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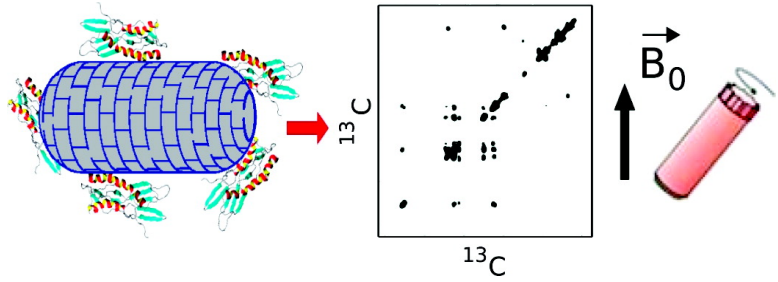

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Toward the Characterization of Peptidoglycan Structure and Protein−Peptidoglycan Interactions by Solid-State NMR Spectroscopy

Thomas Kern,[†] Sabine Hediger,[*,‡] Patrick Müller,[§] Cécile Giustini,[†] Bernard Joris,[¶] Catherine Bougault,[†] Waldemar Vollmer,[#] and Jean-Pierre Simorre[*,†]

Institut de Biologie Structurale, UMR5075 (CEA/CNRS/UJF), 38027 Grenoble, France, Laboratoire de Chimie Inorganique et Biologique, UMR-E3 (CEA/UJF), INAC, CEA, 38054 Grenoble, France, Mikrobielle Genetik, Universität Tübingen, 72076 Tübingen, Germany, Centre d'ingénierie des protéines, Institut de Chimie B6A, Université de Liège, Sart-Tilman B4000, Belgium, and Institute for Cell and Molecular Biosciences, Newcastle University, NE2 4HH Newcastle Upon Tyne, U.K.

Received December 10, 2007; E-mail: jean-pierre.simorre@ibs.fr; sabine.hediger@cea.fr

The peptidoglycan (murein) sacculus is an essential component of the bacterial cell wall. Surrounding the cytoplasmic membrane, it plays a crucial role in allowing cells to withstand osmotic pressure[1] and in defining cellular shape.[2] Unique to the bacterial world, this large polymer network is also the principal target of many antibiotics[3] and one of the main microbial products recognized by the immune system.[4] Consequently, great efforts have been invested in the past decade to determine its architecture and biosynthesis.[5,6] While chemical composition and structure of fragments have been established, and several enzymes involved in peptidoglycan assembly have been isolated, the 3D organization of this biopolymer is still debated[7] (orientation of the glycan strands parallel[5,8] or perpendicular[9,10] to the cell membrane has been proposed), and its growth mechanism remains an area of active research.

Peptidoglycan is a heteropolymer made of linear glycan strands of two alternating β-1,4-linked carbohydrates, *N*-acetylglucosamine (Glc*N*Ac) and *N*-acetylmuramic acid (Mur*N*Ac), that are cross-linked by short peptides (Figure 1a). In the Gram-negative model bacterium *Escherichia coli*, the glycan strands vary in length from 1 to ~80 disaccharide units, with an average length of 21−35. They are terminated with a 1,6-anhydro-Mur*N*Ac instead of a Mur*N*Ac residue.[11] Peptides are covalently linked to the lactyl group of the muramic acid and can vary between different bacteria in the nature of the amino acids involved (for example, *m*-DAP versus L-Lys in position 3), the number of cross-linked peptides (for example, in *E. coli*, from monomers to tetramers), and in the structure of the peptide cross-links (for example, in *E. coli*, D-Ala-D-*m*-DAP or L-*m*-DAP-D-*m*-DAP).[5]

The molecular weight of a sacculus from *E. coli* is in the range of 3×10^6 kDa, making the nonfragmented molecule inaccessible to most analytical methods. In this work, we show for the first time that solid-state NMR spectroscopy can successfully be applied to intact hydrated and fully labeled *E. coli* sacculi. Almost complete NMR assignment of the repetitive polymer has been obtained, allowing the subsequent study of protein−peptidoglycan interactions.

^{13}C,^{15}N-isotopically labeled *E. coli* BL21(DE3) cells were grown in M9 minimal medium and harvested by centrifugation. Sacculi were then purified according to previously published procedure.[11] After centrifugation, 25 mg of the resulting paste was finally loaded into a 4 mm solid-state NMR rotor. Different ^{13}C-correlation experiments were performed, and spectra of astonishing good resolution were obtained, considering the size and noncrystallinity of the sample. Figure 1c illustrates the high spectral quality obtained using a through-bond correlation experiment. The spectrum was acquired using the sequence in Figure S1, which is closely related to previously published experiments.[12,13] Interestingly, the signals observed for the glycan part present symmetric line shapes, without any distortion due to chemical shift inhomogeneity, as has been observed, for example, in cellulose.[14] This suggests a structural inhomogeneity which is small enough to be averaged out by the amplitude-restricted molecular dynamics present in the cross-linked polymer.

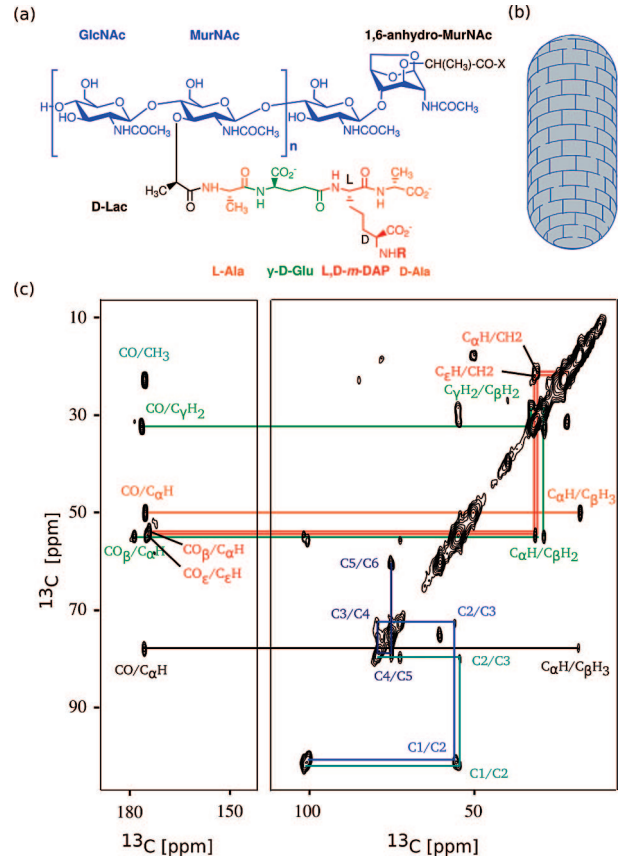

Figure 1. (a) Chemical structure of *E. coli* peptidoglycan. The letter R stands for a hydrogen atom or the peptide stem of another peptidoglycan chain; X stands for a peptide stem. (b) Schematic drawing of the peptidoglycan sacculus. (c) Through-bond ^{13}C-correlation spectrum of ^{13}C-labeled peptidoglycan, measured on a 400 MHz (^{1}H frequency) spectrometer. The resonance assignment follows the color code defined in panel (a) for the different spin systems: shades of blue for glycan, black for lactic acid, orange for alanines, green for glutamate, and red for diaminopimelic acid.

[†] Institut de Biologie Structurale, UMR5075 (CEA/CNRS/UJF).
[‡] Laboratoire de Chimie Inorganique et Biologique, UMR-E3 (CEA/UJF).
[§] Universität Tübingen.
[¶] Université de Liège.
[#] Newcastle University.

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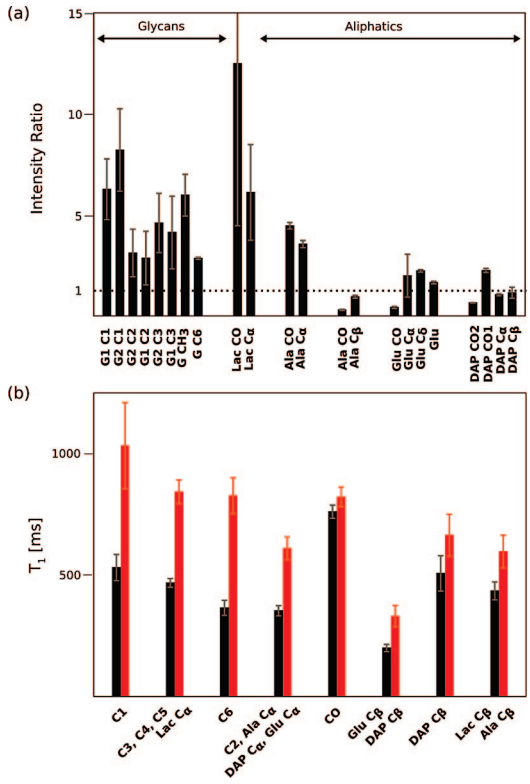

Figure 2. (a) Ratios of cross-peak intensity in through-bond ^{13}C-correlation spectra of *E. coli* sacculi alone and in interaction. (b) T_1 relaxation time values fitted from ^{13}C inversion−recovery experiments collected on peptidoglycan alone (black) and in interaction with the protein (red).

The flexibility of the peptidoglycan polymer can be evaluated from the transfer efficiency observed in proton-driven spin diffusion experiments (PDSD),[15] as an increased mobility yields reduced dipolar couplings. At short contact times (∼2 ms), very few cross peaks are detected, indicating the presence of significant dynamics along the entire polymer network. If the contact time is increased to ∼10 ms, correlations between spatially close carbons in the sugar rings are mainly observed. This suggests that the glycan strands are more rigid than the peptide chains, for which only few correlations are detected even for mixing times as long as 100 ms (Supporting Information Figure S2). The dynamics of the polymer network contribute to the high resolution observed in the NMR spectra.

Essentially complete resonance assignment of peptidoglycan has been obtained (Figure 1c). The glutamate and the lactic acid spin systems were unambiguously assigned. Interestingly, two sets of cross peaks were observed for DAP, which can be explained by the peptide cross-linking biodiversity (D-Ala-D-*m*-DAP and D-DAP-L-DAP) in *E. coli*.[5] Resonances of the two sugar units could not be unambiguously assigned. However, two resolved peaks were observed for the C-1, C-2, and C-3 sites. Additional resonances that could account for the small amount of 1,6-anhydro-MurNAc (3−6%)[5] were not detected. The L- and D-alanine could not be unambiguously assigned. Nevertheless the obtained assignment provides a sufficient base to start screening proteins for interaction sites with intact peptidoglycan sacculi.

To study peptidoglycan−protein interaction, sacculi were incubated with a new peptidoglycan interacting protein (YajG, protein with a yet unknown role in peptidoglycan metabolism[16]) using 50 μL of the previously studied sacculi preparation, incubated for 2 h at 37 °C with 1 mL of a 800 μM protein solution prepared in 50 mM MES at pH 6 (see Supporting Information Figure S3 for more details). After incubation, the sample was centrifuged in the solid-state NMR rotor and a through-bond ^{13}C-correlation experiment was collected. No chemical shift changes were observed upon interaction. However, cross-peak intensities were modified significantly and in a nonuniform manner. Figure 2a shows the ratio between the normalized cross-peak intensities in the spectrum of sacculi alone and in interaction with the protein. Glycan and lactic acid sites are clearly altered by the presence of the protein, while peptide cross-peaks remain nearly unchanged. To confirm the glycan as the preferential interaction site, ^{13}C longitudinal relaxation times (T_1) were measured on both samples. Results, presented in Figure 2b, show an overall increase in the ^{13}C T_1 relaxation times observed for the protein-interacting sacculi, that is, clearly more pronounced for the glycan resonances. Thus, the interaction with the protein induces an overall decrease in the mobility of the peptidoglycan network but targets primarily the glycan chains. Local conformational changes induced by the protein interaction are not observed for the main population as revealed by the absence of chemical shift differences.

In conclusion, we have shown that intact peptidoglycan sacculi of the Gram-negative bacterium *E. coli* give high-quality solid-state NMR spectra, allowing atom-resolved investigation of the peptidoglycan structure and dynamics. The spectra reflect the small heterogeneity of the peptidoglycan fragments constituting the sacculus, the synthesis and remodeling of which involves a large set of cellular proteins. We were able to observe the interaction of the sacculi with one of these proteins. This contribution demonstrates that solid-state NMR can be a powerful tool to screen for cell-wall interacting proteins. Furthermore, this technique has the potential to elucidate the mechanisms of the interactions between peptidoglycan and proteins such as Penicillin-binding proteins or proteins of the innate immunity.[4,17]

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Supporting Information Available: Details on the through-bond ^{13}C-correlation, proton-driven spin diffusion (PDSD) experiments, and sample preparations. This material is available free of charge via the Internet at http://pubs.acs.org.

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