Characterization of Cross-linking of Cell Walls of *Bacillus subtilis* by a Combination of Magic-angle Spinning NMR and Gas Chromatography-Mass Spectrometry of Both Intact and Hydrolyzed $^{13}$C- and $^{15}$N-Labeled Cell-wall Peptidoglycan*

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Cross-polarization magic-angle spinning $^{13}$C and $^{15}$N NMR, rotational-echo double resonance $^{13}$C NMR, and delays alternating with nutation for tailored excitation-difference $^{13}$C NMR spectra have been obtained from lyophilized cell walls of *Bacillus subtilis* grown on a synthetic medium containing D,-L-$^{[2-13]$C,$^{15}$N]$\text{aspartate}$ and D-$^{[1-13]$C}$alanine. Label from aspartate is incorporated into D-glutamic acid and $m$-diaminopimelic acid of cell-wall peptidoglycan, while label from alanine appears in the C-1 positions of both D- and L-alanine residues. The cross-link index (the fraction of peptide stems joined by an iso peptide covalent bond) is obtained directly from analysis of the results of the $^{13}$C NMR experiments. However, specific isotopic enrichments of cell-wall components cannot be obtained from NMR data alone. The latter are determined either from a gas chromatographic-mass spectrometric analysis of the amino acids derived from hydrolysis of cell-wall peptidoglycan, or from a combination of NMR and gas chromatographic-mass spectrometric results. The combined analysis is overdetermined and so involves the least error for evaluations of both the cross-link index and the isotopic enrichments. The cross-link index is 0.33 ± 0.03 for cell walls of *B. subtilis* grown in the presence of the antibiotic, cephalothin.

The structural component of bacterial cell walls is a cross-linked polysaccharide known as peptidoglycan (1). The peptidoglycan polysaccharide is derived from a monomeric unit of $\beta$-1,4-(N-acetylmuramyl N-acetylglycosamine) and is cross-linked through peptide stems pendant on the muramyl carboxyl group. In *Bacillus subtilis* polysaccharide the chain length was found to be about 168 dimer units (2). The most common type of cross-linking occurs as an $\epsilon$-amide or a peptide bridge between the C-terminal D-alanine of one stem and a diamino acid of a neighboring peptide stem. The peptide units may be classified as dimers, trimers, etc., depending upon the number of stems linked together. The peptide stem is biosynthesized as a pentapeptide comprising four alternating L- and D-amino acids (one of which is a diamino acid) plus a terminal D-alanine. The diamino acid is almost always L-lysine or $m$-diaminopimelic acid (Dpm). In some cases the peptidoglycan is covalently linked to polyribitol or polyglycerol phosphate polymers known as teichoic acids.

For different bacteria the sugar dimer is conserved, but the peptide stems and cross-links provide a source of diversity and a basis for classification (3, 4). The peptidoglycan of *B. subtilis*, classified as A1$_{7}$, is derived from the pentapeptide $L$-alanyl-$D$-isoglutaminyl-$m$-diaminopimoyl-$D$-alanyl-$D$-alanine. It is directly cross-linked by an amide bond between a D-alanine in the fourth position of one peptide stem and Dpm of another (5, 6). Additionally, the cell wall of *B. subtilis* contains a ribitol teichoic acid esterified in part by D-alanine (7). By determination of Dpm and dinitrophenyl-Dpm from hydrolysates of dinitrophenylated cell walls and by careful chromatography of autolyzed cell walls Warth and Strominger (5) determined that *B. subtilis* was cross-linked to the extent of 41% with 72% dimers and 7.2% trimers.

In previous work, we developed solids NMR methods to characterize the peptidoglycan of *Aerococcus viridans* (8–10) and the effects of penicillin on its structure (11). The solids NMR methods avoid the loss-prone solubilization and manipulations of cell walls required by other methods of studying peptidoglycan. The peptidoglycan of *A. viridans* has a direct cross-link between D-alanine and L-lysine, two amino acids easily labeled by direct incorporation with little scrambling. Cross-polarization magic-angle spinning $^{15}$N NMR was used to characterize cross-linking of the peptidoglycan by measuring the ratio of the intensity of the $^{15}$N signal from the $\epsilon$-amino of uncross-linked L-lysine to the intensity of the $\epsilon$-amide of cross-linked L-lysine. The peptidoglycan was found to be 49% cross-linked, a value reduced to about 34% when the bacteria were grown in the presence of 0.2 µg/liter penicillin (11).

In this paper we report the results of experiments to extend solids NMR to the characterization of cross-links in *B. subtilis* 6633. A complication in the labeling of *B. subtilis* 6633 is that the organism does not transport Dpm. Aspartate, a precursor of Dpm, is transported into the cell, but both carbon and nitrogen labels of aspartate are metabolized into purines and the amino acids of the aspartate family as well as Dpm. Aspartate is also a substrate for a transaminase which distributes the nitrogen to amino acids outside the aspartate family.

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1 The abbreviations used are: Dpm, diaminopimelic acid; DANTE, delays alternating with nutation for tailored excitation; CPMAS, cross-polarization magic-angle spinning; REDOR, rotational-echo double-resonance; BSSM, *B. subtilis* synthetic medium; MC/MS, mass chromatography-mass spectrometry.
These complications were overcome by isotopic labeling of both D-alanine and Dpm and by using GC/MS to assess site-specific activities of labeled cell-wall amino acids.

**MATERIALS AND METHODS**

**CPMAS 13C and 15N NMR—Cross-polarization transfers from protons to either 13C or 15N were made under matched spin-lock conditions at 38 kHz with magic-angle spinning at 3.205 kHz (12). Transfer times of 2 ms were used for all the spectra displayed in the figures. Peak intensities were corrected for proton rotating-frame relaxation by systematic variation of transfer times (13). Dipolar decoupling was performed at 36 kHz. Powdered samples with weights between 20 and 100 mg were contained in double-bearing zirconia rotors fitted with Kel-F end and drive caps.**

**REDOR 13C NMR—The pulse sequence used for REDOR experiments is described in the supplemental material. The single 13C π pulse is in the middle of the REDOR carbon-magnetization dephasing period refocuses all isotropic chemical shifts at the start of data acquisition (14). Application of 13N π pulses every half rotor cycle causes a net dephasing of the transverse magnetization of those carbons dipolar coupled to 15N (15). Weak REDOR difference signals (the difference in 13C carbon-rotational echo intensities with and without the 13N π pulse) can be obtained reliably because the operating conditions of the observation channel do not change from scan to scan. REDOR dephasing was summed over four rotor cycles with magic-angle spinning at 3.205 kHz. A four rotor-cycle dephasing period is optimal for detection of directly bonded 13C-15N pairs, for which the REDOR dephasing is approximately 90% of the full-echo signal (16). For 13C and 15N separated by 4 Å, the four-rotor cycle dephasing decreases to 0.4% of the full-echo signal. Residual spinning sidebands are not suppressed in REDOR experiments.**

**CPMAS DANTE 13C NMR—Centerband and sideband families can be inverted by asynchronous DANTE pulse trains whose irradiation sidebands are outside the spectrum of interest (17). Polarization imbalances created by selective DANTE irradiation are equilibrated during the delay period, 4, by spin exchange between 13C dipolar-coupled pairs of carbons (18). The extent of the exchange was calibrated using D,L-[1,13C]alanine under conditions in which centerbands and sidebands of carbon pairs are mismatched.**

**B. subtilis Growth on Defined Medium—Peptidoglycan was isolated from mid-log phase cells of B. subtilis (ATCC 6633) grown on a defined synthetic medium (BSSM) containing the following on a per liter basis: 2.81 g of KH2PO4, 34.0 g, 11.94 g of KH2PO4, 5 mg each of adenine, guanine, uracil, and thymine, 1 mg each of calcium pantothenate, riboflavin, and thiamine, 2 mg each of nicotinic acid and pyridoxine-HCl, 0.1 mg of biotin, 10 pg of folic acid, 7.6 mg of MnSO4·H2O, 10 mg of FeSO4·7H2O, 0.2 mg of MgSO4·7H2O, 11.92 g of KCl, 10.0 g of Na-glucose, 1.05 g of (NH4)2SO4, 40 μg of the antibiotic, cephalothin, and 0.1 g of all 20 common amino acids. The pH was adjusted to 7.0 before sterilization. The pH was adjusted to 7.0-7.4 for uniformly 13N-labeled cells the ammonium chloride was replaced by 15NH4Cl.**

**Cell Harvesting and Peptidoglycan Isolation—Bacteria were harvested by centrifugation at 10,000 × g for 10 min at 4°C and washed once by resuspension in cold, sterile 0.025 M potassium phosphate buffer. The cells were pelleted by centrifugation at 10,000 × g for 15 min at 4°C and resuspended in 50 ml of cold, sterile 0.025 M potassium phosphate buffer containing 5 mg of DNase, pH 7.0, and disrupted in the 60-ml chamber of a Bead-Beater ( Biospec Products, Bartlesville, OK) one-third full of 0.5-mm diameter glass beads. The beads were removed with a coarse sintered glass funnel and washed with 1 liter of a solution containing 0.1 M EDTA in sterile 0.025 M potassium phosphate buffer, pH 7.0, 4°C. Centrifugation of the filtrate at 17,700 × g for 30 min at 4°C provided crude cell walls. A suspension of the crude cell-wall pellet in sterile water was added dropwise with stirring to 100 ml of boiling 4% sodium dodecyl sulfate. The suspension was allowed to cool with stirring for 2 h, after which it was allowed to stand unstirred overnight at room temperature, then sedimented by centrifugation at 78,000 × g for 20 min at 20°C, and washed at least three times with sterile acetone. The pellet was incubated at 37°C with stirring for 15 h in 50 ml of 0.01 M Tris buffer, pH 8.2, containing 15 mg of trypsin, 15 mg of chymotrypsin, and 5 mg of DNase, then sedimented by centrifugation at 100,000 × g for 1 h at 20°C and washed three times with water.**

**Peptidoglycan Hydrolysis—**Typically, a weighed 10.0- to 20.0-mg sample of clean peptidoglycan in a capped 5-ml microprol product vial was hydrolyzed in 6 M HCl at 110°C for 48 h under nitrogen. The sample was dried in vacuo and rinsed three times with reagent grade water. L-Pipelic acid, the internal standard, was added to the dry sample, and the entire sample was then derivatized for GC/MS analysis by the method used for amino acid derivatization described below.**

**Amino Acid Derivatization—**N-Trifluoroacetyl n-propyl esters of both amino acid standards and amino acids resulting from the complete hydrolysis of B. subtilis peptidoglycan were prepared by the method of Abe et al. (20).**

**Gas Chromatography/Mass Spectrometry—**GC/MS analyses were performed using a Hewlett-Packard Model 5970B mass selective detector (Palo Alto, CA) equipped with a Hewlett-Packard Model 5998 gas chromatograph, Model 7673A autosampler, and Model 59940 Chemstation (Hewlett-Packard) computer. The gas chromatograph was operated in the electron impact mode at 70 eV. Separation of the D- and L-isomers of the N-trifluoroacetyl n-propyl ester of alanine and glutamic acid was accomplished on a 25 m × 0.25 mm I.d. Chirasil Val-II capillary column (Alltech Associates, Deerfield, IL), the D-isomers eluting first. Helium, at a flow rate of 1.5 ml/min, was used as the carrier gas. The injection port was maintained at 250°C, and the injector was operated in split mode at a ratio of 70:1. Both the N-trifluoroacetyl and N-acetyl n-propyl ester derivatives of diaminomelic acid standards and bacterial samples were run isothermally at 200°C. Typically, 1-μl injections were made using an autosampler.

**Quantification of Alanine, Diaminopimelic Acid, and Glutamic Acid—**Cell-wall amino acid compositions were obtained by mass spectroscopic monitoring of the gas chromatographic separations of the N-trifluoroacetyl n-propyl ester derivatives of hydrolyzed bacterial cell walls (21). The Glu:D-Ala:Dpm ratios were obtained from the total ion current by comparisons to mixtures of known composition. L-Alanine coeluted with an unknown material which accounted for about 10% of the total ion current of the peak. Therefore the D-Ala:L-Ala ratio was obtained by selective ion monitoring of the peak at m/z 175 and the Dpm:L-Ala ratio from the peak at m/z 190. The Glu:D-Ala:Dpm activities of D- and L-[1,13C2]alanine and D- and L-[15N]glutamate were determined using multiple line pair analyses as previously described (22).

**Isotopomer Analysis of Diaminopimelic Acid by GC/MS—**Ion clusters for fragments containing all labeled carbon and nitrogen atoms were found at m/z 406, 379, 353, and 320; for fragments containing both α-carbon atoms and a single nitrogen atom, at m/z 266, 224, 206, and 178; and for fragments containing one of the α-carbon atoms and one of the nitrogen atoms, at m/z 153. Intensities of the p+1, and p+2 peaks obtained in scan mode, after application of a natural abundance correction, could be solved for the isotopomeric specific activities E-I, Table I.

**RESULTS AND DISCUSSION**

**CPMAS 13C and 15N NMR—**The natural abundance CPMAS 13C NMR spectrum of B. subtilis cell walls has prominent peaks for the oxygenated carbons of sugar moieties (α: 65, 80, and 105), methyl carbons (α: 20), aliphatic carbons (α: 30), and carbonyl carbons (α: 175) of peptidoglycans (Fig. 1, top). The CPMAS 13C cell-wall spectrum obtained from B.

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2 Portions of this paper (including "Results" and Figs. S1-S7) are presented in miniprint at the end of this paper. Miniprint is easily readable with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
The pulse sequence of Fig. S2 (with bacteria grown on media containing a combination of D,L-[2-\(^{13}\)C,\(^{15}\)N]aspartate and its spinning sidebands are inverted (Fig. 60 and 175) are observed in the spectrum of cell walls from B. subtilis grown on medium containing D,L-[2-\(^{13}\)C,\(^{15}\)N]aspartate and D-[l-\(^{13}\)C]alanine. The minor peak in the REDOR difference spectrum arises from an overlap of spinning sidebands.

**Table I**

| Entry Structure | Relative concn | Specific activity |
|-----------------|----------------|-------------------|
| A D-[l-\(^{13}\)C]Ala | 0.41 | 0.685 |
| B l-[l-\(^{13}\)C]Ala | 0.99 | 0.625 |
| C [\(^{15}\)N]Glu• | 1.00 | 0.152 |
| D Dpm• | 0.87 | |
| E HOOC\((NH\_2)\_2\)(CH\(_2\)_3(CH\(_2\)_3)COOH | 0.65 | |
| F HOOC\((NH\_2)\_2\)(CH\(_2\)_3(CH\(_2\)_3)COOH | 0.10 | |
| G HOOC\((NH\_2)\_2\)(CH\(_2\)_3(CH\(_2\)_3)COOH | 0.02 | |
| H HOOC\((NH\_2)\_2\)(CH\(_2\)_3(CH\(_2\)_3)COOH | 0.14 | |
| I HOOC\((NH\_2)\_2\)(CH\(_2\)_3(CH\(_2\)_3)COOH | 0.09 | |
| J HOOC\((NH\_2)\_2\)(CH\(_2\)_3(CH\(_2\)_3)COOH | 0.01 | |

*Consists of 93% D-Glu and 7% L-Glu with equal specific activities.

\(^6\)Consists of 4% D-Dpm and 96% of a mixture of mostly \(m\)-Dpm with a trace of \(l\)-Dpm incompletely resolved under the derivatization and separation conditions reported.

**Fig. 1.** 50.3-MHz CPMAS \(^{13}\)C NMR spectra of cell walls of B. subtilis grown in media at natural abundance (top) or containing a combination of D,L-[2-\(^{13}\)C,\(^{15}\)N]aspartate and D-[l-\(^{13}\)C]alanine (bottom). Spinning sidebands are designated by ssb.

**Fig. 2.** 20.3-MHz CPMAS \(^{15}\)N NMR spectra of cell walls of B. subtilis grown in media at natural abundance (top) or containing D,L-[2-\(^{13}\)C,\(^{15}\)N]aspartate (bottom).

**Fig. 3.** 50.3-MHz REDOR \(^{13}\)C NMR spectra of cell walls of B. subtilis grown in media containing a combination of D,L-[2-\(^{13}\)C,\(^{15}\)N]aspartate and D-[l-\(^{13}\)C]alanine. The minor peak in the difference spectrum near 115 ppm arise from an overlap of spinning sidebands.

**Concentrations of Labels**—For the purpose of NMR analysis we describe the distribution of peptidoglycan labels in terms of the concentrations of \(^{13}\)C and \(^{15}\)N per peptidoglycan peptide stem (Fig. 5). This description is independent of the sugar composition of the isolated cell-wall material and is also independent of the number of terminal D-alanines of peptide stems. Neither of these quantities is known with certainty. We define \(C_{1}\) as the number of \(C\)-1 carbons of D-alanines per peptidoglycan peptide stem that are \(^{13}\)C-labeled, and \(C_{11}\) as the number of \(C\)-1 carbons of D-alanines per peptidoglycan peptide stem that are \(^{15}\)N-labeled. If there are on average two D-alanines per stem, and both of these are \(^{13}\)C-labeled, then \(C_{1} = 2.0\). If there is one D-alanine per stem, but on average only half of these are \(^{15}\)N-labeled, then \(C_{11} = 0.5\). If there is on average one-half D-alanine per stem, and only half of these are \(^{15}\)N-labeled, then \(C_{11} = 0.25\). Similarly,
Cross-linking of Cell Walls of *B. subtilis*

**NMR Intensity Ratios**—We describe intensity ratios observed in the $^{13}$C and $^{15}$N NMR experiments in terms of ratios of $C_i$ values.

1. The CPMAS $^{13}$C NMR spectrum (Fig. 1) gives the ratio of the $^{13}$C-labeled carbonyl to $\alpha$-carbon intensities,

$$R_1 = K_1(C_{11} + C_{22})(C_{12} + C_{21})$$  \hspace{1cm} (1)

2. The CPMAS $^{15}$N NMR spectrum (Fig. 2) gives the ratio of $^{15}$N-labeled amide to amine nitrogen intensities,

$$R_2 = K_2(N_{12} + 0.5N + 0.5N)/(0.5(1 - \rho)N)$$  \hspace{1cm} (2)

where $N = N_{12} + N_{22}$.

3. The ratio of the REDOR $^{13}$C difference intensity to full-echo intensity at $\delta_c$ 175 (Fig. 3) gives the ratio of $^{13}$C-$^{15}$N double-labeled peptide bonds to $^{13}$C single-labeled peptide bonds,

$$R_3 = K_3(C_{12}N_{12} + 0.5f_pN_1)/(C_{11} + C_{22})$$  \hspace{1cm} (3)

where $f_p$ is the isotopic specific activity of $\mathrm{D-[1-^{13}C]}$Ala in peptidoglycan. The second term in the numerator for the expression for $R_3$ contains $f_p$ rather than $C_1$ because cross-links do not form to D-Ala units in the fifth position of pentapeptide stems.

4. The ratio of the REDOR $^{13}$C difference intensity to full-echo intensity at $\delta_c$ 60 (Fig. 4) gives the ratio of $^{13}$C-$^{15}$N double-labeled $\alpha$-carbons to $^{15}$N single-labeled $\alpha$-carbons,

$$R_4 = K_4C_{22}/(C_{12} + C_{22})$$  \hspace{1cm} (4)

5. The ratio of the DANTE $^{13}$C difference intensity at $\delta_c$ 60 (Fig. 4) to the carbonyl-$\alpha$ carbon intensity at $\delta_c$ 175 gives the ratio of $^{13}$C-labeled $\alpha$-carbons with $^{13}$C-labeled carbonyl carbons that are two bonds away to the total $^{13}$C-labeled carbonyl carbons,

$$R_5 = 0.5f_pK_5(C_{12} + C_{22})/(C_{11} + C_{12})$$  \hspace{1cm} (5)

In writing these ratios, we assume that all natural abundance contributions to integrated intensities have been removed by subtraction of spectra of unlabeled cell-wall samples. The constants, $K_i$ values, are NMR corrections arising from (Equations 1 and 2) the differential effects of proton relaxation during the matched spin-lock transfer in the two CPMAS experiments (13); (Equations 3 and 4) the percentage dephasing for $^{13}$C-$^{15}$N bond lengths of 1.34 and 1.49 Å, respectively, during a 4-cycle REDOR experiment (16); and (Equation 5) the fraction of $^{13}$C-$^{12}$C polarization transfer in $30$ ms between the amide carbonyl carbon and $\alpha$-carbon separated by 2.49 Å with carbonyl and $\alpha$-carbon $T_1$ values of 6.7 and 6.6 s, respectively, under conditions of mismatched spinning sidebands in a DANTE-difference experiment (22). These constants are $K_1 = 1.00; K_2 = 0.83; K_3 = 0.95; K_4 = 0.66; K_5 = 0.56$. Observed intensity ratios for cell walls of *B. subtilis* grown in the presence of $\mathrm{D-[2-^{13}C,^{15}N]}$aspartate and $\mathrm{D-[1-^{13}C]}$alanine.

**Determination of the Cross-link Index**—The cross-link index, $\rho$, can be determined directly by NMR data using the product, $R_1$-$R_5$. Thus, from the data of Figs. 1 and 4, we obtain $\rho = 0.33$ for cell walls of *B. subtilis* grown in the presence of cephalothin. (In a subsequent paper we will describe the effect on *B. subtilis* peptidoglycan of growth in media with and without added cephalothin).

The distribution of label represented by Fig. 5 involves seven parameters, while only five parameters can be evaluated from NMR data. Thus, although the cross-link index can be determined from NMR data alone, no tests of internal self-consistency are available. A more reliable procedure is to...

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**Fig. 4.** 50.3-MHz DANTE $^{13}$C NMR spectra of cell walls of *B. subtilis* grown in media containing a combination of $\mathrm{D-[2-^{13}C,^{15}N]}$aspartate and $\mathrm{D-[1-^{13}C]}$alanine. The carbonyl-carbon peak in the bottom spectrum has been inverted by a selective train of weak pulses (using the sequence of Fig. 2). Only carbons that are connected by $^{13}$C-$^{13}$C one- or two-bond dipolar coupling contribute to the DANTE difference spectrum.

**Fig. 5.** Description of the incorporation of labels into the peptide stems of cell walls of *B. subtilis* grown in media containing a combination of $\mathrm{D-[2-^{13}C,^{15}N]}$aspartate and $\mathrm{D-[1-^{13}C]}$alanine. $C_i$ is the number of $C_i$ carbons of D-alanine per peptide stem that are $^{13}$C-labeled; $N_i$ is the number of $\alpha$-nitrogens of D-glutamic acid that are $^{15}$N-labeled; $C_2$ is the number of $\alpha$-carbons of Dpm that are $^{15}$N-labeled and are also directly bonded to a Dpm $^{13}$C; $C_{21}$ is the corresponding number of $\alpha$-nitrogens of Dpm that are $^{13}$C-labeled and are also bonded to a Dpm $^{15}$N; $C_{22}$ is the number of $\alpha$-carbons of Dpm that are $^{15}$N-labeled but are not bonded to a Dpm $^{13}$C. The cross-link index is $\rho$.

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**Description**

The carbonyl-carbon peak in the bottom spectrum has been inverted by a selective train of weak pulses (using the sequence of Fig. 2). Only carbons that are connected by $^{13}$C-$^{13}$C one- or two-bond dipolar coupling contribute to the DANTE difference spectrum.
combine the results of NMR and GC/MS experiments. For example, if we use the isotopic specific activities of \( \text{D-} \) and \( \text{L-} \) alanine of Table I, then

\[
C_{11} = c_{1A} \quad \text{and} \quad C_{12} = c_{1B}.
\]

where the \( c_i \) values are relative molar concentrations of amino acids from hydrolyzed peptidoglycan determined by GC, and the \( f_i \) values are isotopic specific activities determined by MS of the \( A, B, \ldots, J \) fragments identified in Table I. For the cell walls of \( \text{B. subtilis} \) grown in the presence of cephalothin on media containing \( \text{D-}[2,13\text{C},15\text{N}] \) aspartate and \( \text{D-}[1,13\text{C},15\text{N}] \) alanine, \( C_{11} = 0.280 \) and \( C_{12} = 0.619 \). Combining these values with the five experimental NMR intensity ratios, \( R_1 - R_5 \), leads to a determination of the remaining five parameters of Fig. 5 for this sample: \( \rho = 0.33; C_{21} = 0.086; C_{22} = 0.081; N_{12} = 0.13; \) and \( N = 0.23 \) (where \( N = N_{02} + N_{22} \)).

Three internal self-consistency checks are now available. From the NMR-determined \( C_i \) and \( N_i \) values and the GC-determined \( c_i \) values, we can predict the isotopic specific activities (\( f_i \)) of all MS-observed fragments (except those of \( \text{D-} \) and \( \text{L-} \) alanine which were used to evaluate the \( R_i \) values). The three observed and predicted isotopic specific activities are in agreement (Table II).

**Strategies for Combining NMR and GC/MS Results**—We rewrite the expressions for the NMR intensity ratios in terms of the GC/MS-determined quantities.

\[
R_i = K_i(c_{iA} + c_{iB})/[c_{iA}(f_i + f_0 + f_1 + f_2)]
\]

(7)

\[
R_1 = K_0(1 + \rho)c_0(f_0 + f_1 + f_2) + \rho c_0 c_f/[0.5(1 - \rho)c_0(f_0 + f_1 + f_2)]
\]

(8)

\[
R_2 = K_0(0.5pc_{0A}f_0 + f_1 + f_2) + \rho c_0 c_f/[c_{iA} + c_{iB}]
\]

(9)

\[
R_0 = K_0(f_0 + f_1)/(f_0 + f_1 + f_2)
\]

(10)

\[
R_5 = K_0(0.5pc_{0A}(f_0 + f_1 + f_2))/[c_{iA} + c_{iB}]
\]

(11)

With combined NMR and GC/MS results, we can determine

**Table II**

Specific activities of isotopes in amino acids of hydrolyzed cell walls of \( \text{B. subtilis} \)

| Isotope | GC/MS isotomers | Isotopic specific activity |
|---------|----------------|---------------------------|
| \( 15\text{N}_{0A} \) | C | 0.15 | 0.13 |
| \( 15\text{N}_{0B} \) | G + H + I + 2J | 0.27 | 0.23 |
| \( 13\text{C}_{0B} \) | P + G + I + J | 0.22 | 0.17 |

* Based upon \( \rho = 0.33, C_{21} = 0.086, C_{22} = 0.081, N_{12} = 0.13, \) and \( N = 0.23 \).

**Table III**

Ratios of intensities in five NMR experiments on hydrolyzed cell walls of \( \text{B. subtilis} \)

| Ratio | Measurement | Quantity | Observed |
|-------|-------------|----------|----------|
| \( R_1 \) | \( 13\text{C} \) CPMAS | Carboxyl to \( \alpha \)-carbon | 5.4 | 4.7 |
| \( R_0 \) | \( 13\text{N} \) CPMAS | Amide to amine | 3.0 | 3.2 |
| \( R_3 \) | \( 13\text{C} \) REDOR | Double-labeled carboxyls | 0.11 | 0.13 |
| \( R_2 \) | \( 13\text{C} \) REDOR | Double-labeled \( \alpha \)-carbons | 0.34 | 0.30 |
| \( R_5 \) | \( 13\text{C} \) DANTE | Carboxyl and \( \alpha \)-carbon | 0.017 | 0.019 |

* For \( \rho = 0.33 \), the data of Table I, and Equations 7-11.

the cross-link index, \( \rho \), with either \( R_0, R_5 \), or \( R_3 \), and the appropriate \( c_i \) and \( f_i \) values. For example, using \( R_1 \) and the GC/MS data of Table I pertaining to \( \text{D-} \) and \( \text{D-} \) glutamic acid and Dpm, we obtain \( \rho = 0.30 \). We believe that this is the most reliable combination of data to use because it involves the least complicated NMR experiment, and only MS fragments (or combinations of fragments) that produce high ion currents. Using \( R_5 \) (a ratio which requires the most complicated correction factor, \( K_5 \)) we also obtain \( \rho = 0.30 \). More reliable evaluations of \( R_5 \) may soon be possible using new magic-angle spinning \( ^{13}\text{C} \) NMR experiments to measure the concentration of isolated homonuclear pairs of spins (23). These experiments rely on dephasing rather than polarization transfer.

The carbonyl-carbon REDOR ratio, \( R_0 \), leads to \( \rho = 0.12 \). This ratio is dominated by the many \( ^{13}\text{C} - ^{15}\text{N} \) bonds formed between \( \text{L-} \)alanine and \( \text{D-} \)glutamic acid (dark lines, Fig. 5) and so is the least sensitive of the three intensity ratios that depend on the cross-link index, \( \rho \). A cross-link index as small as 0.12 seems implausible in view of the other values and the measurements of Warth and Strominger (7, 8).

If we assume the NMR-determined \( \rho = 0.33 \) (from the product of \( R_1 \) and \( R_2 \)), we can use all the GC/MS data to predict the observed intensity ratios from the five types of NMR experiments. The observed and predicted values are in reasonable agreement (Table III). We take the degree of self-consistency of Table III as the measure of NMR and GC/MS experimental accuracy. The cross-link index is therefore determined to \( \pm 0.03 \).

In conclusion, the most reliable determination of the cross-link index for cell walls of \( \text{B. subtilis} \) combines simple CPMAS \( ^{13}\text{C} \) NMR data (the amide to amine intensity ratio, \( R_2 \)) with GC/MS data on the relative concentrations and isotopic enrichments of \( \text{D-} \)glutamic acid and diaminopimelic acid. Results from experiments involving either single- or double-labeled aspartate can be compared directly. Such experiments can be performed in any laboratory equipped with conventional solid state NMR and GC/MS instruments.

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Supplemental Material.

Characterization of Cross-Linking of Cell Walls of Bacillus subtilis by a Combination of Magic Angle Spinning (MAS) NMR and 13C/15N/14N of Both Isotopic and Doped Carbon-13 Wall Pyrolysis.

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NMR Difference Experiments.

The pulse sequence used for DANTE difference experiments is shown in Figure S1. DANTE pulse trains can be used in total NMR to measure specific 13C connectivity in multiple 13C-labeled samples (17). The DANTE pulse trains consisted of 65 75-µs pulses generated by a 31MHz radio frequency. The spacing between the pulses was 1 µs, and a 3.3-msec interval immediately preceding the DANTE pulse train was used to ensure that the 31C resonance frequency in the selected polarization window, and to permit the decay of residual nuclear magnetization arising from pulse imperfections during the 31MHz unlocked spin-echo cross-polarization and retransmission parts of the experiment.

Simple REDOR Difference Spectra of Cell-Wall Pyrolysates. The full 13C NMR spectrum of cell walls of 8 samples grown on through-dialysis 13C labeled (13) pyruvate and (Figure S3, bottom left) is similar to the natural-abundance spectrum shown in Figure S1 (top left) except for the presence of a 13C isotope signal corresponding to the incorporation of label in the carbohydrates of pyrolysis. A significant REDOR difference signal is observed only in the 13C bond of double-labeled samples (Figure S3, top left). This indicates incorporation of some of the double-labeled isocitrate and succinate into cell-wall pyrolysis from DL-labeled than from L-labeled, and the distribution of isotopes from the two samples is the same.

Incorporation of Label from DL-13C-Labeled (13)Pyruvate and 3,4-Dihydroxyproline (3,4-DHP) into Cell-Wall Pyrolysis. According to the Tryp binocular pathways (Figure S4), both nitrogen and carbon derived from 13C pyruvate and the 13C pyruvate should be equally distributed between the D and L-13C carbon's of pyruvate that is used as an antecedent in other pathways by both nitrogen and carbon derived from 13C pyruvate. The carbon nitrogen dipolar decoupling determines the extent of dephasing ammonia or nitrogen derived from pyruvate. On or about loss lines, both 13C and 13N label are incorporated into cell-wall pyrolysis from DL-labeled than from L-labeled, and the distribution of isotopes from the two samples is the same.

A sample also has an active carbon resonance (21), so L-13C-3,4-DHP will form an NMR signal. This means that 13C-carbon bonds will appear as non-equivalent between L-13C-3,4-DHP and D-13C-3,4-DHP.The NMR spectra of these samples were obtained and subsequently labeled (Figures S5, 6). In particular, the 13C label was not incorporated at all in the presence of 13C pyruvate as an antecedent in other pathways. The REDOR difference signal at 10 and 17 ppm of 13C pyruvate was labeled by a combination of 13C pyruvate and D-13C pyruvate. The 13C isotope signal increases by factors of 1 and 2, respectively, as the number of carbon-decoupled pyruvates increases from 0 to 6 (Figure S7). The spectrum is for 13C pyruvate and 13C pyruvate of both samples and shows the 13C-13N and 13C-13C bound carbon of pyruvate.

Absence of Synchronization of Label. The 13N label from apparent extra only specific inclusions and the cell wall as evidenced by the component of the CPMASS-13 NMR spectrum of white cells labeled by L-13N pyruvate at 1 ppm of white cell uniformly labeled (Figure S5). In particular, the 13N label is not incorporated in the presence of 13C pyruvate as an antecedent in other pathways. The REDOR difference signal at 10 and 17 ppm of 13C pyruvate was labeled by a combination of 13C pyruvate and D-13C pyruvate. The 13C isotope signal increases by factors of 1 and 2, respectively, as the number of carbon-decoupled pyruvates increases from 0 to 6 (Figure S7). The spectral sequence is for 13C pyruvate and 13C pyruvate of both samples and shows the 13C-13N and 13C-13C bound carbon of pyruvate.

Determination of Specific Amounts of 13N Labeled by D-13C-Labeled (13)Pyruvate. We have assumed a model for in which the observed 13N content arise directly from the molecular level by a route not involving intramolecular transfer of a nitrogen from one carbon to the other a carbon. The assumption was obtained from the least-squares solution for the following set of equations which describe the normalized intensities of the p, q, and p-q peaks of the four signals containing both carbon and nitrogen, two carbons and one nitrogen, one carbon and two nitrogen.

\[ I_p = e + f \]
\[ I_q = e + g \]
\[ I_{p-q} = f + h \]
\[ I_{p+q} = e + g + h \]

where I is the peak intensity and e, f, g, and h are the peak intensities at the beginning of data acquisition. After the CP transfer, resonant decoupling removes the protons from the experiment. The irradiation for four rotor cycles.
Cross-linking of Cell Walls of B. subtilis

Figure S3. 50.3-MHz REDOR $^{13}$C NMR spectra of cell walls of B. subtilis grown in the absence of cephalothin in media containing D,L-[2-$^{13}$C, $^{15}$N]aspartate (left) or a combination of D-[l-$^{13}$C]alanine and L-[l-$^{15}$N]aspartate (right). The full-echo spectra after 4 rotor cycles are shown at the bottom of the figure, and the REDOR difference (the difference between with and without dephasing $^{15}$N pulse) at the bottom of the figure. The difference signal arises only from those $^{13}$C's directly bonded to $^{15}$N's. Each REDOR difference spectrum has only one peak.

Figure S4. Metabolic pathway showing the incorporation of $^{15}$N label derived from asparagine and D-m-diaminopimelic acid.

Figure S5. Incorporation of $^{13}$C label from alanine and $^{15}$N label from asparagine acid into peptide amines. Papillary micro-cross-links in cell walls of B. subtilis involve amide bond formation between the C-1 carbon of D-alanine and a side-chain amine nitrogen of m-diaminopimelic acid.

Figure S6. 20.3-MHz CPMAS $^{15}$N NMR spectra of whole cells of B. subtilis grown in media containing $^{15}$NH$_4^+$ (top) or L-$[^{15}$N]aspartate (bottom). The peak at 260 ppm in the bottom spectrum arises from labeled purines. Spinning sidebands appear at about -60 and +250 ppm.

Figure S7. 50.3-MHz REDOR $^{13}$C NMR spectra of cell walls of B. subtilis grown in media containing a combination of L-[2-$^{13}$C]alanine and D-[l-$^{15}$N]aspartate as a function of the number of rotor cycles with $^{15}$N dephasing.