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Analysis on the Molecular Interactions between Tobramycin, an Aminoglycoside Antibiotic and Periplasmic Glucans Isolated from *Pseudomonas syringae*

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Tobramycin, an aminoglycoside antibiotic, is produced by *Streptomyces tenebraius* and its usage is increasing in pharmaceutics and medical science.[1] It has an antibiotic activity against a wide range of microorganisms including *Pseudomonas aeruginosa*.[2] Commonly, tobramycin works by binding to a site on the bacterial 30S and 50S ribosomes, preventing formation of the 70S complex.[3] Thus, mRNA cannot be translated into a protein and cell death ensues. Fortunately, multiple cellular mechanisms to reduce the antibacterial effect of tobramycin have been developed in bacteria. Besides enzymatic resistance or efflux pump in cells,[4] it has been reported that bacterial exopolysaccharides (EPS) reduce the penetration of antimicrobial agents to their target sites.[5] The present study considers another possible mechanism of antibiotic resistance, that is, whether bacterial periplasmic glucans play a role in the suppression of antibiotics.

Periplasmic glucans are general constituents of all the Proteobacteria. Among them, *Escherichia coli*, *Erwinia chrysanthemi*, *P. aeruginosa* and *Pseudomonas syringae* produce linear periplasmic glucans.[6-9] The periplasmic glucans of *P. syringae*[7] has the identical structure with those of *P. aeruginosa*.[8,9] Both are linear β-1,6 branched β-1,2 glucans which have the main degree of polymerization from 6 to 10 (DP 6-10). The biological functions of periplasmic glucans in the cell have also been investigated.[6] They are needed for hypoosmotic adaptation of the bacteria, and play roles in host infection or symbiosis. In *E. chrysanthemi*,[10] *P. aeruginosa*,[11] and *P. syringae*,[12] mutants not able to produce the linear periplasmic glucan showed the reduced virulence during infection of the host. This means that periplasmic glucans play an important role in bacterium-host interactions. Recently, a study that *Pseudomonas* periplasmic glucans may prevent the action of tobramycin by sequestering it in the periplasmic space has invoked our NMR study on the molecular interaction between them.[13] In the present study, we suggest interaction of periplasmic glucans with antibiotics.

The structures of tobramycin and linear β-glucans were confirmed with ^{1}H-NMR, ^{13}C-NMR, and HSQC-NMR spectroscopic and MALDI-TOF mass spectrometric analyses, as our previous reports (data not shown).[8,9] The chemical structures of these molecules are shown in Figure 1. Tobramycin (Figure 1A) consists of three rings of nebrosamine (a), 2-deoxystraptamine (b), and kanosamine moiety (c). Linear β-glucans (Figure 1B) are β-1,2 linked and β-1,6 branched glucans containing 6-12 glucose residues.

NMR spectroscopic analysis is a technique providing the evidence of complexation[14,15] and the change in the chemical shifts of some of tobramycin protons could be observed after the complexation with linear β-glucans. ^{1}H-NMR chemical shift changes of tobramycin complexed with linear β-glucans were indicated in Figure 2, where aH1-3, bH2 and cH3 protons of tobramycin showed downfield shifts (Δδ) upon the formation of complexes. This downfield shift indicates that the protons are surrounded by densely located electrons of the linear β-glucans.[16] For comparison, another glucan (β-CD) was investigated

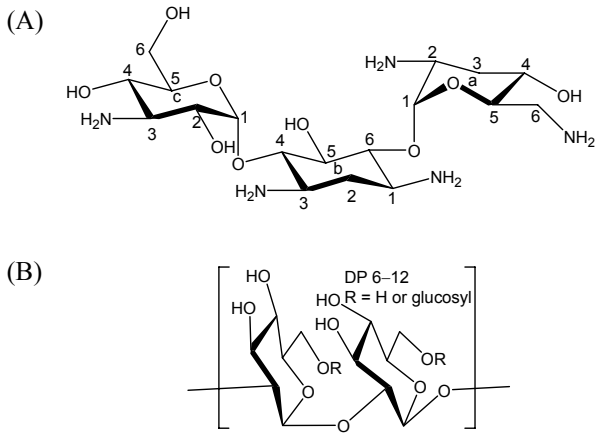

Figure 1. Chemical structures of tobramycin (A) and linear β-glucan from *P. syringae* (B).

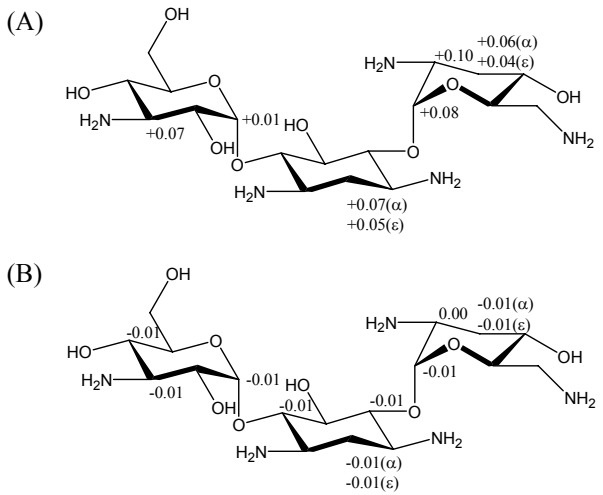

Figure 2. Changes in the ^{1}H-NMR chemical shifts of tobramycin with linear β-glucans (A) and β-CD (B).

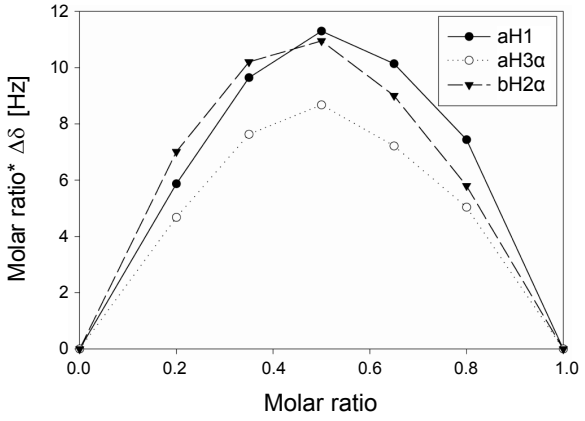

Figure 3. Job plots for the complexes of tobramycin/linear β-glucans.

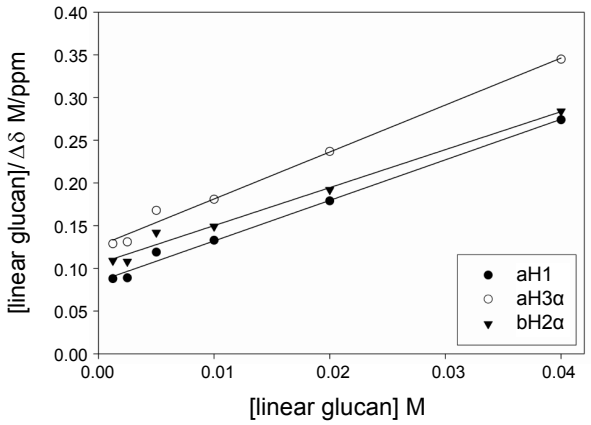

Figure 4. Scott's plots for the complexes of tobramycin/linear β-glucans.

but, there was no or minimal change (Figure 2).

This NMR study was carried out based on the discovery that shows the interaction between tobramycin and *Pseudomonas* periplasmic glucans on hydrophobic interaction (HI) chromatography.[13] Also, the wild type was 16 fold resistant to tobramycin compared with the mutant not able to produce periplasmic glucans, and they suggested that this resistance is due to the periplasmic glucan interactions with tobramycin. Among their tested antibiotics, specially, nalidixic acid sensitivity was not changed between the wild type and mutant. Thus, we analyzed the chemical shift changes for nalidixic acid protons after complexation with linear β-glucans. The differences in chemical shift were negligible, less than 0.02 in all nalidixic acid protons, suggesting that tobramycin interacts physically with linear β-glucans.

To determine the stoichiometry of the complexes, Job plots were obtained with the continuous variation method. The plots were constructed with the aH1, aH3α, and bH2α protons of tobramycin. The result shows the maximum at a molar ratio of 0.5, suggesting the formation of one to one complex between tobramycin and linear β-glucans (Figure 3).

Additionally, Scott's plots were given with the same protons as protons used for Job plot. As presented in Figure 4, they show the excellent linear fit confirming 1:1 stoichiometry for the complex. The equations of the lines for the complexes give fine r^2 values, ranging from 0.987 to 0.993. According to Eq. (1), the slope of the plot is thus equal to $1/\Delta\delta_c$ and the intercept with the vertical axis to $1/K_a\Delta\delta_c$, allowing the estimation of association constant (K_a) to be 47 ± 8 M^{-1} (Table 1). With this association constant, we can gauge the affinity of tobramycin with linear β-glucans. This value would be useful to consider the physiological significance of the interactions observed. For example, K_a and the stoichiometry of the complex can explain how much tobramycin is able to be effectively bound by periplasmic linear β-glucans. Since tobramycin by β-CD or nalidixic acid by linear β-glucans does not show any differentiated chemical shift change, the comparable association constants could not determined.

Table 1. Association constants (K_a) of tobramycin/linear β-glucans complex

Protons	K_a	K_{av}
aH1	56	
aH3α	44	47 ± 8
bH2α	42	

Through the effective interaction between tobramycin and periplasmic glucans, the glucans may inhibit cellular function of tobramycin by sequestering antibacterials in periplasmic space.[13] Therefore, antibiotic resistance might be another novel role played by periplasmic glucans besides known functions such as hypoosmotic adaptation, pathogenecity, and symbiosis.[6] This hypothesis appears reasonable from the viewpoint of some recent studies suggesting that β-1,2 glucans or β-1,3 glucans reduce penetration of antimicrobials into the cell cytoplasm.[13,17]

In conclusion, we have investigated the interaction of periplasmic linear β-glucans isolated from *Pseudomonas* species with tobramycin through NMR spectroscopic analyses. By complexation between tobramycin and linear β-glucans, noticeable chemical shift changes were observed, comparing with negligible ones both from tobramycin/β-cyclodextrin complex and nalidixic acid/linear β-glucan complex. Tobramycin/linear β-glucan complex was determined to be a 1:1 stoichiometry and the association constant (K_a) for this complex was calculated to be 47 ± 8 M^{-1}. The present interaction study provides a direct molecular view and indirect evidence suggesting a role for tobramycin resistance by periplasmic linear β-glucans in *Pseudomonas* strain.

Experimental Section

Materials. Tobramycin was purchased from Sigma-Aldrich Corp. (St. Louis, MO).

Separation and Preparation of *Pseudomonas* Periplasmic Glucans. *P. syringae* pv. syringae (ATCC 19310) were grown at 26 °C in TGY medium. Cell pellets were extracted with 5% trichloroacetic acid and the extracts were applied to a Sephadex G-25 column. After anion exchange chromatography, neutral glucans were collected and desalted on a Bio-Gel P-4 column.[8] The obtained molecules were hydrolyzed with 0.1 M KOH at 37 °C for 1 h to remove nonsugar subtituents of neutral glucans, desalted, and lyophilized.

Structural Analyses of Tobramycin and *Pseudomonas* Periplasmic Glucans. The structure of tobramycin was confirmed

by ^{1}H, ^{13}C, and hetero single-quantum correlation (HSQC) NMR spectroscopic analyses. The structure and molecular weight of *Pseudomonas* periplasmic glucans were confirmed through NMR spectroscopic and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometric analyses.[8] *Pseudomonas* periplasmic glucans are structurally analyzed as linear β-glucans.[7-9]

Preparation of Complexes of Tobramycin with Linear β-Glucans. Tobramycin and linear β-glucans were dissolved at pH 6.4 in 10 mM sodium phosphate[15] with 1:1 molar ratio, and mixed in an incubator at 30 °C for 24 h. After complexation, the solution was lyophilized and analyzed.

Stoichiometry and Association Constant (K_a). The stoichiometry of the complexes was determined with continuous variation method (Job plot). The samples were prepared by mixing the solutions of tobramycin and linear β-glucans in ratios of 0.2, 0.35, 0.5, 0.65 and 0.8. The total molar concentration was kept constant at 10 mM.

The association constants (K_a) of complexes were calculated by using Scott's modification of Benesi-Hildebrand equation.[18] In Scott's equation,

$$[\text{linear } \beta\text{-glucans}]_t/\Delta\delta_{obs} = [\text{linear } \beta\text{-glucans}]_t/\Delta\delta_c + 1/K_a\Delta\delta_c \quad (1)$$

$[\text{linear } \beta\text{-glucans}]_t$ is the molar concentration of the linear β-glucans, $\Delta\delta_{obs}$ is the observed chemical shift change for a given $[\text{linear } \beta\text{-glucans}]_t$ concentration, and $\Delta\delta_c$ is the chemical shift change between a pure sample of complex and the free component at saturation.

NMR Spectroscopy. For NMR spectroscopic analysis, a Bruker Avance 500 spectrometer was used to record the ^{1}H-NMR, ^{1}C-NMR, and HSQC. NMR spectroscopic analyses of tobramycin/linear β-glucans or β-cyclodextrin (β-CD) were performed in D_2O at room temperature. For nalidixic acid/linear β-glucans interaction analysis, D_2O/MeOD (1/1, v/v) was used.

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