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Frequency-selective REDOR and Spin-diffusion Relays in Uniformly Labeled Whole Cells

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Abstract

Solid-state NMR is a powerful and non-perturbative method to measure and define chemical composition and architecture in bacterial cell walls, even in the context of whole cells. Most NMR studies on whole cells have used selectively labeled samples. Here, we introduce an NMR sequence relay using frequency-selective REDOR (fsREDOR) and spin diffusion elements to probe a unique amine contribution in uniformly ^{13}C - and ^{15}N -labeled *Staphylococcus aureus* whole cells that we attribute to the D-alanine of teichoic acid. In addition to the primary peptidoglycan structural scaffold, cell walls can contain significant amounts of teichoic acid that contribute to cell-wall function. When incorporated into teichoic acid, D-alanine is present as an ester, connected via its carbonyl to a ribitol carbon, and thus has a free amine. Teichoic acid D-Ala is removed during cell-wall isolations and can only be detected in the context of whole cells. The sequence presented here begins with fsREDOR and a chemical shift evolution period for 2D data acquisition, followed by DARR spin diffusion and then an additional fsREDOR period. fsREDOR elements were used for ^{13}C observation to avoid complications from ^{13}C - ^{13}C couplings due to uniform labeling and for ^{15}N dephasing to achieve selectivity in the nitrogens serving as dephasers. The results show that the selected amine nitrogen of interest is near to teichoic acid ribitol carbons and also the methyl group carbon associated with alanine. In addition, its carbonyl is not significantly dephased by amide nitrogens, consistent with the expected microenvironment around teichoic acid.

Graphical abstract

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Keywords

REDOR; frequency-selective REDOR; solid-state NMR; whole-cell NMR; cell wall; teichoic acid; *S. aureus*

1. INTRODUCTION

1.1. The bacterial cell wall

The bacterial cell wall is essential to cell viability and is a major target of antibiotics.¹ The cell wall of Gram-positive bacteria such as *Staphylococcus Aureus* (*S. aureus*) has two primary components: peptidoglycan (Figure 1, left), and teichoic acid (Figure 1, right).² The peptidoglycan is a mesh-like network of polymerized N-acetyl glucosamine (GlcNAc) and muramic acid (MurNAc) disaccharides that are highly cross-linked by short peptide stems attached to the lactyl moiety of MurNAc. In *S. aureus*, the peptide stems include a mixture of four or five amino acids that are cross-linked to an adjacent glycan strand through a pentaglycine bridge, providing mechanical strength and elasticity to the cell wall. The peptidoglycan provides the major structural and mechanical framework of the cell wall. The inhibition of cell-wall synthesis results in cell-wall thinning and cell lysis. Teichoic acids are long polymers that decorate and are appended to the peptidoglycan. Teichoic acid can contribute to virulence in *S. aureus* by aiding in adhesion and colonization. In addition, the inhibition of final steps of teichoic acid synthesis, after synthesis has been initiated, arrests cell growth.^{3, 4}

Quantifying alterations in cell-wall composition are important in evaluating drug modes of action, particularly important for human pathogens that are now resistant to multiple antibiotics such as *S. aureus*. Harsh degradative methods are needed to generate digested cell walls for analysis by traditional biochemical methods, including HPLC and mass spectrometry.^{5–7} Yet, for Gram-positive bacteria with the very thick cell wall present outside the cell membrane, complete digestion is usually not possible, compromising quantification. Solid-state NMR has emerged as a powerful tool to examine cell-wall composition in intact isolated cell walls, also known as sacculi, void of cytoplasmic contents, and in whole cells themselves.^{8, 9}

1.2. Selective labeling and REDOR approaches for bacterial cell walls

Bacterial peptidoglycan contains unique compositional linkages that are not found intracellularly. *S. aureus* use D-Ala in the cell wall (Figure 1) which results in D-Ala-Gly crosslinks, for example, that exist only in that particular site out of all the cellular components. Thus, specific labeling strategies have been immensely valuable in measuring features like these crosslinks using selective D-[1-¹³C]Ala and [¹⁵N]Gly labels, for example, during growth in defined medium to select the unique one-bond C-N pairs.¹⁰ [¹⁵N]Gly and L-[ε-¹⁵N]Lys labels have been valuable in quantifying bridgelinks where the pentaglycine bridge is attached to the lysine sidechain, converting the sidechain amine to an amide.^{7, 10} Rotational-echo Double Resonance (REDOR) experiments have been useful for these selections and for measurements of internuclear distances (e.g. from D-Ala-Gly crosslink site to an antibiotic ¹⁹F label) to determine drug modes of action and to generate atomic-level models for cell-wall antibiotic complexes.^{8, 11–13}

1.3. Non-selective natural abundance and uniform labeling NMR approaches for bacterial cell walls

We recently reported that one-dimensional spectra of either unlabeled ¹³C or uniformly ¹³C and ¹⁵N enriched cell walls and whole cells provide valuable compositional profiles of cell-wall and whole-cell samples.¹⁴ We were even able to determine that the polysaccharide contributions to whole cells primarily arise from the cell wall by examining protoplasts in which much of the cell wall is removed. Furthermore, the polysaccharide signatures in whole cells were able to reflect the influence of two antibiotics with different modes of action. A cell-wall inhibitor, fosfomycin, resulted in whole-cell spectra that differed in having reduced peak intensities in the polysaccharide region. Chloramphenicol, on the other hand, inhibits protein synthesis and during the time of treatment has little effect on cell-wall assembly. Whole-cell ¹³C spectra revealed an altered balance of ¹³C contributions, where cell-wall polysaccharides were preferentially increased as protein synthesis was inhibited and protein contributions were reduced.¹⁴ We have been working to extend this approach to other bacterial organisms and to explore the extent of atomic-level compositional detail that can be extracted from uniformly labeled samples, with one of the goals being to examine the full repertoire of cellular changes that accompany treatment with old and new antibiotics. Uniform labeling is typically simpler to implement than specific-labeling strategies. One does not have to quantify scrambling and isotopic dilution due to endogenous synthesis. Uniform ¹³C labeling also provides the opportunity to use homonuclear spin-diffusion mixing to select other nearby resonances, in a manner similar to that widely used for assignment and structure determination of proteins.

1.4. A new approach using relayed frequency-selective REDOR and spin diffusion steps

In this contribution, we report the design and implementation of a relayed frequency-selective REDOR experiment (fsREDOR-DARR-fsREDOR) in one and two dimensions with uniformly labeled whole cells. The experiment was designed to provide atomic-level specificity regarding teichoic acid in *S. aureus* whole cells. The first application of fsREDOR was implemented to identify local order in a polymer system.¹⁵ Following that, there have been several applications of frequency-selective REDOR to biological systems,

including applications to uniformly labeled peptides and proteins^{16–22}, A β fibrils²³, as well as in the characterization of intact plant tissue²⁴, and other systems^{25,26}. In related approaches, particularly in selectively labeled samples, frequency-selective inversions followed by spin diffusion have also been employed to extract connectivity and proximity information in complex assemblies such as plant leaves and bacterial whole cells.^{27–29} In this study we implemented a three-part sequence relay in which we begin with fsREDOR to observe carbons bonded to amine nitrogens of interest, spread magnetization through dipolar-assisted rotational resonance (DARR)³⁰, and then probe whether the observable carbonyls are near to amide nitrogens. As described below, this allowed us to identify whole-cell NMR contributions that we ascribe to teichoic acid D-alanine.

EXPERIMENTAL METHODS

2.1. Uniformly ¹³C- and ¹⁵N-labeled *S. aureus* whole-cell sample preparation

Uniformly labeled *S. aureus* (ATCC 29213) were grown in a modified *S. aureus* synthetic medium (SASM) ^{31–33} in which all amino acids were replaced by 2 g/L ¹⁵N and ¹³C labeled algal amino acid mixture (ISOTEC Cat # 487910). The algal extract contains between 65–95% amino acids by mass and has an isotope enrichment of 99% for ¹³C and ¹⁵N. Labeled (¹⁵NH₄)₂SO₄ (98% ¹⁵N enrichment) and [u-¹³C]glucose (99% ¹³C enrichment) were also used in place of their unlabeled counterparts. *S. aureus* cultures were maintained on Tryptic soy agar (TSA). To begin NMR sample preparations, 5 ml aliquots of [¹⁵N]SASM or [¹³C, ¹⁵N]SASM were inoculated with a single colony and grown overnight at 37 °C, shaking at 200 rpm. A 300 mL culture was prepared in a 1 L growth flask. The growth was started with a 1:300 inoculum (v/v) using 1 mL of the overnight starter culture for the 300 mL media. Cells were harvested at OD₆₀₀ = 1.7 by centrifugation at 10,000 g at 4 °C for 10 min. Cells were subsequently washed three times by resuspension with 5 mM HEPES buffer (pH 7) and centrifugation to remove excess media components. The final cell pellet was frozen and lyophilized.

2.2. Solid-state NMR spectroscopy

Solid-state Cross Polarization Magic Angle Spinning (CPMAS) NMR experiments were performed in an 89 mm bore 11.7 T magnet (Agilent Technologies, Danbury CT) using a home-built four-frequency HPCN transmission line probe (¹H 500.92, ¹³C 125.96 and ¹⁵N 50.76) with a four channel DD2 console (Agilent Technologies), employing high-power linear Transmitter/Receiver (T/R) line switching on the ¹³C and ¹⁵N channels. Samples were spun at 7143 Hz in thin-wall 5mm zirconia rotors and maintained at 5 °C with an FTS Chiller (FTS Thermal Products, SP Scientific, Warminster, PA) supplying nitrogen at –25 °C. Field strengths for ¹³C and ¹⁵N cross polarization (CP) were all 50 kHz with hard π pulses of 10 μ s and with a 10% ¹H linear ramp centered at 57 kHz. SPINAL ¹H decoupling was applied at 72 kHz during acquisition and at 90 to 100 kHz during indicated selective REDOR periods. CW decoupling was applied with field strength $\gamma B_1 = \omega_r$ for DARR mixing. The CPMAS mixing time was 1.5 ms and the recycle time was 2.0 s for all experiments. ¹³C chemical shifts were referenced to tetramethylsilane as 0.0 ppm using a solid adamantane sample at 38.5 ppm. The ¹⁵N chemical shift scale is referenced to ammonia at 0 ppm where solid L-[amide-¹⁵N]Asn appears at 114.5 ppm. Referencing to ammonia at 0 ppm is a

change from some previous uses of solid NH_4SO_4 for which L-[amide- ^{15}N]Asn would be reported as 89.1 ppm.

2.3. Parameters for the fsREDOR-DARR-fsREDOR pulse sequence and individual elements

The sequence of Figure 2 consists of three parts following ^1H - ^{13}C CP: (i) $^{13}\text{C}\{^{15}\text{N}\}$ fsREDOR, followed by an optional period of ^{13}C chemical shift evolution, (ii) a longitudinal ^{13}C mixing period for spin diffusion, and (iii) a second $^{13}\text{C}\{^{15}\text{N}\}$ fsREDOR period. Each fsREDOR component of the pulse sequence in Figure 2 is based on the sequence version of Jaroniec *et al.* applied to uniformly labeled tripeptides and consists of two blocks of $n/2$ rotor periods before and after a pair of ^{13}C and ^{15}N selective π pulses, centered in an even-number of rotor periods.¹⁶ Hard ^{15}N inversion pulses are applied two-per-rotor period with the XY-8 phase cycle for both the control and dephased spectra.

Either selective RSNOB³⁴ or DANTE²⁶ pulses were employed for the frequency-selective pulses in our work, as detailed below. Each fsREDOR control (or full-echo reference) spectrum, S_0 , is obtained with only the center ^{13}C pulse and without the center ^{15}N pulse. The dephased spectrum, S , is obtained with both pulses. Selective DANTE pulses ($\text{pw} = 2240 \mu\text{s}$) applied at 34 and 42 ppm are used to individually invert each resonance for the fsREDOR spectra of Figure 5. A DANTE pulse ($\text{pw} = 1120 \mu\text{s}$) at 38 ppm is used to invert both resonances in Figures 6 and 7. A low-power shaped RSNOB pulse ($\text{pw} = 980 \mu\text{s}$) applied at 42 ppm is used to refocus the 20–60 ppm ^{13}C region for the first fsREDOR period (identified as Part 1 in the pulse sequence) of Figures 5, 6 and 7. For the second fsREDOR period (identified as Part 2 in the pulse sequence) of Figure 7 a low-power RSNOB pulse ($\text{pw} = 980 \mu\text{s}$) was used to refocus the 160–185 ppm ^{13}C carbonyl region and a ^{15}N RSNOB pulse ($\text{pw} = 1120 \mu\text{s}$) was used to invert the 100–140 ppm amide region. Our choice was to use a ^{15}N DANTE pulse in the first fsREDOR period and a low-power shaped pulse in the second, though either could have been used.

The phase cycles of the ^{13}C refocusing pulses for each fsREDOR component (ϕ_{C1} and ϕ_{C2} below) relative to the receiver (ϕ_{R} below) remove all ^{13}C signal outside the refocusing bandwidth, allowing correlations from ^{13}C spin-diffusion mixing to be observed with a one-dimensional experiment. When both fsREDOR periods are used the two refocusing pulses are usually applied at different frequency offsets, ω_1 and ω_2 . The full phase cycle of refocusing pulses and receiver will refocus signal at ω_2 that is correlated by spin diffusion to signal at ω_1 . Often the sequence is applied with only a selective pulse in the first fsREDOR component and the second fsREDOR component is replaced with a hard refocusing pulse for echo detection of the full spectral bandwidth. In this case resonances outside the refocusing bandwidth are correlations with ω_1 due to spin diffusion.

The bandwidths for all refocusing pulses were determined with a CP pulse sequence followed by the selective refocusing period, similar to the fsREDOR sequence without dephasing pulses. For ^{13}C RSNOB refocusing pulses the pulsewidth was set as an integral number of rotor periods to achieve the desired refocusing bandwidth and the amplitude was calibrated to achieve maximum refocusing on resonance. For ^{15}N DANTE pulses the total pulsewidth was set in rotor cycles and the amplitude was set at nominal 25 kHz. The flip angle of the individual pulse in each rotor period was adjusted for maximum refocusing. The

insets of figure 5 were obtained in this way. The calibration of the ^{15}N inversion pulses was also verified with the fsREDOR sequence itself.

1D and 2D cosine phases are $\phi_H = y$, $\phi_{\text{DEC}} = x$; $\phi_C = -y$; $\phi_{C1} = x, y, x, y, -x, -y, -x, -y$; $\phi_{N1} = 0$; $\phi_{C2} = -y, -y, x, x, y, y, -x, -x$; $\phi_{N2} = 0$; $\phi_{M1} = 0$; $\phi_{M2} = -x, -x, -y, -y, x, x, y, y$ and $\phi_R = -x, x, x, -x, -y, y, y, -y, x, -x, -x, x, y, -y, -y, y$; ϕ_{M1} is shifted 90° for the sine data and both ϕ_{M1} and ϕ_R are incremented for States TPPI. Cosine phases are used for 1D data. The dwell time for F1 evolution is synchronized as an integral number of rotor periods to produce the minimum F1 spectral width that is required to span the refocused bandwidth. The two-dimensional ^{13}C - ^{13}C correlation spectra in Figure 5 and Figure 6 were obtained with an F1 spectral width of 7143 Hz with 32 and 16 complex increments, respectively.

In the full fsREDOR-DARR-fsREDOR experiment (Figure 7) four data sets are collected with combinations of S_0 and S for the first and second fsREDOR periods as follows: $[S_0S_0]$, $[S_1S_0]$, $[S_0S_2]$ and $[S_1S_2]$, where subscripts 1 and 2 refer to the first and second fsREDOR periods. The first fsREDOR period permits observation of aliphatic carbons that are dephased selectively by amine nitrogens. The second fsREDOR period, after the DARR correlation, is performed to observe correlated carbonyl carbons that are dephased selectively by amide nitrogens. The final control, S_{0f} , of Figure 7 is $S_{0f} = [S_0S_0] - [S_1S_0]$, the data set with no dephasing in both fsREDOR periods minus that with dephasing in the first (amines) but not the second. The final dephased, S_f , spectrum of Figure 7 is the difference $S_f = [S_0S_2] - [S_1S_2]$ with additional dephasing of amides in the second period of both data sets. The final difference is $S_f = S_{0f} - S_f = [S_0S_0] - [S_1S_0] - [S_0S_2] + [S_1S_2]$.

RESULTS AND DISCUSSION

3.1. CPMAS spectra of $[\text{u-}^{13}\text{C}, \text{u-}^{15}\text{N}]$ -labeled *S. aureus* whole cells

These ^{13}C and ^{15}N CPMAS spectra of uniformly labeled *S. aureus* strain 29213, harvested at OD_{600} 1.7, (Figure 3) are comparable to previously published natural abundance and uniformly labeled *S. aureus* whole-cell spectra.¹⁴ The carbon sugar region that is highlighted in Figure 3A arises primarily from the cell-wall peptidoglycan as demonstrated previously through comparison with protoplasts in which most of the cell wall is removed by digestion. The α -carbon region is highlighted and will be selectively observed in subsequent experiments, as described below. The ^{15}N spectrum contains all the nitrogen contributions in the cell. Two amine populations are indicated. The chemical shift of amine nitrogen B at 34 ppm is consistent with that of the lysine sidechain ϵ nitrogen. This amine peak would arise from all protein lysines in the cell as well as cell-wall precursors termed Park's nucleotide that have a free lysine sidechain. We hypothesized that Amine A at 42 ppm could be attributed to the D-Ala amine present in teichoic acid. Teichoic acid is estimated to make up as much as 50–60% of the cell wall by mass and, with the prevalence of D-Ala as indicated in the chemical structure of teichoic acid, we anticipated that its amine contribution should be prominent in a whole-cell spectrum. Other amino acid nitrogens in the whole cell are expected to be present as peptide amides (for backbone nitrogens) or sidechain amides (Asn, Gln), in the 100–140 ppm region, or as the sidechain guanidinium group in Arg near 80 and 90 ppm (Figure 3), or the imidazole nitrogens in His, near 170 ppm (not shown). Nucleic acid nitrogens can also contribute to the ^{15}N spectrum, particularly as observed between

143–148 ppm and near the Arg sidechains (70–90 ppm), where ribosomal RNA accounts for the majority of the nucleic acid contributions by mass.

3.2. NMR strategy to determine the atomic-level nature of the amine-containing molecules

As described above, we hypothesized that Amine A arises from teichoic acid D-Ala, whereas the chemical shift of Amine B is consistent with lysine sidechains. To provide evidence for the teichoic acid assignment, we designed a new NMR experiment (Figure 2). The experiment includes three major parts with some additional options and variations. The experiment is illustrated schematically in Figure 4 in the context of the specific atomic-level information we were seeking in the cell wall. The D-Ala amine of teichoic acid is proximate to teichoic acid ribitol carbons and not near to other peptide bonds or nitrogen amides, whereas the Lys ϵ nitrogen in proteins are proximate to the sidechain carbons and then to its peptide bond and neighboring peptide bonds. The sequence in Figure 2 was designed to first selectively excite carbons in the 20–60 ppm range, including the D-Ala α -carbon at 50 ppm and the Lys sidechain carbon adjacent to the ϵ - ^{15}N at 40 ppm and uses fsREDOR to dephase only those carbons proximate to either Amine A or Amine B. This is followed by an optional sequence element to allow for chemical shift evolution in a second dimension. After this, spin diffusion for 50 ms transfers the magnetization to neighboring carbons. Finally, a second and final frequency-selective REDOR block was added to selectively dephase the correlated carbonyl carbons with nitrogen amides. In this final step, carbonyls near lysine sidechain amines, as in proteins, should exhibit significant dephasing whereas the carbonyls of the teichoic acid D-Ala should not.

3.3. The one-part fsREDOR experiment and two-part fsREDOR-DARR experiment identifies molecular attributes consistent with Amine A as teichoic acid D-Ala

Two experiments were first performed to probe the immediate environment around each of the amine populations. This is not the complete sequence shown in Figure 2, but just the first fsREDOR element and then an experiment with the first fsREDOR element followed by DARR. First, fsREDOR was used to observe only carbons in the 20–60 ppm range to include all α -carbons and the lysine sidechain ϵ -carbon with frequency-selective dephasing pulses implemented on either of the two amines as dephasers in separate experiments. The results for this REDOR-only experiment are shown in the top of Figure 5. The α -carbons which were dephased by Amine A, the putative teichoic acid amine, were centered at 50 ppm and those dephased by Amine B were centered at 40 ppm. In the next experiment, this fsREDOR preparation was followed by a short period of DARR spin diffusion to spread magnetization to neighboring carbons. As shown in the bottom of Figure 5, this results in three sets of REDOR spectra. Each S_0 control spectrum contains observable carbons after spin diffusion from all carbons in the 20–60 ppm region. The dephased, S , spectrum contains all the observable carbons after spin diffusion initiated from carbons in the 20–60 ppm region except those that were dephased by the amine nitrogens. The difference spectrum, ΔS , thus represents the observed spin diffusion resulting from the carbons that were dephased.

Amine A is bonded to carbons centered at 50 ppm and through spin diffusion (Figure 5, top left) these were demonstrated to be close to a carbonyl centered at 172 ppm and carbons in

the methyl chemical shift range, centered at 18 ppm (Figure 5, bottom left). These are consistent with D-Ala. This spin diffusion spectrum also contains a unique pair of peaks between 60 and 80 ppm which suggests correlation to carbons of sugars, most likely through the ester linkage to ribitol of teichoic acid.

For the experiment with Amine B, fsREDOR identified carbons centered at 40 ppm as being bonded to Amine B (Figure 5, top right) and, after DARR mixing, resulted in observable magnetization consistent with the other carbons of lysine: C α 54 ppm, C β 33 ppm, C γ 25 ppm, C δ 28 ppm, and amide carbon 175 ppm (Figure 5, bottom right). As anticipated, the carbonyl peak intensity is comparatively less than for the Amine A experiment since the lysine carbonyl would be further away from the ϵ -nitrogen and carbon of the sidechain.

The two-part fsREDOR-DARR experiment was repeated with the optional chemical shift evolution period to generate a two-dimensional data set. In this case the selective ^{15}N bandwidth covered the entire amine region to capture both the D-Ala and Lys amines. The resulting two-dimensional difference, $S - S_0$, spectrum is shown in Figure 6 and it represents all the carbons that can be observed after spin diffusion that is initiated from carbons that are adjacent to an amine nitrogen. We provide the annotations for the corresponding D-Ala carbonyl, ribitol sugar and D-Ala methyl groups that can be observed, and for the Lys carbonyl. The 1D and 2D approaches of Figures 5 and 6 establish the same result and summed projections of the 2D D-Ala and Lys traces in F2 of Figure 6 are similar to the 1D spin-diffusion spectra in Figure 5. In practice the 2D plot provides better selectivity between the D-Ala and Lys bands with a shorter band-selective pulse, but the 2D experiment takes more time to achieve similar sensitivity. While a 2D band-selective experiment is used extensively for proteins, where there are many resonances in the band, whole-cell spectra may contain only one or two resonances of interest and resonance-selective 1D spectroscopy can be more time-efficient.

3.4. The complete fsREDOR-DARR-fsREDOR experiment adds additional evidence for assignment of Amine A as teichoic acid D-Ala

The atomic-level environment around each amine, described in the experiments above, is consistent with the assignments of Amine A as teichoic acid D-Ala and Amine B as Lys amines. We next sought to implement one additional analysis to further test the assignment. The D-Ala carbonyls selected above would not be proximate to amide nitrogens if they were part of teichoic acid, since the teichoic acid D-Ala nitrogen is present as an amine (Figure 4). The Lys carbonyl resulting from spin diffusion in Figures 5 and 6, however, should be primarily due to peptide backbone carbonyls and should be dephased by amide nitrogens (as illustrated in Figure 4). Thus, we added a final fsREDOR step to the experiment resulting in the full fsREDOR-DARR-fsREDOR experiment. In the final fsREDOR step, frequency-selective pulses were used to observe only carbonyl carbons and to dephase only with amide nitrogens. The results of the experiment are shown in Figure 7. The 2D REDOR data, including S_0 , S , and $S - S_0$ 2D plots show the significant difference in dephasing between the two carbonyl populations. The carbonyl we attribute to lysine is significantly dephased, whereas the carbonyl assigned to the teichoic acid D-Ala is not dephased significantly. The 2D plots of Figure 7 are displayed with F1 horizontal and so projections permit

quantification of the dephasing of the two bands. The Lys carbonyl is dephased by approximately 70% by amides, while the D-Ala carbonyl band is dephased by less than 20%. A trace through the D-Ala carbonyl is dephased by only 10%. Thus, the full suite of spectroscopic characterization, including a new fsREDOR-DARR-fsREDOR relay supports our assignment of the teichoic acid D-Ala amine in whole cells.

3.5. Conclusions and outlook

In this work, we focused on identifying teichoic acid D-alanine in whole cells. This measurement must be performed in whole cells because cell-wall isolation protocols result in the de-esterification and loss of D-Ala from teichoic acid. We are unaware of any other way to quantify teichoic acid D-Ala in intact cells. In this whole-cell measurement, both wall teichoic acids and lipoteichoic acids are observed. Future work with teichoic acid synthesis mutants can dissect the quantitative contributions of each. More generally, our discoveries here emphasize the new insights that are possible in examining intact uniformly labeled whole cells with appropriately designed NMR pulse sequences.

Solid-state NMR has been a valuable tool for the study of bacterial cell walls since the first bacterial cell-wall and bacterial whole-cell NMR measurements reported in 1985 by Schaefer and coworkers.³⁵ Many compositional and structural analyses can be performed on isolated cell walls, enhancing the experimental sensitivity by removal of cytoplasmic contents. In addition, many whole-cell experiments have verified the relevance of distances measured in lyophilized cell walls.^{12, 36–38} However, there is often desirable information to be gained from whole-cell experiments and that is usually why they are employed. For example, increases in the cytoplasmic precursor, Park's nucleotide, can be observed upon antibiotic treatment for cells labeled specifically with L-[ϵ -¹⁵N]Lys.^{10, 13} We also recently showed that one can monitor the balance of cell-wall versus cytoplasmic carbon contributions in whole cells and that changes can be detected due to the influence of antibiotics with different modes of action (e.g. a cell wall inhibitor versus a protein synthesis inhibitor).¹⁴

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Highlights

- SSNMR is a powerful way to measure cell-wall composition even in whole cells.
- A pulse sequence with fsREDOR and spin-diffusion relays identifies teichoic acid D-Ala in whole cells.
- This approach is generally applicable to heterogeneous, insoluble, complex assemblies.

Peptidoglycan

Wall teichoic acid

Figure 1. Chemical structures of major cell-wall components peptidoglycan and teichoic acid
Wall teichoic acids are covalently attached to peptidoglycan and are substituted with D-Ala through an ester linkage.

Figure 2. The fsREDOR-DARR-fsREDOR pulse sequence

This pulse sequence consists of three parts following ^1H - ^{13}C CP, with description of the targeted carbons and nitrogens in each element in our application: (i) fsREDOR to observe α carbons that are proximate to amine nitrogens, (ii) DARR to spread ^{13}C polarization to neighboring carbons, preceded by an optional ^{13}C chemical shift evolution period, (iii) fsREDOR to dephase the observable carbonyls with amide nitrogens. The colored, open rectangles are simultaneous ^{13}C and ^{15}N selective π pulses at frequencies ω_{C1} , ω_{N1} , ω_{C2} , and ω_{N2} , centered in m_1 or m_2 , even-number of rotor periods, τ_r . Brackets and black open rectangles indicate periods of rotor-synchronized XY8 π pulses for ^{15}N (initial phase = 0) for $n_1/2$ or $n_2/2$ rotor periods to cause REDOR dephasing. Filled rectangles, M_1 and M_2 are $\pi/2$ pulses surrounding the mixing period.

Figure 3. CPMAS spectra of [u- ^{13}C , u- ^{15}N]-labeled *S. aureus* whole cells

The two resonances in the amine region (25-55 ppm) of the ^{15}N CPMAS spectrum (32 scans) are due primarily to the ϵ -nitrogen of lysine (34 ppm), designated “B”, and an amine which we attribute to the amino group of D-Ala in teichoic acids (42 ppm), designated “A.”

1. fsREDOR from amine N to C α

2. DARR from C α to C=O

3. fsREDOR from amide N to C=O

Lys in protein

Figure 5. Characterization of the two amine environments by fsREDOR and the two-part fsREDOR-DARR combination

2.2 ms $^{13}\text{C}\{^{15}\text{N}\}$ fsREDOR data (S_0 , S , and ΔS) were obtained for $[\text{u-}^{13}\text{C}, \text{u-}^{15}\text{N}]$ -labeled *S. aureus* whole cells with frequency selective observation of 20–60 ppm carbons and frequency selective dephasing of either amine A (top left) or amine B (top right) with a 2240 μs DANTE inversion pulse at the appropriate frequency. Insets show selective ^{15}N -observe spectra (in blue) obtained with the same selective pulses and the full ^{15}N CPMAS spectrum of the amine region is shown in dashed grey for reference. The bottom panels show the additional peaks that result from the subsequent 50-ms ^{13}C mixing period using DARR.

Figure 6. Characterization of the two amine environments by fsREDOR-DARR in two dimensions

A 2D ^{13}C - ^{13}C correlation experiment was obtained with the experiment of Figure 5 with an additional chemical shift evolution period before the mixing period. The fsREDOR element in this experiment used a $1120\ \mu\text{s}$ ^{15}N DANTE pulse to selectively dephase the whole amine region, including Amine A and Amine B. The 2D plot corresponds to the fsREDOR S data and reveals all carbons that result from spin diffusion initiated from the carbons that are proximate to the amine nitrogens.

Figure 7. Evaluation of the two amine environments by the three-part fsREDOR-DARR-fsREDOR experiment in two dimensions

A second fsREDOR element was implemented to identify the extent to which the carbonyls that resulted from the spin diffusion shown in Figure 6 were near to amide nitrogens (within two bonds). Plots corresponding to the control (S_0), dephased (S), and difference (ΔS) data are shown. The F1 dimension is shown horizontally in this figure along with F1 projections of S_0 , S and ΔS to show REDOR dephasing for the separate peaks attributed to D-Ala (F1 50 ppm) and Lys (F1 40 ppm) in a single set of projections.