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Characterization of the dTDP-D-fucofuranose biosynthetic pathway in *Escherichia coli* O52

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Summary

D-fucofuranose (D-Fuc*f*) is a component of *Escherichia coli* O52 O antigen. This uncommon sugar is also the sugar moiety of the anticancer drug – gilvocarcin V produced by many streptomycetes. In *E. coli* O52, *rmlA*, *rmlB*, *fcf1* and *fcf2* were proposed in a previous study by our group to encode the enzymes of the dTDP-D-Fuc*f* (the nucleotide-activated form of D-Fuc*f*) biosynthetic pathway. In this study, Fcf1 and Fcf2 from *E. coli* O52 were expressed, purified and assayed for their respective activities. Novel product peaks from enzyme-substrate reactions were detected by capillary electrophoresis and the structures of the product compounds were elucidated by electro-spray ionization mass spectrometry and nuclear magnetic resonance spectroscopy. Fcf1 was confirmed to be a dTDP-6-deoxy-D-*xylo*-hex-4-ulopyranose reductase for the conversion of dTDP-6-deoxy-D-*xylo*-hex-4-ulopyranose to dTDP-D-fucopyranose (dTDP-D-Fuc*p*), and Fcf2 a dTDP-D-Fuc*p* mutase for the conversion of dTDP-D-Fuc*p* to dTDP-D-Fuc*f*. The K_m of Fcf1 for dTDP-6-deoxy-D-*xylo*-hex-4-ulopyranose was determined to be 0.38 mM, and of Fcf2 for dTDP-D-Fuc*p* to be 3.43 mM. The functional role of *fcf1* and *fcf2* in the biosynthesis of *E. coli* O52 O antigen were confirmed by mutation and complementation tests. This is the first time that the biosynthetic pathway of dTDP-D-Fuc*f* has been fully characterized.

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Introduction

A group of 6-deoxyhexoses, including L-rhamnose (6-deoxy-L-mannose), L-6dTal (6-deoxy-L-talose), D-fucose (6-deoxy-D-galactose), D-fucofuranose (D-Fuc*f*) (6-deoxy-D-galactofuranose), D-Fuc3NAc (3-acetamido-3,6-dideoxy-D-galactose), D-Fuc4NAc (4-acetamido-4,6-dideoxy-D-galactose), D-Qui3NAc (3-acetamido-3,6-dideoxy-D-glucose) and D-Qui4NAc (4-acetamido-4,6-dideoxy-D-glucose), are synthesized in the nucleotide-activated form (dTDP-sugars) from α-D-glucose-1-phosphate (D-Glc-1-P) (http://www.mmb.usyd.edu.au/BPGD/sugarpathways.htm) (Pföstl *et al.*, 2008). For their syntheses, D-Glc-1-P is converted to dTDP-6-deoxy-D-*xylo*-hex-4-ulopyranose, which is the branching point in different dTDP-sugar biosynthetic pathways. The biosynthetic pathway of dTDP-L-rhamnose was the first in this group to be biochemically characterized, which includes four reaction steps catalysed by RmlABCD proteins (Giraud *et al.*, 1999). Of the Rml proteins, RmlA (a glucose-1-phosphate thymidylyltransferase) catalyses the conversion of D-Glc-1-P to dTDP-D-glucose (dTDP-D-Glc) and RmlB (a dTDP-glucose 4, 6-dehydratase) catalyses the conversion of dTDP-D-Glc to dTDP-6-deoxy-D-*xylo*-hex-4-ulopyranose. Biosynthetic pathways of other dTDP-sugars in this group with the exception of D-Fuc*f* and D-Fuc4NAc have also been characterized (Giraud *et al.*, 1999; Yoshida *et al.*, 1999; Nakano *et al.*, 2000; Pfoestl *et al.*, 2003; Wang *et al.*, 2007; Pföstl *et al.*, 2008). The methodology for the identification of enzyme products with novel structures, including the use of capillary electrophoresis (CE), mass spectroscopy (MS) and nuclear magnetic resonance (NMR) has been well established (Kneidinger *et al.*, 2003a,b; Obhi and Creuzenet, 2005; McNally *et al.*, 2006; Vijayakumar *et al.*, 2006).

D-fucofuranose is an uncommon sugar found in several bacterial surface polysaccharides, including *Escherichia coli* O52 O antigen (Zdorovenko *et al.*, 2001; Sato *et al.*, 2003; Feng *et al.*, 2004). It is also the sugar moiety of gilvocarcin V (GV), which is the most important member of the benzo[*d*]naphtho[1,2-*b*]pyran-6-one *C*-glycoside

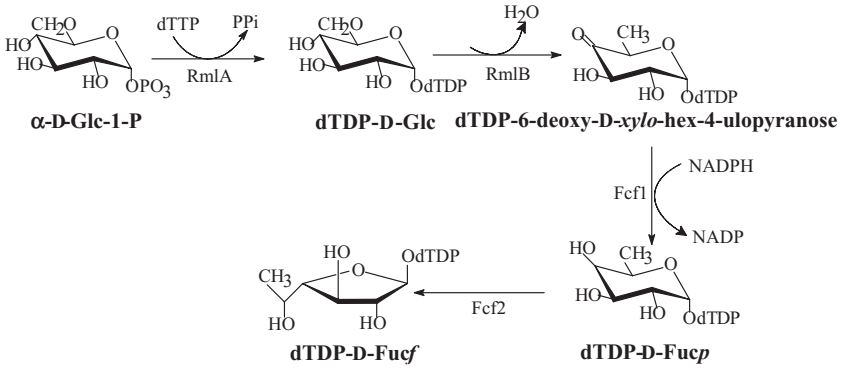

Fig. 1. Pathway for the biosynthesis of dTDP-D-Fuc*f*.

anticancer drug produced by a variety of streptomycetes (Breiding-Mack and Zeeck, 1987; Fischer *et al*., 2003; Liu *et al*., 2004). The biosynthetic pathway of this sugar has never been fully characterized.

In our previous study, four genes (*rmlA*, *rmlB*, *fcf1* and *fcf2*) from the *E. coli* O52 O antigen gene cluster were proposed to encode the enzymes for the synthesis of dTDP-D-Fuc*f* (Feng *et al*., 2004). While RmlA and RmlB have been well characterized in many bacteria including *E. coli* and *Salmonella* (Melo and Glaser, 1968; Melo *et al*., 1968; Marumo *et al*., 1992; Lindqvist *et al*., 1993), Fcf1 was proposed to be a reductase for the conversion of dTDP-6-deoxy-D-*xylo*-hex-4-ulopyranose to dTDP-D-Fuc*p*, and Fcf2 a mutase for the conversion of dTDP-D-Fuc*p* to dTDP-D-Fuc*f* (Fig. 1). The functions of Fcf1 and Fcf2 have not yet been confirmed.

In this study, *fcf1* and *fcf2* from *E. coli* O52 were cloned, and the gene products were overexpressed in *E. coli* BL21 and purified as His_6-tagged fusion proteins. The catalytic activities of Fcf1 on dTDP-6-deoxy-D-*xylo*-hex-4-ulopyranose and of Fcf2 on dTDP-D-Fuc*p* were assayed, and kinetic parameters of the two proteins were measured. Mutation and complementation analyses for *fcf1* and *fcf2* were also carried out to confirm the role of those two genes in the synthesis of *E. coli* O52 O antigen. This is the first report on the characterization of the dTDP-D-Fuc*f* biosynthetic pathway.

Results

Overexpression and purification of Fcf1 and Fcf2

The genes *fcf1* and *fcf2* from *E. coli* O52 were cloned into *E. coli* BL21, and the expression of the genes was induced by IPTG. As estimated by SDS-PAGE analysis, Fcf1 and Fcf2 accounted for 50% and 80% of total proteins, respectively, of which 80% and 60% were found in the soluble fractions. The two proteins were purified to near homogeneity as His_6-tagged fusion proteins. The estimated molecular mass was 40.7 kDa for Fcf1 and 50.6 kDa for Fcf2, corresponding well to the calculated masses (39.4 and 48.0 kDa) (Fig. S1).

Detection of Fcf1 and Fcf2 products by CE

Due to the unavailability of the sugars required for the Fcf1 and Fcf2 activity assays, the RmlB reaction was carried out to produce dTDP-6-deoxy-D-*xylo*-hex-4-ulopyranose from dTDP-D-Glc (Wang *et al*., 2007), thereby providing the substrate for Fcf1. The Fcf1 reaction product was subsequently used as the substrate for Fcf2. Enzyme products were detected by CE (Fig. 2). As expected, the reaction catalysed by RmlB converted dTDP-D-Glc to dTDP-6-deoxy-D-*xylo*-hex-4-ulopyranose, which eluted at 11.1 min (Fig. 2B). In the follow-up reaction catalysed by Fcf1, dTDP-6-deoxy-D-*xylo*-hex-4-ulopyranose was converted to a new product eluting at 10.2 min (Fig. 2C). The Fcf1 reaction required the presence of NADH or NADPH, which were concomitantly converted to NAD^+ or $NADP^+$, indicating that the enzyme is a NAD(P)H-dependent reductase. In the final reaction catalysed by Fcf2, another new product (eluting at 9.8 min) was detected (Fig. 2D and E). The conversion ratio was low for the Fcf2 reaction, with less than 10% of the Fcf1 product (the 10.2 min peak) being converted to the new product (the 9.8 min peak) under standard assay conditions, and less than 20% of the conversion ratio being obtained when the enzyme concentration was increased from 3.9 to 19.7 μM. Therefore, the Fcf2 reaction is apparently in favour of the reverse reaction. To further confirm the activity of Fcf2, reactions were carried out using different amounts of the enzyme (0.625–20 μg), and the dependency of the Fcf2 activity on the enzyme concentration was detected (Fig. S2).

Identification of Fcf1 and Fcf2 products by electro-spray ionization mass spectrometry and NMR

Fcf1 and Fcf2 products were purified by reverse-phase high-performance liquid chromatography (HPLC) (data

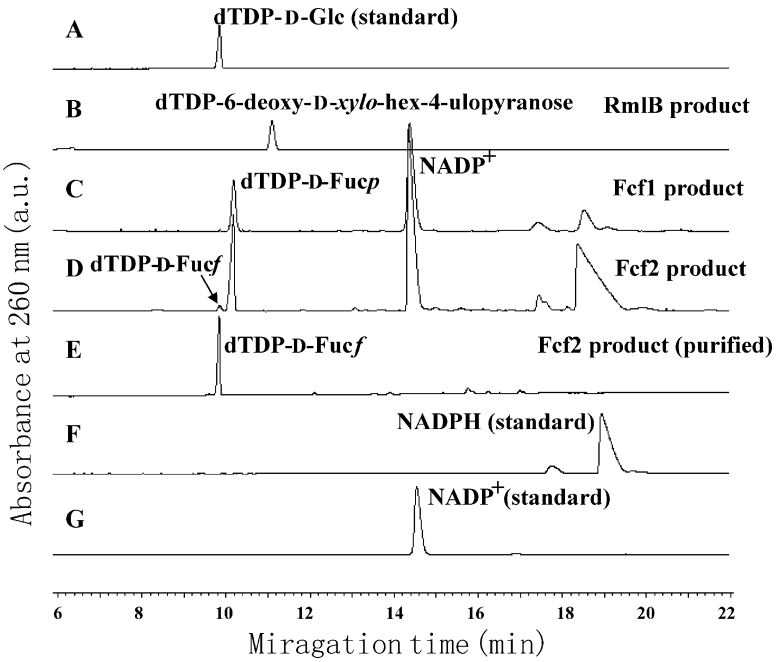

Fig. 2. CE chromatographs of the Fcf1 and Fcf2 products.
A. dTDP-D-Glc standard.
B. RmlB reaction product.
C. Fcf1 reaction product.
D. Fcf2 reaction product.
E. Purified Fcf2 product.
F. NADPH standard.
G. $NADP^+$ standard. The RmlB and Fcf1 and Fcf2 reaction products were diluted by fivefold and twofold respectively, prior to CE analysis.

not shown), and analysed by electro-spray ionization mass spectrometry (ESI-MS). Ion peaks at *m/z* 547.04 (Fig. 3A) and 547.00 (Fig. 4A) were obtained, which are in agreement with the expected masses for dTDP-D-Fuc*p* (548.33) and dTDP-D-Fuc*f* (548.33) respectively. MS/MS (MS2) analysis of the two ion peaks and the follow-up MS/MS/MS (MS3) analysis of selected MS2 ion peaks resulted in the detection of ion peaks matching the fragments derived from dTDP-D-Fuc*p* and dTDP-D-Fuc*f* respectively (Figs 3B, C, 4B and C). Fragments corresponding to each peak are depicted in Table 1.

The identities of the Fcf1 product as dTDP-D-Fuc*p* and the Fcf2 product as dTDP-D-Fuc*f* were structurally confirmed by NMR analysis. In the total correlation spectroscopy (TOCSY) spectrum of the purified Fcf1 product (Fig. 5A), there were correlations of H1 at δ 5.57 with H2 to H4 at δ 3.76, 3.92 and 3.83; H4 with H5 at δ 3.83/4.29, and H6 with H5 and H4 at δ 1.22/4.29 and 1.22/3.83 respectively, in the Fuc moiety; H1′ δ 6.35 with H2′ to H5′ at δ 2.38 (H2), 4.63 (H3) and 4.18 (H4, H5a, H5b) in 2′-deoxyribose; and H6 with CH_3 at δ 7.75/1.94 in thymine. The assigned chemical shifts were in good agreement with published data for dTDP (Nakano *et al.*, 2000) and α-fucopyranose (Jansson *et al.*, 1989). ^3J coupling constant values, $J_{1,2}$ 3.8, $J_{2,3}$ 10.2, $J_{3,4}$ 3.4 and $J_{4,5} < 1$ Hz, confirmed the α-*galacto* configuration of the sugar moiety (Jansson *et al.*, 1989), which is thus 6-deoxy-α-galactopyranose (α-Fuc*p*). The ^{31}P NMR spectrum of dTDP-D-Fuc*p* contained signals for a diphosphate group at δ −11.2 and −12.5, which showed correlations with the H5′ signal of dTDP at δ −11.2/4.18 and H1 of α-Fuc*p* at δ −12.5/5.57 in the $^1H,^{31}P$ heteronuclear multiquantum coherence spectrum. These data proved unambiguously the structure of the Fcf1 product as dTDP-α-D-Fuc*p* as depicted in Fig. 5A.

The TOCSY spectrum of the purified Fcf2 product (Fig. 5B) showed correlations of H1 at δ 5.65 with H2 to H6 at δ 4.14, 4.07, 3.62, 3.89 and 1.95, respectively, in the Fuc*f* moiety; H1′ at δ 6.35 with H2′ to H5′ at δ 2.38 (H2), 4.63 (H3) and 4.18 (H4, H5a, H5b) in 2′-deoxyribose; and H6 with CH_3 at δ 7.75/1.94 in thymine. The assigned chemical shifts and ^3J coupling constants, $J_{1,2}$ 4.4, $J_{2,3} \approx J_{3,4}$ 7.3, $J_{4,5}$ 6.9, $J_{5,6}$ 6.5 Hz for Fuc*f*, were in good agreement with published data for dTDP (Nakano *et al.*, 2000) and α-fucofuranose (Hanniffy *et al.*, 1999). The ^{31}P NMR spectrum of the product contained signals for a diphosphate group at δ −11.1 and −12.4, which showed clear correlations with H5′ signal of dTDP at δ −11.1/4.18 and H1 of α-Fuc*f* at δ −12.4/5.65 in the $^1H,^{31}P$ heteronuclear multiquantum coherence spectrum. These data confirmed unambiguously the structure of the Fcf2 product as dTDP-α-D-Fuc*f* as depicted in Fig. 5B.

Kinetic parameters, temperature optima and cation requirements of Fcf1 and Fcf2

Kinetic parameters of Fcf1 for the substrate (dTDP-6-deoxy-D-*xylo*-hex-4-ulopyranose) and cofactor (NADH and NADPH), and of Fcf2 for the substrate (dTDP-D-Fuc*p*) were measured. The initial velocities were measured and used for the calculation of kinetic parameters. The kinetics of the reaction catalysed by Fcf1 and by Fcf2 fit reasonable well to the Michaelis–Menten model (data not shown), and the kinetic parameters of the two

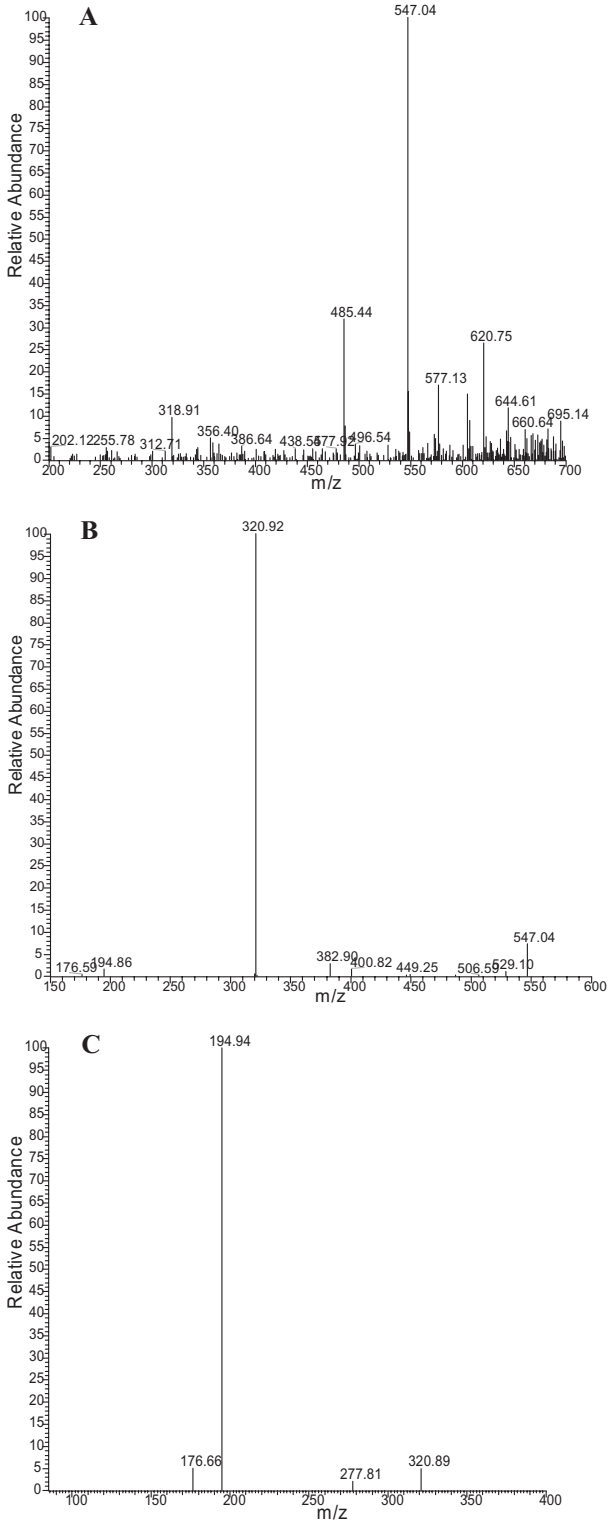

Fig. 3. A. ESI-MS of dTDP-D-Fuc*p*.
B. MS2 analysis of ions with *m/z* 547.04 in (A).
C. MS3 analysis of ions with *m/z* 320.92 in (B).

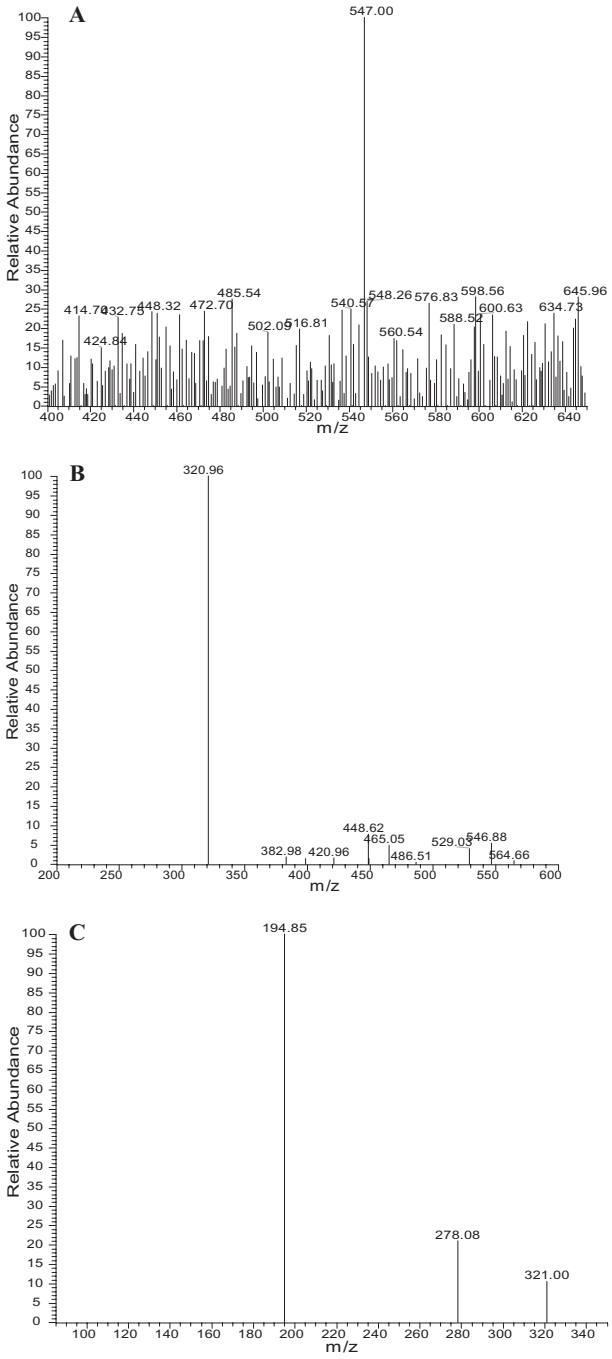

Fig. 4. A. ESI-MS of dTDP-D-Fuc*f*.
B. MS2 analysis of ions with *m/z* 547.00 in (A).
C. MS3 analysis of ions with *m/z* 320.96 in (B).

enzymes are listed in Table 2. The K_m values of Fcf1 were 0.38 mM for dTDP-6-deoxy-D-*xylo*-hex-4-ulopyranose, 0.84 mM for NADPH and 0.89 mM for NADH. Fcf1 showed similar affinities to NADH and NADPH, but the V_{max} value was higher for NADH (0.15 versus 0.037 mM min^{-1}). Therefore, NADH is a better cofactor for Fcf1. The K_m value of Fcf2 for dTDP-D-Fuc*p* was

Table 1. Interpretations of ion peaks present in ESI-MS and MSn.

Composition of fragment	Molecular formula	Molecular mass	Mass (negative)
dTDP-D-Fuc*p* (full scan)			
dTDP-D-Fuc*p*	$C_{16}H_{26}P_2O_{15}N_2$	548.33	547.04
dTDP-D-Fuc*p* (MS2-547)			
dTDP-D-Fuc*p* minus H_2O	$C_{16}H_{24}P_2O_{14}N_2$	530.31	529.01
dTDP plus H_2O	$C_{10}H_{15}P_2O_{11}N_2$	401.18	400.82
dTDP	$C_{10}H_{13}P_2O_{10}N_2$	383.17	382.90
Fucose + PO_3 + PO_3	$C_6H_{11}P_2O_{11}$	321.09	320.92
PO_3 + PO_3 + $2H_2O$	$P_2O_8H_6$	195.99	194.86
PO_3 + PO_3 + H_2O	$P_2O_7H_4$	177.97	176.59
dTDP-D-Fuc*p* (MS3-321)			
Fucose + PO_3 + PO_3 minus the group of CH3-CH-O	$C_4H_9P_2O_{10}$	279.05	277.81
PO_3 + PO_3 + $2H_2O$	$P_2O_8H_6$	195.99	194.94
PO_3 + PO_3 + H_2O	$P_2O_7H_4$	177.97	176.66
dTDP-D-Fuc*f* (full scan)			
dTDP-D-Fuc*f*	$C_{16}H_{26}P_2O_{15}N_2$	548.33	547.00
dTDP-D-Fuc*f* (MS2-547)			
dTDP-D-Fuc*f* minus H_2O	$C_{16}H_{24}P_2O_{14}N_2$	530.31	529.03
dTDP-D-Fuc*f* minus H_2O and CH3-CH-O	$C_{14}H_{20}P_2O_{13}N_2$	486.26	486.51
dTDP-D-Fuc*f* minus thymine	$C_{11}H_{19}P_2O_{13}$	421.21	420.96
dTDP	$C_{10}H_{13}P_2O_{10}N_2$	383.17	382.98
Fucose + PO_3 + PO_3	$C_6H_{11}P_2O_{11}$	321.09	320.96
dTDP-D-Fuc*f* (MS3-321)			
Fucose + PO_3 + PO_3 minus the group of CH3-CH-O	$C_4H_7P_2O_{10}$	279.05	278.08
PO_3 + PO_3 + $2H_2O$	$P_2O_8H_6$	195.99	194.85

3.43 mM. The lower affinity of the enzyme to the substrate is apparently the reason for the lower conversion ratio of the Fcf2 reaction, as the V_{max} values of Fcf1 and Fcf2 are similar (0.015 and 0.012 mM min^{-1}).

The activity of Fcf1 was detected over a broad range of temperatures from 4°C to 80°C with 100% of the conversion ratio obtained at 15°C, 25°C and 37°C (Table S3). The activity of Fcf2 was detected at temperatures ranging from 4°C to 50°C, but the overall conversion ratio was low (1.6–9.6% under standard assay conditions) with the maximum obtained at 15°C, 25°C and 37°C (Table S3). The enzyme can be maintained in 50% glycerol at –20°C for at least 5 months (Fcf1) or 2 months (Fcf2) without significant loss of the activity (data not shown).

Effects of Co^{2+}, Fe^{2+}, Ca^{2+}, Mn^{2+}, Mg^{2+} and Cu^{2+} on the activity of Fcf1 and Fcf2 were examined under standard assay conditions. With the exception of Cu^{2+}, which showed high level of inhibition, all of the cations tested had no obvious effects on the enzyme activity (Table S3). Therefore, both Fcf1 and Fcf2 are divalent cation-independent.

Mutation and complementation analysis of fcf1 *and* fcf2 *in* E. coli *O52*

To confirm the biological role of *fcf1* and *fcf2* in the synthesis of *E. coli* O52 O antigen, mutant strains H1862 lacking *fcf1* and H1863 lacking *fcf2* were constructed by using the Red recombinase-mediated gene replacement method of Datsenko and Wanner (2000) and examined for their lippolysaccharide (LPS) phenotypes. The mutant strains produced semirough (SR-type) LPS with only one O unit attached to the core-lipid A moiety while the wild-type strain produced normal LPS (Fig. 6). When the mutants were complemented by plasmid pLW1468 (containing *fcf1*) and pLW1469 (containing *fcf2*) respectively, the normal LPS phenotype was restored (Fig. 6). These results indicated that both *fcf1* and *fcf2* are required for the synthesis of the O antigen in *E. coli* O52.

Discussion

In this study, the biosynthetic pathway of dTDP-D-Fuc*f* was fully characterized. The fact that the activity of Fcf2 is apparently in favour of the reverse reaction indicates that the Fcf2 reaction is the limiting step for the synthesis of dTDP-D-Fuc*f*. The biological roles of *fcf1* and *fcf2* in the synthesis of the O antigen in *E. coli* O52 were confirmed. The rapid utilization of the synthesized dTDP-D-Fuc*f* for building up *E. coli* O52 O units presumably allows the Fcf2 reaction to proceed under physiological conditions.

Fcf1 is the second dTDP-6-deoxy-D-*xylo*-hex-4-ulopyranose reductase that has been characterized biochemically up to now. The other is Fcd from *Actinobacillus actinomycetemcomitans* Y4, which has a D-Fuc*p*-containing capsular polysaccharide (Yoshida *et al*., 1999). However, the two proteins share only 16% DNA identity and belong to different protein families,

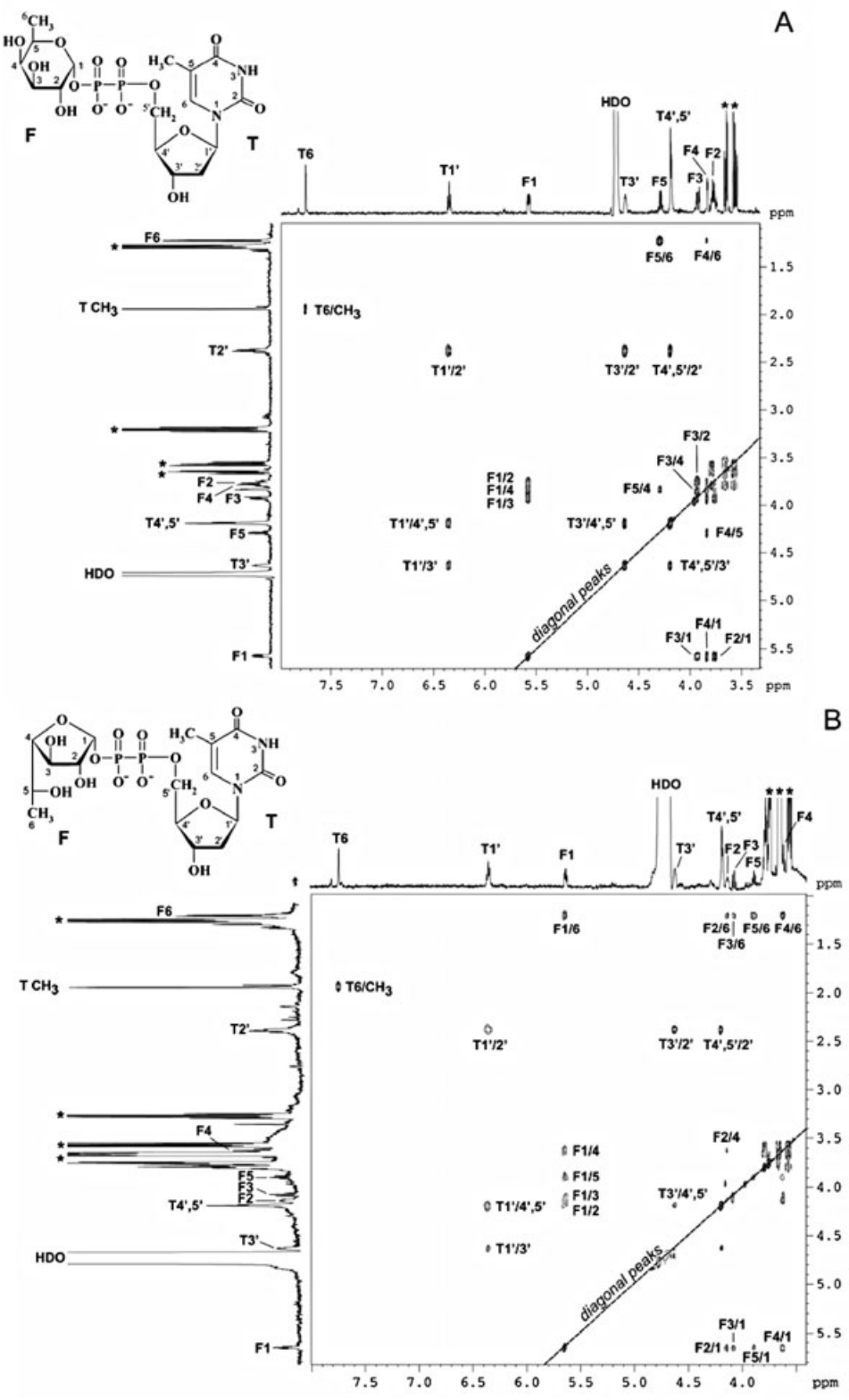

Fig. 5. Parts of ^{1}H, ^{1}H TOCSY spectra of dTDP-α-D-Fuc*p* (A) and dTDP-α-D-Fuc*f* (B). The corresponding parts of the ^{1}H NMR spectra are displayed along the axes. Arabic numerals refer to protons in the Fuc and dTDP moieties designated as F and T respectively. Signals of contaminating substances from HPLC buffer are indicated by asterisks. The structures of the products are shown in the insets.

Table 2. Kinetic parameters.

Enzyme	Substrate	K_m (mM)	V_{max} (mM min^{-1})	k_{cat} (min^{-1})
Fcf1	dTDP-6-deoxy-D-*xylo*-hex-4-ulopyranose	0.38 ± 0.03	0.015 ± 0.005	166.67 ± 55.78
Fcf1	NADPH	0.84 ± 0.05	0.037 ± 0.008	405.80 ± 91.16
Fcf1	NADH	0.89 ± 0.03	0.15 ± 0.02	1594.20 ± 251.02
Fcf2	dTDP-D-Fuc*p*	3.43 ± 0.30	0.012 ± 0.002	3.37 ± 0.68

the NDP-dependent epimerase/dehydratase family (PF01370) for Fcf1 and GtrA-like protein family (PF04138) for Fcd. Furthermore, the NAD(P)H binding motifs at the N terminus are also different: GXGXXG in Fcf1 (Jiang *et al*., 1995) and GXGXXA in Fcd (Yoshida *et al*., 1999). These disparities indicate distinct evolutionary origins for the two genes; thus, *fcf1* is a novel gene coding for dTDP-6-deoxy-D-*xylo*-hex-4-ulopyranose reductase.

To our knowledge, Fcf2 is the first dTDP-D-fucopyranose mutase that has been characterized. It shares high DNA identities (39–60%) with and belongs to the same protein family (PF03275, *E*-value = $9.5 \times e^{-98}$) as UDP-galactopyranose mutases. UDP-galactopyranose mutase catalyses the reversible reaction between pyranose and furanose galactose, and the reaction was also shown to be biased strongly in favour of the pyranose isomer with less than 10% of conversion ratio towards the furanose isomer (Nassau *et al*., 1996; Köplin *et al*., 1997; Zhang and Liu, 2000). In future studies, it will be important to perform the reactions of Fcf2 to evaluate the conversion ratio between dTDP-D-Fuc*p* and dTDP-D-Fuc*f* upon availability of dTDP-D-Fuc*p* and dTDP-D-Fuc*f* from commercial suppliers or by taking approaches to scale up the Fcf2 reaction and purify the product. Alignment of Fcf2 and 13 available UDP-galactopyranose mutase sequences revealed that the ADP-binding βαβ-fold, which is involved in FAD binding (Wierenga *et al*., 1983), is conserved among all sequences (Fig. S3). Nine-amino-acid residues present in all 13 UDP-galactopyranose mutases are substituted by different residues in Fcf2 (Fig. S3), suggesting a possible role for these residues in substrate binding.

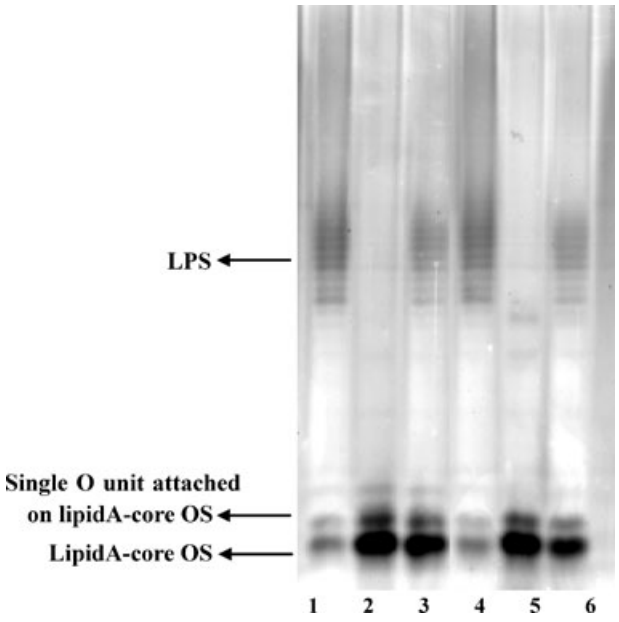

Fig. 6. LPS profiles of *E. coli* O52 wild-type, *fcf1* mutant, *fcf2* mutant and trans-complemented mutant strains. Membrane extracts were run on SDS-PAGE gels and visualized by silver staining. Lane 1, G1066 (*E. coli* O52 type strain); lane 2, H1862 (G1066 lacking *fcf1*); lane 3, H1864 (H1862 with plasmid pLW1468 containing *fcf1*); lane 4, G1066 (*E. coli* O52 type strain); lane 5, H1863 (G1066 lacking *fcf2*); lane 6, H1865 (H1863 with plasmid pLW1469 containing *fcf2*). OS, oligosaccharide.

The *gil* gene cluster is required for the synthesis of GV in *Streptomyces griseoflavus* Gö 3592, and five genes (*gilD*, *gilE*, *gilU*, *gilR* and *gilM*) have been proposed to encode the enzymes responsible for biosynthesis of the D-Fuc*f* moiety (Fischer *et al*., 2003). GilD and GilE are RmlA and RmlB homologues respectively, and are predicted to be responsible for the first two reaction steps. Either GilU, predicted to be a NDP-dependent epimerase/dehydratase, or GilR, a putative oxidoreductase, is expected to convert dTDP-6-deoxy-D-*xylo*-hex-4-ulopyranose to dTDP-D-Fuc*p*. GilM, predicted to be a conserved hypothetical protein, is proposed to be the enzyme for the conversion of dTDP-D-Fuc*p* to dTDP-D-Fuc*f*. We compared the similarity of GilU and GilR with Fcf1 and of GilM with Fcf2. GilU shares 42% similarity (22% identity) with and belongs to the same protein family (PF01370) as Fcf1, indicating the same function for the two proteins. In contrast, GilR shares no similarity with Fcf1. Therefore, it is most likely that GilU is responsible for the conversion of dTDP-6-deoxy-D-*xylo*-hex-4-ulopyranose to dTDP-D-Fuc*p* in *S. griseoflavus* Gö 3592. No similarity was detected between GilM and Fcf2, and none of the other Gil proteins shares similarity to Fcf2 (*E*-value < 0.1), suggesting that the function of *fcf2* is substituted by other non-orthologous genes in *S. griseoflavus*.

Despite their great potential for applications in drug development in the pharmaceutical industry, uncommon sugars such as dTDP-D-Fuc*f* are difficult to produce by chemical synthesis due to the requirement for multistep reactions of protection and de-protection. The Fcf1 and Fcf2 enzymes characterized in this study, together with the previously characterized RmlA and RmlB proteins,

provide good enzyme sources for the biological synthesis of dTDP-D-Fucf.

Experimental procedures

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table S1.

Cloning and plasmid construction

Genes *fcf1* and *fcf2* from *E. coli* O52 (G1066) were amplified by PCR using the primer pair wl-5658/5659 and wl-5660/5661 respectively (Table S2). A total of 30 cycles were performed using the following conditions: denaturation at 94°C for 15 s, annealing at 50°C for 30 s and extension at 72°C for 1 min, in a final volume of 25 μl. The amplified genes were cloned into pET28a$^+$ to construct pLW1203 (containing *fcf1*) and pLW1204 (containing *fcf2*). The presence of the inserts in the plasmids was confirmed by sequencing using an ABI 3730 Sequencer.

Protein expression and purification

Escherichia coli BL21 carrying pLW1203 or pLW1204 was grown in LB medium containing 50 μg ml^{-1} kanamycin overnight at 37°C. The overnight culture (5 ml) was inoculated into 500 ml of fresh LB and grown at 37°C until the A_{600} reached 0.6. Expression of *fcf1* or *fcf2* was induced with 0.1 mM IPTG for 4 h at 25°C and 20°C respectively. After the IPTG induction, the cells were harvested by centrifugation, washed with binding buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl and 10 mM imidazole), re-suspended in 5 ml of the same buffer containing 1 mM phenylmethanesulfonyl fluoride and 1 mg ml^{-1} lysozyme, and sonicated. The cell debris was removed by centrifugation, and the supernatant containing soluble proteins was collected. The His_6-tagged fusion proteins in the supernatant were purified by nickel ion affinity chromatography with a Chelating Sepharose Fast Flow column (GE Healthcare) according to the manufacturer's instruction. Unbound proteins were washed out with 100 ml of wash buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl and 25 mM imidazole). Fusion proteins were eluted with 3 ml of elution buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl and 250 mM imidazole), and dialysed overnight against 50 mM Tris-HCl buffer (pH 7.4) at 4°C. Protein concentration was determined by the Bradford method. Purified proteins were stored at −20°C in the presence of glycerol (50%).

Enzyme activity assays

Unless specified elsewhere, standard reactions were carried out as follows. The RmlB reaction was carried out at 37°C for 2 h in a reaction mixture containing 4 mM dTDP-D-Glc (Sigma-Aldrich), 50 mM Tris-HCl (pH 7.4) and 1.8 μM purified RmlB (Wang *et al.*, 2007), in a total volume of 25 μl. The Fcf1 reaction was carried out at 37°C for 2 h in a reaction mixture containing 3 mM NADPH (Sigma-Aldrich), 25 mM Tris-HCl (pH 7.4), 10 μl of the finished RmlB reaction mixture (containing 1.6 mM dTDP-6-deoxy-D-*xylo*-hex-4-ulopyranose), and 0.25 μM purified Fcf1, in a total volume of 20 μl. The Fcf2 reaction was carried out at 37°C for 3 h in the finished Fcf1 reaction mixture (20 μl, containing 1.6 mM dTDP-D-Fuc*p*) containing 3.9 μM purified Fcf2. The concentration of dTDP-6-deoxy-D-*xylo*-hex-4-ulopyranose and dTDP-D-Fuc*p* were calculated based on 100% of conversion ratio from dTDP-D-Glc to dTDP-6-deoxy-D-*xylo*-hex-4-ulopyranose and from dTDP-6-deoxy-D-*xylo*-hex-4-ulopyranose to dTDP-D-Fuc*p* respectively. Reaction products were detected by CE, and identified by ESI-MS and NMR (see below). Enzyme activity was indicated by the conversion of the substrate to the product.

CE analysis

Capillary electrophoresis was performed using a Beckman Coulter P/ACE MDQ Capillary Electrophoresis System with a PDA detector (Beckman Coulter, CA). The capillary was bare silica of 75 μm i.d. × 57 cm, with the detector at 50 cm. The capillary was conditioned before each run by washing with 0.1 M NaOH first, with deionized water next, and with 25 mM borate-sodium hydroxide (pH 9.6) (used as the mobile phase) last for 2 min each. Samples were loaded by pressure injection at 0.5 p.s.i. for 10 s, and separation was carried out at 20 kV. Peak integration and trace alignment were done using the Beckman P/ACE station software (32 Karat version 5.0). Conversion ratios were calculated by comparing the peak areas of the substrate and product.

Reverse-phase HPLC and ESI-MS analysis

The Fcf1 and Fcf2 reaction products were purified by reverse-phase HPLC using a BioCAD 700E Perfusion Chromatography Workstation (Applied Biosystems, CA) with a Venusil MP-C18 column (5 μm particle, 4.6 mm × 250 mm) (Agela Technologies). The mobile phase used was composed of 3.3% of acetonitrile and 96.7% of 50 mM triethylamine-acetatic acid (pH 6.8). The flow rate was 0.6 ml min^{-1}. Fractions containing expected products were collected, lyophilized and re-dissolved in 50% of methanol before injecting into a Finnigan LCQ Advantage MAX ion trap mass spectrometer (Thermo Electron, CA) at negative mode (4.5 kV, 250°C) for ESI-MS analysis. For MS2 and MS3 analyses, nitrogen was used as collision gas and helium as auxiliary gas and collision energies used were typically 20–30 eV.

NMR spectroscopy

Samples of purified Fcf1 product (0.25 mg) or Fcf2 product (0.055 mg) were deuterium-exchanged by freeze-drying from D_2O, dissolved in 99.96% D_2O (150 μl) and examined using a Shigemi microtube (Japan). NMR spectra were recorded on a Bruker DRX-500 spectrometer (Germany) at 30°C using internal sodium 3-trimethylsilyl-[2,2,3,3–$^2H_4$]propanoate (δ_H 0.00) and external aqueous 85% H_3PO_4 (δ_P 0.0) as references. Two-dimensional NMR spectra were obtained using standard pulse sequences from the manufacturer. The

XWINNMR 2.6 program (Bruker) was used to acquire and process the NMR data. A mixing time of 200 ms was used in TOCSY experiments.

Sugar analysis by gas–liquid chromatography

A sample of purified Fcf2 product (0.05 mg) was hydrolysed with 2 M CF_3CO_2H (120°C, 2 h). The products were reduced with 0.25 M $NaBH_4$ in aq 1 M ammonia (25°C, 1 h), acetylated with a 1:1 (v/v) mixture of pyridine and acetic anhydride (120°C, 0.5 h), and analysed by gas–liquid chromatography using a Hewlett-Packard 5880 instrument equipped with an Ultra-2 column (0.20 mm × 25 m) and a temperature programme of 160–290°C at 3°C min^{-1}.

Measurement of kinetic parameters

To measure K_m and V_{max} values, reactions for Fcf1 were carried out with various concentrations of dTDP-6-deoxy-D-*xylo*-hex-4-ulopyranose (0.1–1.6 mM), various concentrations of NADPH or NADH (0.1–2 mM), and a fixed concentration of Fcf1 (0.092 μM). Reactions for Fcf2 were carried out with various concentrations of dTDP-D-Fuc*p* (0.5–4.5 mM) and a fixed concentration of Fcf2 (3.5 μM). The final volume of reaction mixtures was 20 μl, and reactions were terminated by adding an equal volume of chloroform. Conversion of dTDP-6-deoxy-D-*xylo*-hex-4-ulopyranose to dTDP-D-Fuc*p* and of dTDP-D-Fuc*p* to dTDP-D-Fuc*f* were monitored by CE. K_m and V_{max} values were calculated based on the Michaelis–Menten equation. The data shown are the averages of three independent experiments.

Determination of temperature optima and divalent cation requirements

To determine the temperature optima for Fcf1 and Fcf2, standard reactions were carried out at 4°C, 15°C, 25°C, 37°C, 50°C, 65°C and 80°C respectively. To test effects of different cations on the enzyme activities, standard reactions were carried out in the presence of 5 mM $MgCl_2$, $MnCl_2$, $FeCl_2$, $CuCl_2$, $CaCl_2$, $CoCl_2$ respectively.

Construction of fcf1 *and* fcf2 *mutant strains of* E. coli *O52*

To generate *fcf1* and *fcf2* mutant strains of *E. coli* O52, *fcf1* and *fcf2* were replaced, respectively, by a chloramphenicol acetyltransferase gene using the Red recombination system of phage lambda (Datsenko and Wanner, 2000; Yu *et al.*, 2000). The detailed protocol used for the replacement was described previously (Senchenkova *et al.*, 2006), except for the use of different primers (Table S2). To complement the mutants, *fcf1* and *fcf2* were PCR-amplified from the wild-type *E. coli* O52 strain using the primer pair wl-13780/wl-13781 and wl-13786/wl-13787 respectively (Table S2). The resulting PCR products were cloned into pTRC99A (Amann *et al.*, 1988) to make the plasmid pLW1468 (containing *fcf1*) and pLW1469 (containing *fcf2*), which were transformed into the corresponding mutants by electroporation. Membrane preparation, SDS-PAGE and silver staining for visualization of LPS were carried out as described by Wang and Reeves (1994).

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