TM0416, a Hyperthermophilic Promiscuous Nonphosphorylated Sugar Isomerase, Catalyzes Various C₅ and C₆ Epimerization Reactions

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ABSTRACT There is currently little information on nonphosphorylated sugar epimerases, which are of potential interest for producing rare sugars. We found a gene (the TM0416 gene) encoding a putative D-tagatose-3-epimerase-related protein from the hyperthermophilic bacterium *Thermotoga maritima*. We overexpressed the TM0416 gene in *Escherichia coli* and purified the resulting recombinant protein for detailed characterization. Amino acid sequence alignment and a structural similarity search revealed that TM0416 is a putative nonphosphorylated sugar epimerase. The recombinant enzyme exhibited maximal C-3 epimerization of L-ribulose to L-xylulose at ~80°C and pH 7 in the presence of 1 mM Mn²⁺. In addition, this enzyme showed unusually high activity for the epimerization of D-tagatose to D-sorbose, with a conversion yield of 20% after 6 h at 80°C. Remarkably, the enzyme catalyzed the isomerization of D-erythrose or D-threose to D-erythrulose significantly, with conversion yields of 71% and 54.5%, respectively, after 6 h at 80°C at pH 7. To further investigate the substrate specificity of TM0416, we determined its crystal structures in complex with divalent metal ions and L-erythrulose at resolutions of 1.5 and 1.6 Å. Detailed inspection of the structural features and biochemical data clearly demonstrated that this metalloenzyme, with a freely accessible substrate-binding site and neighboring hydrophobic residues, exhibits different and promiscuous substrate preferences, compared with its mesophilic counterparts. Therefore, this study suggests that TM0416 can be functionally classified as a novel type of L-ribulose 3-epimerase (R3E) with D-erythrose isomerase activity.

IMPORTANCE Rare sugars, which occur naturally in small amounts, have attracted considerable attention in the food and drug industries. However, there is little information on nonphosphorylated sugar epimerases, which might potentially be applied for the production of rare sugars. This study describes the characterization and functional annotation of a putative nonphosphorylated sugar 3-epimerase from a hyperthermophilic bacterium. Furthermore, we determined its crystal structures in complex with divalent metal ions and L-erythrulose, highlighting its metal-dependent, bifunctional, sugar-isomerizing activity. This hyperthermophilic R3E exhibited D-erythrose/D-threose isomerase activity, with structural features near the substrate-binding site distinct from those of its mesophilic counterparts. Moreover, this metalloenzyme showed unusually high activity for the epimerization of D-tagatose to D-sorbose at 70°C. Therefore, TM0416 can be functionally classified as a novel type of promiscuous R3E with a potential for the production of rare sugars for the food and pharmaceutical industries.

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Low-calorie sweeteners have positive medical and economic effects when they are used to treat obesity and diabetes mellitus (1). Rare sugars (e.g., D-tagatose, D- and L-sorbose, D-psicose, and L-ribose), which occur naturally in small amounts, have attracted considerable interest in the food and drug industries because they are potent sugar substitutes and important building blocks for pharmaceutically active ingredients (2). D-Tagatose, which is classified as generally recognized as safe (GRAS) in the United States, can serve as a low-calorie sweetener and an antidiabetes and obesity-control drug (3–5). In recent years, D-tagatose has been produced commercially using a thermostable L-arabinose isomerase (6, 7); D-sorbose, L-sorbose, and L-galactose are vitamin C precursors and have been used as low-cariogenesis sweeteners (8, 9). D-Sorbose, a C-3 epimer of D-tagatose, is of special interest as a low-calorie sweetener (10, 11), an insect-control agent (12), and a starting material for L-threo-2, 5-hexodiulose (13). Izumori (14) proposed “Izumoring” as a plausible strategy to biologically produce rare L- and D-ketohexose sugars from abundant raw materials (e.g., starch, whey, or hemicellulose). In this regard, D-tagatose 3-epimerase (T3E) (EC 5.1.3.31) has received attention as a potential biocatalyst for the epimerization of all ketohexoses at the C-3 position, producing rare sugars such as D-sorbose and D-psicose (15–20). T3E has not been investigated intensively, however, and its structural and functional relationships, including its substrate specificity, remain unclear. This has hampered its development and application as a novel enzyme for the industrial production of rare sugars.

Epimerases are a subgroup of isomerases that catalyze configurational reversal at an asymmetric carbon atom, usually by a deprotonation/protonation mechanism (21, 22). The catalytic mechanism and biochemical properties of phosphorylated sugar epimerases have been reviewed extensively (22, 23). Phosphorylated sugar epimerases can be divided into several groups, according to their mechanism of catalysis, i.e., (i) proton abstraction/addition (24, 25), (ii) formation of a transient keto intermediate (26, 27), (iii) carbon-carbon bond cleavage (28, 29), (iv) mutarotation (ring opening) (30), and (v) nucleotide elimination and readdition (31). However, little is known about the non-phosphorylated L-ribulose 3-epimerase (R3E). Furthermore, T3E-like epimerases have unique substrate specificity for rare sugars, but only a few T3E-related epimerases have been characterized biochemically and/or structurally (15, 32, 33).

To date, a variety of microbial genome sequences (>70,000 sequences) have revealed a large number of genes encoding putative ketohexose 3-epimerases, highlighting the need for functional characterization. The hyperthermophilic bacterium *Thermotoga maritima* (34), which grows optimally at 80°C, represents one of the deepest and most slowly evolving lineages in the family *Eubacteriaceae* (35, 36). Moreover, 10 to 15% of the predicted coding sequences in the 1.86-Mbp genome of *T. maritima* are involved in simple and complex sugar metabolism (36). For this study, we chose a gene encoding a putative T3E-related protein (TM0416) from *T. maritima*, with an amino acid sequence similar to the sequences of D-psicose 3-epimerase (P3E) from *Clostridium cellulolyticum* (20.7% identity) (37) and T3E from *Pseudomonas cichorii* (20.0% identity) (15).

To determine whether the protein encoded by the TM0416 gene can epimerize nonphosphorylated sugars at the C-3 position, we functionally and structurally characterized it and annotated it as a R3E with D-erythrose/D-threose isomerase activity. Subsequently, we determined the substrate spectrum of TM0416 and compared it with those of its homologs.

RESULTS

Cloning, expression, and purification of TM0416. A search for nonphosphorylated sugar 3-epimerases revealed that the *Thermotoga maritima* genome contains a gene (TM0416) that was initially annotated as encoding a D-tagatose-3-epimerase-

FIG 1 Biochemical properties of TM0416. (A) Purification of recombinant TM0416. The purified enzyme was analyzed by 12% SDS-PAGE. Lane M, molecular weight markers; lane 1, whole-cell lysate of *E. coli* BL21(DE3); lane 2, whole-cell lysate of *E. coli* BL21(DE3) containing pET-15b-TM0416; lane 3, cytosolic fraction of *E. coli* BL21(DE3) containing pET-15b-TM0416; lane 4, lysate after heat treatment at 70°C; lane 5, protein after Ni²⁺ affinity chromatography; lane 6, protein after size exclusion chromatography. (B) Size exclusion chromatography of recombinant TM0416. Chromatography was performed using a Superdex 75 pg column, at a flow rate of 0.3 ml min⁻¹, and the elution profile was monitored at 280 nm. The column was calibrated with blue dextran (2,000 kDa), conalbumin (75 kDa), ovalbumin (44 kDa), and carbonic anhydrase (29 kDa) as standards. (Inset) Calibration curve. ○, molecular size standards; ●, TM0416. (C) Effect of temperature on recombinant TM0416 activity. (D) Effect of pH on recombinant enzyme activity. □, sodium acetate-acetic acid buffer (pH 4 to 6); △, HEPES buffer (pH 6 to 8); ○, glycine-NaOH buffer (pH 8 to 10). The enzyme activity data were plotted as a function of pH and fitted to a bell-shaped activity profile with the equation $V = V_m \times [1 / (1 + (10^{-pH} / 10^{-pK_{a1}}) + (10^{-pK_{a2}} / 10^{-pH}))]$, where the apparent pK_a values corresponding to the acidic (pK_{a1}) and basic (pK_{a2}) limbs were determined by nonlinear curve fitting (OriginPro8). (E) Effects of divalent metal ions on the activity of recombinant TM0416. (F) Differential scanning calorimetry data for recombinant TM0416.

related protein (36). The TM0416 gene (816 bp), encoding a 271-amino-acid protein with a calculated molecular weight of 30,464, was amplified from *T. maritima* genomic DNA by PCR and cloned into the pET-15b expression vector to yield pET-15b_TM0416. As shown in Fig. 1A, the TM0416 gene was successfully expressed as an N-terminal hexahistidine-tagged fusion protein in *Escherichia coli* BL21(DE3) upon isopropyl- β -D-thiogalactopyranoside (IPTG) induction. After disruption of the protein-expressing *E. coli* BL21(DE3) cells by sonication, the cell lysates were heated to 70°C to remove the majority of endogenous proteins, while the expressed protein remained soluble (Fig. 1A). A single step of Ni²⁺-chelate affinity chromatography served to purify the recombinant protein to >90% purity, with a yield of 76%. Subsequent thrombin digestion to remove the hexahistidine tag, followed by gel filtration chromatography, yielded an intact protein with an apparent molecular mass of 30 kDa estimated by SDS-PAGE, consistent with the theoretical molecular mass of 30,464.1 Da calculated from the presumptive amino acid sequence (Fig. 1A). Gel filtration chromatography on Superdex 75 pg resin yielded an estimated molecular mass of 60 kDa, suggesting a homodimeric structure (Fig. 1B).

Primary sequence of TM0416 and structural comparisons with its homologs.

The amino acid sequence alignment of several TM0416 homologs showed that TM0416 exhibited poor sequence identity (16.7 to 20.7%) with respect to known epimerases (Table 1). However, a structural similarity search of the DALI server (see Table S2 in the supplemental material) (38) using the partially solved three-dimensional structure of TM0416 (PDB accession no. 2ZVR) (39) revealed that its 89-amino-acid sequence is quite similar to highly conserved structural regions from *Mesorhizobium loti* R3E (MLR3E) (PDB accession no. 3VYL), *Pseudomonas cichorii* T3E (PCT3E) (PDB accession no. 2QUL), and *Agrobacterium tumefaciens* P3E (ATP3E) (PDB accession no. 2HK0). In addition, TM0416

TABLE 1 Biochemical properties of TM0416 and its homologs

Parameter	TM0416	CCP3E ^a	PCT3E	ATP3E	MLR3E
Sequence identity (%) ^b	100	20.7	20.0	16.7	17.4
Optimal temperature (°C)	80	55	60	50	60
Optimal pH	7.0	8.0	7.5	8.0	8.0
Molecular mass (kDa)	30 (dimer)	32 (tetramer)	32.5 (dimer)	33 (tetramer)	32 (tetramer)
Cofactor	Mn ²⁺	Co ²⁺	Not reported	Mn ²⁺	Mn ²⁺
Substrate specificity ^c	D-Erythrose, D-threose, L-ribulose, L-xylulose	D-Psicose, D-fructose, D-tagatose, D-sorbose	D-Tagatose, D-sorbose, D-fructose, D-psicose	D-Psicose, D-fructose, D-tagatose, D-ribulose	L-Ribulose, L-xylulose, D-tagatose, D-ribulose
Reference	This study	37	15	60	61, 62

^aCCP3E, *C. cellulolyticum* P3E; PCT3E, *P. cichorii* T3E; ATP3E, *A. tumefaciens* P3E; MLR3E, *M. loti* R3E.

^bIdentity of the amino acid sequences of various epimerases with respect to TM0416, based on CLUSTAL alignment using BioEdit software.

^cAccording to Table 2.

was found to have the manganese-binding motif (DXXH), which is widely distributed among epimerases (21, 28–30, 40, 41). Therefore, the structural similarity search using the DALI server, based on the primary and tertiary structures of TM0416 homologs, suggested that, despite the low level of amino acid sequence identity to the homologs, a conserved structural feature of those sugar epimerases is descended from a common structural fold responsible for the C-3 epimerization of ketoses.

Biochemical characterization of recombinant TM0416. To determine whether TM0416 epimerizes either ketohexose or ketopentose, we performed enzymatic reactions with various C₄, C₅, and C₆ sugars as substrates and then analyzed the reaction products by high-performance liquid chromatography (HPLC) (Fig. 2A). TM0416 was able to catalyze the C-3 epimerization of L-ribulose, D-tagatose, and D-psicose, resulting in the production of L-xylulose (66.3%), D-sorbose (20.4%), and D-fructose (20%), respectively, under the assay conditions used. However, this enzyme exhibited no activity for C-4 epimerization of D-tagatose to D-fructose. Thus, it was tentatively concluded that

FIG 2 Nonphosphorylated sugar epimerization and isomerization activities of TM0416. (A) HPLC analysis (left) of C-3 epimerization and isomerization products (right). The reaction mixtures (1 ml) contained 50 mM HEPES buffer (pH 7.0), 10 mM substrate (L-ribulose, D-fructose, D-tagatose, or D-erythrose), 1 mM Mn²⁺, and 2 mg of TM0416. (B) Time course of L-xylulose, D-sorbose, D-fructose, and D-erythrulose production during TM0416-catalyzed epimerization and isomerization. Aliquots were withdrawn after various periods of incubation at 60°C or 70°C. The experiments were performed in duplicate.

TABLE 2 Initial screening for 3-epimerization activities of TM0416 with various sugars^a

Sugar	Relative activity (%)	Specific activity (U/mg)
D-Erythrose (C ₄)	100.0 ± 1.0	18.7 ± 1.0
D-Threose (C ₄)	22.0 ± 2.0	4.1 ± 0.4
L-Ribulose (C ₅)	27.0 ± 2.0	5.0 ± 0.9
L-Xylulose (C ₅)	10.0 ± 1.0	2.0 ± 0.4
D-Ribulose (C ₅)	24.0 ± 3.0	4.4 ± 0.1
D-Xylulose (C ₅)	13.0 ± 4.0	2.5 ± 0.6
D-Tagatose (C ₆)	5.0 ± 0.5	1.0 ± 0.1
D-Psicose (C ₆)	0.5 ± 0.1	0.1 ± 0.01
D-Fructose (C ₆)	0.5 ± 0.1	0.1 ± 0.01
D-Sorbose (C ₆)	0.5 ± 0.1	0.1 ± 0.01

^aExperiments were carried out for 3 h at 80°C at pH 7.0 in the presence of 1 mM Mn²⁺. The reaction mixture (120 μl) contained 50 mM HEPES buffer (pH 7.0), 1 mM MnCl₂, 40 mM each monosaccharide as the substrate, and the enzyme (1 mg ml⁻¹). The data are mean ± standard deviation (SD).

the recombinant TM0416 might be an R3E that prefers L-ribulose over D-tagatose or D-psicose, and we used L-ribulose as a substrate to study the biochemical properties of TM0416. The temperature dependence and pH dependence of the recombinant TM0416 were also investigated, using HPLC to detect L-xylulose after 4 h of incubation at various temperatures and pH values. The optimal temperature for the recombinant TM0416 was ~80°C (Fig. 1C), and the purified enzyme showed optimal activity at pH 6.5 to 7.0 (Fig. 1D).

Several sugar epimerases that are involved in the pentose phosphate pathway require divalent metal ions for epimerization via a *cis*-enediolate-stabilized intermediate (29). Therefore, to investigate the effects of divalent metal ions on TM0416 activity, we purified a metal-depleted enzyme by using EDTA treatment followed by dialysis to yield the apo form of TM0416 (with an estimated 0.015 ± 0.001 equivalents of Mn²⁺ per subunit), which was verified using high-resolution inductively coupled plasma mass spectrometry (ICP-MS). The as-isolated enzyme (with 0.08 ± 0.001 equivalents of Mn²⁺ per subunit) showed pronounced activity, but the apo enzyme showed no activity in the absence of divalent metal ions (Fig. 1E). However, enzyme activity was significantly restored by the addition of divalent metal ions such as 1 mM Mn²⁺ and Ni²⁺, indicating that TM0416 activity is absolutely dependent on divalent metal ions. Ni²⁺, Mg²⁺, Co²⁺, and Zn²⁺ acted as strong metal cofactors, whereas TM0416 activity was inhibited by Ca²⁺, Cu²⁺, and Fe²⁺ (Fig. 1E). The thermal unfolding profile of the recombinant TM0416 revealed that this enzyme is hyperthermostable, with an apparent melting temperature (*T_m*) of 102.4°C (Fig. 1F).

Substrate specificity of TM0416. To investigate the substrate preferences of TM0416, we first determined the equilibrium ratios between D-tagatose and D-sorbose (or D-fructose and D-psicose) and between L-ribulose and L-xylulose. As shown in Fig. 2A, TM0416 catalyzed the epimerization of L-ribulose to L-xylulose with an equilibrium ratio of 40:60, whereas the enzyme exhibited equilibrium ratios of 80:20 and 20:80 for D-tagatose to D-sorbose and D-fructose to D-psicose, respectively. These results indicate that this enzyme might have a strong substrate preference for ketopentoses rather than ketohexoses, compared with PCT3E and ATP3E. Next, we further investigated the substrate spectrum of TM0416 by monitoring its C-3 epimerization activity with a variety of ketoses as substrates (e.g., D-fructose, D-psicose, D-sorbose, D-tagatose, D/L-ribulose, and D/L-xylulose). As indicated in Table 2, TM0416 showed the highest activity with L-ribulose and pronounced activity with other nonphosphorylated sugars, in the following order: L-ribulose, L-xylulose, D-ribulose, D-xylulose, D-tagatose, D-psicose, D-fructose, and D-sorbose. Since L-ribulose and D-tagatose have a common structure from C-1 to C-4, such differential preferences for a variety of sugars might be caused by differences in the molecular sizes of the substrates or interactions between the hydroxyl groups of sugars at C-5 or C-6 and the corresponding sugar-binding residues of TM0416. In particular, this enzyme reversibly converted ketoses with the *cis*-configuration at C-3 and C-4 (L-ribulose, D-ribulose, D-psicose, and D-tagatose) to their

TABLE 3 Kinetic parameters of TM0416 with various sugars^a

Sugar	K_m (mM)	V_{max} (U mg ⁻¹)	k_{cat} (min ⁻¹) ^b	k_{cat}/K_m (mM ⁻¹ min ⁻¹)
D-Erythrose (C ₄)	452 ± 19	1.23 ± 0.13	74.8 ± 7.8	0.17 ± 0.01
D-Threose (C ₄)	232 ± 7	0.64 ± 0.03	39.0 ± 1.9	0.17 ± 0.01
D-Erythrulose (C ₄)	78 ± 22	0.08 ± 0.01	4.7 ± 0.7	0.06 ± 0.01
L-Ribulose (C ₅)	46 ± 8	0.23 ± 0.05	13.7 ± 2.9	0.40 ± 0.07
D-Ribulose (C ₅)	348 ± 35	0.62 ± 0.07	37.9 ± 4.0	0.11 ± 0.01
L-Xylulose (C ₅)	459 ± 6	1.4 ± 0.16	86.3 ± 3.9	0.19 ± 0.01
D-Xylulose (C ₅)	232 ± 31	0.26 ± 0.02	15.9 ± 1.0	0.07 ± 0.01

^aThe kinetic parameters were obtained by fitting the experimental data to the Michaelis-Menten equation, as follows: $V = V_{max}[S]/(K_m + [S])$. All data were obtained in duplicate. The data are mean ± SD.

^b k_{cat} is the number of substrate molecules reacting per minute.

C-3 epimer *trans*-ketoses (L-xylulose, D-xylulose, D-fructose, and D-sorbose, respectively), among the sugars tested in this study (Fig. 2B). Serendipitously, we also examined C₄ sugars, such as D-erythrose and D-threose, and their epimerization reactions (Table 2). Although TM0416 did not show C-3 epimerization activity with those sugars, it showed the highest activity for the conversion of either D-erythrose or D-threose to D-erythrulose, with poor activity for the reverse reactions (Fig. 2). These results suggest that TM0416 is a bifunctional sugar isomerase (i.e., C-3 epimerization and aldose-ketose isomerase).

Based on these data, we determined the kinetic parameters of TM0416 with various C₄, C₅, and C₆ sugars as the substrates, at 70°C and pH 7, using Michaelis-Menten kinetics (Table 3). TM0416 had lower K_m values, higher turnover numbers (k_{cat} values), and higher catalytic efficiency (k_{cat}/K_m) values for ketopentoses than for ketohexoses, which is consistent with its substrate preferences (Table 2). We noticed that the catalytic efficiency (k_{cat}/K_m) values of TM0416 for L-ribulose and D-ribulose were approximately 10 to 20 times higher than those for D-tagatose and D-psicose (data not shown), indicating that this enzyme prefers C₅ ketoses over C₆ ketoses as substrates. Notably, isomerization of D-erythrose and D-threose to D-erythrulose exhibited the highest catalytic efficiency and lower K_m values than with the C₅ ketoses we tested (Table 3). Therefore, although direct comparison is difficult, TM0416 appears to be closer to MLR3E than to PCT3E, *C. cellulolyticum* P3E (CCP3E), and ATP3E with respect to substrate specificity, suggesting that this enzyme can be classified as a novel type of R3E with strong isomerization activity for C₄ sugars.

Crystal structure of TM0416 in complex with divalent metal ions and L-erythrulose. To understand in detail the structural features of TM0416 and the molecular mechanism by which it carries out epimerization, we determined its crystal structures in complex with divalent metal ions (i.e., Mn²⁺, Ni²⁺, Co²⁺, and Cu²⁺) and L-erythrulose (Table 4; also see Table S1). The asymmetric unit contained two R3E molecules, and the model included complete ordered residues 1 to 270 of a subunit and two metal atoms (Fig. 3A). As shown in Fig. 3B and D, the subunit structure of TM0416 comprises an (α/β)₈ barrel fold encompassing three additional helical segments, i.e., $\alpha 3'$, $\alpha 4'$, and $\alpha 6'$, which are positioned at the forefront of $\alpha 3$, $\alpha 4$, and $\alpha 6$, respectively. As shown in Fig. 3C, the subunit structure of TM0416 was superimposed on four homologous proteins with the greatest structural similarity (as described above), i.e., CCP3E (PDB accession no. 3VNI; root mean square deviation [RMSD], 2.0 Å), PCT3E (PDB accession no. 2QUL; RMSD, 2.2 Å), ATP3E (PDB accession no. 2HK0; RMSD, 2.1 Å), and MLR3E (PDB accession no. 3VYL; RMSD, 1.9 Å), indicating that the main-chain coordinates of TM0416 are quite similar to those of its bacterial homologs, except for the absence of a short $\alpha 8'$ helix.

Based on the B-factor analysis and the atomic absorption spectrum, the bound metal ions were defined as Mn²⁺, Ni²⁺, Co²⁺, or Cu²⁺ (Fig. 4A). The crystal structures of PCT3E in complex with Mn²⁺ (PDB accession no. 2QUL) and MLR3E in complex with Mn²⁺ (PDB accession no. 3VYL) were superimposed on the structures of TM0416, with RMSD values of 0.96 to 0.95 Å for Mn²⁺-, Ni²⁺-, Co²⁺-, and Cu²⁺-bound forms of the

TABLE 4 Statistics for data collection and refinement of Mn²⁺- and L-erythrulose-bound TM0416

Parameter	Mn ²⁺ -R3E ^a	Mn ²⁺ -R3E with L-erythrulose
PDB accession no.	5H6H	5H1W
Data collection		
Beamline	PLS-5C	PLS-7A
Wavelength (Å)	0.97960	0.97933
Resolution (Å)	50–1.4 (1.48–1.45)	50–1.6 (1.66–1.63)
Space group	P1	P1
Unit cell dimensions		
<i>a</i> (Å)	50.32	50.24
<i>b</i> (Å)	55.28	55.41
<i>c</i> (Å)	58.70	59.00
α (°)	107.22	107.48
β (°)	102.71	102.14
γ (°)	91.75	91.71
No. of total reflections	382,373	272,876
No. of unique reflections	98,787 (4,913)	70,454 (2,733)
<i>R</i> _{merge} (%) ^b	5.0 (32.3)	4.9 (22.5)
Completeness (%)	94.5 (93.8)	95.6 (74.4)
Redundancy	3.9 (3.9)	3.9 (3.6)
Average <i>I</i> / σ (<i>I</i>)	25.79 (4.04)	37.01 (7.46)
Matthews coefficient (Å ³ Da ^{−1})	2.32	2.32
Solvent (%)	46.99	47.00
Refinement		
<i>R</i> _{work} / <i>R</i> _{free} (%)	17.77/20.31	15.98/18.08
No. of protein residues	537	540
No. of water molecules	570	531
No. of metal ions	2	2
RMSD		
Bond angle (°)	1.166	1.209
Bond length (Å)	0.007	0.009
Average <i>B</i> -factor (Å ²)	19.00	21.50
Ramachandran plot		
Favored (%)	97.75	98.14
Allowed (%)	1.50	1.30
Outliers (%)	0.75	0.56

^aThe values in parentheses correspond to the shell with highest resolution.

^b $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the observed intensity and $\langle I(hkl) \rangle$ is the average intensity of symmetry-related observations.

C- α atoms of 270 residues; those structures enabled us to identify the metal-binding residues in TM0416 (Fig. 4A). As shown in Fig. 4A, the Mn²⁺ ion bound to TM0416 is coordinated by four amino acid residues (Glu149, Asp182, His208, and Glu243), which are also highly conserved in CCP3E, ATP3E, and PCT3E (Fig. 4C). In addition, three water molecules (W1, W2, and W3) proximal to Mn²⁺ are hydrogen bonded with Glu149, His185, Arg214, and Glu243, forming a distorted octahedral structure. W2 in the center of the network is linked directly to Mn²⁺ and Glu243 and indirectly to Arg214 and His185 via W3, which forms three additional hydrogen bonds to further stabilize the active site. Such a water-mediated hydrogen-bonding network may facilitate the protonation/deprotonation function of divalent metal ions during catalysis. Although we cannot rule out the possibility that differences in the atomic sizes of metal ions could affect the metal-binding architecture, the loss of W2 loosened the coordination of the authentic bond network in the metal-binding site, due to the flexibility of Glu243 and Arg214 (Fig. 4A; also see Fig. S1). Indeed, Glu243 and Arg214 displayed higher *B*-factors (17.7 Å² and 13.1 Å², respectively) than the *B*-factors for Co²⁺ (11.5 Å² and 11.7 Å²), Ni²⁺ (11.2 Å² and 11.0 Å²), and Mn²⁺ (10.6 Å² and 11.3 Å²). These structural features may explain the scarce catalytic activity of TM0416 in the presence of Cu²⁺ (Fig. 1E).

The difference Fourier maps (2*F*_o−*F*_c maps) revealed a high electron density of bound L-erythrulose as a substrate analogue proximal to the metal-binding site with coordi-

FIG 3 Three-dimensional structure of TM0416 bound to Mn^{2+} and alignments of amino acids encoding putative 3-epimerases. (A) Ribbon representation of the dimeric TM0416, with subunits shown in different colors. (B) Subunit structure of TM0416. The α -helices (numbered $\alpha 1$ to $\alpha 8$) and β -strands (numbered $\beta 1$ to $\beta 8$) are indicated in red and green, respectively. (C) Superposition of the monomeric structures of TM0416, CCP3E, PCT3E, ATP3E, and MLR3E. (D) Structure-based amino acid sequence alignment of TM0416 and its homologs. The secondary structure assignments for TM0416 are shown above the alignment. Amino acid residues that are identical in all of the displayed sequences are marked by asterisks; strongly conserved and weakly conserved residues are indicated by colons and dots, respectively. Metal-binding residues are shown in green. Four residues (Glu149, Asp182, His208, and Glu243) in TM0416 are responsible for the deprotonation/protonation at the C-3 epimerization center; the corresponding residues in CCP3E (Glu150, Asp183, His209, and Glu244), PCT3E (Glu152, Asp185, His211, and Glu246), ATP3E (Glu150, Asp183, His209, and Glu244), MLR3E (Glu147, Asp180, His206, and Glu241), and *Mesorhizobium loti* T3E (Glu141, Asp173, His199, and Glu234) are depicted in green.

nation distances of atoms in the enzyme-substrate complexes (Fig. 4B). However, we were unable to determine the crystal structure of TM0416 with such potential substrates as D-tagatose, D-fructose, and D-sorbose. The O-1 atom of L-erythrulose coordinates with Mn^{2+} and Glu149 and forms a hydrogen bond with Glu149 (2.5 Å) (Fig. 4B). The O-2 atom of L-erythrulose coordinates with Mn^{2+} , His185, and Arg214 through hydrogen bonds, and the O-3 atom forms hydrogen bonds with Glu155, His185, and Arg214. Similar to the substrate-binding mode found in other sugar-converting enzymes, water molecules (W2 and W3) in the metal-only active site are displaced when

FIG 4 Metal- and substrate-binding sites of TM0416. (A) Structures of the TM0416 binding site with different metals. The residues interacting with the divalent metal ions Mn^{2+} , Ni^{2+} , Co^{2+} , and Cu^{2+} in TM0416 are depicted as orange, green, salmon, and cyan sticks, respectively. (B) Closeup views of water-mediated hydrogen bonds in the active sites of holo-TM0416 and L-erythrulose-bound TM0416. The residues interacting with the metal are depicted as sticks, and the bound L-erythrulose is shown as green sticks. The electron density map around ligands contoured at 2.0σ is depicted as blue lines. Mn^{2+} and water molecules are shown as orange and red spheres, respectively. Hydrogen bonds are shown as black dashed lines. The ligands and side chains are colored according to atom type (oxygen in red and nitrogen in blue). (C) Closeup views of substrate binding sites of TM0416 and its homologs. Superposition of the TM0416 substrate-binding site (red) on CCP3E (PDB accession no. 3VNJ) (olive) bound to D-psicose, ATP3E (PDB accession no. 2HK1) (light green and green labels) bound to D-fructose, or PCT3E (PDB accession no. 2QUM) (blue) bound to D-tagatose is shown. The ligands and side chains are colored according to atom type (oxygen in red, nitrogen in blue, and phosphorus in orange). The Mn^{2+} ion and water molecules are shown as spheres.

L-erythrulose residues in the active site. An additional water molecule (W4), which was observed in Co^{2+} -, Ni^{2+} -, and Mn^{2+} -bound structures, forms a hydrogen bond with the O-4 atom of L-erythrulose (Fig. 4B). Taken together, these findings indicate that substrate binding by TM0416 involves the residues Glu149, Glu155, His185, Arg214, and W4. Notably, compared with the substrate-bound forms of CCP3E (PDB accession no. 3VNJ), ATP3E (PDB accession no. 2HK1), and PCT3E (PDB accession no. 2QUM), TM0416 revealed a distinct structural feature for coordinating substrate binding near the active site. As shown in Fig. 4C, Glu158 (O-1), Arg217 (O-1 and O-2), His188 (O-2), Glu152 (O-3), and Glu246 (O-3) in PCT3E, which may interact with O-1, O-2, and O-3 of D-fructose, were also found in TM0416. These residues are highly conserved in other homologs as well.

However, the residues of CCP3E, ATP3E, and PCT3E that are responsible for the creation of a hydrophobic pocket around the C-4, C-5, and C-6 positions of the substrate are variable in TM0416 (i.e., Val6, Gly68, Gly112, Leu113, and Leu245). In particular, Tyr6 in CCP3E, which was proposed as a key residue for direct hydrogen bonding to the substrate at C-6 (42), is replaced by hydrophobic Val6 in TM0416, with displacement 4.5 Å away from Tyr6. The structural features mentioned above suggest that TM0416 has potentially greater specificity for C₅ ketoses than C₆ ketoses, owing to the differences in the hydrophobic pocket. Moreover, such distinct substrate binding coordination may facilitate C₄ sugars fitting into the pocket.

DISCUSSION

TM0416 homologs seem to play a key role in catalyzing the C-3 epimerization of various ketohexoses (e.g., D-tagatose, D-sorbose, D-psicose, and D-fructose), but their structures and true functions remain unclear. Very recently, TM0416 was proposed to be a plausible enzyme that catalyzes the C-4 epimerization of 5-keto-L-gluconate to D-tagaturonate, which is involved in the *myo*-inositol pathway (43). Our biochemical and structural characterization clearly demonstrated that TM0416 might be a bifunctional R3E. Intriguingly, our equilibrium studies and kinetic data clearly showed that, although the recombinant TM0416 exhibited pronounced activity of C-3 epimerization of D-ketohexoses, it showed a greater substrate preference for L-ketopentoses, with an isomerization activity with C₄ sugars (Tables 2 and 3). As shown in Fig. 4, two strictly conserved residues, i.e., Glu149 and Glu243, near the active site of TM0416 are probably responsible for the deprotonation/protonation at the C-3 epimerization center, suggesting that this enzyme follows a two-base catalytic mechanism (22). Regarding this mechanism, it is proposed that the H-3 of the bound ketose faces the carboxyl group of Glu243; deprotonation at C-3 in the forward reaction can be achieved by Glu243. Substrate binding is further stabilized by an interaction with a bound metal in an octahedral coordination (Fig. 4). Indeed, superposition of PCT3E (PDB accession no. 2QUN) onto TM0416 (RMSD values of 1.9 to 2.0 Å for the C-α atoms of 270 amino acid residues) enabled us to propose the residues involved in the divalent metal ion and substrate binding of TM0416. As shown in Fig. 3D, the metal-ligand-binding residues of TM0416 are well conserved in PCT3E. Based on the structural and mechanistic features of TM0416 and PCT3E, it is suggested that the two enzymes share a binding niche for ketoses as substrates, in the sense that their substrate-binding residues for C-1 to C-3 of ketoses are highly conserved (Fig. 4). This hypothesis fits well with the observation that both enzymes show higher activity for *cis*-ketoses at the C-3 and C-4 positions (e.g., L-ribulose and D-tagatose) than for *trans*-ketoses (e.g., L-xylulose and D-sorbose). Notably, the L-erythrulose-bound R3E structure with Mn²⁺ shows that C-1 to C-3 of L-erythrulose are well superimposed on the corresponding carbons in the hexoses in CCP3E, PCT3E, and ATP3E, supporting a common binding site for ketoses (Fig. 4C). However, TM0416 showed higher activity with L-ribulose and D-erythrose than D-tagatose, whereas PCT3E exhibited the opposite activity (15, 44). Such a discrepancy in substrate preferences may be ascribed to the variable substrate-binding residues at the C-5 and C-6 positions of bound substrates (Fig. 4C). In addition, some of the major differences with respect to other homologs are that TM0416 exhibits a unique solvent-accessible substrate-binding pocket because of the absence of an α8' helix and the residues forming a hydrophobic environment around the substrate in TM0416 differ from those in the other enzymes (Fig. 5A). Indeed, other structures, including PCT3E, ATP3E, and MLR3E, have an α8' helix between the β8 strand and the α8 helix that covers the active-site cleft, effectively sealing off the bound substrate (45). However, it is notable that the C-terminal α8 helix of TM0416 lacks the short α8' helix that is present in the other four enzymes (Fig. 5B). TM0416 does not seem to undergo a substrate-induced conformational change like its mesophilic counterparts. As shown in Fig. 5B, the β4-α4 loop of ATP3E, serving as the lid over the active site, seems to be absent in the corresponding β4-α4 loop (R115 to Y123) of TM0416, because the loop length is four amino acids shorter than that of ATP3E. Displacement of the α1'-α1 loop

FIG 5 Comparison of structures of TM0416 and its homologs. (A) Electrostatic potential surfaces of the proximal sides of TM0416 (TMR3E) and its homologs (CCP3E, PCT3E, ATP3E, and MLR3E). The electrostatic potential surfaces were calculated using the PDB2PQR server (58) and PyMOL with the APBS 2.1 plugin (59). The electrostatic potentials are mapped on the surface of each enzyme at pH 7.0 for the positively charged residues ($+3K_bT/e_c$) (blue) and negatively charged residues ($-3K_bT/e_c$) (red). The substrate-binding site is located in the vicinity of the metal-binding site. (B) Structural comparison of TM0416 (red) with PCT3E (blue), ATP3E (green), and MLR3E (orange). The dashed circle highlights the absence of an $\alpha 8'$ helix at the C terminus of TM0416 (right). The $\beta 1$ - $\alpha 1$ and $\beta 4$ - $\alpha 4$ loops induced by substrate binding are shorter in TM0416 than in the other enzymes (left and middle).

of ATP3E, which is responsible for induced conformational changes in association with the $\beta 4$ - $\alpha 4$ loop of ATP3E for substrate binding (44, 45), was not observed in TM0416 (Fig. 5B). Overall, residues in the substrate-binding pockets, including Phe7 (Tyr6), Trp15 (Trp14), Cys66 (Gly65), Trp113 (Trp112), and Phe248 (Phe246) in PCT3E (corresponding to those in ATP3E), which are located on the $\alpha 1'$ - $\alpha 1$ and $\beta 4$ - $\alpha 4$ loops and the $\alpha 8'$ helix described above, were replaced by Val6, Phe19, Gly68, Leu113, and Leu245, respectively, in TM0416. This structural feature can explain the low C_e -substrate-binding affinity of TM0416. Thus, although the key residues involved in the catalytic reaction (i.e., metal binding and O-1, O-2, and O-3 bonding) are highly conserved, comparison of the crystal structure of TM0416 with those of PCT3E and ATP3E to determine substrate binding supports the idea that TM0416 catalyzes epimerization in a manner similar to that of other 3-epimerases, with clear differences in the hydrophobic pocket around the substrate and the presence of a unique solvent-accessible active site. These structural features also suggest that the substrate specificity of TM0416 differs substantially from those of other T3E-family enzymes, based on the fact that this enzyme can convert either D-erythrose or D-threose to D-erythrulose (Fig. 2 and 4). Moreover, additional physical differences in the substrate size, binding pocket volume, and/or interactions between the binding residues and bound substrates at the C-5 and C-6 positions might explain these enzymes' promiscuous substrate preferences. Notably, several residues on the globular surface of TM0416 that were bound to Mn^{2+} , Ni^{2+} ,

Co^{2+} , and Cu^{2+} showed significant displacements, which may explain why the C-3 epimerization activity of the enzyme was specific for these species of metal ions (Fig. 4A).

The catalytic activity of TM0416 for the C-3 epimerization of nonphosphorylated ketoses absolutely required divalent metal ions (Fig. 2). It has been noted that the phosphate groups of phosphosugars play a key role in the orientation and direction of sugars bound to the substrate-binding site of sugar-converting enzymes (46). However, phosphosugar epimerases, such as D-ribulose-5-phosphate 3-epimerase (EC 5.1.3.1) and L-ribulose-5-phosphate 4-epimerase (EC 5.1.3.4), absolutely depend on divalent metal ions for epimerization activity (28, 30, 47). Thus, we speculate that sugar-epimerizing enzymes that use phosphosugars and nonphosphorylated sugars as substrates share common structural features through divergent evolution from common superfamily enzymes. TM0416 appears to be the most thermostable R3E among the homologs reported to date, and such thermophilicity could be advantageous for producing rare C_4 sugars on an industrial scale. Moreover, TM0416, with very broad and/or even promiscuous substrate specificity toward a wide variety of sugars, could be used as a robust and thermostable starting enzyme for the engineering of new enzymes for industrial applications. Recently, Bosshart et al. (48) carried out directed evolution of PCT3E through saturation mutagenesis, to increase the thermostability to meet industrial demands at elevated temperatures, which suggests that the residue resulting from the F157Y mutation could form a hydrogen bond with the strictly conserved N155 residue on the other subunit, thereby stabilizing the dimer interface. Remarkably, native hyperthermophilic TM0416 seems to form a hydrogen bond between Y154 (corresponding to F157 in PCT3E) and N152 (N155), consistent with the mutational result for PCT3E. Several groups have developed synthetic methods for the production of rare sugars but the yields were poor and the procedures were characterized by enantioselectivity and regioselectivity, a laborious process, and expensive starting materials (7, 17, 49, 50). Alternatively, some biological approaches using microbial enzymes were exploited to produce rare sugars (6, 7, 14). In recent years, successful production of rare sugars was carried out using hyperthermophilic sugar-metabolizing enzymes (6, 7). In light of this, TM0416 is an excellent model system for investigating the molecular evolution between phosphorylated and nonphosphorylated sugar epimerases, which will also provide insight into the design and development of customized enzymes for the production of rare sugars.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *T. maritima* MSB8 was obtained from Deutsche Sammlung von Mikroorganismen (DSM) (Braunschweig, Germany). *T. maritima* was grown for 48 h at 80°C in an artificial seawater-based medium (DSM medium 343), in sealed serum bottles, under N_2 gas. *Escherichia coli* DH5 α cells were used as the host for the construction of cloning and expression vectors. *E. coli* BL21(DE3) was used as the expression host for the recombinant TM0416 from *T. maritima*. *E. coli* strains were grown at 37°C in Luria-Bertani (LB) medium with ampicillin (100 $\mu\text{g ml}^{-1}$), in a rotary shaker. The pTOP Blunt V2 vector (Enzymomics, Daejeon, South Korea) was used for cloning and sequencing, and the pET-15b vector (Novagen, Madison, WI) was used for expression. Growth was monitored by determining the optical density at 600 nm (OD_{600}) with an Ultra 8000 spectrophotometer (GE Healthcare, Piscataway, NJ).

Cloning and expression of *T. maritima* D-tagatose-3-epimerase-related protein TM0416. A search of the microbial genome sequences in GenBank revealed a TM0416 gene encoding a putative *T. maritima* D-tagatose 3-epimerase (T3E) (36). Genomic DNA was isolated from *T. maritima* and purified using a genomic DNA extraction kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The TM0416 gene was amplified by PCR from *T. maritima* genomic DNA using the following forward and reverse primers (with NdeI and BamHI sites, respectively, underlined): TM_T3E F, 5'-CATA TGAAGCTATCTCTGGTGATCAGTAC-3'; TM_T3E R, 5'-GGATCCTCATGTAAGTTAATAATCAGTTC-3'. NdeI and BamHI sites were integrated into the primers for cloning into pET-15b. The PCR mixture (50 μl) contained 2 mM MgCl_2 , 20 ng of genomic DNA, 10 pmol of each primer, 0.2 mM deoxynucleoside triphosphate (dNTP) mix, and 2.5 U of PrimeSTAR polymerase (TaKaRa Co., Shiga, Japan). After the initial denaturation for 5 min at 95°C, the DNA was amplified in 30 cycles of 30 s of denaturation at 95°C, 5 s of annealing at 58°C, and 1 min of extension at 72°C; this was followed by a final extension step of 10 min at 72°C. The PCR product was cloned into pTOP Blunt V2 and was used to transform competent *E. coli* DH5 α cells. Transformants containing pTOP Blunt V2 harboring the gene encoding TM0416 were selected on LB plates containing ampicillin. Plasmid DNA was isolated from the transformants with

inserts and digested with NdeI and BamHI. The digested DNA was purified and inserted into the NdeI and BamHI sites of pET-15b, yielding pET-15b-TM0416, which was used to transform *E. coli* BL21(DE3) cells. The expression vector also encodes an N-terminal polyhistidine sequence. For expression of the recombinant enzyme, *E. coli* BL21(DE3) cells transformed with pET-15b-TM0416 were grown at 37°C in LB medium (1.0 liter) containing 100 $\mu\text{g ml}^{-1}$ ampicillin, to an OD_{600} of 0.5 to 0.6. After induction with 1 mM IPTG, the cells were grown for additional 6 h and harvested by centrifugation at $10,000 \times g$ for 20 min at 4°C. Bacterial pellets were stored at -80°C .

Purification of recombinant TM0416. Recombinant enzymes were purified as described elsewhere (26, 51). Briefly, centrifuged cells were resuspended in 50 ml of $1\times$ His-binding buffer (20 mM Tris-HCl, 500 mM NaCl, 5 mM imidazole [pH 7.9]) and disrupted by sonication. The lysate was centrifuged at $10,000 \times g$ for 20 min to remove cell debris, and the soluble supernatant was heated at 70°C for 20 min. After the heat treatment, the suspension was centrifuged at $10,000 \times g$ for 20 min to remove insoluble denatured *E. coli* proteins, and the soluble fraction was filtered (pore size, 0.45 μm). The filtrate was loaded onto a His-bound resin (Novagen) column (10 ml) that had been equilibrated previously with $1\times$ His-binding buffer, and 250 mM imidazole was applied to elute the recombinant protein. The fractions containing R3E were pooled and subjected to digestion with thrombin (EMD Millipore Corp., Billerica, MA) to remove the N-terminal hexahistidine tag, as described elsewhere (26). Further purification was carried out on a HiLoad 16/600 Superdex 75 pg column (GE Healthcare) that had been equilibrated with 50 mM Tris-HCl (pH 7.0) containing 150 mM NaCl. Protein concentrations were determined by the bicinchoninic acid method (52), with bovine serum albumin as the standard. Enzyme fractions were analyzed by 12% SDS-PAGE and visualized with Coomassie blue (53).

Enzyme assays. R3E activity was determined by measuring the accumulation of L-xylulose using HPLC. Unless otherwise noted, the standard reaction mixture (120 μl) contained 50 mM HEPES buffer (pH 7.0 at room temperature), 1 mM MnCl_2 , 10 mM L-ribulose, and the enzyme (1 mg ml^{-1}). After incubation at 80°C for 4 h, the reaction was stopped by cooling on ice. The mixture was centrifuged at $12,000 \times g$ for 5 min, and an aliquot (20 μl) was analyzed using a Waters Alliance HPLC system (model 2695; Waters, Milford, MA) equipped with a Waters refractive index (RI) detector (Waters 410) and a Carbosep CHO-620 column (300 by 6.5 mm, 10- μm particle size; Transgenomic, Inc., Omaha, NE). The column and RI detector temperatures were 90°C and 35°C , respectively. Deionized water was used as the mobile phase, at a flow rate of 0.3 ml min^{-1} . The amount of sugar produced was estimated from the ratio of peak areas, based on the curves for L-ribulose and L-xylulose (Carbosynth, Compton, United Kingdom) as calibration standards. One unit of epimerase activity was defined as the amount of enzyme that produced 1 μmol of product per minute under the assay conditions. When other sugars were examined for their efficiency as substrates for the enzyme, each sugar produced was confirmed by comparison of its elution time, as follows: D-erythrose, 27.6 min; D-erythrulose, 23.1 min; D-threose, 19.3 min; L-ribulose, 25.4 min; L-xylulose, 19.4 min; D-tagatose, 20.7 min; D-sorbose, 17.6 min; D-fructose, 19.2 min; D-psicose, 24.9 min.

Characterization of TM0416. To determine the effect of temperature on R3E activity, the enzyme (1 mg ml^{-1}) was incubated at different temperatures, ranging from 40°C to 100°C , in 50 mM HEPES buffer (pH 7.0) containing 1 mM MnCl_2 and 5 mM substrate (L-ribulose). For the pH studies, R3E activity was measured with L-ribulose as the substrate as described above, except that the HEPES buffer was replaced with 50 mM sodium acetate-acetic acid (pH 4.0 to 6.0), 50 mM HEPES buffer (pH 6.0 to 8.0), or 50 mM glycine-NaOH (pH 8.0 to 10.0). All pH values were adjusted at room temperature, and the $\Delta pK_a/\Delta T$ value for each buffer was taken into account when the results were analyzed.

To investigate the effects of various metal ions on enzyme activity, we prepared apo-TM0416 by treatment with 20 mM EDTA for 2 h at 60°C , followed by overnight dialysis against 20 mM Tris-HCl buffer (pH 7.0) at 4°C , with several buffer changes. Removal of metals was confirmed by ICP-MS. After preincubation of the dialyzed enzyme (the apo form) for 15 min at 70°C in the presence of 1 mM $\text{CoCl}_2\cdot 6\text{H}_2\text{O}$, $\text{MnCl}_2\cdot 4\text{H}_2\text{O}$, $\text{MgCl}_2\cdot 6\text{H}_2\text{O}$, $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$, $\text{ZnCl}_2\cdot 6\text{H}_2\text{O}$, $\text{CuCl}_2\cdot 2\text{H}_2\text{O}$, $\text{FeCl}_2\cdot 6\text{H}_2\text{O}$, or $\text{NiCl}_2\cdot 6\text{H}_2\text{O}$ and assay of TM0416 activity under standard assay conditions, the substrate conversion was estimated using HPLC analysis, as described above. Heat denaturation measurements were carried out on a MicroCal VP-DSC microcalorimeter (GE Healthcare). A scan was run at pH 7.4 in 10 mM HEPES buffer in a temperature range of 15 to 120°C , with a heating rate of 90°C/h . The cell volume was 0.8 ml, and the protein concentration was 200 μM in 10 mM HEPES buffer. To obtain T_m values for TM0416, curves were fit using MicroCal Origin software.

To determine the kinetic parameters, all enzymatic assays were performed for 30 min at 80°C in 50 mM HEPES buffer (pH 7.0) containing 1 mM Mn^{2+} and 5 to 100 mM substrate (D-erythrose, D-threose, L-ribulose, L-xylulose, D-ribulose, or D-xylulose) or 100 to 500 mM substrate (D-sorbose, D-tagatose, D-fructose, or D-psicose). The kinetic parameters were determined by fitting data to Michaelis-Menten equations using Origin 8.0 software.

Crystallization and structure determination of TM0416. The purified enzyme was concentrated to 30 mg ml^{-1} using a Vivaspin centrifugal concentrator (molecular weight cutoff value, 10,000; Sartorius), in 10 mM potassium phosphate (pH 7.0) with 10 mM NaCl. The apo enzyme was prepared and crystallized by the hanging-drop vapor-diffusion method, using protein crystallization kits (Hampton Research and Wizard), as described previously (39). Improved crystals were obtained by modifying the reservoir solution (0.1 M morpholineethanesulfonic acid [MES] [pH 6.0], 22% polyethylene glycol [PEG] 200, 5% PEG 1000). Crystals of TM0416 were soaked in 0.5 mM MnCl_2 , NiCl_2 , CoCl_2 , CuCl_2 , or L-erythrulose for 30 min to ~2 days and then frozen in a cryosolution containing 20% glycerol in the reservoir solution. The diffraction data for TM0416 bound to Mn^{2+} , Ni^{2+} , Co^{2+} , Cu^{2+} , or L-erythrulose were collected at resolutions of 1.3 to 1.7 Å using PLS (Pohang Light Source, Pohang, South Korea) 5C and 7A beamlines and were processed with the HKL2000 program (HKL Research Inc.) (54). The structure of Mn^{2+} -bound

TM0416 was solved initially by molecular replacement (MR) with PDB accession no. 2ZVR as the search model and was refined using the CCP4i program REFMAC5 (55). Subsequent model building and refinement were performed with the refinement tool in PHENIX software (56) and Coot (57). The R_{free} and R_{work} values, which are measures of the ability of the final model, were 18.4% and 19.4%, respectively. The Mn^{2+} -bound and L-erythrulose-bound TM0416 structures were used as models for MR of the other metal-bound TM0416 structures. All illustrations of the structures of TM0416 and its homologs were generated using PyMOL v1.8 (DeLano Scientific).

Accession number(s). The structures of TM0416 with Mn^{2+} , Ni^{2+} , Co^{2+} , Cu^{2+} , and L-erythrulose were deposited in the Protein Data Bank under accession numbers 5H6H, 5B7Z, 5B7Y, 5B80, and 5H1W, respectively (Table 4; also see Table S1).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.03291-16>.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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S.-M.S., T.-P.C., S.H.L., and D.-W.L. formulated the research plan, coordinated the research team, carried out experiments, performed result interpretation and data analysis, and drafted the manuscript. J.M.C., S.-B.K., and S.-J.L. participated in the design of the study, helped to conduct experiments, and performed result interpretation and data analysis. S.H.L. and D.-W.L. conceived, planned, supervised, and managed the study and helped to draft the manuscript. All authors read and approved the final manuscript.

REFERENCES

- Poonperm W, Takata G, Ando Y, Sahachaisaree V, Lumyong P, Lumyong S, Izumori K. 2007. Efficient conversion of allitol to D-psicose by *Bacillus pallidus* Y25. *J Biosci Bioeng* 103:282–285. <https://doi.org/10.1263/jbb.103.282>.
- Li Z, Gao Y, Nakanishi H, Gao X, Cai L. 2013. Biosynthesis of rare hexoses using microorganisms and related enzymes. *Beilstein J Org Chem* 9:2434–2445. <https://doi.org/10.3762/bjoc.9.281>.
- Nabors LO. 2002. Sweet choices: sugar replacements for foods and beverages. *Food Technol* 56:28–45.
- Zehner LR. November 1988. D-Tagatose as a low-calorie carbohydrate sweetener and bulking agent. US patent 4,786,722.
- Lu Y, Levin GV, Donner TW. 2008. Tagatose, a new antidiabetic and obesity control drug. *Diabetes Obes Metab* 10:109–134. <https://doi.org/10.1111/j.1463-1326.2008.00944.x>.
- Lee DW, Jang HJ, Choe EA, Kim BC, Lee SJ, Kim SB, Hong YH, Pyun YR. 2004. Characterization of a thermostable L-arabinose (D-galactose) isomerase from the hyperthermophilic eubacterium *Thermotoga maritima*. *Appl Environ Microbiol* 70:1397–1404. <https://doi.org/10.1128/AEM.70.3.1397-1404.2004>.
- Kim BC, Lee YH, Lee HS, Lee DW, Choe EA, Pyun YR. 2002. Cloning, expression and characterization of L-arabinose isomerase from *Thermotoga neapolitana*: bioconversion of D-galactose to D-tagatose using the enzyme. *FEMS Microbiol Lett* 212:121–126. <https://doi.org/10.1111/j.1574-6968.2002.tb11254.x>.
- Wheeler GL, Jones MA, Smirnoff N. 1998. The biosynthetic pathway of vitamin C in higher plants. *Nature* 393:365–369. <https://doi.org/10.1038/30728>.
- Furneaux RH, Tyler PC, Whitehouse LA. 1993. Synthesis of 1,5-dideoxy-1,5-imino-D-galactitol from L-sorbose. *Tetrahedron Lett* 34:3609–3612. [https://doi.org/10.1016/S0040-4039\(00\)73649-5](https://doi.org/10.1016/S0040-4039(00)73649-5).
- Noma A, Sato M, Suzuki Y. 1974. Taste effectiveness of anomers of sugars and glycosides as revealed from hamster taste responses. *Comp Biochem Physiol A Comp Physiol* 48:249–262. [https://doi.org/10.1016/0300-9629\(74\)90706-3](https://doi.org/10.1016/0300-9629(74)90706-3).
- Oku T, Murata-Takenoshita Y, Yamazaki Y, Shimura F, Nakamura S. 2014. D-Sorbose inhibits disaccharidase activity and demonstrates suppressive action on postprandial blood levels of glucose and insulin in the rat. *Nutr Res* 34:961–967. <https://doi.org/10.1016/j.nutres.2014.09.009>.
- Draganova S, Pilarska D, Takov D, Doychev D. 2011. Utilization of carbohydrates by *Beauveria bassiana* isolates obtained from forest pests. *J Plant Prot Res* 51:349–354. <https://doi.org/10.2478/v10045-011-0057-1>.
- Dhawale MR, Szarek WA, Hay GW, Kropinski AMB. 1986. Preparation of L-fructose and D-sorbose by the bacterial oxidation of L-mannitol and L-glucitol, respectively. *Carbohydr Res* 155:262–265. [https://doi.org/10.1016/S0008-6215\(00\)90156-3](https://doi.org/10.1016/S0008-6215(00)90156-3).
- Izumori K. 2002. Bioproduction strategies for rare hexose sugars. *Naturwissenschaften* 89:120–124. <https://doi.org/10.1007/s00114-002-0297-z>.
- Itoh H, Okaya H, Khan AR, Tajima S, Hayakawa S, Izumori K. 1994. Purification and characterization of D-tagatose 3-epimerase from *Pseudomonas* sp. ST-24. *Biosci Biotechnol Biochem* 58:2168–2171. <https://doi.org/10.1271/bbb.58.2168>.
- Itoh H, Sato T, Izumori K. 1995. Preparation of D-psicose from D-fructose by immobilized D-tagatose 3-epimerase. *J Ferment Bioeng* 80:101–103. [https://doi.org/10.1016/0922-338X\(95\)98186-O](https://doi.org/10.1016/0922-338X(95)98186-O).
- Takeshita K, Suga A, Takada G, Izumori K. 2000. Mass production of D-psicose from D-fructose by a continuous bioreactor system using

- immobilized D-tagatose 3-epimerase. *J Biosci Bioeng* 90:453–455. [https://doi.org/10.1016/S1389-1723\(01\)80018-9](https://doi.org/10.1016/S1389-1723(01)80018-9).
18. Itoh H, Sato T, Takeuchi T, Khan AR, Izumori K. 1995. Preparation of D-sorbose from D-tagatose by immobilized D-tagatose 3-epimerase. *J Ferment Bioeng* 79:184–185. [https://doi.org/10.1016/0922-338X\(95\)94091-5](https://doi.org/10.1016/0922-338X(95)94091-5).
19. Matsuo T, Tanaka T, Hashiguchi M, Izumori K, Suzuki H. 2003. Metabolic effects of D-psicose in rats: studies on faecal and urinary excretion and caecal fermentation. *Asia Pac J Clin Nutr* 12:225–231.
20. Matsuo T, Suzuki H, Hashiguchi M, Izumori K. 2002. D-Psicose is a rare sugar that provides no energy to growing rats. *J Nutr Sci Vitaminol* 48:77–80. <https://doi.org/10.3177/jnsv.48.77>.
21. Jelakovic S, Kopriva S, Suss KH, Schulz GE. 2003. Structure and catalytic mechanism of the cytosolic D-ribulose-5-phosphate 3-epimerase from rice. *J Mol Biol* 326:127–135. [https://doi.org/10.1016/S0022-2836\(02\)01374-8](https://doi.org/10.1016/S0022-2836(02)01374-8).
22. Samuel J, Tanner ME. 2002. Mechanistic aspects of enzymatic carbohydrate epimerization. *Nat Prod Rep* 19:261–277. <https://doi.org/10.1039/b100492l>.
23. Allard STM, Giraud MF, Naismith JH. 2001. Epimerases: structure, function and mechanism. *Cell Mol Life Sci* 58:1650–1665. <https://doi.org/10.1007/PL00000803>.
24. Kopp J, Kopriva S, Suss KH, Schulz GE. 1999. Structure and mechanism of the amphibolic enzyme D-ribulose-5-phosphate 3-epimerase from potato chloroplasts. *J Mol Biol* 287:761–771. <https://doi.org/10.1006/jmbi.1999.2643>.
25. Wee TG, Davis J, Frey PA. 1972. Studies on the mechanism of action of uridine diphosphate-galactose-4-epimerase. I. An ambiguity in the chemical trapping of a proposed keto-intermediate by NaBH₄. *J Biol Chem* 247:1339–1342.
26. Shin SM, Choi JM, di Luccio E, Lee YJ, Lee SJ, Lee SH, Lee DW. 2015. The structural basis of substrate promiscuity in UDP-hexose 4-epimerase from the hyperthermophilic eubacterium *Thermotoga maritima*. *Arch Biochem Biophys* 585:39–51. <https://doi.org/10.1016/j.abb.2015.08.025>.
27. Thoden JB, Frey PA, Holden HM. 1996. Molecular structure of the NADH/UDP-glucose abortive complex of UDP-galactose 4-epimerase from *Escherichia coli*: implications for the catalytic mechanism. *Biochemistry* 35: 5137–5144. <https://doi.org/10.1021/bi9601114>.
28. Deupree JD, Wood WA. 1972. L-Ribulose 5-phosphate 4-epimerase from *Aerobacter aerogenes*: evidence for a role of divalent metal ions in the epimerization reaction. *J Biol Chem* 247:3093–3097.
29. Lee LV, Poyner RR, Vu MV, Cleland WW. 2000. Role of metal ions in the reaction catalyzed by L-ribulose-5-phosphate 4-epimerase. *Biochemistry* 39:4821–4830. <https://doi.org/10.1021/bi9928952>.
30. Timson DJ, Reece RJ. 2003. Identification and characterisation of human aldose 1-epimerase. *FEBS Lett* 543:21–24. [https://doi.org/10.1016/S0014-5793\(03\)00364-8](https://doi.org/10.1016/S0014-5793(03)00364-8).
31. Morgan PM, Sala RF, Tanner ME. 1997. Eliminations in the reactions catalyzed by UDP-N-acetylglucosamine 2-epimerase. *J Am Chem Soc* 119:10269–10277. <https://doi.org/10.1021/ja971718q>.
32. Ishida Y, Kamiya T, Itoh H, Kimura Y, Izumori K. 1997. Cloning and characterization of the D-tagatose 3-epimerase gene from *Pseudomonas cichorii* ST-24. *J Ferment Bioeng* 83:529–534. [https://doi.org/10.1016/S0922-338X\(97\)81132-4](https://doi.org/10.1016/S0922-338X(97)81132-4).
33. Bosshart A, Hee CS, Bechtold M, Schirmer T, Panke S. 2015. Directed divergent evolution of a thermostable D-tagatose epimerase towards improved activity for two hexose substrates. *Chembiochem* 16:592–601. <https://doi.org/10.1002/cbic.201402620>.
34. Huber R, Langworthy T, König H, Thomm M, Woese C, Sleytr U, Stetter K. 1986. *Thermotoga maritima* sp. nov. represents a new genus of unique extremely thermophilic eubacteria growing up to 90°C. *Arch Microbiol* 144:324–333. <https://doi.org/10.1007/BF00409880>.
35. Achenbach-Richter L, Gupta R, Stetter KO, Woese CR. 1987. Were the original eubacteria thermophiles? *Syst Appl Microbiol* 9:34–39. [https://doi.org/10.1016/S0723-2020\(87\)80053-X](https://doi.org/10.1016/S0723-2020(87)80053-X).
36. Nelson KE, Clayton RA, Gill SR, Gwinn ML, Dodson RJ, Haft DH, Hickey EK, Peterson JD, Nelson WC, Ketchum KA, McDonald L, Utterback TR, Malek JA, Linher KD, Garrett MM, Stewart AM, Cotton MD, Pratt MS, Phillips CA, Richardson D, Heidelberg J, Sutton GG, Fleischmann RD, Eisen JA, White O, Salzberg SL, Smith HO, Venter JC, Fraser CM. 1999. Evidence for lateral gene transfer between Archaea and bacteria from genome sequence of *Thermotoga maritima*. *Nature* 399:323–329. <https://doi.org/10.1038/20601>.
37. Mu W, Chu F, Xing Q, Yu S, Zhou L, Jiang B. 2011. Cloning, expression, and characterization of a D-psicose 3-epimerase from *Clostridium cellulolyticum* H10. *J Agric Food Chem* 59:7785–7792. <https://doi.org/10.1021/jf201356q>.
38. Holm L, Rosenstrom P. 2010. Dali server: conservation mapping in 3D. *Nucleic Acids Res* 38:W545–W549. <https://doi.org/10.1093/nar/gkq366>.
39. Sakuraba H, Yoneda K, Satomura T, Kawakami R, Ohshima T. 2009. Structure of a D-tagatose 3-epimerase-related protein from the hyperthermophilic bacterium *Thermotoga maritima*. *Acta Crystallogr F Struct Biol Commun* 65:199–203. <https://doi.org/10.1107/S1744309109002115>.
40. Deupree JD, Wood WA. 1970. L-Ribulose 5-phosphate 4-epimerase of *Aerobacter aerogenes*: evidence for nicotinamide adenine dinucleotide-independent 4-epimerization by the crystalline enzyme. *J Biol Chem* 245:3988–3995.
41. Khrustalev VV, Barkovsky EV, Khrustaleva TA. 2016. Magnesium and manganese binding sites on proteins have the same predominant motif of secondary structure. *J Theor Biol* 395:174–185. <https://doi.org/10.1016/j.jtbi.2016.02.006>.
42. Chan HC, Zhu Y, Hu Y, Ko TP, Huang CH, Ren F, Chen CC, Ma Y, Guo RT, Sun Y. 2012. Crystal structures of D-psicose 3-epimerase from *Clostridium cellulolyticum* H10 and its complex with ketohexose sugars. *Protein Cell* 3:123–131. <https://doi.org/10.1007/s13238-012-2026-5>.
43. Rodionova IA, Leyn SA, Burkart MD, Boucher N, Noll KM, Osterman AL, Rodionov DA. 2013. Novel inositol catabolic pathway in *Thermotoga maritima*. *Environ Microbiol* 15:2254–2266. <https://doi.org/10.1111/1462-2920.12096>.
44. Yoshida H, Yamada M, Nishitani T, Takada G, Izumori K, Kamitori S. 2007. Crystal structures of D-tagatose 3-epimerase from *Pseudomonas cichorii* and its complexes with D-tagatose and D-fructose. *J Mol Biol* 374: 443–453. <https://doi.org/10.1016/j.jmb.2007.09.033>.
45. Kim K, Kim HJ, Oh DK, Cha SS, Rhee S. 2006. Crystal structure of D-psicose 3-epimerase from *Agrobacterium tumefaciens* and its complex with true substrate D-fructose: a pivotal role of metal in catalysis, an active site for the non-phosphorylated substrate, and its conformational changes. *J Mol Biol* 361:920–931. <https://doi.org/10.1016/j.jmb.2006.06.069>.
46. Banerjee S, Anderson F, Farber GK. 1995. The evolution of sugar isomerases. *Protein Eng* 8:1189–1195. <https://doi.org/10.1093/protein/8.12.1189>.
47. Lee LV, Vu MV, Cleland WW. 2000. ¹³C and deuterium isotope effects suggest an aldol cleavage mechanism for L-ribulose-5-phosphate 4-epimerase. *Biochemistry* 39:4808–4820. <https://doi.org/10.1021/bi992894+>.
48. Bosshart A, Panke S, Bechtold M. 2013. Systematic optimization of interface interactions increases the thermostability of a multimeric enzyme. *Angew Chem Int Ed Engl* 52:9673–9676. <https://doi.org/10.1002/anie.201304141>.
49. Itoh K, Mizuno S, Hama S, Oshima W, Kawamata M, Hossain A, Ishihara Y, Tokuda M. 2015. Beneficial effects of supplementation of the rare sugar “D-allulose” against hepatic steatosis and severe obesity in *Lep^{ob}/Lep^{ob}* mice. *J Food Sci* 80:H1619–H1626. <https://doi.org/10.1111/1750-3841.12908>.
50. Liang M, Chen M, Liu X, Zhai Y, Liu XW, Zhang H, Xiao M, Wang P. 2012. Bioconversion of D-galactose to D-tagatose: continuous packed bed reaction with an immobilized thermostable L-arabinose isomerase and efficient purification by selective microbial degradation. *Appl Microbiol Biotechnol* 93:1469–1474. <https://doi.org/10.1007/s00253-011-3638-z>.
51. Choi JM, Lee YJ, Cao TP, Shin SM, Park MK, Lee HS, di Luccio E, Kim SB, Lee SJ, Lee SJ, Lee SH, Lee DW. 2016. Structure of the thermophilic L-arabinose isomerase from *Geobacillus kaustophilus* reveals metal-mediated intersubunit interactions for activity and thermostability. *Arch Biochem Biophys* 596:51–62. <https://doi.org/10.1016/j.abb.2016.02.033>.
52. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. 1985. Measurement of protein using bicinchoninic acid. *Anal Biochem* 150:76–85. [https://doi.org/10.1016/0003-2697\(85\)90442-7](https://doi.org/10.1016/0003-2697(85)90442-7).
53. Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685. <https://doi.org/10.1038/227680a0>.
54. Otwinowski Z, Minor W. 1997. Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol* 276:307–326.
55. Murshudov GN, Skubák P, Lebedev AA, Pannu NS, Steiner RA, Nicholls RA, Winn MD, Long F, Vagin AA. 2011. REFMAC5 for the refinement of macromolecular crystal structures. *Acta Crystallogr D Biol Crystallogr* 67:355–367. <https://doi.org/10.1107/S0907444911001314>.

56. Adams PD, Grosse-Kunstleve RW, Hung LW, Ioerger TR, McCoy AJ, Moriarty NW, Read RJ, Sacchettini JC, Sauter NK, Terwilliger TC. 2002. PHENIX: building new software for automated crystallographic structure determination. *Acta Crystallogr D Biol Crystallogr* 58:1948–1954. <https://doi.org/10.1107/S0907444902016657>.
57. Emsley P, Cowtan K. 2004. Coot: model-building tools for molecular graphics. *Acta Crystallogr D Biol Crystallogr* 60:2126–2132. <https://doi.org/10.1107/S0907444904019158>.
58. Dolinsky TJ, Nielsen JE, McCammon JA, Baker NA. 2004. PDB2PQR: an automated pipeline for the setup of Poisson-Boltzmann electrostatics calculations. *Nucleic Acids Res* 32:W665–W667. <https://doi.org/10.1093/nar/gkh381>.
59. Baker NA, Sept D, Joseph S, Holst MJ, McCammon JA. 2001. Electrostatics of nanosystems: application to microtubules and the ribosome. *Proc Natl Acad Sci U S A* 98:10037–10041. <https://doi.org/10.1073/pnas.181342398>.
60. Kim HJ, Hyun EK, Kim YS, Lee YJ, Oh DK. 2006. Characterization of an *Agrobacterium tumefaciens* D-psicose 3-epimerase that converts D-fructose to D-psicose. *Appl Environ Microbiol* 72:981–985. <https://doi.org/10.1128/AEM.72.2.981-985.2006>.
61. Uechi K, Sakuraba H, Yoshihara A, Morimoto K, Takata G. 2013. Structural insight into L-ribulose 3-epimerase from *Mesorhizobium loti*. *Acta Crystallogr D Biol Crystallogr* 69:2330–2339. <https://doi.org/10.1107/S0907444913021665>.
62. Uechi K, Takata G, Fukai Y, Yoshihara A, Morimoto K. 2013. Gene cloning and characterization of L-ribulose 3-epimerase from *Mesorhizobium loti* and its application to rare sugar production. *Biosci Biotechnol Biochem* 77:511–515. <https://doi.org/10.1271/bbb.120745>.