CA) 5-mm outer diameter-zirconia rotor at 7143 Hz, with the speed under active control and maintained to within ±2 Hz. A Tecmag Libra pulse programmer (Houston, TX) controlled the spectrometer. A 2-kW American Microwave Technology (AMT) power amplifier was used to produce radio-frequency pulses for ^{13}C (125 MHz), and a 1-kW AMT amplifier for ^{31}P (202 MHz). The ^{1}H (500 MHz) and ^{19}F (470 MHz) radio-frequency pulses were generated by a 2-kW Creative Electronics tube amplifiers driven by 50-W AMT amplifiers. All final-stage amplifiers were under active control [6]. The π-pulse lengths were 9 μs for ^{13}C and ^{1}H, 6 μs for ^{31}P, and 5 μs for ^{19}F. Proton-carbon-matched cross-polarization transfers were made in 2 ms at 56 kHz. Proton dipolar decoupling was 100 kHz during data acquisition. The S and S_0 alternate-scan strategy compensated for short-term drifts in REDOR experiments. Standard XY-8 phase cycling [7] was used for all refocusing observe-channel π pulses (inserted at the end of each rotor period during dipolar evolution) and dephasing π pulses (inserted in the middle of each rotor period) to compensate for pulse imperfections. Frequency-specific ^{13}C chemical shifts were selected prior to $^{13}C\{^{19}F\}$ REDOR experiments using rotor-asynchronous Dante irradiation, z-axis storage, and mixing times between 200 and 400 ms with no ^{1}H decoupling [8,9]. Typically, spectra from 100-mg cell-wall samples were the result of the accumulation of 16,384 scans at room temperature.

2.4. Calculated REDOR dephasing

REDOR dephasing was calculated using the modified Bessel function expressions given by Mueller et al. [10] and de la Caillerie and Fretigny [11] for a spin-1/2 pair. A plot of $\Delta S/S_0$ with respect to time ($t = NT_r$) yields the dipolar coupling constant and hence the internuclear distance (r_{IS}). The distance and spin-pair concentration (asymptotic dephasing maximum) were allowed to vary to minimize the root-mean-square deviation between the experimental and calculated dephasing [12].

3. Results and discussion

3.1. Glycyl Dante frequency selection for the parent strain of S. aureus

The long-bridge parent *S. aureus* strain has an intense Dante-inverted glycyl peak at 42 ppm (Fig. 2). Spin diffusion in 200 ms from the glycyl label to the D-[1-^{13}C]alanyl label arises only from the nearest-neighbor glycyl unit in the bridge. Two alanyl peaks are partially resolved: a cross-linked D-Ala-4 peptide peak at 175 ppm, and a weaker uncross-linked D-Ala-5 carboxyl-carbon shoulder at 178 ppm [1,2]. The deconvoluted relative intensities are 4:1, which indicates that cross-linking is 80% for this cell-wall

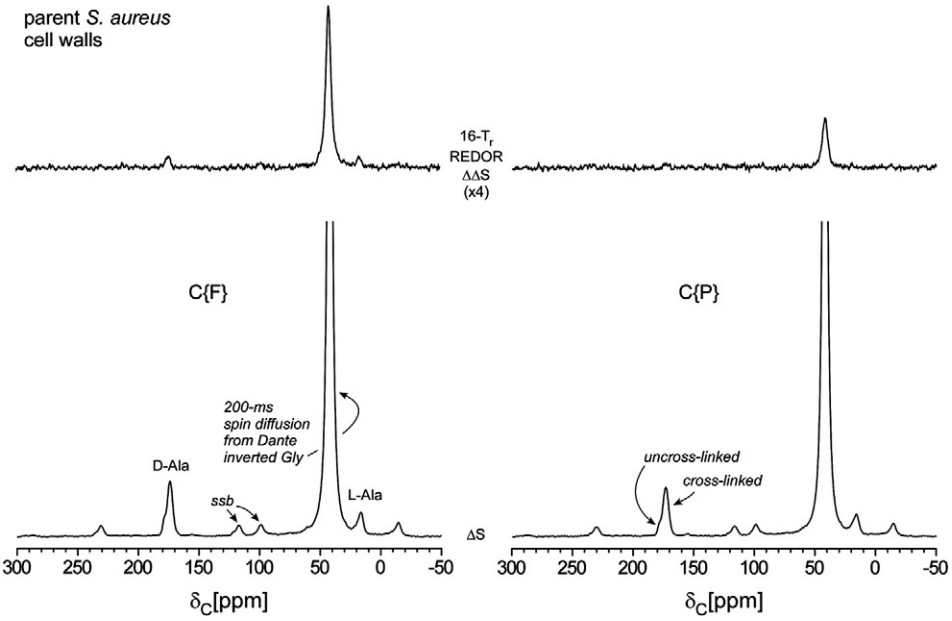

Fig. 2. Dante-selected C{F} (left) and C{P} (right) REDOR of intact cell walls of the parent strain of *S. aureus* grown in media containing D-[1-^{13}C]alanine, L-[3-^{13}C]alanine, [2-^{13}C]glycine, and L-[5-^{19}F]lysine with the alanine racemase inhibitor, alaphosphin. Dante differencing with inversion of the glycyl-carbon peak (42 ppm) preceded REDOR dephasing. Four data blocks were collected resulting in spectra with and without Dante irradiation, each with and without ^{19}F (or ^{31}P) dephasing. The Dante differences (ΔS) are shown at the bottom of the figure and are the reference spectra for Dante-REDOR dephasing ($\Delta\Delta S$) shown above. Spinning sidebands are designated by "ssb".

sample. Based on the lattice structure of the parent strain [2], the only cross-linked D-Ala ^{13}C label within 5 Å of a lysyl fluorine is part of the same stem and this accounts for the minor $\Delta\Delta S/\Delta S$ of 5% (Fig. 2, left), a factor of 4 less than that observed for the FemA cell wall where inter-stem proximities are possible [1]. The C{P} $\Delta\Delta S/\Delta S$ is also small (Fig. 2, right) and is associated only with the cross-linked D-Ala ^{13}C label. This result is consistent with the fact that the surface of the peptidoglycan structural motif of the *S. aureus* parent strain displays mostly bridges (see Fig. 9 of Ref. [1]) with the uncross-linked sites buried within more hydrophobic regions (Fig. 8 of Ref. [2]).

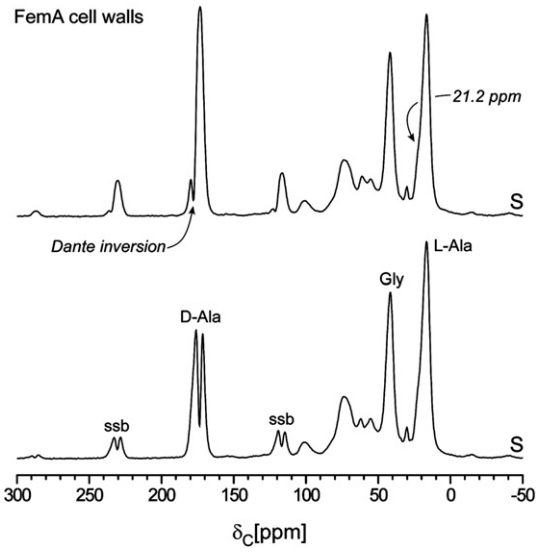

Fig. 3. Dante frequency selection for the FemA cell-wall sample. A train of 1-μs ^{13}C radio-frequency pulses separated by 5 μs, with the carrier frequency centered at 178 ppm (top) or 175 ppm (bottom), was followed by a z-axis storage for 200 ms. The resulting spectra are designated as "S".

3.2. D-Alanyl and L-Alanyl FemA Dante inversions

Dante inversions at 175 (Fig. 3, bottom) and 178 ppm (Fig. 3, top) both result in contacts of similar strengths with Gly and L-Ala ^{13}C labels (Fig. 4, left and right, respectively). However, the glycyl-label chemical shifts are different by 3.3 ppm. The unusual glycyl downfield shift (46.1 ppm) shows (i) the conformational strain associated with an uncross-linked stem (D-alanyl shift of 178 ppm) in the mixed-geometry lattice of FemA (see Fig. 10 of Ref. [1]); and (ii) the ability of Dante selection to make frequency-specific connectivities despite the broad lines of a heterogeneous cell wall.

The L-alanyl ^{13}C methyl contacts are also of similar strengths with the two Dante D-Ala inversions, and this rules out the possibility of substantial D-Ala and L-Ala scrambling. Such scrambling would have resulted in D-[1-^{13}C]Ala-4-D-[3-^{13}C]Ala-5 pairs with a separation of only three bonds between ^{13}C labels. These pairs would have resulted in a much stronger spin-diffusion contact for Dante inversion at 175 ppm than at 178 ppm.

The L-Ala methyl-carbon ^{13}C peak shows an unusual low-field shoulder at 21.2 ppm (Fig. 3, top). When this shoulder is Dante inverted (Fig. 5, right), a stronger contact with the glycyl label is observed than for inversion of the main peak at 17.5 ppm (Fig. 5, dotted lines). We attribute this result to strained conformations resulting from stems that are separated by only a few Ångstroms (Fig. 10, Ref. [1]) where the bridge of one stem is apparently forced near the L-alanyl head of a nearest-neighbor stem.

3.3. Contact of D-alanyl ^{13}C-label with L-alanyl ^{15}N-label in FemA cell walls

We attribute the minor contribution to the C{N} REDOR dephasing of Fig. 6 (top left) that is associated with a one-bond distance [2] to natural-abundance carbonyl carbons near L-Ala ^{15}N labels (see Fig. 1, left). Some minor scrambling of ^{13}C and ^{15}N labels may also be present resulting in D-Ala–D-Ala ^{13}C– ^{15}N bonds. The major component (3.4-Å

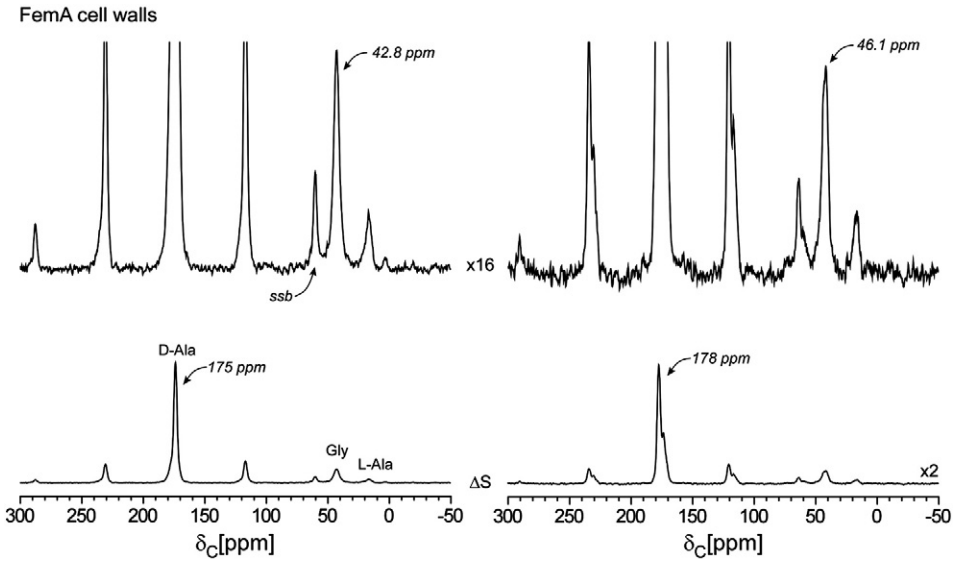

Fig. 4. Dante difference spectra (ΔS) from the FemA cell-wall inversions of Fig. 3 showing $^{13}C-{}^{13}C$ spin diffusion (for 200 ms) from the carbonyl-carbon label (175 ppm, left; 178 ppm, right) to the glycyl label (near 42 ppm) and the L-alanyl label (15 ppm). $\Delta S = S_0 - S$. Spinning sidebands are designated by "ssb".

$^{13}C-{}^{15}N$ separation) arises from the proximities of the heads and tails of adjacent parallel stems (Fig. 7, top left and expanded inset). Both parent and FemA peptidoglycan lattices have high concentrations of locally parallel adjacent stems [1,2]. N{C} REDOR dephasing reveals the same inter-stem contacts (Fig. 6, bottom left). The asymptotic dephasing limit for the C{N} dephasing is 12% (1/2 × 1/2 × 1/2) and depends on the ^{15}N isotopic enrichment (50%, [3]), the fraction of D-Ala label in wall teichoic acid (50%, [13]), and the fraction of D-Ala–D-Ala termini in conformationally strained locations pushed away from the stem head and toward the glycyl bridge (50%, [1]). The N{C} asymptotic limit is twice as large (25%) because only 50% of the stem-head L-Ala ^{15}Ns that are in stems not conformationally strained (50% of the total) are also near a D-Ala (Fig. 9, Ref. [1]).

3.4. Drug binding

Adjacent parallel stems offer the same binding site for eremomycin analogs [14] in FemA (Fig. 7) as in the parent strain of *S. aureus* [2]. FemA has a mixture of parallel and perpendicular stems but LCTA-1110 prefers the parallel-stem sites (Fig. 7, left). The REDOR-measured C – F distances of 4.8 and 8.8 Å from the ^{13}C label of D-Ala to the ^{19}F of LCTA-1110 (Fig. 6, top right), combined with the 3.4-Å $^{15}N-{}^{13}C$

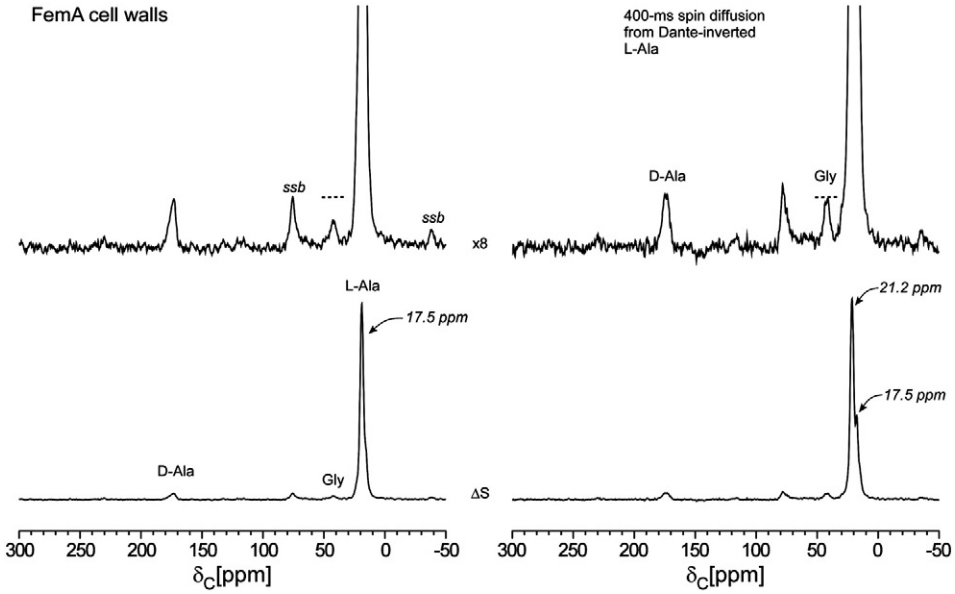

Fig. 5. Dante difference spectra (ΔS) from L-alanyl FemA cell-wall inversions showing $^{13}C-{}^{13}C$ spin diffusion (for 400 ms) from the methyl-carbon label (17.5 ppm, left; 21.2 ppm, right) to the glycyl label (near 42 ppm) and the D-alanyl label (near 175 ppm). Spinning sidebands are designated by "ssb".

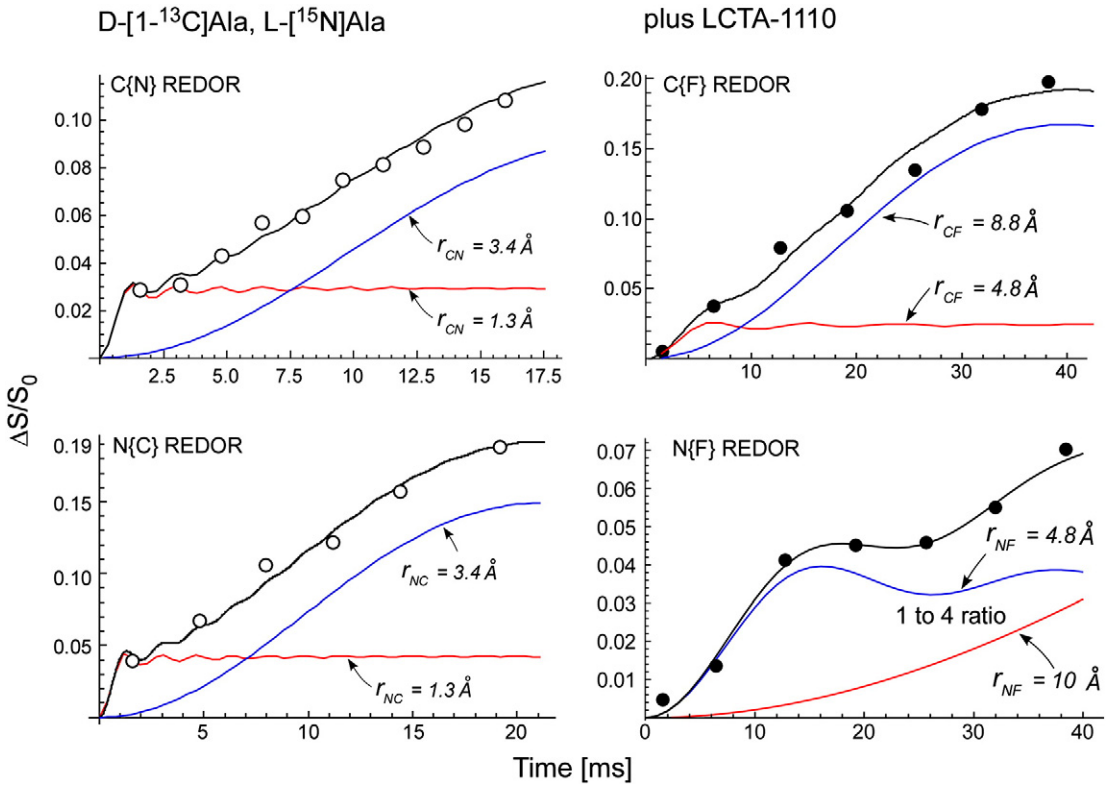

Fig. 6. (Left) C{N} and N{C} REDOR dephasing ($\Delta S/S_0$) of ^{19}F-labeled LCTA-1110 complexed to cell walls of the FemA mutant of *S. aureus* grown on media containing D-[1-^{13}C]alanine and L-[^{15}N]alanine, with the alanine racemase inhibitor, alaphosphin (10 μg/ml), as a function of the dipolar evolution (open circles). The error in the integrated REDOR difference is estimated as the diameter of the open-circle symbols. (Right) C{F} and N{F} REDOR dephasing of the same sample (closed circles). The calculated dephasings for two single-distance components are shown in red and blue, and the combined dephasing curve (25% shorter-distance component and 75% longer-distance component), as a solid line.

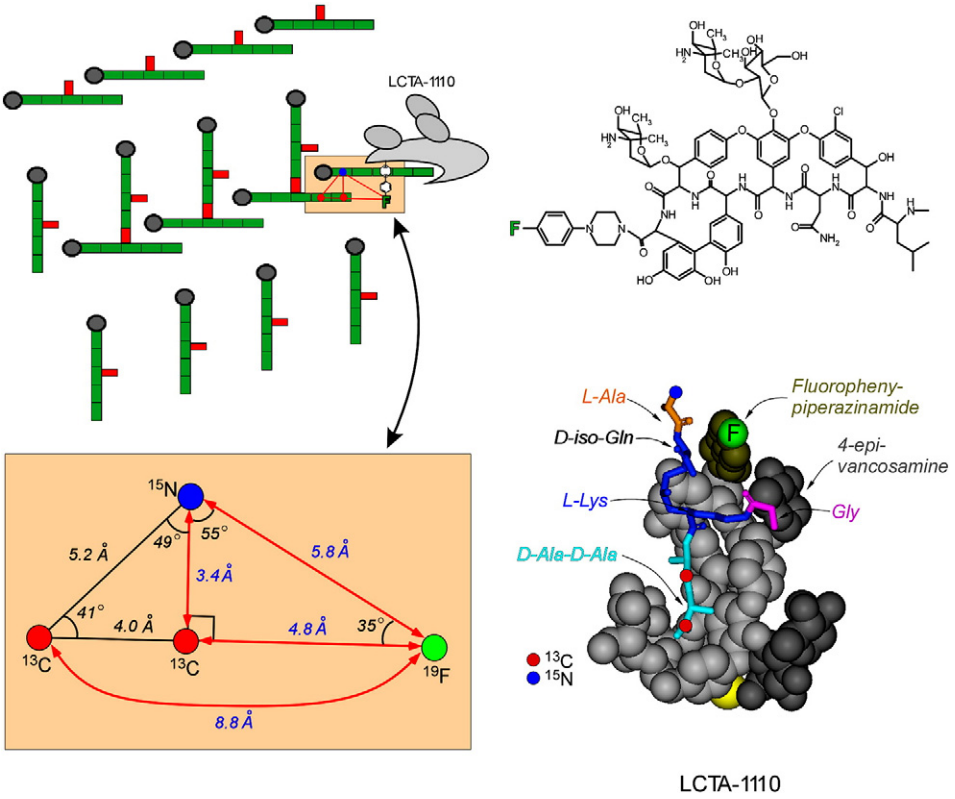

Fig. 7. (Top left) A cross-section of the proposed peptidoglycan-tertiary structure for the FemA mutant of *S. aureus* (Ref. [1]). The cross-section consists of sixteen glycan chains in a 4 × 4 matrix where the glycan backbones (represented by gray circles) are propagating perpendicular to the plane of the paper. The stems and bridges are represented by green and red rectangles, respectively. A *cartoon* of the glycopeptide LCTA-1110 (bottom right) is shown bound to a D-Ala–D-Ala uncross-linked peptide stem based on the lattice model of Ref. [1]. Details of the highlighted region of the top-left panel are shown in the light-orange insert (bottom left). REDOR distance measurements (blue numbers) connect labels (red arrows). (Top right) Chemical structure of LCTA-1110.

D-Ala – L-Ala interstem distance (Fig. 6, left), combine for a calculation of 5.8 Å for the distance between the ^{15}N label of L-Ala and the ^{19}F of LCTA-1110 (Fig. 7, bottom left inset). This value is in reasonable agreement with the observed REDOR distance of 4.8 Å (Fig. 6, bottom right). We therefore conclude that the glycopeptide drug is bound in a hydrophobic region of the peptidoglycan lattice [1,2].

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References

[1] S.J. Kim, M. Singh, S. Shasad, J. Schaefer, Cross-link formation and peptidoglycan lattice assembly in the FemA mutant of *Staphylococcus aureus*, Biochemistry (2014), http://dx.doi.org/10.1021/bi4016742.
[2] S.J. Kim, M. Singh, M. Preobrazhenskaya, J. Schaefer, *Staphylococcus aureus* peptidoglycan stem packing by rotational-echo double-resonance NMR spectroscopy, Biochemistry 52 (2013) 3651–3659.
[3] S. Sharif, S.J. Kim, H. Labischinski, J. Schaefer, Characterization of peptidoglycan in fem-deletion mutants of methicillin-resistant *Staphylococcus aureus* by solid-state NMR, Biochemistry 48 (2009) 3100–3108.
[4] T. Gullion, J. Schaefer, Rotational-echo double-resonance NMR, J. Magn. Reson. 81 (1989) 196–200.
[5] T. Gullion, J. Schaefer, Detection of weak heteronuclear dipolar coupling by rotational-echo double-resonance NMR, Adv. Magn. Reson. 13 (1989) 57–83.
[6] D. Stueber, A.K. Mehta, Z. Chen, K.L. Wooley, J. Schaefer, Local order in polycarbonate glasses by ^{13}C{^{19}F} Rotational-Echo Double-Resonance NMR, J. Polym. Sci. Part B Polym. Phys. 44 (2006) 2760–2775.
[7] T. Gullion, D.B. Baker, M.S. Conradi, New, compensated Carr–Purcell sequences, J. Magn. Reson. 89 (1990) 479–484.
[8] V. Bork, J. Schaefer, Measuring ^{13}C– ^{13}C connectivity in spinning solids by selective excitation, J. Magn. Reson. 78 (1988) 348–354.
[9] L. Cegelski, J. Schaefer, Glycine metabolism in intact leaves by *in vivo* ^{13}C and ^{15}N labeling, J. Biol. Chem. 280 (2005) 39238–39245.
[10] K.T. Mueller, T.P. Jarvie, D.J. Aurentz, B.W. Roberts, The REDOR transform: direct calculation of internuclear couplings from dipolar-dephasing NMR data, Chem. Phys. Lett. 242 (1995) 535–542.
[11] J.-B.d.E. de la Caillerie, C. Fretigny, Analysis of the REDOR signal and inversion, J. Magn. Reson. 133 (1998) 273–280.
[12] R.D. O'Connor, J. Schaefer, Relative CSA-dipolar orientation from REDOR sidebands, J. Magn. Reson. 154 (2002) 46–52.
[13] L. Cegelski, D. Steuber, A.K. Mehta, D.W. Kulp, P.H. Axelsen, J. Schaefer, Conformational and quantitative characterization of oritavancin–peptidoglycan complexes in whole cells of *Staphylococcus aureus* by in vivo ^{13}C and ^{15}N labeling, J. Mol. Biol. 357 (2006) 1253–1262.
[14] S.E. Solov'eva, S.S. Printsevskaya, E.N. Olsuf'eva, G. Batta, M.N. Preobrazhenskaya, New derivatives of eremomycin containing ^{15}N or F atoms for NMR study, Russ. J. Bioorg. Chem. 34 (2008) 747–754.