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Control of Bacteria Adhesion by Cell-Wall Engineering

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Abstract: UDP-MurNAc-pentapeptide derivative bacterial cell-wall precursors were synthesized as effective tools for surface display on living bacteria. *Lactobacilli* were incubated in the ketone-modified precursor-containing medium, and the ketone moiety was displayed on the bacterial surface through cell-wall biosynthesis. Oligomannose was coupled with the ketone moiety on the bacterial surface via a aminooxyl linker, thereby displaying this oligosaccharide on the surface of the bacteria. The increase in the adhesion of the sugar-displaying bacteria onto a concanavalin A-attached film compared to that of native bacteria was confirmed by microscopic observation and surface plasmon resonance measurement. The incorporation of the artificial cell-wall precursors was enhanced when incubated with fosfomycin, an inhibitor of cell-wall precursor biosynthesis.

Introduction

Bacterial adhesion is an important event in the infection of host cells and in the interaction between bacteria, and adhesion is known to be mainly mediated by interactions involving carbohydrates.[1−6] Therefore, controlling adhesion properties of the bacterial surface by adding extraneous carbohydrate molecules to the surface of living bacteria to be taken orally as probiotics would be a useful technique for the prevention of infection in the intestine by blocking the carbohydrate−protein binding of pathogens. Lactic acid bacteria have certain advantages over other bacteria in their application to surface display in that they can be easily used in food and for medical purposes because they are generally recognized as safe.[7,8] Isolauri et al. have reported the potential benefits of the use of *Lactobacilli* in the development of vaccines against infectious diseases and in the treatment of autoimmune or other immune disorders.[9] Therefore, the addition of an oligosaccharide on the surface would allow for targeted vaccination against intestinal pathogens. We focused on lactic acid bacteria and herein introduce a novel function afforded by the artificial surface display of carbohydrates on the bacterial surface. The first example of the chemical engineering of living cells was reported by Bertozzi's group,[10−13] who attached a ketone group to *N*-acetyl-D-mannosamine. That compound was then metabolically converted to ketone-attached sialic acid on the surface of mammalian cells. In the case of bacteria and yeast, almost all methods of cell-surface engineering are based on the genetic approach.[14−16] The target proteins are displayed on the surface of bacteria through fusion to surface proteins, such as GPI anchor-type proteins. However, only proteins can be displayed through this method, and the genetically engineered bacteria are not suited for use in pharmaceuticals because of the inability to regulate the amount of expression of the target proteins.

We have previously reported[17,18] bacterial cell-surface engineering using chemically synthesized cell-wall precursor derivatives (Figure 1). Our approach is based on bacterial cell-wall biosynthesis (Figure 2). As almost all bacteria have a cell wall

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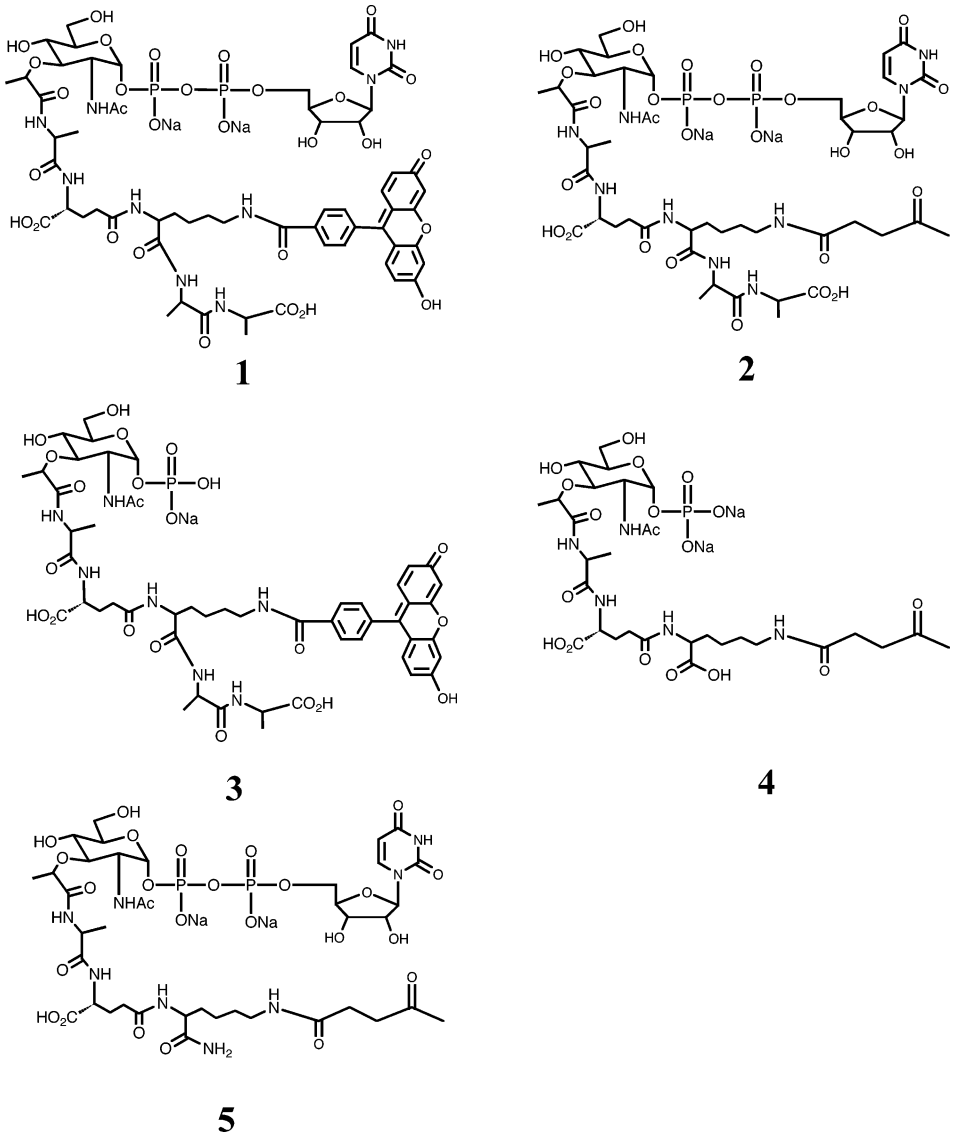
(1) *Handbook of Bacterial Adhesion: Principles, Methods, and Applications*; An, Y. H., Friedman, R. J., Eds.; Humana Press: Totowa, NJ, 2000.
(2) Schengrund, C.-L. *Biochem. Pharmacol.* **2003**, *65*, 699−707.
(3) Sharon, N.; Ofek, I. *Glycoconjugate. J.* **2001**, *17*, 659−664.
(4) Sharon, N.; Ofek, I. *Critic. Rev. Food Sci. Nutr.* **2002**, *42*, 267−272.
(5) Ofek, I.; Sharon, N. *Cell. Mol. Life Sci.* **2002**, *59*, 1666−1667.
(6) Lee, Y.-K.; Puong, K.-Y. *Br. J. Nutr. Med.* **2002**, *88*, S101−S108.
(7) Leenhouts, K.; Buist, G.; Kok, J. *Antonie van Leeuwenhoek* **1999**, *76*, 367−376.
(8) Havenith, C. E. G.; Seegers, J. F. M. L.; Pouwels, P. H. *Food Res. Int.* **2002**, *35*, 151−163.
(9) Isolauri, E.; Joensuu, J.; Suomalainen, H.; Luomala, M.; Vesikari, T. *Vaccine* **1995**, *13*, 310−312.
(10) Hang, H. C.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2001**, *123*, 1242−1243.
(11) (a) Jacobs, C. L.; Goon, S.; Yarema, K. J.; Hinderlich, S.; Hang, H. C.; Chai, D. H.; Bertozzi, C. R. *Biochemistry* **2001**, *40*, 12864−12874. (b) Yarema, K. J.; Goon, S.; Bertozzi, C. R. *Nat. Biotechnol.* **2001**, *19*, 553−558. (c) Yarema, K. J.; Mahal, L. K.; Bruehl, R. E.; Rodriguez, E. C.; Bertozzi, C. R. *J. Biol. Chem.* **1998**, *273*, 31168−31179. (d) Mahal, L. K.; Yarema, K. J.; Bertozzi, C. R. *Science* **1997**, *276*, 1125−1128. (e) Yarema, K. J.; Bertozzi, C. R. *Curr. Opin. Chem. Biol.* **1998**, *2*, 49−61.
(12) Saxon, E.; Bertozzi, C. R. *Science* **2000**, *287*, 2007−2010.
(13) Vocadlo, D. J.; Hang, H. C.; Kim, E.-J.; Hanover, J. A.; Bertozzi, C. R. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 9116−9121.
(14) Ståhl, S.; Uhlén, M. *Trends Biotechnol.* **1997**, *15*, 185−192.
(15) Medof, M. E.; Nagarajan, S.; Tykocinski, M. L. *FASEB J.* **1996**, *10*, 574−86.
(16) (a) Ye, K.; Shibasaki, S.; Ueda, M.; Murai, T.; Kamasawa, N.; Osumi, M.; Shimizu, K.; Tanaka, A. *Appl. Microbiol. Biotechnol.* **2000**, *54*, 90−96. (b) Abe, H.; Shimma, Y.; Jigami, Y. *Glycobiology* **2003**, *13*, 87−95.
(17) Sadamoto, R.; Niikura, K.; Sears, P. S.; Liu, H.; Wong, C.-H.; Suksomcheep, A.; Tomita, F.; Monde, K.; Nishimura, S.-I. *J. Am. Chem. Soc.* **2002**, *124*, 9018−9019.
(18) Sadamoto, R.; Niikura, K.; Monde, K.; Nishimura, S.-I. *Methods Enzymol.* **2003**, *362*, 273−286.

Chart 1



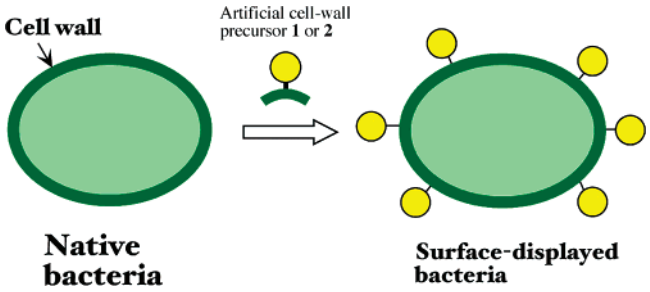


Figure 1. Surface display of living bacteria through cell-wall biosynthesis.

consisting of similar conserved structures, the bacterial cell wall provides a good platform for surface display using chemical techniques.

Chart 1 shows the structures of the modified UDP-MurNAc pentapeptide derivatives. Precursors were synthesized according to the procedure described in refs 19 and 20 with minor modifications. The synthetic procedures were reported in our first communication.[17] Our previous research[17,18] shows that UDP-MurNAc pentapeptide derivatives (**1** and **2**) were incorporated into the cell wall of *Lactobacilli*, while the monophosphate compound (**3**) was not incorporated. For *Escherichia coli*, the precursors were incorporated into the cell wall only after treatment with an EDTA-containing buffer. The EDTA treatment increased the permeability of the outer membrane of the *E. coli* cell wall.

In this article, we expand this method to the control of the adhesion of living lactic acid bacteria by means of the display of target oligosaccharides. Lactic acid bacteria are gram-positive bacteria, with a thick cell-wall layer with no significant outer membrane, indicating the possibility of effective surface display. For clear detection of changes in the adhesion of the substrate, we chose mannopentaose as the sugar moiety and investigated the interaction with concanavalin A (Con A).

Results and Discussion

A. Preparation of Sugar-Displaying Lactic Acid Bacteria. In our previous study, the hydrazine-attached fluorescent dye

(19) Hitchcock, S. A.; Eid, C. N.; Aikins, J. A.; Zia-Ebrahimi, M.; Blaszczak, L. C. *J. Am. Chem. Soc.* **1998**, *120*, 1916−1917.
(20) (a) Men, H.; Park, P.; Ge, M.; Walker, S. *J. Am. Chem. Soc.* **1998**, *120*, 2484−2485. (b) Ha, S.; Chang, E.; Lo, M.-H.; Men, H.; Park, P.; Ge, M.; Walker, S. *J. Am. Chem. Soc.* **1999**, *121*, 8415−8426.

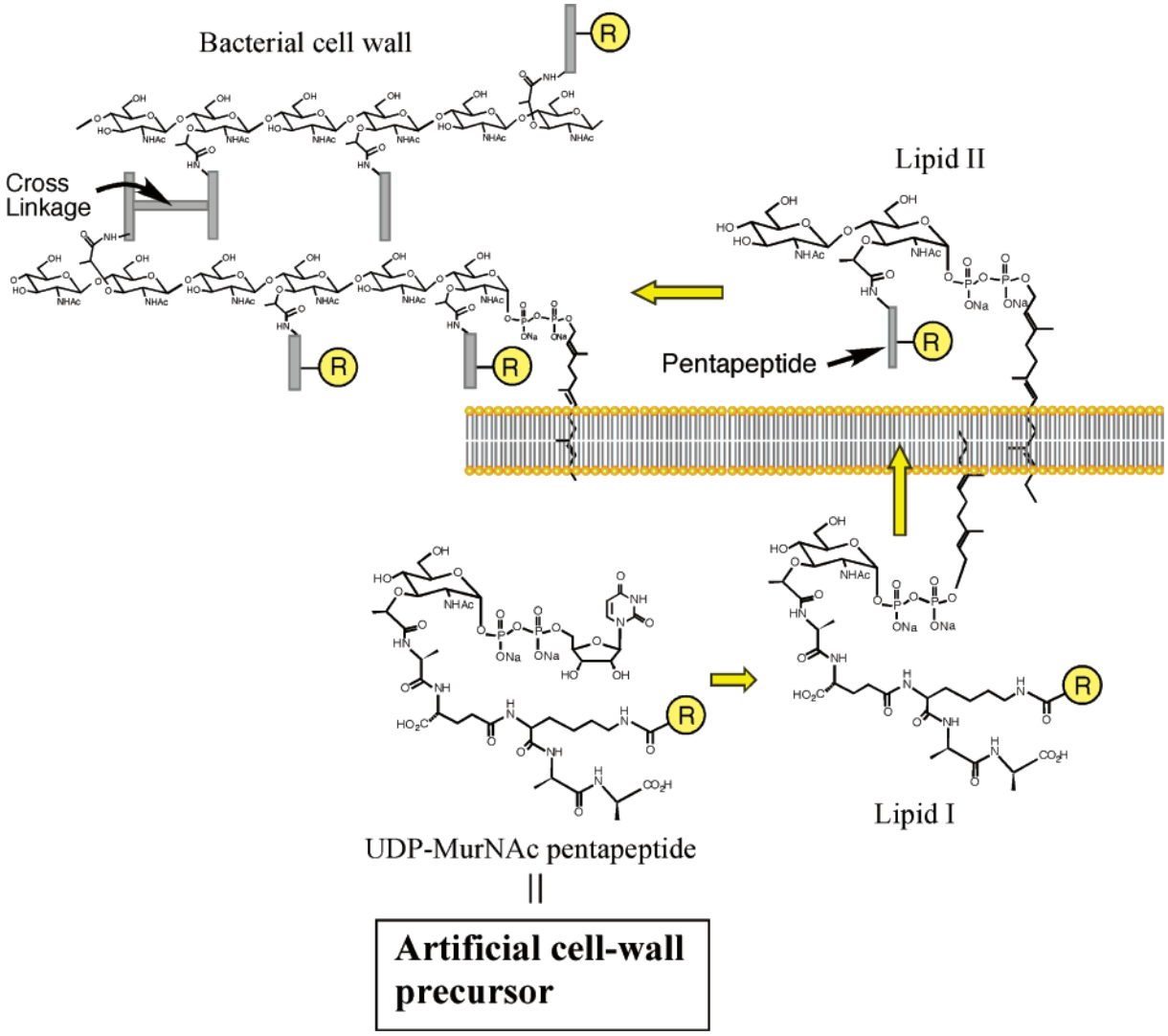


Figure 2. Metabolic incorporation of artificial precursors into the cell wall via the cell-wall biosynthetic route.

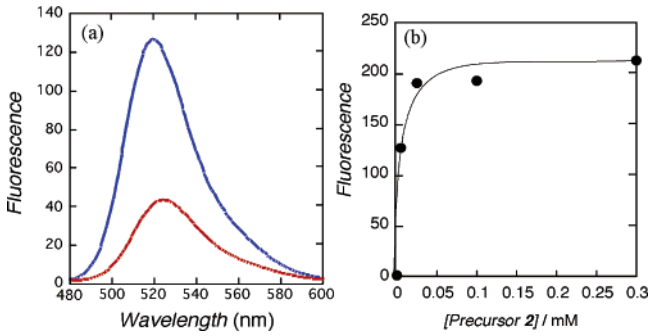


Figure 3. Fluorescence of the extracted cell-wall fractions after treatment with hydrazine-fluorescent dye. (a) Ketone-displaying lactic acid bacteria (blue line) and natural lactic acid bacteria (red line, control). (b) Concentration dependence of **2** on fluorescence intensities.

could be displayed on the surface of engineered lactic acid bacteria via ketone−hydrazine coupling. After culturing in the presence of the desired concentration of **2** for 15 h, the lactic acid bacteria were collected. Hydrazine-attached fluorescent dye was added to the bacterial suspension, and the cell-wall component was isolated to measure fluorescent intensities. Figure 3a shows the fluorescent spectra of the cell-wall component when treated with 5 μM of **2** (blue line). As a control experiment, the fluorescent dye was added to natural bacteria (red line). Fluorescence intensity from the ketone-displaying bacterial cell-wall fraction was larger than that from the control. The fluorescence-intensity dependency on precursor **2** concentration is shown in Figure 3b. The fluorescence intensity was saturated at approximately 10 μM.

On the basis of this method, a sugar moiety instead of fluorescent dye was displayed on the bacterial surface. As a target oligosaccharide we chose a mannose derivative. The control of the adhesion of the bacteria could be observed via the strong, specific interaction between mannopentaose and Con A. We prepared the linker **7**, having aminooxyl residues on both ends, from a dibromo derivative (Scheme 1). Mannopentaose was attached to the linker by one of the aminooxyl residues to generate **8** in 65% yield. This chemoselective coupling was performed in methanol at room temperature. This strategy has the great advantage of simplicity in that unprotected sugars can be used. The aminooxyl group located on the opposite end of the molecule can react selectively with the ketone group on the bacterial surface.

Ketone-displaying bacteria were prepared by the incubation of lactic acid bacteria (*L. plantarum* JCM1149) in a *Lactobacilli* MRS broth (Difco Laboratories, Detroit, MI) containing the precursor **2** (0.5 mM) under anaerobic conditions for 15 h at

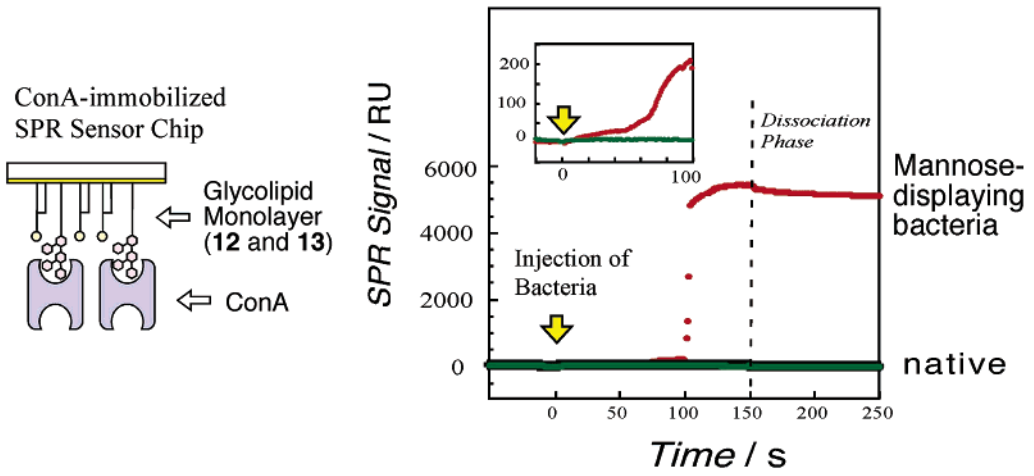
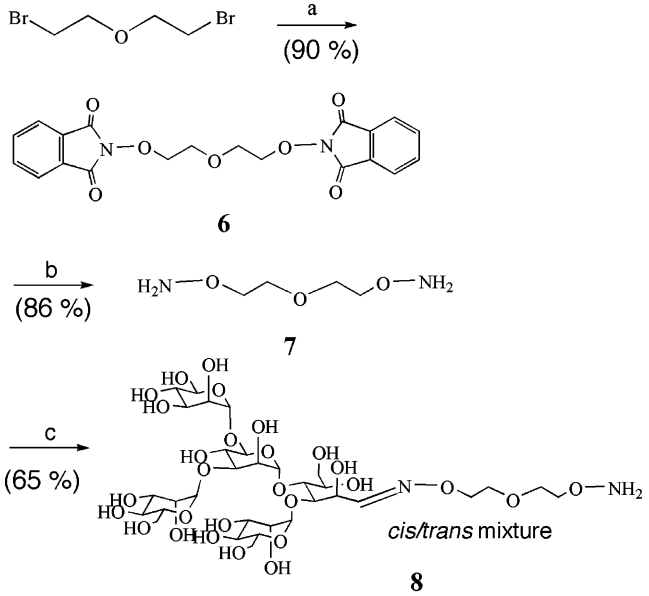


Figure 4. SPR detection of the adhesion of oligomannnose-displaying bacteria and natural bacteria onto the Con A-immobilized film.

Scheme 1. Synthesis of Aminooxyl-Functionalized Mannopentaose Derivative[a]



[a] (a) *N*-Hydroxyphthalimide, TEA, DMF. (b) Hydrazine, MeOH. (c) Mannopentaose, MeOH.

37 °C. On the basis of colony counting, the addition of the artificial precursor into the broth was observed to have no effect on bacterial growth.

After the collection of the bacteria by centrifugation, the bacteria were resuspended in a PBS buffer. The mannopentaose derivative **8** (1.2 mM) was added to the suspension of the ketone-displaying bacteria in a PBS buffer, and the mixture was allowed to stand for 1 h, was washed with PBS buffer three times, and was used for adhesion property experiments.

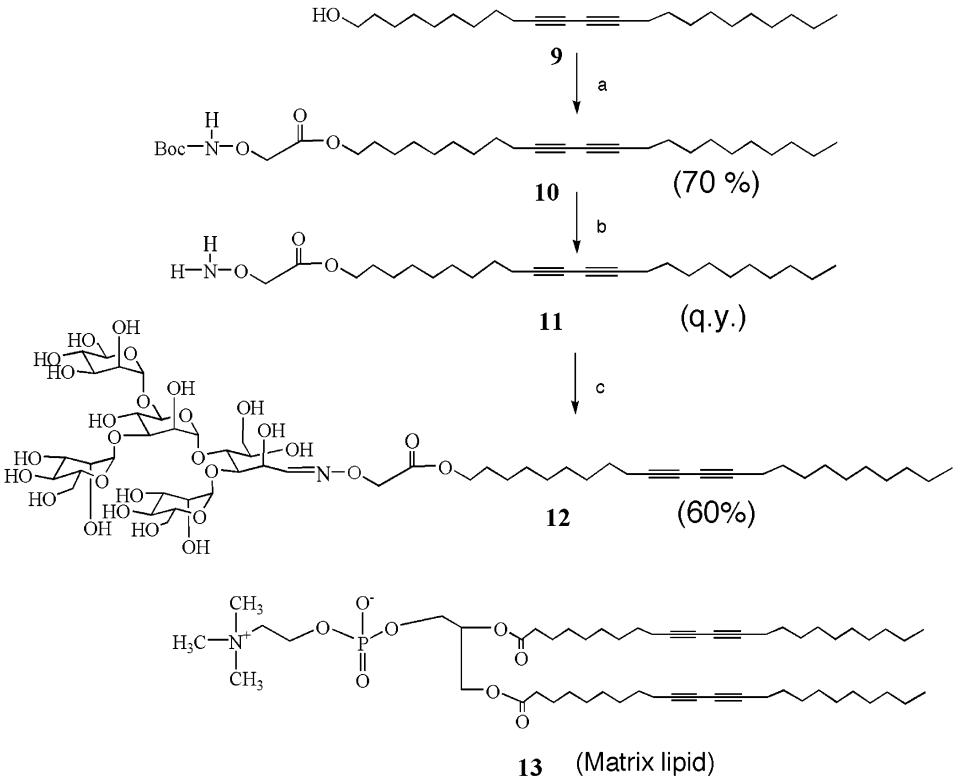
B. Adhesion of Oligosaccharide-Displaying Bacteria to the Con A-Immobilized Film. Adhesion experiments of engineered bacteria onto lectin films were carried out on a surface plasmon resonance (SPR) spectrometer (BIAcore 2000, Biacore, Sweden) using an HPA gold sensor chip covered by a 1-octadecanethiol self-assembled monolayer designed to facilitate liposome-mediated hydrophobic adsorption.[21] The modulation of the bacterial adhesion by cell-wall engineering was tested using the Con A (mannose-binding protein)-immobilized surface (Figure 4). Con A was immobilized on the mixed-lipid monolayer (containing mannose-attached lipid **12** and matrix phospholipid **13** at 1:9 mol/mol. The mannose-attached lipid **12** was synthesized from 10,12-pentacosadiyn-1-ol **9** (Scheme 2). Con A has four binding sites; therefore, at least two binding sites are still available after binding to the sensor chip. At time zero, engineered bacteria in 15 mM PBS buffer at 25 °C were injected onto the sensor surface. The red line represents the binding of the mannose-displaying bacteria. A large response was obtained after an initial small sigmoidal response with good reproducibility, whereas the injection of natural bacteria gave no response. These results indicate that mannose-displaying bacteria readily adhered to the Con A film.

Binding was confirmed directly by microscopic observation (Figure 5). The lectin plate was prepared on a slide glass by a similar procedure as in the SPR experiment. A suspension of engineered bacteria was mounted on the Con A-immobilized film for 10 min and washed with PBS buffer. The mannose-displaying bacteria bound more readily to the Con A-immobilized substrate than did natural bacteria, indicating that bacterial adhesion can be manipulated by surface display.

C. Truncated Cell-Wall Precursors. We speculated that the precursor would be incorporated more efficiently when more penetrative precursors were used. As an artificial cell-wall precursor with a shorter peptide moiety, UDP-MurNAc tripeptide derivative **5** was synthesized by a method similar to that described in our previous report.[17,18] With its smaller molecular weight, it was expected to be accessible to the cytoplasm through the cell wall and inner membrane. Lactic acid bacteria (*L. plantarum* JCM1149) were incubated for 15 h in a *Lactobacilli* MRS broth in the presence of the synthesized cell-wall precursor **5** (0.5 mM). As a control experiment, the bacteria were incubated with **4**, which could not be incorporated into the cell wall. After fluorophore labeling of the ketone group, the cells were collected and the cell-wall component was isolated according to the procedure described in the Experimental Section. The fluorescence intensity from the degraded cell-wall components was measured. Figure 6 shows a comparison of incorporation between the pentapeptide-type precursor **2** and

(21) (a) MacKenzie, C. R.; Hirama, T. *Methods Enzymol.* **2000**, *312*, 205−216. (b) Kabl, E.; Frey, S.; Tamm, L. K. *Biochim. Biophys. Acta* **1992**, *1103*, 307−316. (c) Mann, D. A.; Kanai, M.; Maly, D. J.; Kiessling, L. L. *J. Am. Chem. Soc.* **1998**, *120*, 10575−10582. (d) Ariga, T.; Kobayashi, K.; Hasegawa, A.; Kiso, M.; Ishida, H.; Miyatake, T. *Arch. Biochem. Biophys.* **2001**, *388*, 225−230.

Scheme 2. Synthetic Route to Mannose-Attached Lipid **12** and the Chemical Structure of the Matrix Lipid **13**



(a) Boc-amino-oxyacetic acid, DMAP, CH_2Cl_2; (b) TFA, CH_2Cl_2; (c) Mannnopentaose, MeOH

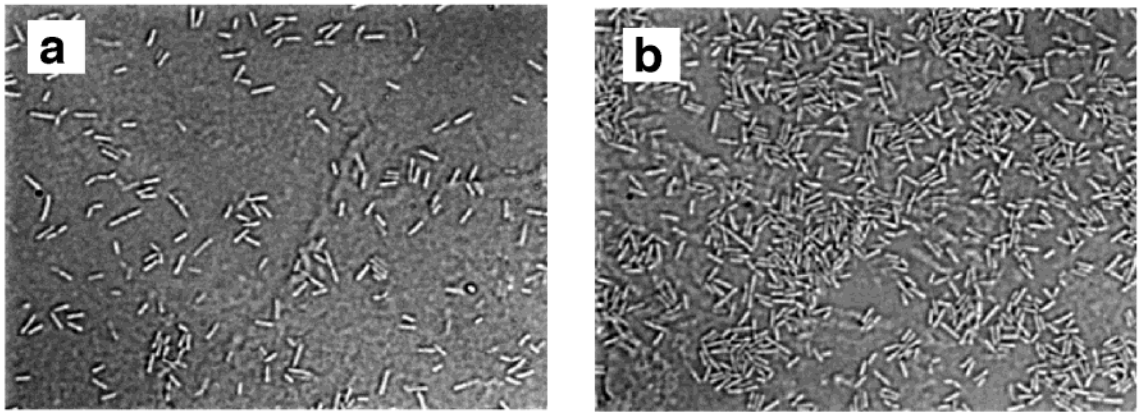


Figure 5. Binding of (a) natural bacteria and (b) mannose-displaying bacteria onto a Con A-immobilized substrate.

the tripeptide-type precursor **5**. In the case of the tripeptide-type precursor, the cell-wall fraction did not show a strong fluorescence intensity compared to the negative control. Truncation of the peptide moiety did not improve the incorporation ratio. UDP-MurNAc tripeptide was reported to be a poor substrate of translocase,[22] which transfers natural occurring precursors to the outside of the inner membrane of bacteria. The precursor **5** needs to be attached to a D-Ala-D-Ala dipeptide before being incorporated into the cell wall. This reaction might not occur in the bacteria. It is considered that the structure of the precursor was merely not adequate for surface displaying.

D. Effect of Antibiotics on Incorporation Efficiency. To increase the incorporation of the artificial precursor, we investigated decreasing the availability of native cell-wall precursors. We expected that the incubation of lactic acid bacteria in the presence of antibiotics known to inhibit biosynthesis of cell-wall precursors would result in a significant reduction in the availability of the native precursors. To survive under these conditions, bacteria must incorporate the artificial precursors, which had been added to the medium. As an antibiotic, we used fosfomycin, a well-known inhibitor of the transformation of UDP-GlcNAc to UDP-MurNAc.[23] Lactic acid bacteria (*L. plantarum* JCM1149, *L. salivarious* JCM1044, and *L. fermentum* No. 20) were incubated in a *Lactobacilli* MRS broth containing the ketone-attached UDP-MurNAc pentapeptide (0.2 mM) and fosfomycin (1.8 mg/mL) for 8 h. At this

(22) Rogers, H. J.; Perkins, H. R.; Ward, J. B. *Microbial Cell Walls and Membranes*; Chapman and Hall: London, 1980; pp 253−254.

(23) Ritter, T. K.; Wong, C.-H. *Angew. Chem., Int. Ed.* **2001**, *40*, 3508−3533 and references therein.

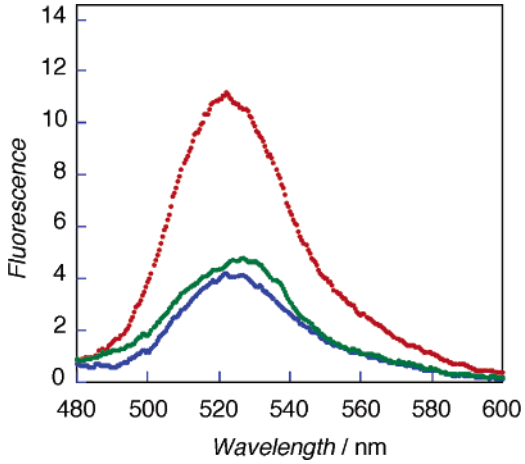


Figure 6. Fluorescence intensity of the cell-wall fraction of lactic acid bacteria. Lactic acid bacteria were incubated with the synthesized precursors and labeled with hydrazine-attached fluorescent dye. Red line: precursor **2**; green line: precursor **5**; blue line: precursor **4** (negative control).

concentration, bacterial growth was not totally inhibited. Figure 7 shows the fluorescent intensity of the cell-wall fractions of the three strains of bacteria. For all three strains, the fluorescence intensity of bacteria incubated with the antibiotic was larger than that from the bacteria incubated without the antibiotic. Thus, the artificial precursor was more effectively incorporated into the cell wall by incubation with fosfomycin than by fosfomycin-free incubation. When cycloserine, which is known to inhibit the peptide-elongation step in cell-wall biosynthesis,[23] was used instead of fosfomycin, no effect was observed for the *L. plantarum* JCM1149 and *L. salivarius* JCM1044 strains (data not shown). Further study on the mechanism of incorporation enhancement by antibiotics is now underway.

Conclusion

Using the modified cell-wall precursors as a carrier, we succeeded in displaying the target oligosaccharide sugars on the surface of living bacteria. Addition of fosfomycin increased the incorporation of the modified cell-wall precursor. Our study demonstrated that by displaying the oligosaccharides on the surface of bacteria, bacterial adhesion to the target substrate can be manipulated through protein−carbohydrate interactions. This method is widely applicable to various kinds of ligand−receptor interactions. The ability to control adhesion on the bacterial surface provides potential benefits to development of novel bacterial drugs. Effective and strong interaction with the mucosal wall of the intestine would be possible using the engineered bacteria. Therefore, functional molecule-displaying bacteria provide a potential model for the development of a novel range of oral vaccines.

Experimental Section

General. Mannopentaose was purchased from Funakoshi Co. (Japan). Con A was purchased from Seikagaku Co. (Japan). 10,12-Pentacosadiyn-1-ol was purchased from Tokyo Kasei Co. (Japan). Boc-amino-oxyacetic acid was purchased from Novabiochem. Reactions were monitored by TLC on 250-μm silica gel plates (Merck, NJ) using UV light and a cerium molydate solution (10% cerium(IV) sulfate, 15% H_2SO_4 aqueous solution). NMR spectra (500 MHz) were recorded on a Bruker AMX-500 instrument. All NMR measurements were carried out at room temperature in $CDCl_3$, D_2O, or methanol-d_4. The synthesized precursors were sterilized by filtration before being added to *Lactobacilli* MRS broth (Difco-Laboratories) for bacterial incubation.

Compound 6. 2-Bromoethyl ether (2 g, 8.7 mmol), *N*-hydroxyphthalimide (5.5 g, 34 mmol), and TEA (5 mL) were dissolved in DMF. After being stirred for 24 h at 40 °C, the solvent was evaporated in vacuo. The residue was dissolved in chloroform and washed with brine.

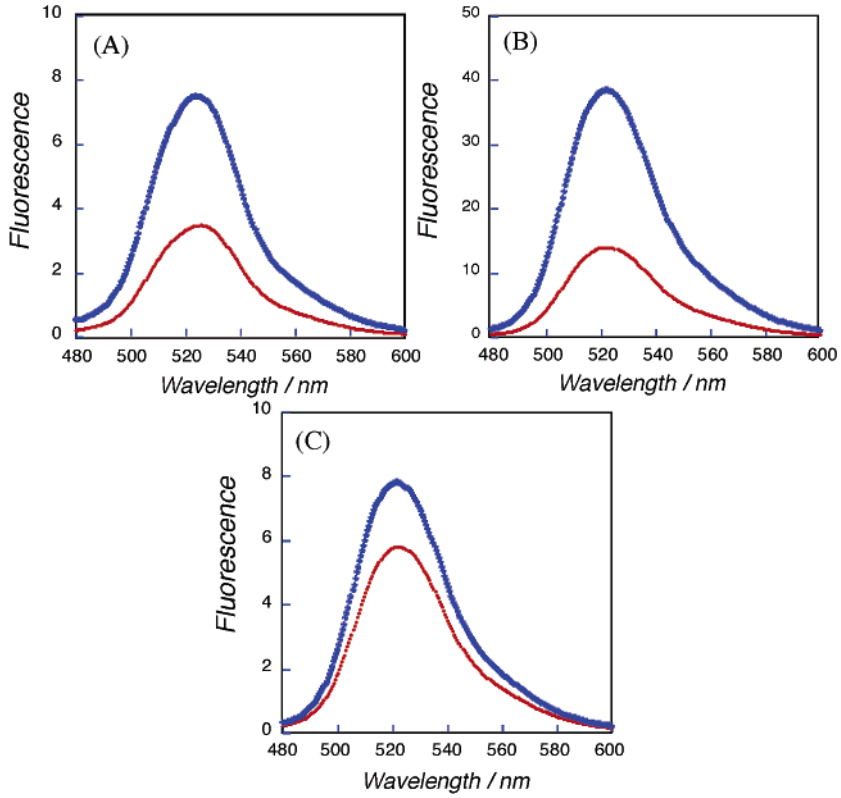


Figure 7. Fluorescence intensity of the cell-wall fraction from three strains of bacteria incubated with and without fosfomycin. The blue lines represent the intensity after incubation with fosfomycin, and the red lines show that without fosfomycin.

The organic layer was dried over Na_2SO_4 and concentrated. The residue was purified using silicagel column chromatography eluted with dichloromethane/EtOAc (7/3) to generate pure **6** as white solid in 90% yield. ^{1}H NMR (CD_3OD, 500 MHz) 7.81 (4H, dd, J =3.16 and 5.36 Hz), 7.75 (4H, dd, J = 3.16 and 5.67 Hz), 4.28 (4H, m), 3.89 (4H, m).

***O*-[2-(2-Aminooxy-ethoxy)-ethyl]-hydroxylamine 7.** Hydrazine (2 mL) was dropped into a solution of compound **6** (2.8 g) in methanol (40 mL) at room temperature. The solution was warmed to 40 °C and stirred for 12 h. After the solvent was evaporated in vacuo, the residue was purified using silicagel chromatography eluted with dichloromethane/methanol (7/3) to generate pure **7** as a free form. The free form of **7** was dissolved in 3% TFA aqueous solution, and the solution was then lyophilized to produce **7** as a TFA salt in 86% yield. ^{1}H NMR (CD_3OD, 500 MHz) 4.47 (4H, bs), 3.43 (4H, bs). ESI-MS: Calcd for $C_4H_{12}N_2O_3$: m/z = 137 $[M + H]^+$; found: m/z = 136.

Aminooxyl-Functionalized Mannopentaose Derivative 8. Mannopentaose (10 mg) and compound **7** were dissolved in methanol for 24 h at room temperature. The reaction mixture was purified by Biogel P-4 column to generate **8** as a white power in 65% yield. ESI-MS: Calcd for $C_{34}H_{63}N_2O_{28}$: m/z = 945 $[M - H]^-$; found: m/z = 945.

Compound 10. To a mixture of 10,12-pentacosadiyn-1-ol **9** (200 mg, 0.56 mmol), Boc-amino-oxyacetic acid (104 mg, 0.55 mmol), and (dimethylamino)pyridine (20 mg) in anhydrous CH_2Cl_2, 1-ethyl-3(3′-dimethylaminopropyl)carbodiimide-HCl (123 mg, 0.64 mmol) was added at 0 °C. The reaction mixture was stirred for 1 h at 0 °C and for 8 h at room temperature. The mixture was washed with water and brine and then dried (Na_2SO_4). The solvent was evaporated in vacuo. The residue was purified by silica gel chromatography (9.5:0.5 CH_2Cl_2−EtOAc) to give a colorless liquid **10** in 70% yield. ^{1}H NMR ($CDCl_3$): 4.41 (s, 2H), 4.13 (t, 2H), 2.23 (t, 4H), 1.47 (s, 9H), 1.46−1.22 (m, 36H), 0.87 (t, 3H). ESI-Mass (pos): Calcd for $C_{32}H_{55}NO_5$: m/z 556.8 $[M + H]^+$; found: m/z 556.3 $[M + H]^+$.

Aminooxyl-Functionalized Lipid 11. To a solution of compound **10** (0.5 g) in 50 mL of CH_2Cl_2, trifluoroacetic acid (10 mL) was added at 0 °C. The reaction mixture was stirred for 5 h at 0 °C. Toluene (10 mL) was added to the reaction mixture, and solvents were evaporated in vacuo to generate a pure compound **11** in a quantitive yield. ^{1}H NMR (500 MHz, $CDCl_3$): 7.43 (bs, 3H), 4.38 (s, 2H), 4.16 (t, J = 7.10 Hz, 2H), 2.23 (t, 4H, J = 7.25 Hz), 1.6−1.1 (m, 36H), 0.857 (t, J = 7.25 Hz, 3H). ESI-MS (pos): Calcd for $C_{27}H_{48}NO_3$: m/z = 434.6 $[M + H]^+$; found: m/z 434.3.

Mannopentaose-Attached Lipid 12. Lipid **11** (5 mg, 9.1 μmol) and mannopentaose (5 mg) were mixed in methanol (0.7 mL) at room temperature. The mixture was stirred for 24 h, and the solvent was evaporated. The residue was purified by silica gel chromatography and eluted with 6:4 (v/v) chloroform/methanol to afford glycolipids **12** in 60% yield. The ratio of trans- to cis-isomers for **12** was 20:1 as judged from the area of signals corresponding to oxime protons [trans-isomer: d 7.73 (d, J = 5.65 Hz); cis-isomer: d 6.98 (d, J = 5.00 Hz)] in the NMR spectra in a mixed solvent of $CDCl_3$ and CD_3OD. ^{1}H NMR ($CD_3OD/CDCl_3$ = 1:1): [trans-isomer: d 7.73 (d, J = 7.55 Hz); cis-isomer: d 7.00 (brs)], 7.62 (d, 5.13 (1H, H-1), 5.11 (1H, H-1), 4.85 (1H, H-1), 4.79 (1H, H-1), 2.25 (t, 3H, J = 6.05, 6.60 Hz), 0.89 (t, 3H, CH_3). TOF MS m/z 1267.57 $[M + Na]^+$ (Calcd for $C_{58}H_{97}N_3O_{25}Na^+$: 1266.61).

Incorporation Experiments of UDP-MurNAc Derivatives. Lactic acid bacteria were grown in 1 mL *Lactobacilli* MRS broth containing **2** (0−0.5 mM, sterilized by filtration before being added to the culture media) under anaerobic conditions for 18 h at 37 °C. Cells were collected by centrifugation at 1500g for 3 min and washed three times with PBS buffer to remove culture media. Alexa 488 hydrazide (Molecular Probes, Eugene, OR) was added to the bacterial suspension to a final concentration of 0.17 mM in PBS buffer (pH 7) and incubated for 1 h at room temperature. After the cells were disrupted by ultrasonication, the residue was applied to a centrifuge column (Microcon YM-10 Millipore, Billerica, MA) and washed three times with PBS. Then, the residue was treated with 4 μL of lysozyme solution (50 mg/mL in water, lysozyme from chicken egg white, ICN Pharmaceuticals, Costa Mesa, CA) for 5 h at 37 °C. After adding a PBS buffer containing 10% DMSO to the residue, the mixture was filtered through a Microcon YM-10. The filtrate, containing the digested cell wall, was diluted with the buffer to the same volume for each sample with the buffer and used for fluorescence measurement.

SPR Detection of Bacterial Adhesion on Glycolipid Membranes. Mannose-attached glycolipid **12** (5.0 mmol) and 10,12-tricosadiynoyl-containing phospholipid **13** (Avanti Polar Lipids, Alabaster, AL, 45.2 mmol) were dissolved in $CHCl_3$/MeOH (9/1), and the solvent was evaporated to yield a thin lipid film on the inner surface of the flask. Deionized water was added to the flask to adjust the concentration of the lipid to 0.3 mM. The solution in the flask was heated to 75 °C and sonicated using a probe-type sonicator (Sonifier II, Branson, CT) for 10 min. The warm, clear solution containing the liposome was then cooled to 4 °C. Lactic acid bacteria-binding experiments were carried out on an SPR system (Biacore 2000, Biacore, Sweden). The liposome solution was injected onto a hydrophobic chip (Biacore HPA chip) that was covered with a 1-octadecanethiol self-assembled monolayer designed to facilitate liposome-mediated hydrophobic adsorption. After coating the surface of the sensor chip with the glycolipid, the sensor surface was rinsed with buffered solution until the baseline became flat. The lactic acid bacteria suspensions were applied to the sensor surface at a flow rate of 20 μL/min at 30 °C.

Microscopic Observation. For microscopic observations, lactic acid bacteria were incubated in *Lactobacilli* MRS broth containing **2** (0.5 mM) for 15 h under anaerobic conditions. Compound **2** was dissolved in PBS and sterilized by filtration before addition to the media. After culturing, the cells were collected and washed three times with PBS to remove media and compounds not incorporated into the cell wall. Mannopentaose derivative **8** was added (1 mM) to the bacteria suspended in a PBS solution and incubated for 30 min. The bacteria collected by centrifugation were washed three times with PBS, resuspended in a mixture of PBS and ethanol (1:1 v/v) and applied onto a glycolipid-coated slide glass prepared by a similar procedure to that described above, and observed by microscope in differential interference mode (Olympus BX51, Japan).

Incorporation Experiments in the Presence of Fosfomycin. Three strains of lacticacid bacteria *(L. plantarum* JCM1149, *L. salivarious* JCM 1044, *L. fermentum* No. 20) were grown in *Lactobacilli* MRS broth broth containing **2** (0.5 mM) with or without fosfomycin (1.8 mg/mL) under anaerobic conditions for 8 h at 37 °C. Cells were treated using the same procedure as that described above, and the fluorescence intensity was measured (Hitachi F-2500, Japan).

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