Rotational-Echo Double Resonance Characterization of the Effects of Vancomycin on Cell Wall Synthesis in *Staphylococcus aureus*†

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ABSTRACT: Cross-polarization magic-angle spinning and rotational-echo double resonance ^{13}C and ^{15}N NMR experiments have been performed on intact cells of *Staphylococcus aureus* labeled with D-[1-^{13}C]alanine and [^{15}N]glycine or with [1-^{13}C]glycine and L-[ϵ-^{15}N]lysine. The cells were harvested during stationary or exponential growth conditions, the latter in media with and without the addition of vancomycin. The results of these experiments allowed the in situ determination of the relative concentrations of peptidoglycan cross-links (the number of peptide-stem D-alanines covalently linked to a pentaglycyl bridge) and bridge-links (the number of peptide-stem lysines covalently linked to a pentaglycyl bridge). The concentration of cross-links remained constant in the presence of vancomycin, whereas the number of bridge-links decreased. These changes suggest that vancomycin (at therapeutic levels) interrupts peptidoglycan synthesis in *S. aureus* by interference with transglycosylation.

Vancomycin and other closely related glycopeptide antibiotics inhibit the biosynthesis of the peptidoglycan of the cell wall of Gram-positive bacteria (*1*). The antibiotics do not penetrate into the cytoplasm of the cell but apparently form complexes with the D-Ala-D-Ala carboxyl termini of peptidoglycan precursors outside the cell membrane. This binding presumably interferes with transglycosylase activity (*2*, *3*) and possibly transpeptidase activity as well (*1*), both of which are essential for the synthesis of new cell wall. The former extends the glycan chain and the latter cross-links the peptide stems with subsequent elimination of the terminal D-Ala. Recent experiments on permeabilized cells of Gram-negative *Escherichia coli* suggested that vancomycin inhibited transpeptidase rather than transglycosylase activity (*4*). In general, however, it is difficult to determine whether just one or the other of the vancomycin inhibition mechanisms predominates in vivo or whether both mechanisms are operating (*5*).

Stable isotope labeling of Gram-positive *Staphylococcus aureus* is efficient because of the ready incorporation of exogenous alanine, glycine, and lysine (*6*). This means that ^{13}C and ^{15}N labels in these amino acids are incorporated in the bridges, bridge-links, and cross-links of the cell wall peptidoglycan (Figure 1). Detection of the labels is possible by magic-angle spinning ^{13}C and ^{15}N NMR and their through-space proximities by $^{13}C\{^{15}N\}$ and $^{15}N\{^{13}C\}$ rotational-echo double resonance (REDOR)[1] (*7*−*11*). Such experiments are well suited to explore the effects of vancomycin on cell wall metabolism in vivo.

In this paper we describe the use of solid-state NMR to determine the effect of vancomycin on cell wall assembly in normal *S. aureus* during active cell division. The results of solid-state NMR experiments performed on ^{13}C- and ^{15}N-labeled cell walls and cell wall precursors in whole cells are interpreted in terms of the effect of therapeutic levels of vancomycin on the relative concentrations of peptidoglycan cross-links and bridge-links. These are direct measurements, not inferences, made in situ, without perturbation of the cell wall. The experiments are performed on intact cells so that cell wall and cytoplasmic peptidoglycan precursors are measured together. The combination of results on cross-links and bridge-links suggests that vancomycin (at therapeutic levels) interrupts peptidoglycan synthesis in *S. aureus* by interference with transglycosylation.

EXPERIMENTAL PROCEDURES

Growth and Labeling of Cells. Stable isotope labeling was performed with *S. aureus* (ATCC 6538P) grown on a defined medium (SASM) as described by Tong et al. and Kim et al. (*6*, *11*). Bacteria were grown in SASM in which the natural abundance D-alanine, glycine, or L-lysine was replaced by D-[1-^{13}C]alanine and [^{15}N]glycine or by [1-^{13}C]glycine and L-[ϵ-^{15}N]lysine. In the D-[1-^{13}C]alanine and [^{15}N]glycine experiments, each flask contained 380 mL of SASM.

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[1] Abbreviations: CPMAS, cross-polarization magic-angle spinning; ΔS, $S_0 - S$, where S and S_0 are rotational-echo double-resonance signal intensities with and without dephasing pulses, respectively; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; REDOR, rotational-echo double resonance; SASM, *Staphylococcus aureus* synthetic medium; ssb, spinning sideband.

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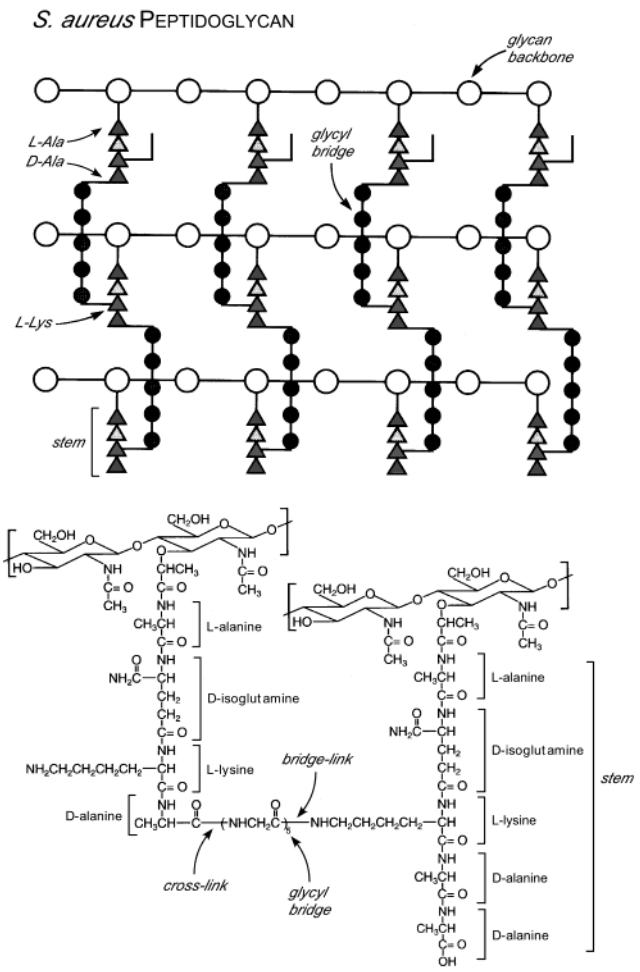

FIGURE 1: (Top) Schematic representation of an idealized version of the cell wall peptidoglycan of *S. aureus* (after Stryer). A four-unit peptide stem (triangles) having the sequence L-Ala-D-Glu-L-Lys-D-Ala is attached to every second sugar of the glycan backbone (open circles). Cross-linking between glycans occurs through pentaglycyl bridges (dark circles) connecting the carbonyl carbon of D-Ala of the fourth position of one stem with the ϵ-nitrogen of L-Lys of the third position of another. (Bottom) Chemical structure of the peptidoglycan of *S. aureus*, with three sites identified for potential labeling: cross-link, bridge-link, and pentaglycyl bridge. The five-residue stem on the right has no cross-link to its D-Ala.

Exponential phase cells grown without vancomycin (one flask) were harvested at $OD_{660} = 0.7$ and stationary phase cells (four flasks) at $OD_{660} = 1.3$. When vancomycin was added during growth, it was added (to five flasks) for a final concentration of 20 μg/mL at $OD_{660} = 0.2$ and harvested 100−120 min later at $OD_{660} \cong 0.4$. In the [1-^{13}C]glycine and L-[ϵ-^{15}N]lysine experiments, each flask contained 300 mL of SASM. Vancomycin at a final concentration of 0, 6, or 23 μg/mL was added at $OD_{660} = 0.2$, and the cells were harvested at 50 min, which corresponded to one doubling time of the nontreated cells. Bacteria were harvested by centrifugation at 10000*g* for 20 min at 4 °C and washed three times by resuspension and repelleting in cold, sterile 0.025 M potassium phosphate buffer, pH 7.0. The final pellets were frozen and lyophilized for NMR.

CPMAS and REDOR NMR. Cross-polarization magic-angle spinning ^{13}C and ^{15}N NMR spectra of cells were acquired with a Chemagnetics/Varian CMX-300 spectrometer operating at 300.02 MHz for ^{1}H, 75.45 MHz for ^{13}C, and 30.40 MHz for ^{15}N. Experiments were performed at ambient temperature using a four-frequency transmission probe. Other experimental conditions included 2 ms 50 kHz ^{1}H−^{13}C and ^{1}H−^{15}N matched cross-polarization transfers, 50 kHz B_1 fields for the ^{13}C and ^{15}N π pulses, and 80 kHz proton decoupling. The chemical shift scale of the ^{13}C NMR spectra was referenced to external liquid tetramethylsilane and that of the ^{15}N NMR spectra to external solid ammonium sulfate.

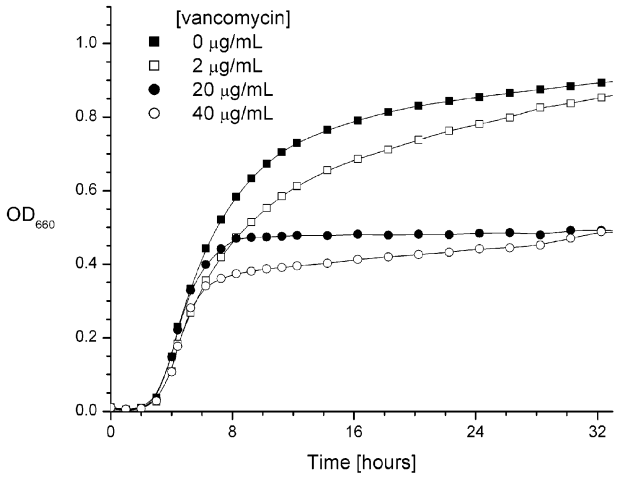

FIGURE 2: Growth of *S. aureus* showing absorbance at 660 nm as a function of time and vancomycin conentration. Vancomycin was added to the growth media at 0.2 OD.

REDOR experiments are always done in two parts (*12*), once with rotor-synchronized dephasing pulses (*S*) and once without (S_0). The dephasing pulses change the sign of the heteronuclear dipolar coupling, and this interferes with the spatial averaging resulting from the motion of the rotor. The difference in signal intensity ($\Delta S = S_0 - S$) for the observed spin in the two parts of the REDOR experiment is directly related to the corresponding distance to the dephasing spin. REDOR dephasing is generally plotted as S/S_0 or $\Delta S/S_0$ as a function of the dipolar evolution time (the number of rotor cycles multipled by the rotor period) so that dependence on the homogeneous decay (T_2) of the observed magnetization is removed. Typical REDOR pulse sequences are found in the literature in both simple (*12*) and refined forms (*7*−*10*). The REDOR spectra of intact cells of *S. aureus* reported here were obtained with ^{15}N dephasing pulses for ^{13}C{^{15}N} experiments and with ^{13}C dephasing pulses for ^{15}N{^{13}C} experiments, as described by Tong et al. (*6*).

RESULTS

Growth and Labeling of S. aureus. The time course for growth of *S. aureus* in the presence of vancomycin is shown in Figure 2. Normal growth is arrested for a vancomycin concentration of about 20 μg/mL approximately 2 h after administration of the drug. The K_D of vancomycin for *S. aureus* (ATCC 6538P) cell wall binding was estimated by Kim et al. (*6*) at 3.6 μM.

Labeling by D-*[1-^{13}C]Alanine and [^{15}N]Glycine.* Under stationary growth conditions, some glycine is used in purine synthesis, and this accounts (*13*) for the aromatic nitrogen peak at 215 ppm (Figure 3, bottom left). However, most glycine is incorporated into the pentaglycyl bridges of the cell wall peptidoglycan. The glycyl ^{15}N label incorporated into cytoplasmic protein is minor, as has been established

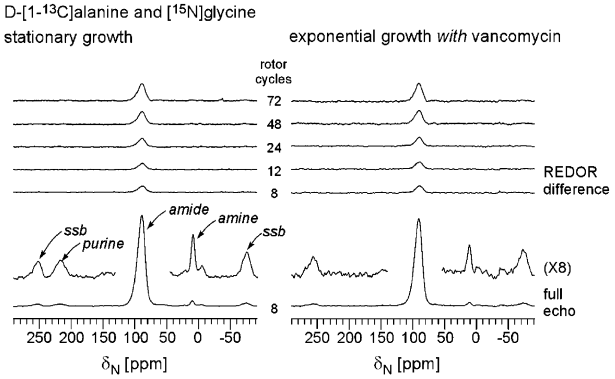

FIGURE 3: 30 MHz $^{15}N\{^{13}C\}$ REDOR spectra for cells of *S. aureus* labeled by D-[1-^{13}C]alanine and [^{15}N]glycine and harvested under stationary growth conditions (left) and exponential growth conditions in the presence of 23 μg/mL vancomycin (right). The REDOR differences are shown as a function of the number of rotor cycles of dephasing. The full-echo spectra after eight rotor cycles are at the bottom of the figure. The REDOR differences have been scaled so that $\Delta S/S_0$ is determined by comparison to the full-echo spectrum for eight rotor cycles. Magic-angle spinning was at 5 kHz.

Table 1: 30 MHz $^{15}N\{^{13}C\}$ REDOR Dephasing of *S. aureus* Whole Cells Labeled by L-[1-^{13}C]Alanine and [^{15}N]Glycine

no. of rotor cycles[a]	$\Delta S/S_0$ (amide nitrogen)		
	stationary growth	exponential growth	
		−vancomycin	+vancomycin[b]
8	0.08	0.07	0.07
12	0.08	0.09	0.07
24	0.10	0.10	0.09
48	0.15	0.15	0.14
72	0.20	0.20	0.19

[a] Magic-angle spinning at 5 kHz. [b] 20 μg/mL.

by direct comparison of spectra of whole cells and cell walls extracted from whole cells (*6*). The bridge amide glycyl ^{15}N label appears at about 90 ppm. The minor peak near 10 ppm is due to the bridge amine glycyl ^{15}N label, and the even smaller peak near 0 ppm is due to free amine in the cytoplasm (*6*). The intensity of the 10 ppm peak relative to the 90 ppm peak is 2−3%, which, because there are five glycines in a bridge, indicates that some 10−15% of the pentaglycyl bridges are open (not cross-linked). Open bridges end in a glycylamine rather than a glycyl peptide cross-link to a D-Ala carbonyl carbon (see Figure 1).

The $^{15}N\{^{13}C\}$ $\Delta S/S_0$ of the cross-linked amide nitrogen peak of the stationary growth sample after eight rotor cycles is 0.08 (Table 1). This establishes a lower limit for the ^{13}C isotopic enrichment at the D-Ala cross-link as 40% (5×0.08). The factor of 5 arises because only the directly bonded $^{13}C-^{15}N$ pair contributes to the eight rotor cycle ΔS (the ^{15}N signal for this pair is totally dephased) even though all five labeled glycyls contribute to S_0 (*6*, *12*). The D-Ala ^{13}C isotopic enrichment is not dependent on vancomycin (Table 1). Only a lower limit can be established for enrichment because glycine can be incorporated intact into cytoplasmic protein, and this fraction contributes to S_0 but not to ΔS. This utilization appears to be minor. The 40% lower limit for D-Ala is close to the 45−50% ^{13}C isotopic enrichment determined for similarly labeled isolated cell walls for which there was no cytoplasmic interference (*6*).

For longer evolution times (more rotor cycles), glycyl bridge nitrogens distant from the cross-link site also contribute to the dephasing. Changing the dipolar evolution time from 1.6 ms (8 rotor cycles) to 14.4 ms (72 rotor cycles) increases the $^{15}N\{^{13}C\}$ dephasing by a factor of 2−3 (Figure 3, top left, and Table 1). This result is consistent with previous measurements (*6*) and indicates that the bridge is in a compact conformation (*11*).

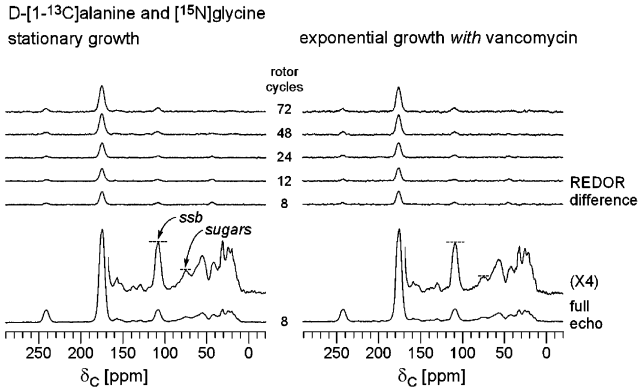

FIGURE 4: 75 MHz $^{13}C\{^{15}N\}$ REDOR spectra for the samples of Figure 3. The dotted lines in the expansions of the full-echo spectra compare the relative intensities of a spinning sideband of the ^{13}C-labeled carbonyl carbon peak at 175 ppm with the natural abundance ^{13}C sugar carbon peak at 75 ppm.

Table 2: 75 MHz $^{13}C\{^{15}N\}$ REDOR Dephasing of *S. aureus* Whole Cells Labeled by L-[1-^{13}C]Alanine and [^{15}N]Glycine

no. of rotor cycles[a]	$\Delta S/S_0$ (carbonyl carbon)		
	stationary growth	exponential growth	
		−vancomycin	+vancomycin[b]
8	0.12	0.13	0.12
12	0.11	0.14	0.11
24	0.14	0.16	0.13
48	0.20	0.19	0.18
72	0.26	0.24	0.22

[a] Magic-angle spinning at 5 kHz. [b] 20 μg/mL.

In general, cells harvested under exponential growth conditions with and without the presence of vancomycin result in $^{15}N\{^{13}C\}$ and $^{13}C\{^{15}N\}$ REDOR spectra and labeling patterns similar to those of cells harvested under stationary growth conditions in the absence of vancomycin (Figures 3 and 4, Tables 1 and 2). An exception is that incorporation of labeled [^{15}N]glycine into purines is totally suppressed by vancomycin (Figure 3, bottom expansion). Vancomycin-induced pressure on cell wall synthesis in *S. aureus* apparently resulted in the routing of all available glycine to peptidoglycan precursors.

The $^{13}C\{^{15}N\}$ $\Delta S/S_0$ for cells harvested under exponential growth conditions with the alanine−glycine-labeled pair has no dependence on the administration of vancomycin (Figure 4 and Table 2). The $^{13}C\{^{15}N\}$ REDOR dephasing for this pair of labels measures important cross-links between stems (see Figure 1), as will be described in detail in the Discussion.

Labeling by [1-^{13}C]Glycine and L-*[ϵ-^{15}N]Lysine.* The CPMAS ^{15}N NMR spectra of whole cells labeled by [1-^{13}C]glycine and L-[ϵ^{15}N]lysine (Figure 5) provide a measure of the ^{15}N label incorporated as an amide nitrogen (95 ppm) in peptidoglycan and peptidoglycan precursor bridge-links and as an amine nitrogen (10 ppm) primarily in peptidoglycan precursors. Previous work on whole cells and cell walls labeled by L-[ϵ-^{15}N]lysine (*6*, *11*, *14*) has

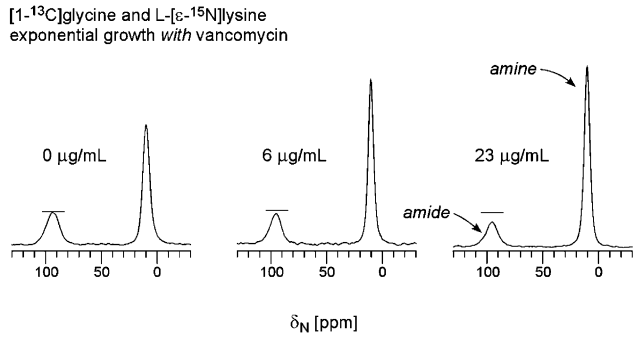

FIGURE 5: 30 MHz ^{15}N CPMAS NMR spectra of *S. aureus* cells labeled by [1-^{13}C]glycine and L-[ϵ-^{15}N]lysine and harvested under exponential growth conditions as a function of the concentration of vancomycin present in the growth medium. The spectra were scaled according to sample weight and total number of scans.

Table 3: 30 MHz ^{15}N{^{13}C} REDOR Dephasing of *S. aureus* Whole Cells Labeled by [1-^{13}C]Glycine and L-[ϵ-^{15}N]Lysine

	$\Delta S/S_0$					
no. of rotor cycles[a]	amide nitrogen (95 ppm) at vancomycin concn (μg/mL)[b]			amine nitrogen (10 ppm) at vancomycin concn (μg/mL)[b]		
	0	6	23	0	6	23
8	0.75	0.65	0.60	0	0	0
12	0.68	0.67	0.56	0	0	0
24	0.71	0.63	0.58	0.04	0.03	0.03
48	0.78	0.69	0.63	0.08	0.06	0.07
72	0.79	0.73	0.64	0.16	0.13	0.14

[a] Magic-angle spinning at 5 kHz. [b] Exponential growth.

established that in mature cells (i) only a minor part of the 95 ppm peak intensity is due to the natural abundance ^{15}N of cytoplasmic proteins, (ii) the fraction of the 10 ppm peak that is due to stems in mature peptidoglycan with no bridges attached is only 15% of the intensity of the 95 ppm peak, (iii) the free labeled lysine levels in the cytoplasm are very small and can be ignored, (iv) only about half of the lysine label is used in non-cell wall synthesis, and (v) isotopic enrichment of the ϵ-nitrogen is close to 100%.

Increasing the concentration of vancomycin in the growth medium from 0 to 23 μg/mL increased the amine to amide intensity ratio by about a factor of 2 (Figure 5). However, this treatment did not affect total ^{15}N label utilization. The absolute sum of amide and amine integrated peak intensities per mg of cell (dry weight) was within 7% across the treatment conditions shown in Figure 5.

The eight rotor cycle ^{15}N{^{13}C} dephasing of the 95 ppm peak (Table 3), which is due exclusively to directly bonded ^{13}C$-^{15}$N cell wall bridge-links (see Figure 1), is a direct measure of the glycyl carbonyl carbon ^{13}C isotopic enrichment of the pentaglycyl bridge (*6*). This value changes from 0.75 for cells harvested under exponential growth conditions in the absence of vancomycin to 0.60 when vancomycin is present (Table 3). This 20% reduction in the ^{13}C isotopic enrichment of the glycyl carbonyl carbon under vancomycin treatment indicates that some natural abundance ^{13}C sources were utilized to produce additional glycine for cell wall synthesis.

Only the directly bonded ^{13}C$-^{15}$N pair contributes significantly to the bridge-link dephasing even for longer dephasing times (Table 3). This insensitivity is due to the fact that almost full dephasing has already been realized from the directly bonded ^{13}C$-^{15}$N pair (*12*).

Table 4: 75-MHz ^{13}C{^{15}N} REDOR Dephasing of *S. Aureus* Whole Cells Labeled by [1-^{13}C]Glycine and L-[ϵ-^{15}N]Lysine

no. of rotor cycles[a]	$\Delta S/S_0$ (carbonyl carbon) at vancomycin concn (μg/mL)[b]		
	0	6	23
8	0.10	0.10	0.09
12	0.11	0.10	0.09
24	0.12	0.11	0.11
48	0.15	0.14	0.15
72	0.20	0.18	0.17

[a] Magic-angle spinning at 5 kHz. [b] Exponential growth.

Despite the nearly 100% ^{15}N isotopic enrichment of the ϵ-nitrogen of the bridge-link, the eight rotor cycle ^{13}C{^{15}N} dephasing is 0.10 (Table 4) rather than the expected 0.20 (only one of five carbonyl carbons is directly bonded to ^{15}N and contributes to ΔS). This reduction is due to the incorporation of labeled glycyl fragments (not intact glycine) via pyruvate into cytoplasmic proteins (*15*). These fragments contribute to S_0 but not ΔS. The ^{13}C{^{15}N} dephasing doubles with an increase in dephasing time to 72 rotor periods, mimicking the dephasing behavior for the alanine$-$glycine-labeled pair reported in Tables 1 and 2.

The [1-^{13}C]glycine label is also incorporated as a purine C-4 carbon, and the intensity of this peak was dependent on vancomycin concentration (spectra not shown). The glycyl peak at 150 ppm had full intensity for whole cells harvested under exponential growth conditions in the absence of vancomycin but had zero intensity for cells grown in the presence of 23 μg/mL vancomycin. This result is due to suppression of purine synthesis, as discussed in connection with the [^{15}N]glycine labeling of Figure 2, and clearly indicates that the cells were metabolizing under pressure by vancomycin.

DISCUSSION

Cross-Links. For the D-[1-^{13}C]alanine$-$[^{15}N]glycine-labeled pair, the ^{13}C (or ^{15}N) REDOR difference (ΔS) for eight rotor cycles is a direct measure of the peptidoglycan cross-linking because one-bond ^{13}C$-^{15}$N coupling occurs only at the cell wall D-Ala bond to the pentaglycyl bridge (Figure 1). These ^{13}C and ^{15}N REDOR differences (scaled by the whole cell S_0's) of samples grown with and without vancomycin are essentially the same (Figures 3 and 4). Furthermore, in the ^{13}C{^{15}N} REDOR experiment, even though a *whole cell* alanyl carbonyl carbon S_0 is measured, this value is equivalent to a *cell wall* alanyl carbonyl carbon S_0 because the distribution of ^{13}C label between cell wall and cytoplasm is unchanged. This conclusion is based on the similarity of spectra of cells harvested under stationary growth conditions with no vancomycin and exponential growth conditions in the presence of vancomycin (Figure 4). For example, there is no significant change in the intensity of the alanyl ^{13}C label relative to that of the natural abundance ^{13}C sugar peak (Figure 4, inset, dotted lines), which depends on the concentration of peptidoglycan (including the teichoic acid component), or to that of the natural abundance ^{13}C aliphatic carbon region (Figure 4, 20$-$40 ppm), which depends on the concentration of cytoplasmic protein. This means that the distribution of the ^{13}C alanyl label within the carbon pools of immature and mature cell walls, cell wall

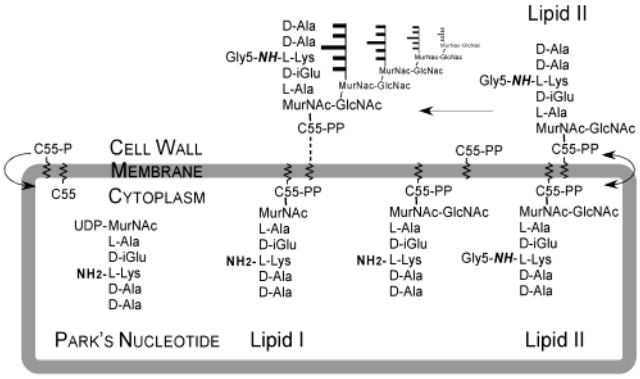

FIGURE 6: Location of the major precursors in the peptidoglycan synthesis of *S. aureus* prior to cross-linking [adapted from Navarre and Schneewind (*15*)]. The cytoplasmic C_{55} lipid species with two sugars but no pentaglycyl bridge is grouped with lipid I and Park's nucleotide because all three have a lysylamine nitrogen. The term lipid II is reserved for the fully competent peptidoglycan precursor with the pentaglycyl bridge attached, as shown at the right of the figure. Transglycosylation transforms lipid II into immature cell wall, which is initially still membrane bound (dotted line). The transformation of immature cell wall to mature (cross-linked) cell wall by transpeptidation is not shown.

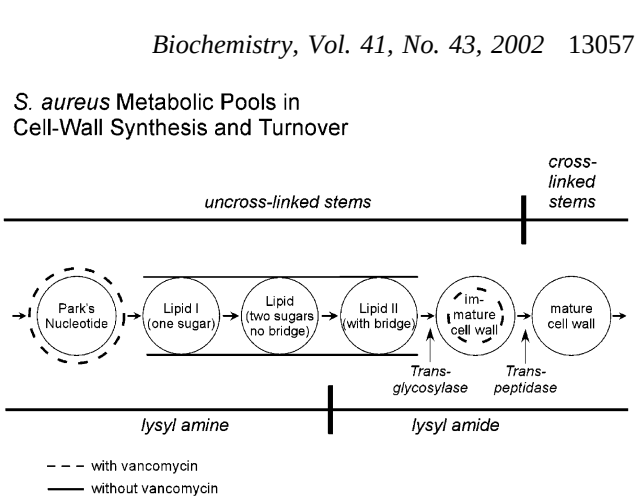

FIGURE 7: Schematic representation of the initial relative changes in the connected metabolic pools associated with peptidoglycan synthesis and turnover in *S. aureus* resulting from therapeutic levels of vancomycin. The structural formulas for the cell wall precursors are shown in Figure 6. The NMR detection of cross-links and bridge-links for the precursors, immature cell wall, and mature cell wall is shown at the top and bottom of the figure, respectively. The pools whose sizes are constrained by the availability of the C_{55} lipid transporter are bounded by light parallel lines. The transpeptidase step is assumed to follow transglycosylation, but this is not essential to the NMR analysis. The changes in pool sizes expected for an interference with transglycosylation by vancomycin are indicated by the dotted lines. The true absolute pool sizes are not necessarily represented by the sizes of the circles.

precursors, and cytoplasmic proteins has not changed in response to vancomycin. Thus, the observed insensitivity to vancomycin of the $^{13}C\{^{15}N\}$ $\Delta S/S_0$ ratio for whole cells harvested under both stationary and exponential growth conditions (Figure 4 and Table 2) shows that the average number of cell wall cross-links does not change in the early response of *S. aureus* to vancomycin treatment.

Bridge-Links. Although the number of cross-links is not dependent on the concentration of vancomycin in the growth medium, the number of bridge-links is. The latter decreases in whole cells of *S. aureus* by one-third in the presence of 23 μg/mL vancomycin, as measured in the [1-^{13}C]glycine−L-[ϵ^{15}N]lysine labeling experiment by the decrease of the 95 ppm amide peak intensity (Figure 5). There is also about a one-third increase in the 10 ppm ϵ-^{15}N amine peak intensity (Figure 5), which means that the total ^{15}N label incorporation is approximately constant. Because the level of cytoplasmic protein did not change in response to vancomycin (see discussion of the expanded insets to Figure 4), the increase in the ϵ-^{15}N amine peak intensity must be associated with cell wall synthesis.

Metabolic Pools. A simple interpretation of the observed combination of constant cross-links and decreasing bridge-links can be made in terms of peptidoglycan synthesis (Figure 6) and changes in the relative concentrations of the mature cell wall and the major cell wall precursors (Figure 7). If vancomycin were to inhibit transpeptidase activity, the concentration of cross-links would decrease as the mature cell wall was degraded and thinned in the actively dividing cell (*16*). At the same time, the concentration of bridge-links would increase as newly synthesized peptidoglycan was not cross-linked and so accumulated as immature cell wall. This pattern is not observed; instead, the number of cross-links is constant and the number of bridge-links decreases. On the other hand, if vancomycin were to inhibit transglycosylation, immature cell wall would continue to be cross-linked to form new mature cell walls, as old cell walls were degraded. This combination would maintain the total number of cross-links approximately constant (see Figure 7). In addition, the immature cell wall concentration would decrease (or, equivalently, the average glycan chain length would be reduced) because the glycan chain extension was blocked. That is, blockage would decrease the total number of bridge-links. This is true even though vancomycin has no direct effect on the cytoplasmic enzymes responsible for attaching the bridge to the peptide stem. Blocking transglycosylation would also effectively sequester the C_{55} lipid transporter, resulting in an accumulation of Park's nucleotide in the cytoplasm. Thus, as the number of lysyl ϵ-nitrogens observed as amides would decrease, the number observed as amines would increase. This combination is, in fact, observed: cross-links are constant (Figure 4), lysyl amides decrease, and lysylamines increase (Figure 5). We therefore believe that vancomycin (at therapeutic levels) interrupts peptidoglycan synthesis in *S. aureus* by interference with transglycosylation.

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