Identification of a New α1,2-Fucosyltransferase Involved in O-Antigen Biosynthesis of *Escherichia coli* O86:B7 and Formation of H-Type 3 Blood Group Antigen

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ABSTRACT: *Escherichia coli* O86 possesses high human blood group B activity because of its O-antigen structure, sharing the human blood group B epitope. In this study, the *wbwK* gene of *E. coli* O86:B7 was expressed and purified as the GST fusion protein. Thereafter, the *wbwK* gene was biochemically identified to encode an α1,2-fucosyltransferase through radioactivity assays, as well as mass spectrometry and NMR spectroscopy. WbwK shows strict substrate specificity and only recognizes Gal*β*1,3GalNAcα-OR (T-antigen and derivatives) as the acceptor to generate the H-type 3 blood group antigen. In contrast to other α1,2-fucosyltransferases, WbwK does not display activity toward the simple substrate Gal*β*-OMe. Comparison with another recently characterized α1,2-fucosyltransferase (WbsJ) of *E. coli* O128:B12 indicates a low level of amino acid identity between them; however, they share a common acceptor substrate, Gal*β*1,3GalNAcα-OR. Domain swapping between WbwK and WbsJ revealed that the smaller variable domains located in the C-terminus determine substrate specificity, whereas the larger variable domain in the N-terminus might play a role in forming the correct conformation for substrate binding or for localization of the α1,2-fucosyltransferase involved in O-antigen biosynthesis. In addition, milligram scale biosynthesis of the H-type 3 blood group antigen was explored using purified recombinant WbwK. WbwK may have potential applications in masking T-antigen, the tumor antigen, *in vivo*.

The O-antigens, also called O-polysaccharides, are important components of the outer membrane of Gram-negative bacteria (*1*) and virulence factors of pathogenic bacteria (*2*). The O-antigens are extremely variable, represented by their different sugar composition, order, and linkage between the sugar residues (*1, 3*). The O-antigens are also strongly immunogenic. Extensive variations of O-antigens contribute to a wide variety of antigenic types (O-serotype) between species and strains of Gram-negative bacteria (*2*−*4*). Growing evidence suggests that the O-antigen plays an important role in effective colonization of host tissues and resistance to host complementary reactions and is crucial for specific interactions with the host immune system (*2, 5*).

Many pathogens display blood group antigens on their cell surface, such as *Escherichia coli* O86, O90, O127, and O128 O-antigens (*6*), and these strains utilize molecular mimicry to evade the immune system of the human host (*7, 8*). To combat infectious diseases efficiently, it is important to understand the molecular mechanisms of the pathogens causing the diseases. Genetic and biochemical studies of O-antigen biosynthesis genes of pathogenic bacteria would increase our knowledge of the biosynthesis pathway. Such information is crucial in order to elucidate the molecular mechanisms of pathogenesis.

E. coli O86:B7, a Gram-negative enteropathogenic bacteria, was the predominant serogroup causing diarrhea in children in developing countries (*9*). During the 1960s, Springer and co-workers suggested that naturally occurring anti-A and -B antibodies are raised in response to the constant enteric bacterial stimulation within the intestinal flora (*10*). These studies found that many Gram-negative bacteria showed blood group activities, among which *E. coli* O86 possesses high human blood group B activity (*11*). Immunological studies revealed that humans and germ-free chickens were able to generate a high titer of anti-B antibody upon feeding with killed *E. coli* O86:B7. This work supports the hypothesis that the origin of human blood group anti-AB antibody is from immune stimulation by bacteria rather than parental inheritance (*12*). Later, Andersson et al. (*13*) determined the O-antigen structure of *E. coli* O86:H2 by nuclear magnetic resonance (NMR).[1] Their results revealed that the O-antigen of *E. coli* O86 and human blood B antigen share a similar oligosaccharide epitope. Recently, in the same manner, Yi and co-workers demonstrated that the O-antigen structure of *E. coli* O86:K62:B7 (Figure 1) shares the

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[1] Abbreviations: FucT, fucosyltransferase; GDP, guanosine 5′-diphosphate; Fuc, fucose; Fru, fructose; Gal, galactose; Glc, glucose; Man, mannose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; GST, glutathione *S*-transferase; IPTG, isopropyl 1-thio-*β*-D-galactosylpyranoside; DTT, dithiothreitol; HRP, horseradish peroxidase; TLC, thin-layer chromatography; MS, mass spectrometry; NMR, nuclear magnetic resonance; Bn, benzyl; Me, methyl; *p*NP, *p*-nitrophenyl.

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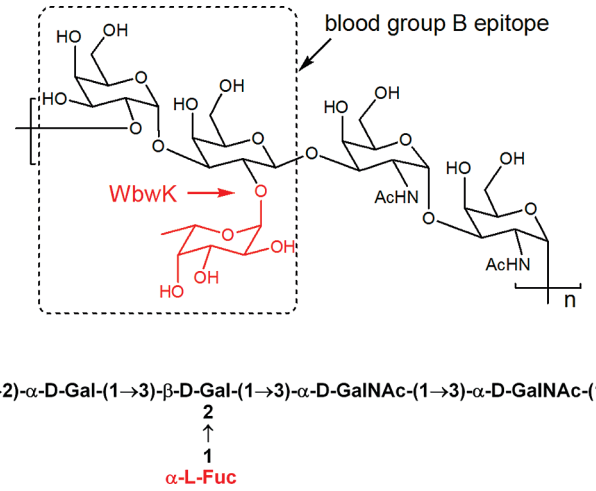

FIGURE 1: The O-antigen repeat unit structure of *E. coli* O86:B7. The fucose residue is highlighted in red, and the human blood group B antigen epitope is labeled in a dashed box.

identical O-antigen unit structure with *E. coli* O86:K2:H2, but with different polymerization sites on different sugar residues (*14*). Recent studies also found that the O-antigen gene cluster of *E. coli* O86 is closely related to those of *E. coli* O127 and *Samonella* serogroup O13 (*15, 16*).

The genes involved in O-antigen biosynthesis include nucleotide sugar biosynthesis genes, sugar transferase genes, and O-antigen processing genes. These genes are generally found on the chromosome as an O-antigen gene cluster (*1*). In our previous study, to cover all the genes involved in O-antigen biosynthesis, we cloned and sequenced the chromosomol sequence of *E. coli* 086:B7 between *galF* and *hisI* (GenBank accession number AY220982 (*17*). The homology study indicates that all the genes involved in O-antigen biosynthesis are located between *galF* and *hisI*. The *wbwK* gene is one of glycosyltransferases that was identified within this O-antigen gene cluster. Therefore, there is no doubt this gene is responsible for O-antigen biosynthesis. However, further evidence is needed to validate the biological function of the putative glycosyltransferases and the assumption made based on homology analysis. Herein, we focus on revealing the function of the *wbwK* gene (putative α1,2-FucT) using a biochemical approach. In this study, we demonstrated the function of WbwK as an α1,2-fucosyltransferase by determining the enzyme activity of purified protein encoded by the *wbwK* gene as well as the enzymatic product using MS and NMR analysis. We also demonstrated that the enzymatic product is in agreement with the O-antigen repeating unt structure of *E.coli* 086:B7. Therefore, WbwK is involved in biosynthesis of the O-antigen repeating unit of *E. coli* 086:B7 by adding a fucose to galactose, the third residue of the O-antigen repeating unit, in an α1,2-linkage (Figure 1).

The α1,2-fucosyltransferase catalyzes transfer of a fucose from GDP-fucose (donor) to galactose-*β*-OR (acceptor) via an α1,2-linkage (Figure 2). Five different human H-type blood group antigens have been identified, which are catalyzed by α1,2-FucTs: type 1, Fucα1,2Gal*β*1,3GlcNAc*β*1-R; type 2, Fucα1,2Gal*β*1,4GlcNAc*β*1-R; type 3, Fucα1,2-Gal*β*1,3GalNAcα1-R; type 4, Fucα1,2Gal*β*1,3GalNAc*β*1-R; and type 5, Fucα1,2Gal*β*1,4Glc*β*1-R (*18*). In human, there are two α1,2-FucTs, FUT1 and FUT2, participating in the synthesis of these antigens. In bacteria, only two bacterial α1,2-fucosyltransferases have been identified biochemically and characterized, the Hp FucT2 of *Helicobacter pylori* (*19*) and WbsJ of *E. coli* O128:B12 (*20*). These two α1,2-FucTs are both involved in the biosynthesis of O-antigens containing the blood group antigen epitopes, Lewis X and B-antigen, respectively. Their different substrate specificities reflect the variations of their O-antigen structures. In this study, the α1,2-fucosyltransferase activity of WbwK was biochemically identified and characterized. WbwK showed strict substrate specificity and was only active with substrates containing the Gal*β*1,3GalNAcα- structure (T-antigen) to form H-type 3 blood group antigen (Fucα1,2Gal*β*1,3GalNAcα-OR). In contrast to known α1,2-fucosyltransferases, WbwK is not active to the simple substrate Gal*β*-OMe. In addition, the substrate specificity determinant of α1,2-fucosyltransferase was investigated by domain swapping between WbwK of *E. coli* O86:B7 and WbsJ of *E. coli* O128:B12, a recently discovered bacterial α1,2-fucosyltransferase. The results in this study provide insight into the mechanism of bacterial α1,2-fucosyltransferase and pathway of O-antigen repeating unit synthesis of *E. coli* O86:B7. This study demonstrated that the sequential synthesis of B-antigen epitope in *E. coli* O86:B7 is the same as that of human blood group B antigen; i.e., H-antigen is synthesized first as a precursor followed by B-antigen. In addition, WbwK may have biomedical potential in masking the tumor antigen, T-antigen.

MATERIALS AND METHODS

Reagents. Restriction endonucleases and T4 DNA ligase were obtained from Promega (Madison, WI). Glutathione Sepharose 4B was from GE Healthcare (Piscataway, NJ). GDP-L-fucose (GDP-Fuc) was from Kyowa Hakko (Tokyo, Japan), GDP-L-[^{13}C-(U)]fucose was from PerkinElmer (Waltham, MA). GDP affinity gel, mouse anti-GST monoclonal antibody, Gal*β*-OMe, Gal*β*1,3GalNAcα-OBn, and Gal*β*1,3GalNAcα-OMe were purchased from Calbiochem (San Diego, CA). Lactose, lactulose, Gal*β*1,4Man, reduced L-glutathione, and ampicillin were from Sigma (St. Louis, MO). Bio-Gel P-2, horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody, HRP color reagent kit, and protein concentration assay kit were purchased from Bio-Rad (Hercules, CA). All other chemicals were obtained from Sigma or Fisher (Pittsburgh, PA).

Bacterial Strains and Plasmids. E. coli competent cell DH5α [*lacΔM15 hsdR recA*] was purchased from Gibco-BRL Life Technology (Carlsbad, CA). Plasmid pGEX-4T-1 was purchased from GE Healthcare (Piscataway, NJ). *E. coli* competent cells BL21 (DE3)[F^- *ompT gal dcm hsdS*$_B$ ($r_B^-m_B^-$) (DE3)] were acquired from Novagen (Madison, WI). *E. coli* O86:K62:B7 strain (ATCC 12701) was purchased from American Type Culture Collection (ATCC) (Manassas, VA).

Construction of the Expression Plasmid of wbwK. The *wbwK* gene, putatively encoding α1,2-FucT, was cloned into plasmid pGEX-4T-1 with a GST tag fused at the N-terminus. Primers wbwK-Fa and wbwK-Rc (Table 1) were used to amplify the *wbwK* gene through the polymerase chain reaction (PCR) from chromosomal DNA of *E. coli* O86:B7. The PCR product was digested with *Bam*HI/*Xho*I and inserted into the *Bam*HI/*Xho*I sites of pEGX-47-1 by ligation. Following transformation into *E. coli* DH5α with ampicillin

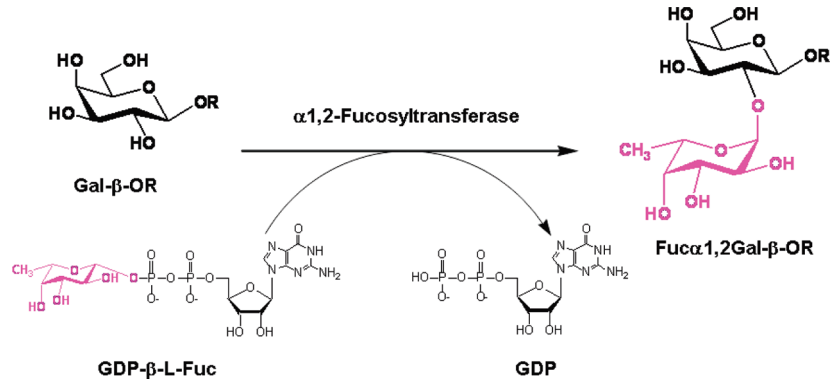

FIGURE 2: Scheme of α1,2-fucosyltransferase-catalyzed reaction. The donor is GDP-Fuc, and the acceptor is Galβ-OR.

Table 1: Primers for Construction of Plasmids Carrying Chimeric α1,2-FucTs

primer	sequence
wbwK-Fa	5′ CGCGGATCCATGTATAGTTGTTTGTCTGGTGGAT 3′
wbwK-Ra	5′ CTCTTCTAATATGTAATGAAGTTGTGTTAGCATTCTCA 3′
wbwK-Fb	5′ TTAAAGAAAATGATACTTGTTCACTACATATCAGGCGTGGAGA 3′
wbwK-Rb	5′ TTATATTATTTTTACACTTTGACATTAAAAACAT 3′
wbwK-Fc	5′ TGCTGCTTATGAGTAAATGTAAAAACAATATAATAGCCAATAG 3′
wbwK-Rc	5′ CCGGAATTCTCGAGTCAAAGATTAATCCAACTATCCAT 3′
wbsJ-Fa	5′ CGCGGATCCATGGAAGTTAAAATTATTGGGGGGCT 3′
wbsJ-Ra	5′ ACGCCTGATATGTAGTGAACAAGTATCATTTTCTTTAA 3′
wbsJ-Fb	5′ TGAGAATGCTAACACAACTTCATTACATATTAGAAGAGGTGAT 3′
wbsJ-Rb	5′ TATTATATTGTTTTTACATTTACTCATAAGCAGCA 3′
wbsJ-Fc	5′ GTTTTTAATGTCAAAGTGTAAAAATAATATAATTTCAAATAG 3′
wbsJ-Rc	5′ CGGAATTCTCGAGTCATAATTTTACCCACGATTCG 3′

selection (100 μg/mL), the resulting recombinant plasmid pGEX-wbwK was characterized by restriction mapping, and the cloned *wbwK* gene was confirmed by DNA sequencing. The correct constructs were subsequently transformed into *E. coli* BL21 (DE3) for protein expression.

Construction of Chimeric FucTs. The templates for chimera construction were the plasmids pGEX-wbwK (this work) and pGEX-wbsJ (*20*). A set of six chimeric α1,2-FucTs were constructed by a two-step PCR approach, in which differently sized and positioned elements of one parental gene (*wbwK*, *E. coli* O86) were replaced by the equivalent of the second parental gene (*wbsJ*, *E. coli* O128) or vice versa. The regions to be swapped within the two genes were defined by the conserved motif sites. Primers used to construct the chimera genes were designed based on identical sequences within the motifs. The primers had at least 25 overlapping base pairs for the second-step PCR, and the chimeric genes were all generated with the *Bam*HI/*Xho*I sites at the termini and cloned into plasmid pGEX-4T-1. The sequences of primers used to construct chimeric FucTs are listed in Table 1. The constructed expression plasmids of chimeric FucTs were sequenced to confirm the identity of the swapped domains.

Overexpression and Purification of GST-Tagged Proteins. *E. coli* BL21 (DE3) harboring the recombinant plasmids, including the wild type and chimeric mutants, was grown in 1 L Luria−Bertani (LB) medium at 37 °C, 220 rpm, with ampicillin selection. When OD_{600nm} reached 0.8, isopropyl 1-thio-β-D-galactosylpyranoside (IPTG) (1 mM) was added to induce protein expression. The cultures were grown for an additional 6 h at 30 °C and then harvested by centrifugation at 5000*g* for 20 min at 4 °C.

The cell pellets were suspended in GST binding buffer A (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) with addition of 1 mM dithiothreitol (DTT) and disrupted by sonication on ice for 2 min at 50% pulse using a Branson Sonifier 450. The cell lysate was clarified by centrifugation (10000*g*, 30 min), and the supernatant was applied onto a GST affinity column packed with 5 mL of glutathione Sepharose 4B resin. The bound protein was washed with 5 bed volumes of buffer A followed by elution with buffer B (50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0).

Fucosyltransferase Activity Assay. Enzyme activities were determined at 37 °C for 2 h in a final volume of 50 μL containing 20 mM Tris-HCl, pH 7.0, 1 mM ATP, 0.3 mM GDP-β-L-fucose, GDP-L-[U-^{14}C]fucose (10000 cpm), 20 μL of enzyme, and 10 mM acceptor. Acceptor was omitted in the control blank. The reaction was stopped with addition of 150 μL of ice-cold water and Dowex 1X 8−200 chloride anion-exchange resin in a water suspension (0.8 mL, v/v = 1/1). After vortexing and centrifugation, the supernatant (0.5 mL) was collected in a 20 mL plastic scintillation vial and mixed thoroughly with ScintiVerse BD (10 mL). The radioactivity of the mixture was counted in a liquid scintillation counter (Beckmann LS-3801 counter).

Protein concentrations were determined by the Bradford method using a Bio-Rad protein assay kit with BSA as standard protein.

Western Blot Analysis. Protein samples were first separated by 4−12% gradient SDS−PAGE and then electrophoretically transferred onto a nitrocellulose membrane followed by blocking with 3% gelatin in TBS-T buffer (20 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20, pH 7.4). The GST-tagged protein was first probed with mouse anti-GST monoclonal antibody (1:3000 dilution) for 1 h followed by wash with TBS-T buffer. Subsequently, the blot was probed with horseradish peroxidase (HRP)-conjugated goat anti-

mouse antibody (1:3000 dilution) for 1 h, washed, and developed in HRP color reagent.

GDP Bead Binding. Purified protein (25 μg) was incubated with 30 μL of GDP beads (glycosyltransferase affinity gel-GDP, 10 μmol of GDP/mL of gel slurry) which was washed and equilibrated with buffer C (50 mM Tris-HCl, 5% glycerol, 100 mM NaCl, 5.0 mM $MgCl_2$, 1 mM DTT, pH 7.0) in a final volume of 100 μL at 4 °C for 1 h with gentle agitation. After washing the unbound protein with buffer C, the bound protein on the beads was released from GDP beads by boiling with 2 × SDS loading buffer and analyzed by SDS−PAGE followed by Western blotting as described above.

Enzymatic Synthesis of Blood Group H Antigen. Synthesis of trisaccharide Fucα1,2Galβ1,3GalNAcα-OMe (H-type 3 blood group antigen) was carried out using purified protein GST-WbwK. The reaction was conducted for 3 days at 30 °C in a final volume of 1.0 mL containing 20 mM Tris-HCl (pH 7.5), 1 mM $MnCl_2$, 1 mM DTT, 10 mM GDP-Fuc, 15 mM acceptor Galβ1,3GalNAcα-OMe, and ca. 1.0 mg of purified GST-WbwK protein. The progress of the reaction was monitored by thin-layer chromatography (TLC) in 2-propanol/H_2O/NH_4OH (8:2:2 v/v/v) conducted on Baker Si250F silica gel TLC plates. Products were visualized by staining solution (anisaldehyde/methanol/H_2SO_4=1:15:2 (v/v/v)). The reaction was stopped by brief boiling and centrifugation (12000*g*, 5 min). The supernatant was purified by gel filtration chromatography Bio-Gel P2. The desired fractions were pooled, lyophilized, and stored at −20 °C.

Mass Spectrometry (MS) and Nuclear Magnetic Resonance (NMR) Analysis. Mass spectrometry (ESI) was run in the negative mode on ESI-QTOF (Micromass). The product structure was identified through one-dimensional ^1H NMR using a Bruker Avance 500 MHz NMR spectrometer. The oligosaccharide product was exchanged with D_2O prior to NMR spectrometry.

RESULTS

Characterization of WbwK. In a previous study, the gene cluster for O-antigen *E. coli* O86:B7 biosynthesis was cloned. Homologous studies identified the genes responsible for nucleotide sugar biosynthesis, O-antigen processing, and four putative glycosyltransferases. Among them, *wbwK* was identified to be a putative α1,2-fucosyltransferase (α1,2-FucT) (GenBank accession number AAO37719). A BLAST search of WbwK indicated a putative conserved domain, which was found in the glycosyltransferase family 11 of the CAZY system (http://www.cazy.org/fam/GT11.html). This family contains α1,2-FucTs cloned from various species of human, animal, bacteria, and virus (*21*). The DNA length of *wbwK* is 909 base pairs, corresponding to a 302 amino acid protein. Sequence comparison of WbwK with α1,2-FucTs belonging to the glycosyltransferase family 11 showed a high level of amino acid identity to α1,2-FucTs from certain bacteria strains, of which O-antigen clusters are closely related, such as WfbI of *Salmonella enterica* O13 (51%) (*15*) and WbiQ of *E. coli* O127:K63:B8 (48%) (AAR90894). WbwK shares a low level of amino acid identity to the functionally characterized α1,2-FucTs, including WbsJ of *E. coli* O128:B12 (34%) (*20*), *Hp* FucT2 of *H. pylori* (30%) (*22*), and human FUT2 (20%) (*23*). Topology analysis of WbwK protein using two different servers, DAS (Dense Alignment Surface) (*24*) and TMpred (Prediction of Transmembrane Regions and Orientation), resulted in the same predictions. Both analyses showed that a short transmembrane domain located approximately in the region from Y^{80}(or L^{82}) to P^{101}(or Y^{99}) with a significantly high score. This result is consistent with the fact that glycosyltransferases for O-antigen repeating unit biosynthesis are located in the inner membrane facing the cytoplasmic side (*1*). Since this predicted transmembrane domain is very short, WbwK was predicted to be a soluble protein.

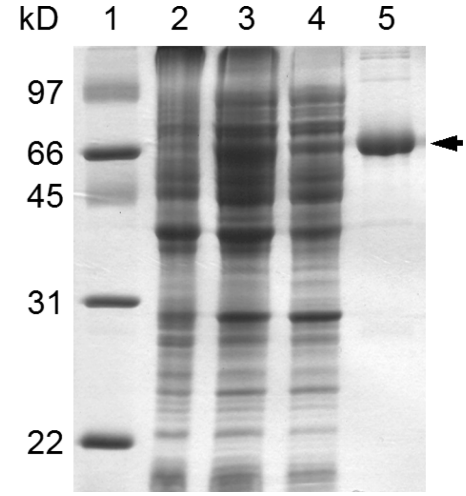

FIGURE 3: Expression and purification of recombinant WbwK (GST-WbwK) analyzed on 12% SDS−PAGE. Lane 1, protein marker; lane 2, cell extract of empty vector (pGEX-4T-1); lanes 3 and 4, whole cell extract and lysate of vector carrying gene *wbwK*; lane 5, elution of GST-WbwK through GST affinity chromatography.

Expression and Purification of WbwK of E. coli O86:B7. Expression of WbwK with a GST tag was carried out in 1 L of LB under induction of IPTG. The expression level of fusion GST-WbwK was high, consisting of about 50% of the total cell protein; however, most of the protein expressed in the form of inclusion bodies and only 30% of the GST-WbwK protein was expressed in soluble form as analyzed by SDS−PAGE analysis (Figure 3). One-step purification of the fusion GST-WbwK protein using GST affinity chromatography resulted in >98% purity (Figure 3). The size of the recombinant WbwK protein (GST-WbwK) is about 63 kDa based on 12% SDS−PAGE and close to calculated molecular mass of 61.8 kDa based on the amino acid sequence. The purified GST-WbwK was tested for α1,2-FucT activity and investigation of substrate specificity.

Determination of α1,2-FucT Activity and Substrate Specificity of WbwK. WbwK protein belongs to glycosyltransferase family 11 (http://www.cazy.org/fam/acc_GT.html). Enzymes in glycosyltransferase family 11 are α1,2-FucTs, which transfer L-fucose from GDP-β-L-fucose to β-D-Gal-R through an α1,2 linkage (Figure 2). Based on the structure of the O-antigen repeating unit of *E. coli* O86:B7 (Figure 1), a panel of substrates having the β-D-Gal residue was selected to detect α1,2-FucT activity using purified GST-WbwK and a radiolabeled donor GDP-fucose (Table 2). The results show that WbwK is highly active with Galβ1,3GalNAcα-R (derivatives of blood group T-antigen) but not with any other substrates tested. In contrast to other known α1,2-FucTs, enzyme activity was not detected using Galβ-OMe as the acceptor. Furthermore, WbwK did not

Table 2: Acceptor-Substrate Specificity of Purified GST-WbwK

acceptor	specific activity[a] (nmol min^{-1} mg^{-1})
Galβ1,3GalNAc$_C$$\alpha$-OBn (T-antigen)[c]	852
Galβ1,3GalNAc$_C$$\alpha$-OMe (T-antigen)	873
Galβ1,4Glc (lactose)	ND[b]
Galβ1,4Fru (lactulose)	ND
Galβ1,4Man	ND
Galα1,4Galβ1,4Glc (Gb_3)	ND
Galα1,3Galβ1,4Glc (α-Gal)	ND
Galβ-OMe	ND

[a] The activities were measured using radioactive GDP-Fuc (7000 cpm, 0.3 mM) incubated with 5 mM acceptors at 37 °C for 1 h. [b] ND: not detectable. [c] Abbreviations: Bn, benzyl; Me, methyl.

FIGURE 4: Thin-layer chromatography of the enzymatic product of recombinant WbwK at reaction times of 0, 24, and 36 h. The arrow indicates the direction of chromatography.

recognize disaccharides such as lactose, lactulose, or Galβ1,4Man as acceptors, even though they have a β-D-Gal residue at the nonreducing end. Oligosaccharides having β-D-Gal but not at the nonreducing end did not serve as acceptors either. WbwK is only active toward Galβ1,3GalNAcα-R structures to form the H-type 3 blood group antigen Fucα1,2Galβ1,3GalNAcα-R, indicating a strict substrate specificity. This result is in agreement with the O-antigen repeating unit structure of *E. coli* O86. Although the natural substrate of WbwK is not available for substrate specificity studies, blood group T-antigen derivatives acted as good substrates, indicating that the identity of the reducing end beyond the disaccharide Galβ1,3GalNAcα structure is not crucial for enzyme activity. Furthermore, this result also gives us a hint that blood group B epitope synthesis of the *E. coli* O86 O-antigen is similar to that of humans (starting from H-antigen to form the B-antigen).

To further demonstrate FucT activity of recombinant WbwK, a 50 μL reaction mixture was set up using Galβ1,3GalNAcα-OMe as the acceptor and GDP-Fuc as the donor, coincubated with purified GST-WbwK protein. Product formation was detected by using a TLC plate assay (Figure 4). After 24 h at room temperature, a third spot that migrated slower than the acceptor disaccharide was clearly detected, indicating formation of a trisaccharide. In addition, under the stated reaction conditions, more than 80% of the disaccharide was converted to trisaccharide in 24 h. This result further demonstrated the FucT activity of GST-WbwK.

Enzymatic Synthesis of the Trisaccharide on a Milligram Scale. A milligram-scale reaction was carried out with the aim of obtaining adequate amounts of the trisaccharide product for identification of the linkage type formed upon fucose transfer onto the disaccharide Galβ1,3GalNAcα-OMe. After a 2 day reaction, a total of 2.3 mg of Fucα1,2Galβ1,3-GalNAcα-OMe was synthesized using Galβ1,3GalNAcα-OMe as the acceptor and GDP-Fuc as the donor, together with purified GST-WbwK protein. Subsequently, the purified trisaccharide product was analyzed by electrospray mass spectrometry (ESI-MS) and NMR spectroscopy.

ESI-MS (negative mode) of Fucα1,2Galβ1,3GalNAcα-OMe showed one prominent peak at *m/z* 542.3 (Supporting Information, Figure S1), which is the $(M - H)^-$ peak of the parent compound, based on the predicted molecular weight of 543.22. The structure of Fucα1,2Galβ1,3GalNAcα-OMe was confirmed using ^{1}H NMR spectroscopy (see Supporting Information, Figure S2). The two methyl groups (protons at C-7 and C-9) on the GalNAc sugar residue can be readily identified (OCH_3 group $\delta = 3.30$ ppm and $COCH_3$ group $\delta = 1.97$ ppm). Meanwhile, the terminal methyl group (C-6) of the Fuc residue appears at high field ($\delta = 1.12$ ppm) as a doublet ($J = 6.6$ Hz) indicating the addition of Fuc residue in the product. A coupling constant of 3.7 Hz for the H-1 resonance of Fuc residue showed that this residue was linked in α-anomeric configuration. The complete assignment of ^{1}H NMR (500 MHz in D_2O at 27 °C) of product trisaccharide (Supporting Information Figure S2) is consistent with that reported previously (*25*). Therefore, the structure of the trisaccharide product was confirmed to be Fucα1,2Galβ1,3GalNAcα-OMe. Likewise, the identity of WbwK was further demonstrated as an α1,2-FucT.

Comparison of WbwK of E. coli O86:B7 to WbsJ of E. coli O128:B12. A recent study found that the *wbsJ* gene of *E. coli* O128:B12 encodes an α1,2-FucT with broad substrate specificity (*20*). WbsJ shows higher enzyme activity to four different disaccharides (lactose, lactulose, Galβ1,4Man, and Galβ1,3GalNAcα-R) and lower activity to the simple substrate Galβ-OMe. Contrary to WbsJ, WbwK shows strict acceptor specificity and is only active with Galβ1,3GalNAcα-R, but not Galβ-OMe. It is noteworthy that WbwK and WbsJ are involved in O-antigen biosynthesis and share a common acceptor containing the Galβ1,3GalNAcα structure with high enzymatic activity. Amino acid sequence comparison of WbwK and WbsJ revealed that there are three highly conserved motifs shared by them (Figure 5A), one located at the N-terminus and the other two located in the C-terminus. These three highly conserved motifs share identical sequences between WbwK and WbsJ and are separated by three variant regions named as domain a, b, and c. Domain a is a bigger variant than either domain b or c. A higher percentage of identical sequences are located in the C-terminus, revealing the catalytic domain. The N-terminus has more variability, suggesting that this region might determine substrate specificity. So far, functional characterized α1,2-FucTs from bacteria such as *H. pylori* and *E. coli* O128 are all involved in the LPS O-antigen synthesis. The complex O-antigen sugar structures of LPS might be reflections of substrate specificities of α1,2-FucTs from different species.

Construction, Expression, and Enzymatic Activity of Chimeric FucT. To demonstrate the hypothesis that the variable regions determine substrate specificity and to investigate which of the variable regions of α1,2-FucT is responsible, six chimeric α1,2-FucTs were constructed by domain swapping between WbsJ and WbwK as shown in Figure 5B. Domain a swapping generated chimeras Ka/Jbc and Ja/Kbc, whereas domain b swapping yielded chimeras Ka/Jb/Kc and Ja/Kb/Jc, and domain c swapping resulted in chimeras Kab/Jc and Jab/Kc.

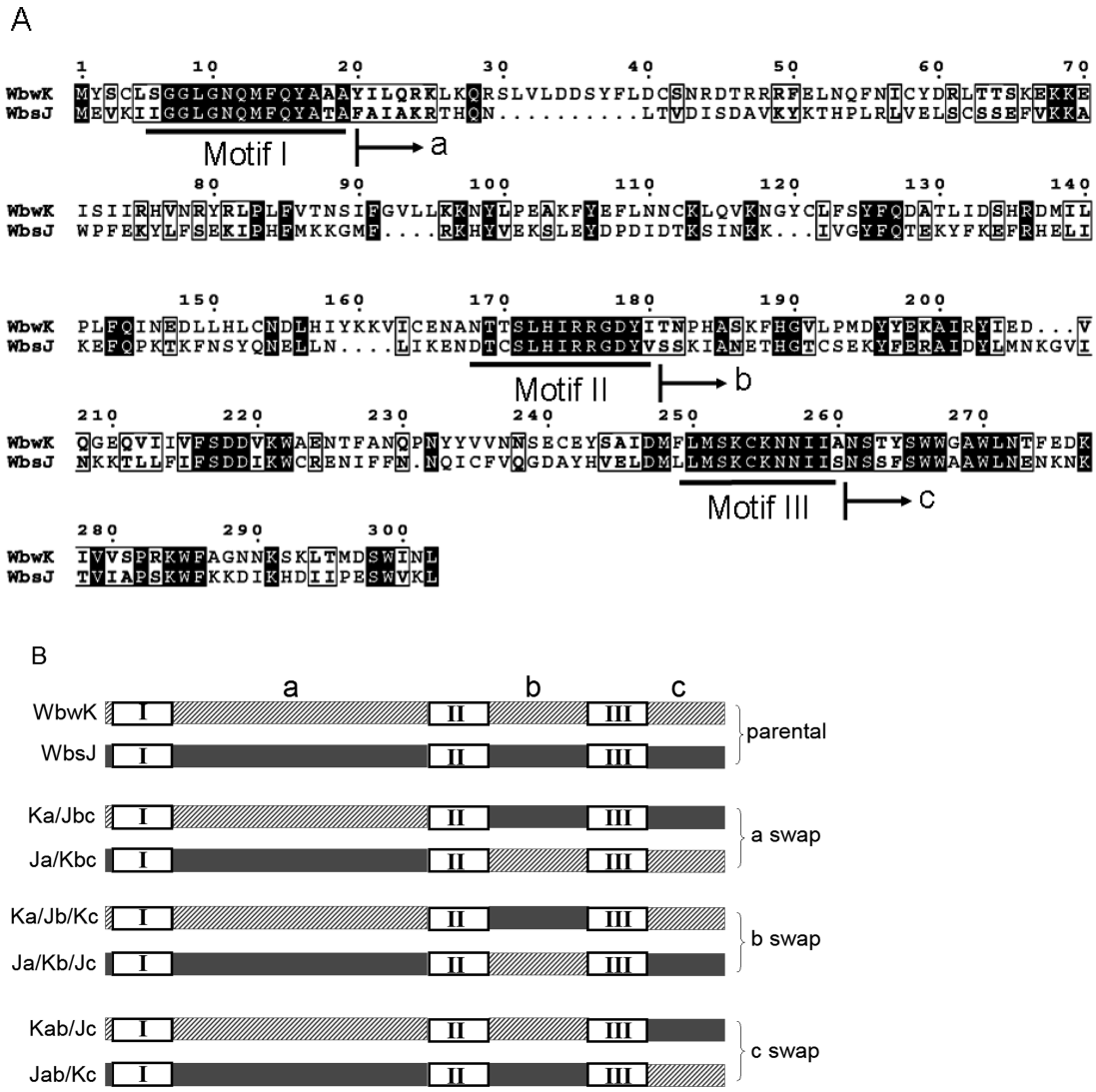

FIGURE 5: (A) The amino acid sequence alignment of WbwK (AAO37719, *E. coli* O86:B7) and WbsJ (AAO37698, *E. coli* O128:B12) is shown. The white letters with black background and boxed represent identical amino acids, while black letters in bold and boxed represent similar amino acids which are conserved in aligned sequences. The highly identical motifs are underlined and labeled as motifs I, II, and III. The variant regions were separated by the three motifs into a, b, and c domains. (B) Schematic diagram of engineering chimeric α1,2-FucTs through domains a, b, and c swapping between WbwK and WbsJ. W represents WbwK and the sequence of WbwK is in the slashed bar, whereas J represents WbsJ and the sequence of WbsJ is in the gray bar.

Each chimeric FucT was expressed with a GST tag and purified in one step by GST affinity chromatography (Figure 6A). The purified chimeric FucTs were subjected to determination of FucT activity and compared with two parental FucTs (WbwK and WbsJ), using lactulose, lactose, Galβ1,4Man, and Galβ1,3GalNAcα-OMe as acceptors. These four disaccharides were chosen because they are good substrates for WbsJ. In addition, Galβ1,3GalNAcα-OMe is the common substrate shared by both WbsJ and WbwK. No enzyme activity was detected for chimera Ja/Kbc, Ja/Kb/Jc, and Jab/Kc to any of the four substrates, indicating that WbsJ requires its own domain b and/or c combined with domain a. In contrast, WbwK showed moderate tolerance to domain swapping, but with a dramatic decrease of enzyme activity (Figure 6B). Interestingly, chimeras Ka/Jbc, Ka/Jb/Kc, and Kab/Jc, which have one or two domains swapped with WbsJ, displayed broad substrate specificity. They are active with acceptors lactulose, lactose, Galβ1,4Man, and Galβ1,3GalNAcα-OMe, which are specific substrates for WbsJ, with activity in the order of Ka/Jbc > Ka/Jb/Kc > Kab/Jc. This indicates that domains b and c likely determine acceptor substrate specificity, with domain b playing a predominant role.

GDP Bead Binding Ability. It is unclear from the above experiment whether domain swapping disrupts binding ability of chimera FucTs to the acceptor or donor (GDP-Fuc) substrates therefore led to loss of enzyme activity. To answer this question, the same amount of purified chimeric and parental FucTs was used to determine their binding ability to GDP, the dominant moiety of donor GDP-Fuc. The results in Figure 7 indicate that all of the chimeric FucTs are able to bind to GDP moiety of donor substrate with different binding affinity. WbsJ showed 2−3-fold stronger binding affinity to GDP than that of WbwK. Chimeras Ka/Jb/Kc and Ka/Jbc showed decreased binding affinity to GDP compared to parental FucTs. Chimeras Ja/Kbc, Ja/Kb/Jc, and Jab/Kc, of which no enzyme activities were detected to any of four acceptors, showed similar GDP binding ability to WbwK. This experiment demonstrates that inactive chimeric FucTs were still able to bind to donor substrate. The loss of enzyme

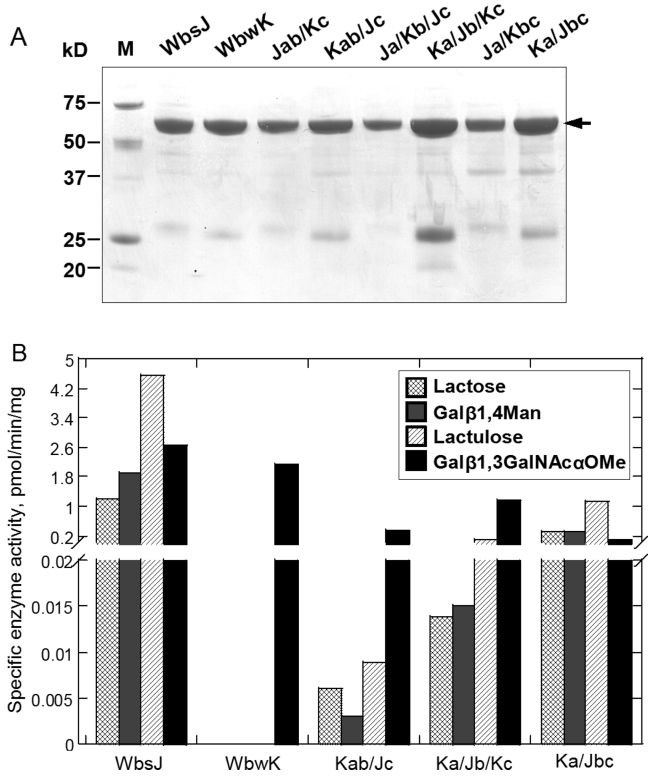

FIGURE 6: (A) 4−12% SDS−PAGE of purified parental and chimeric FucTs. The arrow indicates the recombinant FucTs. (B) Specific enzymatic activities of parental and chimeric FucTs to four acceptor substrates: lactose (cross-hatched bar), Galβ1,4Man (gray bar), lactulose (hatched bar), and Galβ1,3GalNAcα-OMe (black bar). Chimeras Jab/Kc, Ja/Kb/Jc, and Ja/Kbc, which had no detectable activity, were not included in the graph.

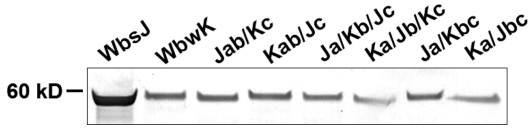

FIGURE 7: Western blot analysis of GDP bead binding abilities of chimeric FucTs with comparison to parental FucTs. Proteins bound to GDP beads were analyzed on 4−12% SDS−PAGE and probed by anti-GST antibody as described in Materials and Methods.

activity of three constructed chimeras Ja/Kbc, Ja/Kb/Jc, and Jab/Kc may be due to the overall disrupted conformation for acceptor binding.

DISCUSSION

Through this study, the putative α1,2-FucT gene *wbw*K, which is involved in O-antigen synthesis of *E. coli* O86:B7, was expressed and purified. The *wbwK* gene was biochemically proved to encode an α1,2-FucT through enzyme activity, MS, and NMR analyses. Although lack of the natural substrate, we demonstrated that WbwK possesses strict substrate specificity and can only recognize T-antigen and its derivatives (Galβ1,3GalNAcα-OR) as a good substrate to generate H-type 3 blood group antigen. This result is in agreement with the known *E. coli* O86 O-antigen repeating unit structure. It also indicates that the reducing end of the O-antigen repeating unit beyond the disaccharide Galβ1,3GalNAcα- is not crucial for WbwK activity. This may be true for many of glycosyltransferases involved in O-antigen repeating unit biosynthesis, since quite a few glycosyltransferases have been found to function well on shorter length substrate compared with their natural substrates, for example, WbsJ from *E. coli* O128:B12 (*20*) and WbnH (α1,3-*N*-acetylgalactosaminyltransferase) from *E. coli* O86:H2 (*26*). Given the strict substrate specificity of WbwK as well as the O-antigen repeating unit structure, it is clear that the last two steps of O-antigen repeating unit biosynthesis of *E. coli* O86:B7 are sequential synthesis of tetrasaccharide Fucα1,2Galβ1,3GalNAcα1,3GalNAcα- (with α1,2-FucT) followed by pentasaccharide Galα1,3(Fucα1,2)-Galβ1,3GalNAc-α1,3GalNAcα- (with α1,3-GalT). Therefore, the biosynthesis of B-antigen epitope in *E. coli* O86:B7 is basically similar to that of human blood group B-antigen, which uses H-antigen as precursor to synthesize B-antigen.

To demonstrate the hypothesis that the highly variant domain determines substrate specificity, the functions of each variant domain were investigated by domain swapping between WbwK and WbsJ. WbwK showed tolerance to the domain changes. On one hand, both domains b and c determine the acceptor specificity; however, domain b plays a predominant role. On the other hand, the larger variant domain a might help domains b and c to form a conformation that facilitates binding to the acceptor or might play a role in anchoring WbwK on the inner membrane for convenient biosynthesis of O-repeating unit. Further investigation through GDP bead binding assay provides evidence that the loss of activity of WbsJ by switching domains b and c is not because of inability of binding to donor substrate but might be due to conformational change which is unfavorable for acceptor binding. Recently, the crystal structures of α1,3-FucT and α1,6-FucTs have been reported (*27*−*29*). However, no crystal structure is available for α1,2-FucT. α1,6-FucTs are evolutionary closely related to α1,2-FucTs. Unfortunately, the published structures of α1,6-FucTs are free form proteins rather than protein−substrate complex and, therefore, lack the direct information about how the proteins interact with their donor and acceptor substrates. Domain swapping in this study provides insights into the mechanism of α1,2-FucT. Nevertheless, crystal structure study combined with further detailed biochemical study of bacterial α1,2-FucT would eventually elucidate the catalytic mechanism of α1,2-FucT.

Based on substrate specificity, the reported α1,2-FucTs could be classified into several subfamilies. Family 1 displays activity to type 1 (Galβ1,3GlcNAc-) and type 2 (Galβ1,4GlcNAc-) acceptors, such as human FUT1 and FUT2. Family 2 is active to type 4 (Galβ1,3GalNAcβ-) acceptors, for example, bovine FUT1, FUT2, and Sec1 (*30*). Family 3 is active to type 3 (Galβ1,3GalNAcα-) and type 5 (Galβ1,4Glc) structures, such as WbsJ of *E. coli* O128:B12. These three families are all active to simple substrate Galβ-O-*p*NP or Galβ-OMe. In this study, we found the fourth subfamily, a unique α1,2-FucT WbwK, prefers type 3 (Galβ1,3GalNAcα-) structure but shows no activity to Galβ-OMe. Recently, newly identified α1,2-FucT from *Caenorhabditis elegans* (*31*) also displays similar substrate specificity to WbwK. Furthermore, Galβ1,3GalNAcα-R disaccharide (Thomsen-Friedenreich or T-antigen) has been well documented as a cancer-related marker on mucin surface and as an important antigen for the detection and immunotherapy of breast carcinomas (*32*−*34*). Extensive research had been focused on synthesis T-antigen conjugates as vaccine to eradicate tumor cells by the immunoresponses and to prevent metastasis of tumor cells (*32*). Early research found that addition of fucose could mask MUC-1 epitopes (T-antigen) in normal and cancerous gastric mucosae and, therefore, plays a role in

prevention of metastasis of cancer cell (*35*). The WbwK exhibits strict substrate specificity and is only active to T-antigen epitope (Galβ1,3GalNAcα-R); therefore, it would be significant to investigate whether this unique α1,2-FucT could mask the tumor antigen, T-antigen, *in vivo*.

SUPPORTING INFORMATION AVAILABLE

MS spectra and NMR spectra of Fucα1,2Galβ1,3GalNAcα-OMe synthesized using GST-WbwK and the assignment of ^{1}H NMR (500 MHz in D_2O at 27 °C) of trisaccharide Fucα1,2Galβ1,3GalNAcα-OMe. This material is available free of charge via the Internet at http://pubs.acs.org.

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