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Bacterial Surface Engineering Utilizing Glucosamine Phosphate Derivatives as Cell Wall Precursor Surrogates

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Surface display on bacteria has attracted much attention due to the potential applications in peptide library screening, and in the syntheses of various bioadsorbants, biosensors, and oral vaccines.[1] Therefore, the cell-surface engineering of bacteria has been an important theme in the field of industry and in particular biotechnology. Several approaches to cell surface engineering have been explored thus far. Genetic techniques have been used to express target proteins as conjugates with their native surface proteins.[2] However, the approaches are limited to the display only of proteins and peptides.

In contrast to the genetic approaches, a chemical approach based on the metabolic pathway has been utilized to display a variety of molecules. Metabolic labeling of cell-surface biomolecules with a non-native chemical tag allows for the covalent attachment of various synthetic epitopes. Bertozzi et al. originally proved that unnatural carbohydrate substrates could be incorporated into an oligosaccharide to introduce novel chemical reactivity to mammalian cell surfaces.[3–6] In the case of bacteria, Tirrell and co-workers successfully incorporated an azide group, using an azido amino acid as a methionine surrogate, into an outer membrane protein of *Escherichia coli* in which additional methionine residues were engineered by site-directed mutagenesis.[5,7,8]

We previously developed an approach to display various kinds of molecules on the bacterial cell surface using chemically synthesized cell-wall (peptidoglycan) precursors.[9,10] Since the composition of peptidoglycan is strikingly similar between bacterial strains,[11] our approach is applicable to a variety of bacteria. In this method, chemically synthesized cell-wall precursors, that is, UDP-MurNAc pentapeptide derivatives, are incorporated into the cell wall through a biosynthetic pathway; this methods does not depend on genetic modifications, thereby presenting a range of potential target moieties, such as oligosaccharides,[9] beyond that of conventional protein display. However, as the synthesis of the UDP-MurNAc pentapeptide derivative is difficult and time-consuming, the large-scale application of this system is problematic.

To overcome these problems, we focused on *N*-acetylglucosamine-1-phosphate (GlcNAc-1-phosphate), which is an early, simple intermediate in the peptidoglycan biosynthetic route (Figure 1), and herein present our results. In bacteria, GlcNAc-1-phosphate can be an intermediate of both the MurNAc-pentapeptide and GlcNAc components of peptidoglycans (see Figure 1). In bacterial cell-wall biosynthesis, GlcNAc-1-phosphate is formed from glucosamine-1-phosphate,[12] whereas in mammalian cells, GlcNAc is directly converted to the phosphorylated form via salvage pathways.[13] Therefore, we chose GlcNAc-1-phosphate, and not GlcNAc, as the platform precursor for the introduction of non-native reactive groups into the bacterial cell wall, and designed compound **1**, in which a ketone group was introduced into the *N*-acetyl group, as a bacterial cell wall pre-

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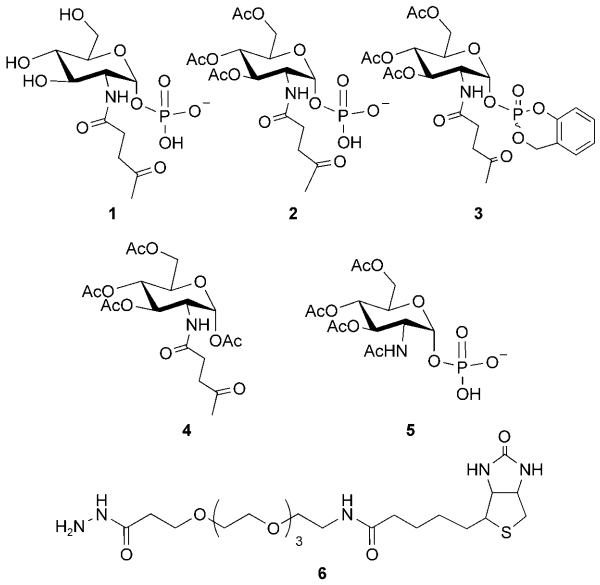
cursor surrogate. We synthesized derivatives of GlcNAc-1-phosphate **1**–**5**, in which **1**–**4** each bear a ketone group. The incorporation of the derivatives into the bacterial cell wall



was estimated by flow cytometry after labeling of the displayed ketone group with biotin-PEO-hydrazide **6** and, subsequently, with streptavidin–Alexa Fluor 488 conjugate. The selective reaction of ketones with the hydrazide group of **6** showed the effective incorporation of **2** into lactic acid bacteria.

Acetylation of the hydroxyl groups of **1** gave **2** with increased hydrophobicity, providing easier access to the cytoplasm of the bacteria. We expected that the protection of the phosphate group by the cyclosaligenyl group would further increase the hydrophobicity of **3**. As the cyclosaligenyl group is known to be removable in basic conditions above pH 7,[14] the phosphate group was expected to be deprotected under the basic environment in bacteria. To confirm the effect of phosphorylation in the bacterial cell wall pathway, 2-ketosugar **4**, which does not possess a phosphate group, was also tested. Peracetylated GlcNAc-phosphate **5** was used as a negative control. The synthesis and characterization of these compounds are described in the Supporting Information.

First, we used the same strain (*Lactobacillus plantarum* JCM1149) as was used in our previous report on the incorporation of UDP-MurNAc-pentapeptide derivatives.[9] Bacteria were incubated in Lactobacilli deMan Rogosa Sharpe (MRS) broth at 37 °C for three hours, then bacteria were collected by centrifuge (3000 rpm, 3 min.), resuspended in a MRS Lactobacilli broth containing each test compound (**1**–**5**, final concentration: 0, 5, 25, or 50 mM), and incubated for a further 15 h. The lactic acid bacteria were washed with acetate buffer (pH 4), and biotin-PEO-hydrazide (**6**), which binds specifically to the ketone group, was added. After washing with phosphate-buffered saline (PBS) (pH 7.2),

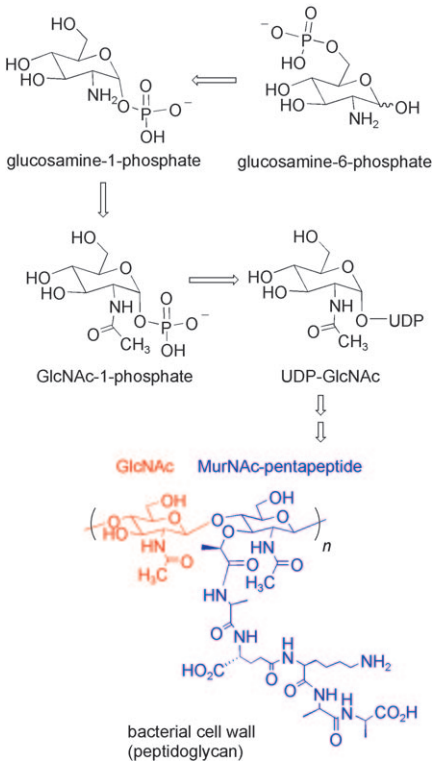


Figure 1. Schematic presentation of the biosynthesis of the bacterial cell wall.

streptavidin–Alexa Fluor 488 conjugate was added and the bacteria were washed with PBS buffer prior to analysis using a flow cytometer.

The fluorescence histograms obtained from the flow cytometric analysis were shifted as the concentration of compound **2** in MRS media increased (Figure 2a). Figure 2b shows the plot of the flow cytometry. FSC-A and SSC-A did not change significantly, indicating that the shape of the bacteria was not changed by the addition of the precursor. In order to estimate more accurately the changes in fluorescence of the labeling, fluorescence intensities were normalized by dividing the intensity by size as shown in Figure 2c. The normalized fluorescence intensity when incubated with compounds **1**–**5** is summarized in Figure 2c. When adding precursor **2** or **3**, the fluorescence intensity was increased as a function of concentration. These increases in fluorescence suggested that the acetylated precursor **2** was incorporated into the bacterial cell wall even at low concentrations, whereas the more hydrophobic precursor **3**, in which the phosphate group was protected, was only incorporated at higher concentrations (Figure 2c). The level of incorporation of **3** was about half that of precursor **2**, which possessed an unprotected phosphate group. The lower level of incorporation of **3** is possibly due to the deprotection process not proceeding in the cytoplasm as expected. No increase in fluorescence was observed for compound **1**, probably due to its highly hydrophilic nature. The negative control **5**, which had no ketone group, showed no increase in fluorescence, indi-

cating that the increase in fluorescence in **2** and **3** was not due to non-specific binding of the precursors to the labeling molecules. In the case of **4**, which has a ketone group but no phosphate group at C-1 position, the fluorescence intensity did not increase. In bacterial cell-wall biosynthesis, the *N*-acetyl group of GlcNAc is once removed and transformed into glucosamine-1-phosphate. Thus, the ketone group of phosphate-free precursor **4** might be removed before incorporation into the cell wall. Notably, the fact that **2**, which has a phosphate group, was incorporated and **4**, which does not have a phosphate group, was not incorporated, indicates that incorporation proceeded via cell-wall biosynthesis and not due to non-specific binding.

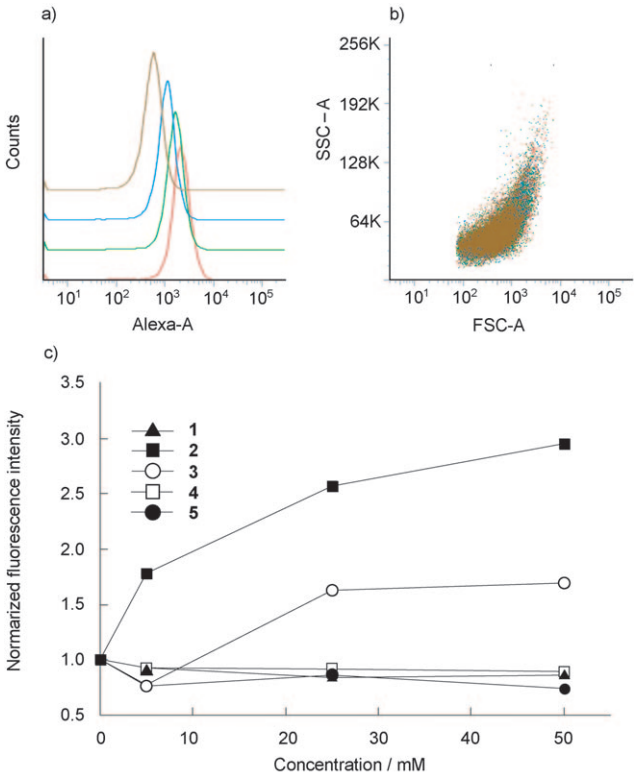


Figure 2. Flow cytometric analysis of lactic acid bacteria. Lactic acid bacteria (*Lactobacillus plantarum* JCM1149) were incubated with GlcNAc-1-phosphate derivative **2** (brown: 0 mM; blue: 5 mM; green: 25 mM; red: 50 mM) and labeled with biotin-PEO-hydrazide and streptavidin–Alexa Fluor 488 conjugate. a) Fluorescence histograms: fluorescence intensity (Alexa Fluor 488 A) versus the number of bacteria. b) Size (FSC-A) versus complexity (SSC-A). c) Concentration of GlcNAc-1-phosphate derivatives versus normalized fluorescence intensity (fluorescence intensity/FSC-A).

Next, we used other strains of bacteria (*Lactobacillus plantarum* JCM11125 and *Weissella confusa* JCM1093) to check the versatility of incorporation of **2** into the cell wall using the same procedure as that described above. The results are shown in Figure 3 together with the results for *Lactobacillus plantarum* JCM1149. Increases in fluorescence intensity due to the binding of dye-conjugated avidin were observed for both strains. These strains have different types of bacterial cell wall in that while the main carbohydrate structure is the same, there are small variations in the peptide moieties.[15] However, our method for the surface display of the reacting ketone group using the cell-wall precursor **2** targets the carbohydrate polymer chain, which is a common structure among bacteria; therefore, it is applicable to a wide variety of bacteria.

To quantify the absolute number of chemically accessible ketone groups on the bacterial surface, the following experiments were performed. Lactic acid bacteria (*Lactobacillus plantarum* JCM1149) were incubated in the presence of **2** and labeled with the streptavidin–Alexa Fluor 488 conjugate, as described above, for preparation of the sample for flow cytometry analysis. The bacteria were washed and then resuspended in PBS buffer and, using an aliquot of the suspension, the number of bacteria in the suspension was determined by colony counting. The remaining bacteria were lyzed by addition of a lyzing buffer containing 1.3 mM Tris, and 0.28 mM EDTA (pH 8.0), and were analyzed by fluorescence spectrophotometer. The results were then compared to the data obtained using the buffer containing free fluorescence dye. By dividing the fluorescence intensity by the number of bacteria, the number of accessible ketone groups was calculated to be more than 10^5 per bacterium. We assumed that the bacteria have a columnar shape (length 5 μm and diameter 1 μm, surface area: 6.6 μm^2) that is covered with a peptidoglycan monolayer since the avidin can only access the ketone groups on the outer layer of the bacterial surface. Assuming the molecular area of the repeating unit of peptidoglycan (GlcNAc-MurNAc pentapeptide) is 1 nm^2, there are about 6.6×10^6 repeating units on the bacterium surface. Based on this simplified model, the ratio of ketone-attached repeating units in the peptidoglycan is roughly 1.5%.

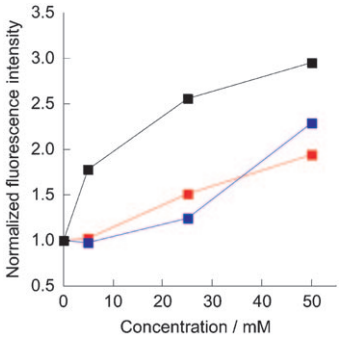


Figure 3. Normalized fluorescence intensity of lactic acid bacteria. The *x* axis is concentration of **2** in the growth culture and the *y* axis is normalized fluorescence intensity (fluorescence intensity/FSC-A). ■: *Lactobacillus plantarum* JCM1149; ■: *Weissella confusa* JCM1093; ■: *Lactobacillus plantarum* JCM11125.

As the natural cell wall precursor, GlcNAc-1-phosphate, is originally made from glucose,[11] the incorporation of the artificial cell-wall precursor **2** is expected to be accelerated when the availability of natural glucose is limited To reduce the glucose concentration in the growth media, phosphate-buffer media (PBM; see the Experimental Section)[16] containing **2** and 0 to 50 mM glucose was used instead of the MRS broth. The PBM media contained only artificial ingre-

dients, apart from peptone. The bacteria were able to grow even in the glucose-free PBM medium for a short period, such as 12 h, although the growth speed was dramatically reduced to only 5% of that in MRS broth. On the basis of flow cytometric analyses (Figure 4), fluorescence due to the incorporated ketone group at 0 mM glucose was found to be three-fold larger than that obtained at other glucose concentrations. Incorporation of **2** in MRS broth was similar to that in PBM media containing 10, 25, or 50 mM glucose. We consider that artificial precursor **2** is incorporated into the cell wall much less effectively compared with native glucose; therefore, the complete removal of glucose was necessary to enhance the incorporation of precursor **2** into the bacterial cell wall. These results also support the hypothesis that precursor **2** is metabolically incorporated through the cell wall synthetic pathway.

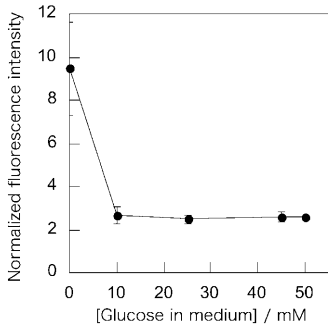


Figure 4. Fluorescence intensity of lactic acid bacteria (JCM 1149) after incubation in PBM broth containing 0, 10, 25, or 50 mM glucose, and labeled using the same procedure as described in Figure 2.

In conclusion, GlcNAc-1-phosphate derivative **2** can be used as a practical precursor for bacterial surface display. However, using GlcNAc derivative **4**, which has no phosphate group, the ketone group cannot be displayed on the bacterial surface. These results suggest that the incorporation of **2** occurred via cell-wall biosynthesis. By limiting the glucose in the growth media, the degree of incorporation was increased about three-fold. Further, this method was superior to our prior system using UDP-MurNAc pentapeptide derivatives and is applicable to large-scale in vivo studies aimed at the development of oral vaccines.

Experimental Section

General: NMR spectra were recorded on a Bruker AMX-500 spectrometer. All NMR measurements were carried out at 27 °C in $CDCl_3$, D_2O, or $[D_4]$MeOH. Unless otherwise noted, reagents were obtained from commercial suppliers, Wako Pure Chemical Industries Ltd. (Osaka, Japan), Tokyo Kasei Kogyo Co. (Tokyo, Japan), and Aldrich Chemical Co. (Milwaukee, WI), and were used without further purification. Synthetic details are described in the Supporting Information.

Composition of PBM: Peptone (5.0 g), ammonium sulfate (2.0 g), bipotassium phosphate (1.4 g), monopotassium phosphate (0.6 g), citric acid (0.1 g), magnesium sulfate (0.12 g), sodium chloride (5.0 g), calcium chloride (0.7 g), manganese sulfate (0.07 g), D-glucose (0–50.0 mM).

Incorporation experiments: The precursors synthesized were sterilized by filtration before being added to the growth media for bacterial incubation. Lactic acid bacteria were incubated for 8 h under anaerobic conditions and the cell culture was diluted with MRS broth (BD) to give OD values (at 600 nm) of 0.05. The diluted culture (270 μL) was then incubated for 3 h. After the addition of 30 μL of MRS broth containing **2** (0, 1.5, 7.5, or 15 μM), the bacteria were incubated for a further 15 h. An aliquot of the culture (30 μL; suspended before collection) was washed with acetate buffer (pH 4, 3×100 μL), and then 100 μM biotin–PEO-hydrazide (15 μL), which binds specifically to the ketone group, was added before further incubation for 2 h at 40 °C. The cells were collected by centrifugation (3000 g, 3 min), washed PBS buffer (pH 7.2, 3×100 μL), and then streptavidin–Alexa Fluor 488 conjugate (Molecular Probes, 28 $\mu g\,mL^{-1}$, 100 μL) was added before incubation for 20 min at 4 °C. After washing two times with PBS buffer (100 μL), PBS buffer (300 μL) was added and the bacteria were analyzed using a flow cytometer (FACScant, BD). Ten thousand signal counts were analyzed using FlowJo software (Tree star Inc.). As a change in size was observed for bacteria incubated in media supplemented with the test compounds, the fluorescence intensity was normalized using average of FITC-A/average of FSC-A.

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Keywords: carbohydrates · glycoconjugates · peptidoglycans

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