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Dynamic structure of whole cells probed by nuclear Overhauser enhanced nitrogen-15 nuclear magnetic resonance spectroscopy

*(Escherichia coli/Bacillus licheniformis/*baker's yeast/Friend leukemic cells/*Staphylococcus aureus)*

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ABSTRACT **The proton-decoupled ^{15}N Fourier transform nuclear magnetic resonance (NMR) spectra of ^{15}N-enriched *Escherichia coli, Bacillus licheniformis,* baker's yeast, and Friend leukemic cells were obtained. The ^{15}N NMR spectra of whole cells displayed ^{15}N resonances originating from (*i*) protein backbones with lysine, arginine, and histidine side chains, (*ii*) ribonucleic acids, (*iii*) peptidoglycan, and (*iv*) phospholipids. Several additional amino and amide resonances were observed but not identified. In bacteria and yeast, the cell wall was found to be the site of a relatively mobile group of molecules, whose resonances dominate the proton-decoupled ^{15}N NMR spectra of whole cells. ^{15}N NMR chemical shifts and nuclear Overhauser effects have provided information on the *in vivo* structure of cell wall peptidoglycan. In *Staphylococcus aureus* the pentaglycine cross-bridge of cell wall peptidoglycan was found to have a random coil conformation. In *B. licheniformis* considerable segmental motional freedom was detected in teichuronic acid and peptidoglycan polysaccharide chains in the wall of the intact cell.**

Nuclear magnetic resonance (NMR) spectroscopy is a powerful tool for obtaining information on the electronic structure, conformation, solvation, and dynamics of molecules and can be applied to systems as complex as biological cell cultures and tissues. The NMR technique has proved to be particularly useful for monitoring the metabolic reactions of small molecules in living cells (1–4). NMR measurements have also provided information on the motional freedom of small molecules in different cellular compartments and cell fractions (5). The NMR spectra of molecules that comprise the structural components of cells are not easily obtained (6).

Although the ^{1}H, ^{13}C, and ^{31}P NMR spectra of complex biological systems are being actively studied, the ^{15}N spectrum of only one cell system, the fungus *Ustilago sphaerogena*, has been reported (7). The scope of ^{15}N NMR spectroscopy of whole cells remains to be determined with respect to (*i*) the cellular components that can be studied, (*ii*) the ability of the ^{15}N-{^{1}H} nuclear Overhauser effect nulling (8) to simplify complex spectra and provide dynamic information, and (*iii*) the need of specific isotopic labeling for making specific assignments and reducing the number of observed resonances. ^{15}N NMR spectroscopy appears to be well suited to probing the structural organization of cells for several reasons. The nitrogen atoms are often found in close proximity to the sites of the intermolecular interactions that play important roles in determining the structure and function of biological molecules. ^{15}N chemical shifts are particularly sensitive to molecular conformation and noncovalent bonding interactions and correlations are actively being sought in small molecules (9–11). ^{15}N NMR spectra of structural molecules may be better resolved than ^{1}H and ^{13}C NMR spectra, because the smaller gyromagnetic moment of the ^{15}N nucleus makes its NMR resonances less susceptible to dipolar line broadening. The low sensitivity of ^{15}N can now be overcome by isotopic labeling and the use of high sensitivity Fourier transform and wide gap spectrometers.

In this paper we survey the ^{15}N NMR spectra of a series of ^{15}N-enriched Gram-positive and Gram-negative bacteria, yeast, and murine Friend leukemic cells. These spectra demonstrate the types of cellular components whose ^{15}N resonances can be detected in proton-decoupled ^{15}N NMR of whole cells and indicate which problems in cell organization lend themselves to ^{15}N NMR techniques. Interestingly, the majority of resonances observed in whole cells of bacteria and yeast originate not from small molecule metabolites but from a relatively mobile group of cell wall components. These latter resonances have provided insights into the organization of the Gram-positive bacterial cell wall.

Abbreviations: NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect.

MATERIALS AND METHODS

Isotopically Labeled Compounds. [^{15}N]Ammonium chloride, enriched to 90–95% ^{15}N, was prepared by standard methods (12) from [^{15}N]nitric acid (obtained from the Isotope Separation Plant of the Weizmann Institute). [^{15}N]Glycine (95%) was prepared by the method of Schoenheimer (13). ^{15}N-labeled algal amino acid mixture was prepared from *Chlorella pyrenidoza* grown on $K^{15}NO_3$ (14).

Cells, Media, and Growth Conditions. *Escherichia coli* B/r was grown at 37° with aeration to the early stationary phase (OD 0.55 at 680 nm) in Spizizen salts medium (15), in which 0.2% $(NH_4)_2SO_4$ was replaced by 0.1% $^{15}NH_4Cl$. *Bacillus licheniformis* ATCC 9945 was grown at 37° with aeration to the mid-logarithmic (OD 0.21), late logarithmic (OD 0.37), early stationary (OD 0.55), and stationary (4 hr at OD 0.67) phases in ^{15}N-labeled Spizizen salts medium. *Staphylococcus aureus* H was grown at 37° with aeration to the late logarithmic phase (OD 0.49) in basal medium (16), in which 0.1% $(NH_4)_2SO_4$ was replaced with 0.05% NH_4Cl and supplemented with the Coutinho and Nutini amino acid/vitamin mixture (17), which contained [^{15}N]glycine (95% ^{15}N, 200 mg/liter) in place of nonlabeled glycine. Baker's yeast (*Saccharomyces cerevisiae*) was grown to the stationary phase at 37° with aeration on basal media, in which 0.1% $(NH_4)_2SO_4$ was replaced with 0.1% $^{15}NH_4Cl$. Friend-virus-induced murine leukemic cells (clone 707) (18) were grown in a humidified 5% CO_2 atmosphere at 37° in minimum essential medium with Earl's salts and antibiotics, in which the amino mixture was replaced with ^{15}N-labeled algal amino acid mixture, supplemented with arginine (100 mg/liter), cysteine (24 mg/liter), methionine (13 mg/liter), threomine (35 mg/liter), phenylalanine (27 mg/liter), and glutamine (240 mg/liter) and harvested after 4 days (3×10^6 cells per ml).

Intact Cell Samples. *E. coli* and baker's yeast cells were harvested by centrifugation in the cold ($6000 \times g$, 10 min) and washed three times with 100 ml of distilled water and packed

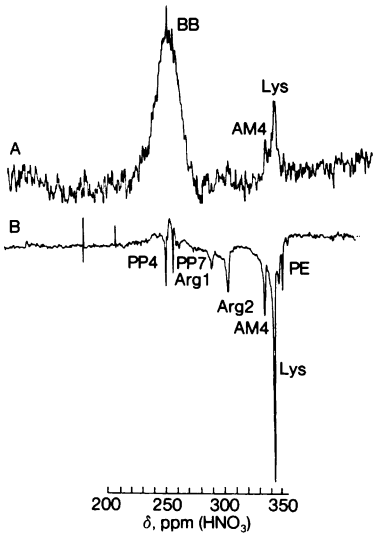

FIG. 1. The undecoupled (*A*) and proton-decoupled (*B*) 9.12 MHz ^{15}N NMR spectra of ^{15}N-enriched *E. coli* cells. Spectral conditions: 90° pulse angle; 121,000 accumulations; 6000 Hz spectrum range; ±0.16 ppm resolution; 1.8 Hz exponential decay filter; 27°C. Resonance frequencies (ppm): BB, 230–270; PP4, 258.5; PP7, 264.2; Arg1, 291.2; Arg2, 304.4; AM4, 335.6; Lys, 343.0; PE, 350.0.

(10,000 × *g*, 0.5–3 hr) into an 10 mm NMR tube fitted with a 5 mm concentric tube containing D_2O. *B. licheniformis* and *S. aureus* cells were harvested and washed thoroughly with distilled water, after 4% sodium dodecyl sulfate had been added to the growth medium to inactivate autolysins. Friend leukemic cells were harvested in the cold (2000 × *g*, 15 min) and washed three times with 25 ml portions of 310 isotonic milliosmolar sodium phosphate buffer, pH 7.

^{15}N NMR Measurements. ^{15}N NMR spectra were obtained on a Bruker HFX-90 spectrometer operating at 9.12 MHz. The ^{15}N NMR spectra represent the Fourier transform (exponential filtering = 0.94 Hz) of the 30,000–60,000 averaged free induction decays following 90° pulses collected in 4096 data points at a dwell time of 166 μsec, unless otherwise stated. ^{15}N chemical shifts were determined from an external reference of 2 M $^{15}NH_4Cl$ in 2 M HCl. However, the spectra are presented and chemical shifts are given in terms of the $H^{15}NO_3$ chemical shift scale of Roberts (19), using a conversion factor $\delta_{HNO_3} - \delta_{NH_4Cl} = -352.7$ ppm.

RESULTS

E. coli. The proton-nondecoupled ^{15}N NMR spectrum (Fig. 1*A*) of ^{15}N-enriched intact *E. coli* cells consists of a lysine-N_ϵ resonance, a protein backbone amide envelope of resonances (BB), and an unidentified amino resonance (AM4). The assignment of these resonances to globular cellular proteins is consistent with the similarity of the spectrum to the proton-nondecoupled spectrum of nonspecifically labeled [^{15}N]hemoglobin (20, 21).

The proton-decoupled ^{15}N spectrum of *E. coli* cells displays narrow and inverted resonances originating from protein side-chain lysine-N_ϵ, arginine-N_ϵ, and arginine-$N_{\omega,\omega}$ nitrogens (Lys, Arg2, and Arg1, respectively), phosphatidylethanolamine nitrogen (PE), and unidentified amino (AM4) and amide (PP4 and PP7) resonances. *The envelope of protein backbone amide resonances (BB) has been nulled by proton broad band noise decoupling.*

The Lys, Arg1, and Arg2 resonances were assigned by comparison to natural abundance ^{15}N NMR spectra of free amino acids at pH 5.0 and to the chemical shifts reported in the literature (19, 22). The PE resonance was assigned on the basis of its disappearance from the ^{15}N spectrum of the cell envelope after chloroform/methanol extraction. Only the amide PP4 and PP7 resonances are exclusively associated with the cell protoplast, because the other resonances were present in the ^{15}N NMR spectrum of the cell envelope. No free amino acid resonances were observed and it is likely that the ^{15}N resonances of amino acids and other small nitrogenous molecules, with T_1 relaxation times longer than 1 sec, had been saturated by the fast repetition times used in these experiments. The resonances that were observed were not saturated, because their relative intensities did not change on decreasing the pulse length from 90° to 30° or increasing the recycle time from 332 to 664 msec. We conclude that intact cell proton-decoupled ^{15}N NMR spectra obtained with fast repetition rates (<1 sec) will be dominated by the resonances of cellular structural components associated with the cell wall, rather than by free metabolites.

B. licheniformis. In Gram-positive bacteria, the protein- and phospholipid-rich outer membrane of the cell envelope is replaced by a thicker cell wall consisting of peptidoglycan, teichoic acid, and teichuronic acid. The different chemical composition of the Gram-positive cell wall is amply demonstrated by the proton-decoupled ^{15}N NMR spectrum of the Gram-positive *B. licheniformis* cells (Fig. 2*B*). The phosphatidylethanolamine resonance is absent. The Lys resonance is weak and broadened. The Arg1 and Arg2 resonances are also weak and vary in intensity among different batches of cells. However, the spectrum displays a relatively intense set of resonances (AM4, P5, and P9) that originate from cell wall components. The P5 resonance is assigned to the acetamido nitrogen of cell *N*-acetylglucosamine, *N*-acetylmuramic acid, and *N*-acetylgalactosamine residues of cell wall peptidoglycan and teichuronic acid, because (*i*) one of the two ^{15}N resonances observed in the proton-decoupled natural-abundance ^{15}N spectrum of *N*-acetylglucosamine in aqueous solution corresponds to P5, (*ii*) cells grown in the presence of nonlabeled *N*-acetylglucosamine had a P5 resonance that was significantly reduced in intensity, and (*iii*) the P5, P9, and AM4 resonances are found in the ^{15}N NMR spectra of the lysozyme digests of *B. licheniformis* cell wall particles freed of proteins and teichoic and teichuronic acids.

The assignment of the intact cell ^{15}N resonances to cell wall structural components rather than to labile metabolite molecules is supported by the fact that (*i*) the intact cell and isolated cell wall spectra are identical, (*ii*) two consecutive intact cell spectra, obtained over the course of 24 hr, did not differ, and (*iii*) the spectra of cells harvested in mid-logarithmic phase, late logarithmic phase, and 4 hr into the stationary phase did not differ significantly. The observed intact cell resonances were not partially saturated, because decreasing the pulse angle from 90° to 30° did not change the relative amplitudes of the resonances.

Increasing the temperature of the cell sample to 47° produced dramatic changes in the *B. licheniformis* cell ^{15}N spectrum, which reflect the thermal denaturation of proteins and the unfolding of ribonucleic acids. The ^{15}N NMR spectrum of *B. licheniformis* cells at 47° (Fig. 3) displays (*i*) relatively intense Arg1 and Arg2 resonances, (*ii*) a noninverted histidine-$N_{\delta,\epsilon}$ nitrogen resonance (His) (22), and (*iii*) a set of ribonucleic acid resonances (G1, A1, C1, U1, U2, C2, A2, and G3) (14), in addition to the Lys, AM4, P5, and P9 resonances that were observed at 27°.

Baker's Yeast. The yeast cell wall consists of proteins and nitrogen-free polysaccharides (mannans), as compared to the

nitrogen-rich cell wall components of bacteria. The proton-decoupled ^{15}N NMR spectrum of baker's yeast cells (Fig. 2*C*) is markedly different from the spectra of bacterial cells. It displays relatively intense protein side-chain group resonances (Lys, Arg1, and Arg2). In place of the narrow peptidoglycan resonance (P5) a broad envelope of peptide resonances (PP1) is observed. Amino resonances (AM3 and AM8), and an unusual noninverted amine resonance (AM9) were observed, but not identified. These resonances apparently originated from the cell wall, because the intact cell spectrum was identical to the ^{15}N spectrum of cell walls prepared by freeze-thaw osmotic shock (4). Interestingly, the yeast proteins and ribonucleic acids did not undergo thermal denaturation at 47°, as had occurred in *B. licheniformis* cells.

Friend Leukemic Cells. The proton-decoupled ^{15}N spectrum of Friend leukemic cells (Fig. 2*D*) is relatively simple and consists of Lys resonance and an envelope of peptide resonances (PP2). Because these cells were grown on a medium containing nonlabeled arginine, the Arg1 and Arg2 resonances have not been observed. Trypan-blue staining of cells upon completion of the NMR measurements indicated that about 75% of the cells remained viable during 5 hr of spectrum accumulation at 27°. The simplicity of the spectrum of Friend leukemic cells is consistent with the absence in the mammalian cell of a cell wall, which appears to be the source of many of the resonances observed in the ^{15}N spectra of microorganisms.

Selectively Labeled *S. aureus* Cells. The ^{15}N NMR spectrum of *S. aureus* cells that have been specifically labeled with [^{15}N]glycine (Fig. 4*A*) displays a single intense resonance at 267.8 ppm. This resonance is assigned to the cell wall peptidoglycan pentaglycine cross-bridge with a random coil conformation, because (*i*) an identical resonance is obtained from the isolated cell wall (Fig. 4*B*), (*ii*) glycine is the major amino acid component of *S. aureus* peptidoglycan and forms a pentaglycine cross-bridge, and (*iii*) the 267.8 ppm chemical shift is identical to that of the middle glycyl residue of *N*-acetyltriglycine in aqueous solution and the major resonance of unfolded [*glycyl*-^{15}N]hemoglobin, both of which have random coil, water-solvated conformations (21). Glycyl residues in other conformations would have different chemical shifts, as demonstrated by native [*glycyl*-^{15}N]hemoglobin, whose ^{15}N chemical shifts range from 256 to 276 ppm (21).

DISCUSSION

The Scope of Whole Cell ^{15}N Spectra. The applicability of ^{15}N NMR spectroscopy to problems in cell biology ultimately depends on whether the ^{15}N resonances of a particular nitrogen-containing group can be detected in an intact cell and can be resolved from the resonances of similar nitrogen groups in different types of molecules and from similar molecules in different cellular compartments. The ^{15}N NMR spectra of intact cells might be expected to display resonances originating from polypeptides and their nitrogenous side chain groups, purines and pyrimidines, amino- and acetamidosugars, phospholipids, and various small nitrogenous metabolite molecules. The intensities of the ^{15}N resonances of these molecules are determined primarily by their linewidths and ^{15}N-{^1H} nuclear Overhauser effects. These NMR parameters are related to the dynamic structure of the cell, and to a first approximation can be related to a single correlation time (τ_c) which describes isotropic tumbling or segmental motion.

The correlation time (τ_c) dependence of line broadening by ^{15}N–^1H dipolar interaction for an N—H group indicates that the ^{15}N resonances of molecules with $\tau_c > 100$ nsec will be very broad (>200 Hz), and usually will not be observable. The effect

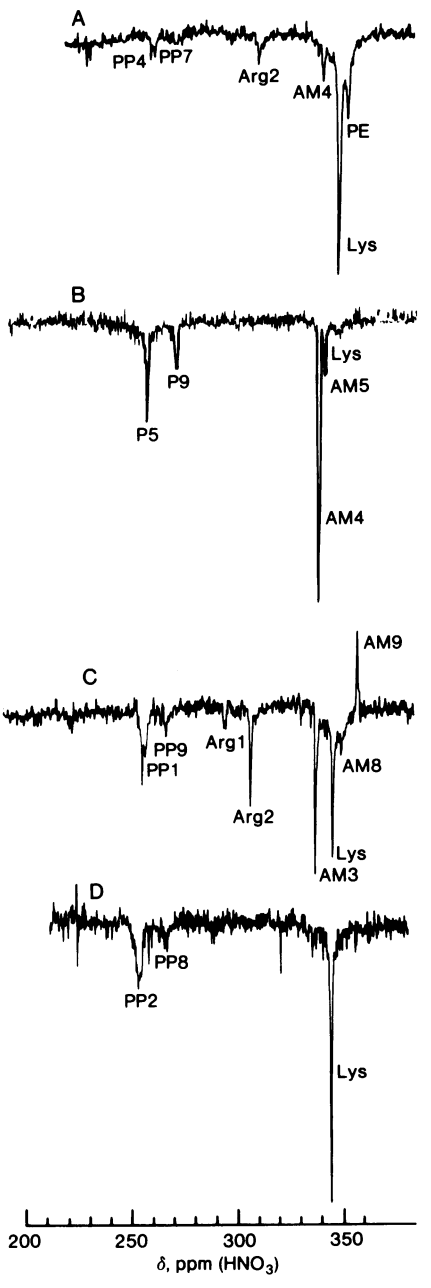

FIG. 2. The proton-decoupled 9.12 MHz ^{15}N NMR spectra at 27°C of ^{15}N-enriched intact cells of (*A*) *E. coli*, (*B*) *B. licheniformis*, (*C*) baker's yeast, and (*D*) Friend leukemic cells. Spectral conditions were as given in *Materials and Methods*. Resonance frequencies (ppm): PP1, 253.5; PP2, 254.2; PP4, 258.5; PP7, 264.2; PP8, 267.7; PP9, 272.8; P5, 253.4; P9, 267.5 Arg1, 291.2; Arg2, 304.4; AM3, 334.3; AM4, 335.6; AM8, 346.5; AM9, 354.9; Lys, 343.0; PE, 258.5.

of correlation time on the ^{15}N-{^1H} nuclear Overhauser effect (NOE = proton-decoupled intensity/proton-nondecoupled intensity) indicates that three types of ^{15}N resonances are expected in proton broad band decoupled spectra of intact cells: (*i*) resonances that are enhanced and inverted (Noe approximately −4) corresponding to molecules with $\tau_c < 1$ nsec; (*ii*) resonances of molecules with τ_c about 5 nsec that are nulled and made unobservable (NOE approximately 0) by proton decoupling; and (*iii*) resonances that are only slightly attenuated (NOE about 0.9) originating from molecules with $\tau_c > 10$ nsec. A fourth possibility is a narrow proton-nondecoupled resonance that is nulled or remains uninverted on proton decoupling, and

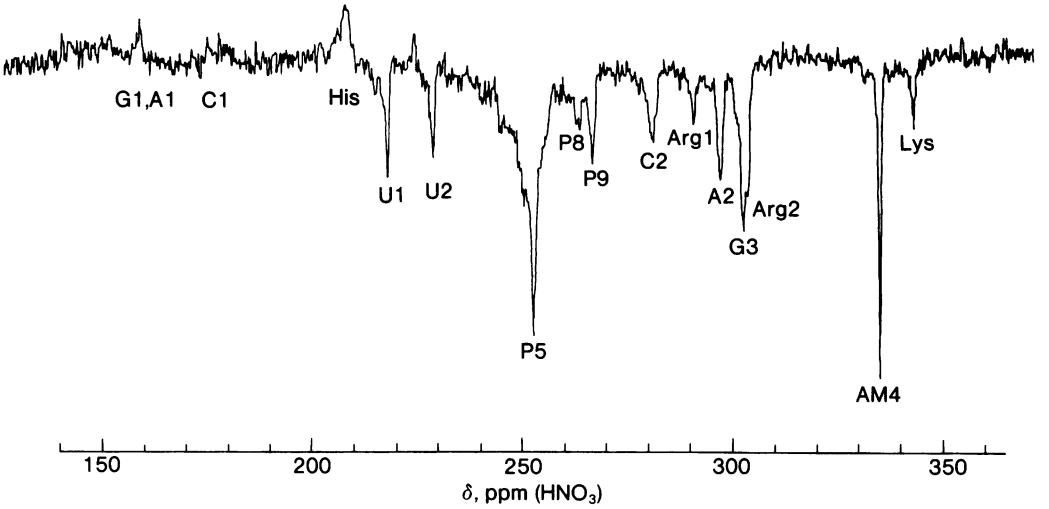

FIG. 3. The proton-decoupled 9.12 MHz ^{15}N NMR spectrum of ^{15}N-enriched *B. licheniformis* cells at 47°C. Spectral conditions were as in *Materials and Methods*. Resonance frequencies (ppm): G1, A1, 158; C1, 177; U1, 218.3; U2, 229.0; C2, 282.0; A2, 297.9; G3, 303.2; His, 207.7; Arg1, 291.2; Arg2, 304.4; Lys, 343.0; AM4, 335.6.

that corresponds to relatively mobile molecules coordinated to or in close proximity to paramagnetic ions (23, 24).

Among the components of the cell protoplast, only the proteins have produced observable ^{15}N NMR resonances. These resonances are nulled on proton decoupling. The nulling of the protein backbone amide ^{15}N resonances in whole cells could be the result of either all cellular proteins having τ_c = approximately 5 nsec and NOE about 0 or the averaging out of inverted resonances of fast tumbling proteins with the noninverted resonances of slower tumbling proteins. The ^{15}N NMR resonances of free small nitrogenous molecules have either been saturated or their NOEs have been nulled by the presence of trace amounts of paramagnetic ions. The use of large samples of highly ^{15}N-enriched cells in wide-gap spectrometers will apparently be required to offset the loss of signal-to-noise ratio associated with longer recycle times required to avoid saturation effects. The absence of purine and pyrimidine resonances from intact cell spectra at 27° indicates that little segmental motion occurs in the tightly hydrogen-bonded, slowly tumbling nucleic acids. Cellular dynamic structure makes the cell protoplast essentially transparent in ^{15}N-{^1H} nuclear Overhauser enhanced ^{15}N NMR measurements. *This restricts ^{15}N NMR observations of intact cells to cell wall components.*

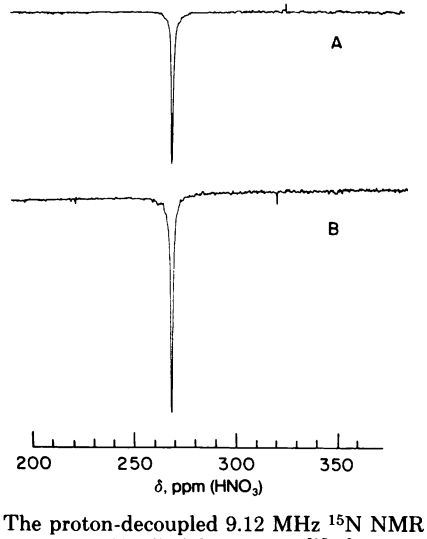

FIG. 4. The proton-decoupled 9.12 MHz ^{15}N NMR spectra at 27°C of *S. aureus* specifically labeled with [^{15}N]glycine: (*A*) intact cells, (*B*) cell walls. Spectral conditions were as in *Materials and Methods*.

Cell Wall Dynamic Structure *In Vivo*. The three-dimensional models proposed for cell wall peptidoglycan (25–29) consist of rigid, extensively hydrogen-bonded mosaics of peptidoglycan subunits, while the x-ray diffraction patterns of *B. licheniformis* and *S. aureus* cell walls (30) as well as the elastic behavior of bacterial cell walls (31) have indicated that peptidoglycan behaves as a randomly crosslinked polymer with much free rotation. The random-coil conformation of a large percentage of the pentaglycine cross-bridges of *S. aureus* cell wall peptidoglycan is more consistent with a mobile conformation than with the extensively hydrogen-bonded β-pleated sheet conformation used in model-building studies (25). The observation of peptidoglycan resonances in the cell walls of intact cells is consistent with the existence of considerable segmental motion, oscillations, conformational jumps, and internal rotations within the native peptidoglycan matrix. Because changes in morphology and tensile strength of the cell wall will most probably affect peptidoglycan τ_c and the relative intensities of its ^{15}N-{^1H} NOE enhanced ^{15}N resonances, ^{15}N NMR promises to be a useful probe of the physiological state of the bacterial cell wall.

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