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Biochemical Characterization of a Glycosyltransferase Homolog from an Oral Pathogen *Fusobacterium nucleatum* as a Human Glycan-Modifying Enzyme

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Bacterial glycosyltransferases have drawn growing attention as economical enzymes for oligosaccharide synthesis, with their easy expression and relatively broad substrate specificity. Here, we characterized a glycosyltransferase homolog (Fnu_GT) from a human oral pathogen, *Fusobacterium nucleatum*. Bioinformatic analysis showed that Fnu_GT belongs to the glycosyltransferases family II. The recombinant Fnu_GT (rFnu_GT) expressed in *Escherichia coli* displayed the highest glycosylation activity when UDP-galactose (Gal) was used as a donor nucleotide-sugar with heptose or *N*-acetylglucosamine (GlcNAc) as an acceptor sugar. Interestingly, rFnu_GT transferred the galactose moiety of UDP-Gal to a nonreducing terminal GlcNAc attached to the trimannosyl core glycan, indicating its potential as an enzyme for human-type *N*-glycan synthesis.

Keywords: Glycosyltransferase, nucleotide-sugar, *Fusobacterium nucleatum*, human-type *N*-linked glycan

The oligosaccharides attached to various glycoconjugates on the cell surface play important roles in biological communication processes and have been recognized as drug candidates or targets for infectious diseases, inflammation, and cancer therapies. Recently, enzymatic synthesis employing glycosyltransferases has been highlighted as a promising method to overcome the limitations of the classical chemical synthesis since they can carry out selectively regio- and stereospecific bond formations in mild condition without any elaborate procedures such as protection and deprotection steps [3, 12]. Some mammalian glycosyltransferases have been shown to be useful for the synthesis of human-type oligosaccharides. However, their functional expressions are generally inefficient and often require to employ a costly expression system such as a mammalian or baculovirus expression system. Therefore, bacterial glycosyltransferases have been considered as economical enzyme sources for the synthesis of oligosaccharides because of their efficient functional expression in bacterial host such as *Escherichia coli* and their broad substrate specificities for the synthesis of valuable oligosaccharides such as human-type *N*-glycan [3, 11, 15, 16].

Many pathogenic bacteria express a wide array of lipooligosaccharide (LOS) and lipopolysaccharide (LPS) structures on their surfaces, many of which mimic mammalian oligosaccharide structures and help them escape attacks by the host immune system [18]. The glycosyltransferase genes responsible for the biosynthesis of the oligosaccharide portion of LOS and LPS structures have been identified and characterized from various pathogens, such as genitourinary (*Neisseria gonorrhoeae* and *N. meningitides*) [22, 24], gastrointestinal (*Helicobacter pylori* and *Campylobacter jejuni*) [8, 13], and respiratory (*Streptococcus pneumoniae*, *S. agalactiae*, *Haemophilus influenzae*, and *H. ducreyi*) [7, 17, 23, 27, 31] pathogens.

In this study, we identified a putative glycosyltransferase Fnu_GT from *Fusobacterium nucleatum,* an oral anaerobic pathogen involved in periodontal and systemic infections. The pathogenic properties of this microorganism have also been reported in periodontitis, pericarditis, bacteremia, urinary tract infection, peritonsillar abscesses, and septic arthritis [1, 11]. *F. nucleatum* is one of the dominant species in biofilm development of the most complex bacterial pathogen flora in human infections [1]. Like other Gram-negative bacteria, its outer surface seems to predominantly consist of polysaccharides including LPS [9], which might

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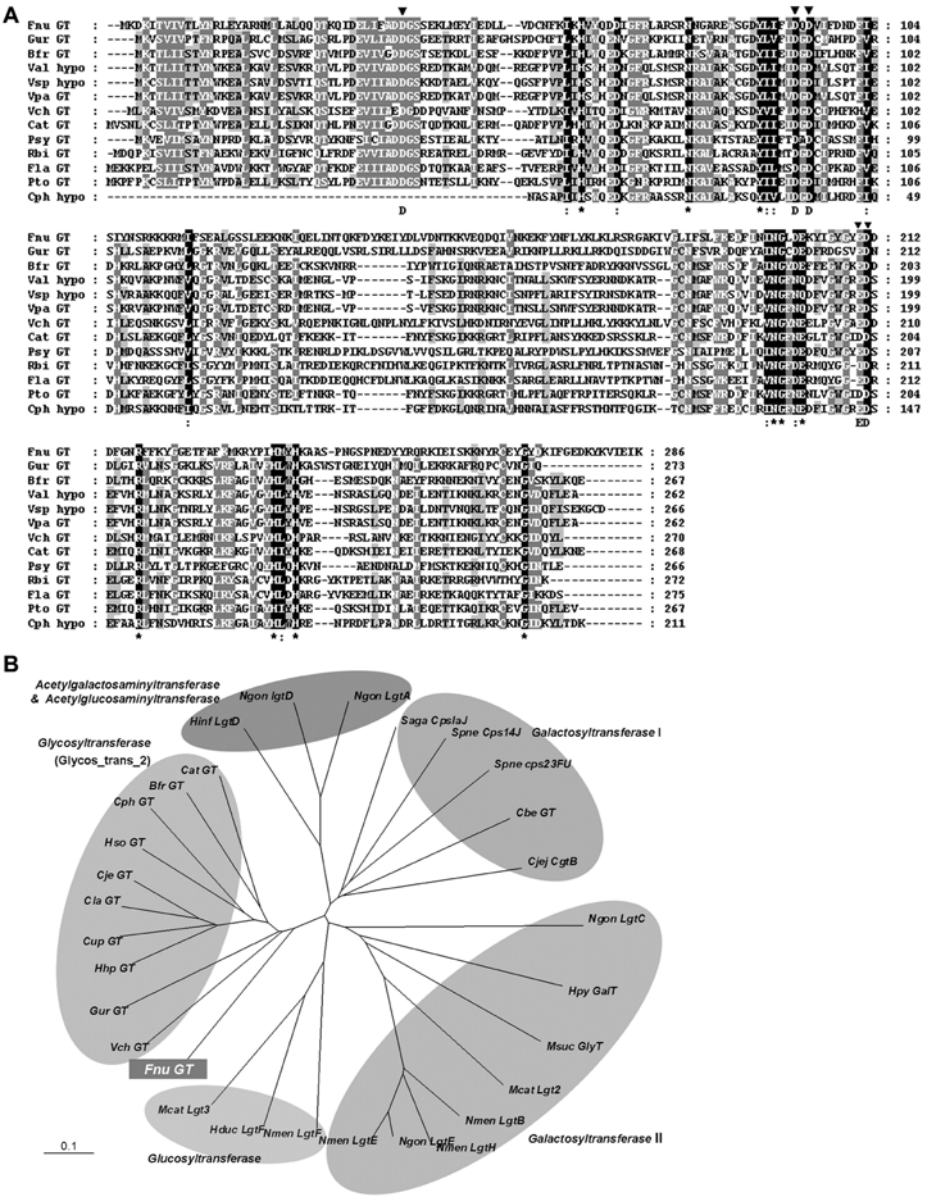


Fig. 1. Bioinformatic analysis of Fnu_GT.

A. Amino acid sequence alignment of Fnu_GT orthologs. The motifs predicted to be involved in the catalytic activity of Fnu_GT are designated by (▼). The symbols (*) and (:) indicate conserved residues and conservative replacement, respectively, among the Fnu_GT orthologs. Abbreviations are as follows: Fnu_GT, *F. nucleatum* glycosyltransferase (NCBI Accession No. AAL95439); Gur_GT, *Geobacter uraniumreducens* glycosyltransferase (ZP_01141487); Bfr_GT, *Bacteroides fragilis* glycosyltransferase (CAH05963); Val_GT, *Vibrio alginolyticus* hypothetical protein (ZP_01261291); Vsp_GT, *Vibrio splendidus* hypothetical protein (ZP_00989898); Vch_GT, *Vibrio cholerae* glycosyltransferase (AAL77367); Cat_GT, *Croceibacter atlanticus* glycosyltransferase (ZP_00949523); Psy_GT, *Psychromonas* sp. glycosyltransferase (ZP_01215722); Rbi_GT, *Robiginitalea biformata* glycosyltransferase (ZP_01121680); Fla_GT, *Flavobacterium* sp. glycosyltransferase (ZP_01060764); Pto_GT, *Psychroflexus torquis* glycosyltransferase (ZP_01253522); Cph_GT, *Chlorobium phaeobacteroides* hypothetical protein (ZP_00532526). **B**. Phylogenetic analysis of Fnu_GT with other bacterial glycosyltransferases belonging to the glycosyltransferase family II and those involved in LPS biosynthesis of pathogenic bacteria. Cup_GT, *Campylobacter upsaliensis* (ZP_00371408); Cje_GT, *Campylobacter jejuni* (AAW35599); Hso_GT, *Haemophilus somnus* (ZP_00132461); Cla_GT, *Campylobacter lari* (ZP_00369503); Cbe_GT, *Clostridium beijerincki* (ZP_00910508); Hhp_GT, *Helicobacter hepaticus* (AAP77370). Galactosyltransferases: Hpy_GalT, *Helicobacter pylori* (BAA88524); Nmen_LgtB, *Neisseria meningitidis* (AAL12841); Ngon_LgtE, *Neisseria gonorrhoeae* (AAA68013); Nmen_LgtE, *N. meningitidis* (2204376C); Nmen_LgtH, *N. meningitidis* (AAX58721); Saga_CpsIaJ, *Streptococcus agalactiae* (BAA82284); Spne_Cps14J, *S. pneumoniae* (CAA59782); Spne_cps23FU, *S. pneumoniae* (NP_358816); Msuc_GlyT, *Mannheimia succiniciproducens* (AAU37053); Ngon_LgtC, *N. gonorrhoeae* (AAF20991); Cjej_CgtB, *C. jejuni* (AAF31770); Mcat_Lgt2, *Moraxella catarrhalis* (AAX11432). Acetylglucosaminyltransferases: Ngon_LgtA, *N. gonorrhoeae* (AAF25876). Galactosaminyltransferases: Ngon_lgtD, *N. gonorrhoeae* (YP_209164); Hinf_LgtD, *Haemophilus influenzae* (NP_439724). Glucosyltransferases: Nmen_LgtF, *N. meningitidis* (AAC44647); Hduc_LgtF, *H. ducreyi* (AAF72876); Mcat_Lgt3, *M. catarrhalis* (AAX11433). Galactosyltransferases are subgrouped as I (galactosyltransferases involved in capsular polysaccharide biosynthesis in Gram-positive strains, except Cjej_CgtB) and II (galactosyltransferases involved in lipopolysaccharide biosynthesis in Gram-negative strains), respectively.

be involved in the adherence to host cells as well as other bacteria [1, 18, 26]. Here, we overexpressed the putative glycosyltransferase Fnu_GT in *E. coli* and investigated its biochemical properties and potentials as an enzyme for the synthesis of human-type *N*-glycan.

Bioinformatic Analysis of Fnu_GT

Our intensive database search for microbial homologs to human β-1,4-galactosyltransferase 1 suggested the putative protein encoded by the FN1243 gene of *F. nucleatum*, designated Fnu_GT (*F. nucleatum* glycosyltransferase), as one of the candidates with galactosyltransferase activities on human-type glycans. It is one of the five putative glycosyltransferases possibly involved in LPS biosynthesis, as predicted from recently published genome sequence data of *F. nucleatum* strain ATCC 25586 [14].

The Position-Specific Iterated-Basic Local Alignment Search Tool (PSI-BLAST) analysis revealed that the Fnu_GT belongs to glycosyltransferase family II (GT-2) in the Pfam Protein Family Database [2, 6], which contains nucleotide-sugar-dependent glycosyltransferases (GTs). The multiple amino acid sequence alignment of Fnu_GT with other bacterial GT-2 enzymes revealed several conserved residues. A highly conserved DXD motif ($D^{93}XD^{95}$) for the divalent metal ion binding was found, which is diagnostic for the glycosyltransferases family [25] (Fig. 1A). Besides this motif, two additional important Asp residue motifs (D^{40} and D^{211}) for glycosyltransferase were predicted. Based on the crystal structure of the *Bacillus subtilis* nucleotide-diphosphosugar transferase SpsA [5], D^{40} appears to bind to the nucleotide of nucleotide-sugar, whereas D^{93} binds at the DXD motif to the pyrophosphate of nucleotide-donor *via* one molecule of divalent metal ion. On the other hand, D^{211} at an acidic amino acid cluster ($E^{210}D^{211}D^{212}$) is expected as a catalytic base in the transferase reaction, as suggested by the point mutation analysis of *Sinorhizobium meliloti* glycosyltransferase ExoM [10].

A database search with Fnu_GT as a query sequence revealed a large number of sequences with GTs mainly involved in LPS or capsular polysaccharide (CP) biosynthesis. Total 29 GTs showing at least 26% identity and 45% similarity with Fnu_GT were used for phylogenetic analysis (Fig. 1B). Seventeen GTs among those analyzed in the phylogenetic tree were previously characterized for their enzyme activities

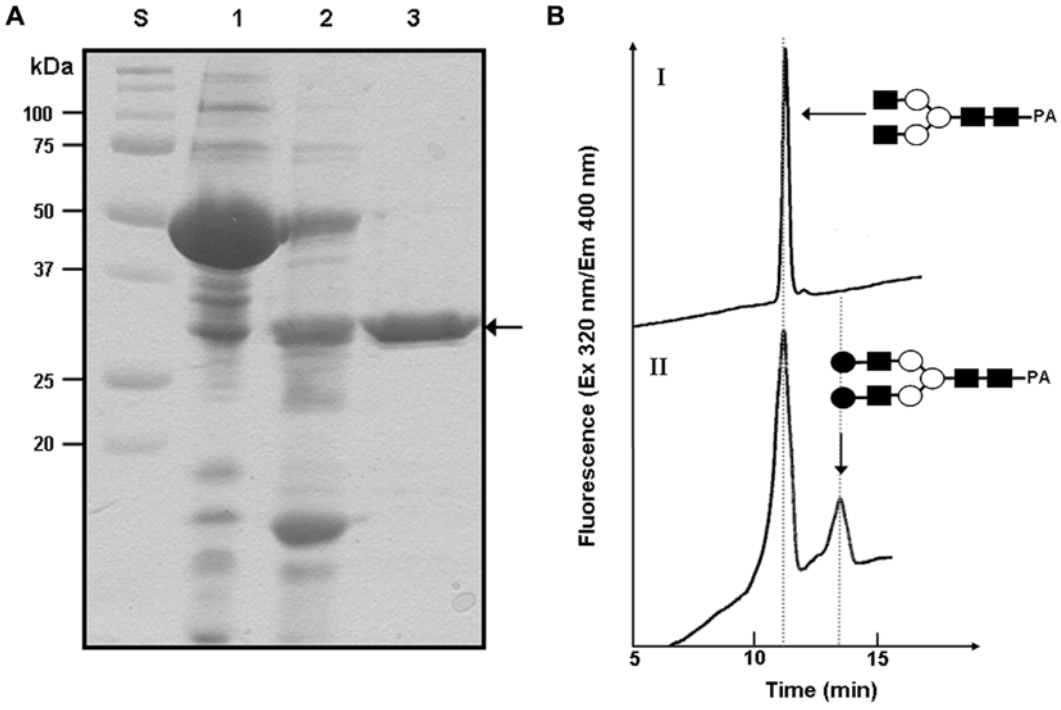


Fig. 2. Purification and galactosyltransferase activity assay of rFnu_GT.

A. The rFnu_GT overexpressed in *E. coli* BL21 (DE3) pLysS was purified by Ni-NTA column and then its N-terminal Trx-His tag was removed by enterokinase digestion. S, size markers; lane 1, Ni-NTA column eluent; lane 2, enterokinase digestion; lane 3, after the removal of enterokinase and Trx-His tag. **B**. HPLC analysis for galactosyltransferase activity of rFnu_GT. The galactosylation reaction was carried out at 37°C for 10 h in 50 μl of 50 mM Tris-HCl (pH 7.5) containing 1 mM $MnCl_2$, 0.1 mM UDP-Gal as donor sugar, 100 pmol PA-labeled oligosaccharide (Takara) as acceptor, and the purified rFnu_GT. The resulting products were analyzed by HPLC system (Waters) equipped with a Waters 2475 Fluorescence Detector using a Shodex Asahipak NH2P-50 normal phase column (4.6×150 mm; Showa Denko). Chromatogram I shows a substrate peak, PA-labeled agalacto-biantennary glycan ($GlcNAc_2Man_3GlcNAc_2$-PA), and the arrow in chromatogram II indicates the peak of the product ($Gal_2GlcNAc_2Man_3GlcNAc_2$-PA) after the enzyme reaction. ■, GlcNAc (*N*-acetylglucosamine); ○, Man (mannose); ●, Gal (galactose).

[3, 8, 10, 13, 24, 28, 29]; galactosyltransferases: *Hpy_GalT*, *Nmen_LgtB*, *Ngon_LgtE*, *Nmen_LgtE*, *Nmen_LgtH*, *Saga_CpsIaJ*, *Spne_Cps14J*, *Spne_cps23FU*, *Ngon_LgtC*, *Cjej_CgtB*, *Mcat_Lgt2*; acetylglucosaminyltransferases: *Ngon_LgtA*; galactosaminyltransferases: *Ngon_lgtD*, *Hinf_LgtD*; glucosyltransferases: *Nmen_LgtF*, *Hduc_LgtF*, *Mcat_Lgt3*. The reported enzyme activities of these are shown to be in good agreement with our result of phylogenetic tree analysis. However, when we compared the amino acid sequences and the sugar-donor specificity, there was no obvious sequence pattern for sugar specificity. Interestingly, Fnu_GT was not clustered with galactosyltransferases (I and II groups), glucosyltransferase, or *N*-acetylglucosaminyl (or *N*-acetylgalactosaminyl) transferases, but was closely clustered to GT in the GT-2 family (Glycos_trans_2) involved in LPS biosynthesis (Fig. 1B), implying that the GT-2 family including Fnu_GT has evolved from different origins in the early evolutionary stages.

Cloning, Expression, and Purification of Recombinant Fnu_GT

The Fnu_GT gene was amplified from *F. nucleatum* genomic DNA by PCR with two primers (forward primer 5'-GCGGATCCATGAAAGATAAAATAACAGTA-3', and reverse primer 5'-GGAATTCTTATTTTATTTCTATCAC-TTTATA-3': underlines indicate the BamHI and EcoRI sites, respectively, added for subcloing) and cloned into the plasmid pET-32a (Novagen), yielding pET32-Fnu_gt. The recombinant Fnu_GT (rFnu-GT) was overexpressed in *E. coli* BL21 (DE3) pLysS harboring pET32-Fnu_gt and purified as a Trx-His-tagged form by using Ni-NTA affinity column. Its N-terminal Trx-His tag was then removed by enterokinase treatment, and the rFnu-GT (34 kDa) was subsequently purified using an enterokinase removal kit (Sigma) and Ni-NTA column (Fig. 2A).

Biochemical Properties of rFnu_GT

The rFnu_GT activity was determined by an enzyme-coupled assay employing pyruvate kinase and L-lactate dehydrogenase [21, 30]. In general, glycosyltransferases involved in the LPS biosynthesis of bacterial cell surface utilize UDP-sugars as sugar nucleotide substrates [4], whereas those associated with the biosynthesis of bacterial secondary metabolites, such as macrolide or glycopeptide antibiotics, preferentially use TDP-sugars as the sugar donor [19]. Based on the bioinformatics analysis indicating Fnu_GT as a glycosyltransferase to be involved in the LPS biosynthesis, we tested the glycosyltransferase activity of Fnu_GT using UDP-sugars as a donor nucleotide. To investigate the donor nucleotide-sugar specificity of rFnu_GT in the glycosyltransferase activity reaction, four UDP-sugars and one GDP-sugar were tested under a standard enzyme assay condition using GlcNAc as an acceptor sugar substrate (Table 1). The rFnu_GT showed significant activity for UDP-Gal (34±2.3 U/mg) and UDP-Glc (3.4±1.0 U/mg), whereas it was not able to use UDP-GalNAc, UDP-GlcNAc, or GDP-Man as a donor substrate. It is noteworthy that rFnu_GT showed 10-fold higher activity with UDP-Gal than UDP-Glc, implying that it prefers UDP-Gal as a donor substrate. To investigate the acceptor sugar specificity of Fnu_GT, the kinetic parameters of rFnu_GT were determined for several acceptor sugars (1 mM) and UDP-sugar donors (0.5 to 4 mM) as substrates under standard assay condition (Table 1). rFnu_GT could transfer the sugar moiety of UDP-Gal or UDP-Glc to either Hep or GlcNAc, whereas no enzyme activity was detected for Gal and Glc (data not shown). Kinetic analysis showed that Fnu_GT had a higher affinity (1.8-fold lower K_m) for UDP-Glc than UDP-Gal when Hep was used as an acceptor. By contrast, the maximum velocity (V_{max}) was 5.2-fold higher for UDP-Gal than UDP-Glc with Hep as an

Table 1. Kinetic parameters of rFnu_GT for monosaccharide acceptors and nucleotide sugar donors.[a]

Acceptor	Donor	K_m (mM)	V_{max} (U·mg^{-1} protein)	k_{cat}/K_m (s^{-1} mM^{-1})
GlcNAc	UDP-Gal	3.2±0.4	34.3±2.3	6.1
	UDP-Glc	2.0±0.1	3.4±0.1	1.0
	GDP-Man	N.D.[b]	ND	
Hep	UDP-Gal	3.4±0.8	45.8±6.1	7.9
	UDP-Glc	1.9±0.5	8.9±1.0	2.9
	UDP-GalNAc	ND	ND	-
	UDP-GlcNAc	ND	ND	-

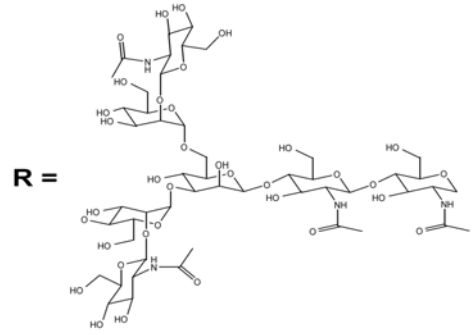
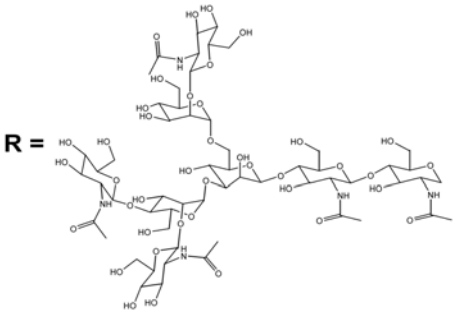
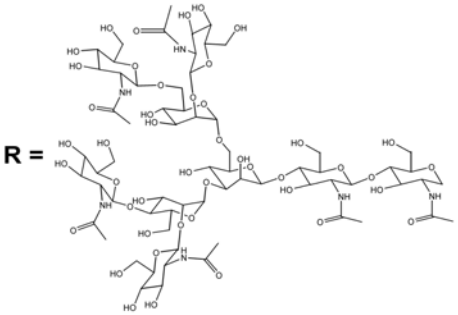
[a]The Fnu_GT reaction was performed at 37°C in 100 µl of 50 mM Tri-HCl buffer (pH 7.6) containing 1 mM $MnCl_2$, with 0.5 mM monosaccharide sugar as an acceptor and 0.5 mM UDP-sugar as a donor, and the purified rFnu_GT. After 30 min, 400 µl of a coupled enzyme reaction mixture in 50 mM Tris-HCl buffer (pH 7.6) containing 1 mM phosphoenolpyruvate, 5 mM $MnCl_2$, 50 mM KCl, 0.5 mM freshly prepared NADH, and lactate dehydrogenase (1.5 U)/pyruvate kinase (1.0 U) was added and incubated at 37°C for 30 min. The reaction mixture was diluted with 500 µl of distilled water and the amount of NADH was measured at 340 nm (ε_{340}=6,220 $M^{-1}cm^{-1}$). One unit was defined as the amount of enzyme required to transfer 1 µmol of sugar donor to acceptor sugar per min. The apparent V_{max} and K_m values (means±SD, $P<0.05$, n=2) were calculated by fitting the initial rate data to the Michaelis-Menten equation with the nonlinear regression analysis program (Sigma Plot, ver. 9.0).

[b]ND, Not detected.

acceptor. When GlcNAc was used as an acceptor, the apparent K_m values for UDP-Gal and UDP-Glc were similar. Interestingly, the V_{max} value for UDP-Gal was 10-fold higher than UDP-Glc when GlcNAc was used as an acceptor sugar, indicating that rFnu_GT has a significantly higher activity for UDP-Gal than UDP-Glc. The k_{cat}/K_m values, the estimates of substrate specificities, were approximately similar for Hep and GlcNAc as acceptor sugars with UDP-Gal donor.

The effect of pH on the rFnu_GT activity was determined at 37°C in 50 mM MES buffer (pH 5.0–7.0), 50 mM MOPS buffer (pH 6.5–8.0), or 50 mM Tris-HCl buffer (pH 7.5–9.0), using the enzyme-coupled assay. The purified rFnu_GT showed the optimal glycosyltransferase activity between pH 7.5 and 8.0 (data not shown). The effects of divalent ions on the rFnu_GT activity were also analyzed by measuring the enzymatic activity after pre-incubation in 50 mM Tris-HCl (pH 7.6) at 37°C for 1 h

Table 2. Conversion of pyridylamino (PA)-labeled oligosaccharides by rFnu_GT.

Acceptor substrate	Retention time ($t_{R, min}$)		Conversion[c] (%)
	Substrate[a]	Product[b]	
R = H	1.2	ND	ND
Pyridylamino (PA) group			
R =  GlcNAc$_2$Man$_3$GlcNAc$_2$	11.4	14.6	21.7
R =  GlcNAc$_3$Man$_3$GlcNAc$_2$	14.5	17.2	49.3
R =  GlcNAc$_4$Man$_3$GlcNAc$_2$	17.5	20.0	61.7

[a]Substrate indicates PA-oligosaccharides used as an acceptor sugar.
[b]Product indicates the galactose-conjugated PA-oligosaccharides produced by the enzyme reaction.
[c]Conversion (%) was calculated by the percentage of the ratio between product and substrate.
ND, not detectable.

in the presence of 1 mM of each divalent ion. Among the tested metal ions, Mn^{2+} was shown to be the most effective activator for rFnu_GT activity, and some alkaline metals, such as Ba^{2+}, Mg^{2+}, and Ca^{2+}, also enhanced the transferase activity at around a 70% level compared with that with Mn^{2+}. However, other divalent metal ions (Co^{2+}, Cu^{2+}, Fe^{2+}, Hg^{2+}, Cd^{2+}, Ni^{2+}, and Zn^{2+}) did not enhance the enzyme activity (data not shown).

Galactosyltransferase Activity on the Human-Type *N*-linked Glycan

To explore the possibility that Fnu_GT can be employed for the synthesis of valuable oligosaccharides, we tested whether its galactosyltransferase activity can work on the human-type *N*-glycans. For the assay, pyridylamino (PA)-labeled oligosaccharides (PA-*N*-acetylglucosamine, PA-agalacto-biantennary, PA-agalacto-triantennary, and PA-agalacto-tetraantennary) (Takara) were used as acceptor substrates with UDP-Gal as a donor sugar. The resulting products of the rFnu_GT reaction were analyzed by HPLC, and it was observed that the peak corresponding to acceptor substrate oligosaccharide was decreased whereas a new peak corresponding to the product oligosaccharide appeared on the HPLC chromatogram (Fig. 2B). The ratio between the oligosaccharide substrate and the galactosylated oligosaccharide product was calculated from their corresponding peak areas on the HPLC (Table 2). Interestingly, the conversion of PA-oligosaccharides was observed to be more efficient with increasing antenna numbers in an acceptor sugar.

To investigate the linkage specificity of galactosylation by rFnu_GT, the enzyme reaction mixtures were treated with linkage-specific galactosidases and analyzed by HPLC. When *S. pneumoniae* β-1,4-galactosidase (Sigma-Aldrich) was treated to reaction mixtures, the amount of product was decreased whereas that of the substrate was increased (data not shown). However, when the recombinant *Xanthomonas manihotis* β-1,3-galactosidase (Calbiochem) was treated, no changes on the amounts of substrate or product were observed. These results imply that rFnu_GT can transfer the galactose of UDP-Gal to the nonreducing terminal, GlcNAc of various acceptor sugars through a β-1,4-linkage which is usually observed in human complex-type *N*-glycan.

In this report, we identified a glycosyltransferase from a human oral pathogen, *F. nucleatum*, by employing extensive bioinformatic analysis and subsequent biochemical characterization. Both phylogenetic analysis and acceptor specificity analysis suggest that this enzyme might be a glycosyltransferase, which could polymerize glucose or galactose by attachment of β-(or α-) linked hexose residues (or probably heptose) onto the LPS acceptor. Interestingly, the rFnu_GT can transfer a galactose moiety of UDP-Gal to a nonreducing terminal GlcNAc attached to trimannosyl core glycan in a β-1,4-linkage-specific manner. Even though it still remains to determine its function *in vivo*, our functional studies suggested its potential application as an enzyme source useful for the synthesis of human-type *N*-glycan.

Acknowledgments

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