Identification of an Extremely Thermostable Enzyme with Dual Sugar-1-phosphate Nucleotidyltransferase Activities from an Acidothermophilic Archaeon, *Sulfolobus tokodaii* strain 7*

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**L-rhamnose** is an essential component of the cell wall and plays roles in mediating virulence and adhesion to host tissues in many microorganisms. Glucose-1-phosphate thymidylyltransferase (RmlA, EC 2.7.7.24) catalyzes the first reaction of the four-step pathway of L-rhamnose biosynthesis, producing dTTP-D-glucose from dTTP and glucose-1-phosphate. Three RmlA homologues of varying size have been identified in the genome of a thermophilic archaeon, *Sulfolobus tokodaii* strain 7. In this study, we report the heterologous expression of the largest homologue (a 401 residue-long ST0452 protein) and characterization of its thermostable activity. RmlA enzymatic activity of this protein was detected from 65 to 100 °C, with a half-life of 60 min at 95 °C and 180 min at 80 °C. Analysis of a deletion mutant lacking the 170-residue C-terminal domain indicated that this region has an important role in the thermostability and activity of the protein. Analyses of substrate specificity indicated that the enzymatic activity of the full-length protein is capable of utilizing α-D-glucose1-phosphate and N-acetyl-D-glucosamine-1-phosphate but not α-D-glucosamine-1-phosphate. However, the protein is capable of utilizing all four deoxyribonucleoside triphosphates and UDP. Thus, the ST0452 protein is an enzyme containing both glucose-1-phosphate thymidylyltransferase and N-acetyl-D-glucosamine-1-phosphate uridylyltransferase activities. This is the first report of a thermostable enzyme with dual sugar-1-phosphate nucleotidyltransferase activities.

Polysaccharides are the outermost structures on a bacterial cell and play a critical role in the interactions between the bacterium and its immediate environment. Such interactions have been implicated as important factors in the virulence of many pathogens (1). L-Rhamnose was found to be a key component in many bacterial polysaccharides. In Gram-negative bacteria, L-rhamnose is an important residue in the lipopolysaccharide O-antigen, which plays a key role in virulence (2). In mycobacteria, L-rhamnose is present in the arabinogalactan moiety that links the lipid mycolic acid layer to the inner peptidoglycan (3). Because the attachment of these two layers by L-rhamnose is important for viability of mycobacteria (4) and L-rhamnose is not present in mammalian polysaccharides, the pathway for L-rhamnose biosynthesis represents a potential target for development of antibacterial drugs.

dTDP-L-rhamnose is synthesized from dTTP and glucose-1-phosphate by a conserved four-step reaction catalyzed by glucose-1-phosphate thymidylyltransferase (RmlA, EC 2.7.7.24), dTDP-D-glucose 4,6-dehydratase (RmlB, EC 4.2.1.46), dTDP-6-deoxy-D-xylono-4-hexulose 3,5-epimerase (RmlC, EC 5.1.3.13), and dTDP-6-deoxy-D-xylono-4-hexulose reductase (RmlD, EC 1.1.1.193).

L-Rhamnose has not previously been identified in any cell wall polymer from archaea (5, 6), and biosynthesis of the nucleotide rhamnose in archaea has not been reported. However, putative RmlA–D genes that may encode the four enzymes of the L-rhamnose biosynthesis pathway, RmlA–D, respectively, have been identified in the genomes of some archaeal species, including *Pyrococcus horikoshii* OT3 (7), *Archaeoglobus fulgidus* (8), *Sulfolobus solfataricus* (9), and *Sulfolobus tokodaii* strain 7 (10).

In *S. tokodaii* strain 7, a gene cluster containing RmlA–D, which includes the ST1971, ST1972, ST1969, and ST1970 ORFs, has been identified. Such a cluster structure for genes encoding RmlA–D is conserved in most microorganisms. In *S. tokodaii* strain 7, in addition to the RmlA included in this cluster, two other ORFs, ST0452 and ST2352, have been identified as RmlA homologues. However, both ST0452 and ST2352 are located at genomic sites remote from the cluster encoding RmlA–D. It is known that RmlA catalyzes the first step in the L-rhamnose biosynthesis pathway, producing dTTP-D-glucose from dTTP and glucose-1-phosphate, and is the key enzyme for feedback control of the entire L-rhamnose biosynthesis pathway (11). Comparison of the putative products of the RmlA-like ORFs indicated that the ST0452 protein would be the largest, mainly because of an additional C-terminal domain. To determine whether the putative protein encoded by ST0452 actually functions as an RmlA enzyme, and what function the extra C-terminal region has, the ST0452 ORF was cloned and expressed in *Escherichia coli*.

Here, we present evidence that ST0452 encodes an extremely thermostable enzyme with RmlA activity. Analysis of substrate specificity indicated that the enzyme possesses two distinct

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¶ The abbreviations used are: ORF, open reading frame; HPLC, high pressure liquid chromatography.
sugar-1-phosphate nucleotidyltransferase activities: glucose-1-phosphate thymidylyltransferase and N-acetylglucosamine-1-phosphate uridylyltransferase. Analysis of a deletion mutant indicated that the 170-residue C-terminal region plays an important role for the thermostability of this protein. This is the first report to identify a thermostable enzyme with dual sugar-1-phosphate nucleotidyltransferase activities.

EXPERIMENTAL PROCEDURES

Materials—ATP, CTP, GTP, UTP, dATP, dCTP, dGTP, dTTP, ADP-β-glucose, GDP-β-glucose, dTDP-β-glucose, UDP-β-glucose, UDP-N-acetyl-β-glucosamine, and all sugar-1-phosphates were purchased from Sigma. The restriction enzymes and ligase used in this work were purchased from New England BioLabs, Inc. (Beverly, MA). The Kod-plus DNA polymerase used for PCR amplification was purchased from Toyobo. Co., Ltd. (Osaka, Japan). The plasmid vector pET21(b) was purchased from Novagen (Madison, WI). S. tokodaii strain 7 (JCM10545) was obtained from the Japan Collection of Microorganisms. E. coli strain DH5α was obtained from Takara Bio Inc. (Osu, Shiga, Japan) for plasmid cloning and the strain BL21-Codon Plus (DE3)-RIL was obtained from Stratagene (La Jolla, CA) for expression of recombinant protein.

Construction of Expression Vectors—To amplify and clone the ST0452 ORF, the primer P1 (ATAGCATATGAAGGCATTTATTCTT-GAGGACCTTGAAAAACTCACC) was designed from the 3′ sequence of ST0452, and primers P2 (TCAACTCGAGTACCTTGAAAAACTCACC) and P3 (TCAACTG-GAGGACCTTGAAAAACTCACC) were designed from the 3′ sequence of ST0452. Primer P1 contained an NdeI site, and primers P2 and P3 contained XhoI sites for cloning into the pET21(b) vector. To enable the expression of wild-type gene product from ST0452, primers P1 and P2 were used for PCR amplification. To obtain recombinant protein fused with a histidine tag at the C terminus, primers P1 and P3 were used for PCR amplification. Ten nanograms of ST0452 ORF, the primer P1 (ATAGCATATGAAGGCATTTATTCTT-GAGGACCTTGAAAAACTCACC) were designed from the 3′ sequence of ST0452, and P3 (TCAACTCGAGTACCTTGAAAAACTCACC) and P3 (TCAACTG-GAGGACCTTGAAAAACTCACC) were designed from the 3′ sequence of ST0452. Primer P1 contained an NdeI site, and primers P2 and P3 contained XhoI sites for cloning into the pET21(b) vector. To enable the expression of wild-type gene product from ST0452, primers P1 and P2 were used for PCR amplification. To obtain recombinant protein fused with a histidine tag at the C terminus, primers P1 and P3 were used for PCR amplification. Ten nanograms of ST0452 strain 7 genomic DNA and 100 pmol of each primer were added to 50 μl of standard PCR mixture. 25 cycles with a temperature profile of 30 s at 94 °C, 30 s at 55 °C, and 1 min at 68 °C were performed with 1 unit of KOD-plus DNA polymerase. The PCR products were digested with NdeI and XhoI and ligated with vector pET21(b) digested with the same restriction enzymes. After confirmation of their nucleotide sequences, the plasmids possessing the ST0452 coding region without and with the histidine tag were referred to as pST0452 and pST0452H, respectively.

To construct the expression vector for a C-terminal deletion deleted ST0452 gene product with the histidine tag at the C terminus, the primer P4 (TCAACTG-GAGGACCTTGAAAAACTCACC) was designed from the nucleotide sequence 510 bp upstream from the stop codon. The product of the PCR using primers P1 and P4 was digested with the restriction enzymes NdeI and XhoI and ligated with vector pET21(b) digested with the same restriction enzymes. After confirmation of their nucleotide sequences, the plasmids possessing the ST0452 coding region without and with the histidine tag were referred to as pST0452 and pST0452H, respectively.

A 50-μl reaction mixture containing 50 mM Tris-HCl (pH 7.5), 2 mM MgCl₂, 10 μM a-β-glucose-1-phosphate, 0.1 mM dTTP, and 0.05 μg of purified recombinant protein. After 2 min of preincubation at 80 °C, the reaction was started by the addition of the recombinant protein and progressed at 80 °C for 5 min as a standard. The reaction mixture was immediately mixed with 300 μl of 500 mM KH₂PO₄ to stop the reaction. A 50-μl aliquot of the solution was analyzed on a Waters LC module I plus (Waters, Milford, MA) HPLC system with a 0.46 × 25-cm column of Wakosil 5C18-200 (Wako, Osaka, Japan). The flow rate of 500 mM KH₂PO₄ was maintained at 1 ml/min. The product of the reaction, dTDP-β-glucose, was monitored by absorbance at 254 nm, and the amount of product was calculated from the area under the peaks.

The assay for the formation of dTDP from dTDP-β-glucose and pyrophosphate, the reverse reaction, was performed in 30 μl of reaction mixture containing 50 mM Tris-Cl (pH 7.5), 2 mM MgCl₂, 1 mM pyrophosphate, 0.1 mM dTDP-β-glucose, and 0.05 μg of purified recombinant protein. The reaction conditions and detection of the product, dTTP, were identical to those used for the forward reaction.

RESULTS

Construction of Expression Vector for ST0452—The 1206-bp ST0452 ORF was predicted to be a homologue of RmlA on the basis of sequence similarity. A putative 401-amino acid residue gene product of ST0452 showed 26–30% identity with the RmlA enzymes from Salmonella enterica (13), Shigella flexneri (14), Mycobacterium tuberculosis (15), Pseudomonas aeruginosa (16), and E. coli (17). Amino acid sequences for the nucleotide and sugar-binding motifs, the exact sequences of which are (G)GXRXX_K and EXXXXKXS, respectively (18), were identified in ST0452 as shown in Fig. 1. In comparison with the RmlA from E. coli and P. aeruginosa, the protein encoded by ST0452 has an additional ~170-residue domain at the C terminus. A putative ribosome-binding site, GGTAA, and a putative promoter consensus sequence, TTTAAC, were identified at 4 and 19 bases, respectively, upstream from the translation initiation codon for ST0452. Based on these elements, ST0452 was expected to be transcriptionally active in S. tokodaii strain 7 cells. To investigate the function of the ST0452-encoded protein, two expression vectors, pST0452 and pST0452H, were constructed according to the procedure described under "Experimental Procedures."
Activity catalyzing the reverse reaction of RmlA, which produces dTTP from dTDP-D-glucose and pyrophosphate, was also detected in this recombinant protein (data not shown). These results demonstrate that the recombinant protein encoded by ST0452 indeed exhibits thermostable RmlA activity.

**Optimal pH, Optimal Temperature, and Thermostability for RmlA Activity—**

The effect of pH on the forward reaction of RmlA activity was analyzed using three different solutions: acetate-NaOH buffer for pH 5, 4-morpholineethanesulfonic acid-NaOH buffer for pH 6, and Tris-HCl buffer for pH 7–10. The recombinant protein exhibited relatively high activity around pH 7.5 and 9, with maximum activity at pH 8.5 (Fig. 4A). This optimal pH is considerably different from that of the surrounding environment for growth of the host cell, which is between pH 2 and 4, indicating that the intracellular environment of *S. tokodaii* strain 7 might be maintained at approximately neutral pH.

Because the recombinant protein was originally cloned from a thermophilic archaeon, *S. tokodaii* strain 7, which grows optimally at 80 °C, the temperature dependence of the enzymatic activity was analyzed between 37 and 100 °C. As shown in Fig. 4B, the enzyme had relatively high activity between 80 and 100 °C with a maximum at 95 °C. Because the activity was greatest at high temperatures, the stability of the protein at high temperatures was assessed.

**Multiple sequence alignment of three proteins corresponding to RmlA.** E., P., and ST0452 indicate the RmlA proteins from *E. coli* (GenBank™ accession number P37744) and *P. aeruginosa* (GenBank™ accession number NP_253850) and ST0452 identified in the genome sequence of *S. tokodaii*. The letters within black boxes indicate residues conserved across these three sequences, and the residues conserved between any two proteins are highlighted in bold type. Underlining indicates the two highly conserved motifs, a motif important for recognition of nucleoside triphosphate, (G)GXXGTRX,K, and a motif for recognition of sugar-1-phosphate, ERQGKPVXG.

**SDS-PAGE analysis of the recombinant proteins.** The recombinant protein expressed in *E. coli* harboring the pST0452H was analyzed on a 12% polyacrylamide gel containing 0.1% SDS. Lane 1, total soluble fraction from *E. coli* harboring the pST0452H. Lane 2, soluble fraction after treatment at 80 °C for 20 min. Lane 3, recombinant protein purified by nickel affinity column chromatography. Lane M, standard molecular mass proteins. The proteins were visualized by Coomassie Brilliant Blue R-250 staining.

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those temperatures was examined. Purified recombinant protein, 0.05 mg/ml in 50 mM Tris-HCl (pH 7.5) and 2 mM MgCl₂ was treated at 80 or 95 °C for the periods indicated in Fig. 4, and then the relative activity was measured by the standard assay. The half-life of the enzyme at 95 °C was about 60 min but was 180 min at 80 °C. This indicates that the protein encoded by ST0452 is an extremely thermostable enzyme.

**Effect of Metal Ions on RmlA Activity**—Because it is well known that the nucleotide- or nucleic acid-modifying enzymes require metal ions for catalytic activity, the effects of different metal ions on the forward reaction catalyzed by the recombinant protein were investigated. As indicated in Table I, the enzymatic activity of the recombinant protein has an absolute requirement for a divalent cation, with no RmlA activity detectable when a divalent cation was absent from the reaction buffer. The order of effectiveness of metal ions on the activity of the enzyme was Co²⁺ > Mn²⁺ > Mg²⁺ and Zn²⁺. Because Mg²⁺ was the most common of these metal ions in the reaction buffer, the effect of Mg²⁺ concentration was measured in reaction mixtures containing 2–12 mM MgCl₂. The results indicated that the optimal Mg²⁺ concentration was 6 mM, but the enzymatic activity did not change substantially between 2 and 12 mM (data not shown).

**Kinetic Constants for RmlA Activity**—To determine the effect of substrate concentration on the enzymatic activity, double reciprocal plots of initial velocity were performed. Calculation of $K_m$ for each substrate was performed under conditions where the concentration of the second substrate was 5–10 times higher than its $K_m$ value. Because Mg²⁺ was the most common of these metal ions in the reaction buffer, the effect of Mg²⁺ concentration was measured in reaction mixtures containing 2–12 mM MgCl₂. The results indicated that the optimal Mg²⁺ concentration was 6 mM, but the enzymatic activity did not change substantially between 2 and 12 mM (data not shown).

**Table I**

| Metal ion | Specific activity | Relative activity |
|----------|------------------|------------------|
| μmol/min/mg protein | %               |
| Co²⁺     | 3.21 ± 0.17      | 243              |
| Mn²⁺     | 2.17 ± 0.07      | 164              |
| Mg²⁺     | 1.32 ± 0.07      | 100              |
| Zn²⁺     | 0.95 ± 0.03      | 72               |
| Ca²⁺     | ND               | ND               |
| None     | ND               | ND               |

**FIG. 3.** HPLC elution profile of the standard substrates and products. A, HPLC elution profile of dTTP and dTDP-D-glucose standards on a Wakosil 5C18–200 column. B, elution profiles for the products of incubation for the indicated period at 80 °C with recombinant ST0452 protein in the standard assay. The scale is automatically modified by HPLC according to the amount of material detected.

**FIG. 4.** RmlA activity of the recombinant protein. Enzyme assays and treatment at high temperature were performed according to the protocols shown under "Experimental Procedures." A, relative activity versus pH. Relative activity is expressed as a percentage of the maximum activity at pH 8.5. B, relative activity versus temperature. Relative activity is expressed as a percentage of the activity at 80 °C. C, relative activity after treatment for the periods indicated at 80 °C (open symbols) and 95 °C (closed symbols). The relative activity is expressed as a percentage of the activity without treatment at high temperature. All of the experiments were repeated four times.
**Substrate Specificity of the Recombinant Enzyme**—The report by Lidquist et al. (12) indicated that the RmlA protein from *S. enterica* was capable of utilizing several different sugar-1-phosphates and nucleoside triphosphates as substrates. Consequently, the specificity of the ST0452 recombinant protein for various nucleoside triphosphates (0.1 mM) and sugar-1-phosphates (10 mM) in the forward reaction was examined under the standard assay conditions.

Initially, the specificity for various nucleoside triphosphates was examined. When α-D-glucose-1-phosphate was utilized as an acceptor substrate, the highest activity was with UTP, which was 1.2 times higher than dTTP. As shown in Table III, enzymatic activity with dCTP, dGTP, and dATP was 110, 47, and 40%, respectively, of the level of activity with dTTP. These results indicate that the enzyme is capable of utilizing all four major deoxyribonucleoside triphosphates as substrates but only UTP of the ribonucleoside triphosphates. Because high activity was obtained with the combination of dTTP or UTP and α-D-glucose-1-phosphate, the effect of various sugar-1-phosphate species on enzymatic activity was analyzed using both dTTP and UTP.

As shown in Table III, the highest activity was detected with N-acetyl-D-glucosamine-1-phosphate in the presence of dTTP or UTP. The activity with N-acetyl-D-glucosamine-1-phosphate combined with dTTP or UTP was 5.8 and 3.4 times higher, respectively, than the corresponding activity with α-D-glucose-1-phosphate. Other sugar-1-phosphates tested, including α-D-galactose-1-phosphate, α-D-glucosamine-1-phosphate, and α-D-mannose-1-phosphate, were not utilized as substrates by the recombinant protein. These results indicated that the high activity of the recombinant protein was detected when dTTP or UTP and α-D-glucose-1-phosphate or N-acetyl-D-glucosamine-1-phosphate were used as substrates. Previous studies have indicated that RmlA is capable of utilizing α-D-galactose-1-phosphate and α-D-glucosamine-1-phosphate as substrates but not N-acetyl-D-glucosamine-1-phosphate (12). In addition, an N-acetylglucosamine-1-phosphate uridylyltransferase (GlmU) enzyme capable of utilizing N-acetyl-D-glucosamine-1-phosphate could only utilize UTP but not dTTP as a nucleotide substrate (19). Therefore, the enzymatic activity possessed by the ST0452 protein is distinctly different from that of other nucleotidylyltransferases (12, 19).

Because the enzymatic activity of the recombinant protein was shown to have a broad substrate specificity in the forward reaction, the substrate specificity in the reverse reaction was analyzed with sugar nucleotides at a concentration of 0.1 mM. Production of dTTP, UTP, or UTP was detected when dTDP-α-glucose, UDP-α-glucose, or UDP-N-acetyl-D-glucosamine, respectively, was used as the substrate. However, GTP was not detected when GDP-α-glucose was used as substrate, as was the case in the corresponding forward reaction. The highest activity was detected with UDP-N-acetyl-D-glucosamine, followed by dTDP-α-glucose and UDP-α-glucose.

**Role of the C-terminal Domain of the Recombinant Protein**—In contrast with RmlA from *E. coli*, the protein encoded by ST0452 has an additional 170-residue C-terminal domain. To determine the function of this region, a plasmid vector containing a truncated gene encoding a protein lacking the 170-residue C-terminal domain was constructed. For this purpose, the 231st amino acid was directly fused to the histidine tag sequence in pET21(b); the resulting plasmid was designated pST0452(N231)H. The truncated protein, ST0452(N231)H, was expressed in *E. coli* and purified by nickel affinity column chromatography. Fig. 5 indicates that ST0452(N231)H remained soluble after treatment at 60 °C or below for 5 min but was precipitated after heating above 70 °C. This indicated that the tertiary structure of this truncated protein was drastically changed between 60 and 70 °C.

This truncated enzyme, with decreased thermostability, was also analyzed for RmlA activity. Although the truncated protein did not precipitate until 60 °C when analyzed by SDS-PAGE, it was not expected to possess the complete RmlA activity exhibited by the full-length enzyme. Therefore, analyses of RmlA activity for both the truncated and the full-length ST0452 proteins were performed at 37 °C, the optimal temperature for the *E. coli* enzyme. It was shown that the truncated protein exhibited RmlA activity with a specific initial velocity of 1.59 ± 0.03 nmol/min/mg protein; this value was 23 times lower than that of the native enzyme when measured at 37 °C.

| Direction of reaction | Substrate A | Substrate B | Specific activity (μmol/min/mg) |
|-----------------------|-------------|-------------|---------------------------------|
| **Forward**           |             |             |                                 |
| dTTP                  | α-D-Glucose-1-phosphate | 1.35 ± 0.09 |
| dCTP                  | α-D-Glucosamine-1-phosphate | ND |
| dGTP                  | α-D-Galactose-1-phosphate | ND |
| dATP                  | α-D-Mannose-1-phosphate | ND |
| UTP                   | N-Acetyl-glucosamine-1-phosphate | 7.87 ± 0.39 |
| ATP/CTP/GTP           | dTTP         | ND |
| **Reverse**           |             |             |                                 |
| dTDP-α-glucose        | UDP-α-glucose | 9.40 ± 0.87 |
| UDP-α-glucose         | UDP-N-acetyl-D-glucosamine | 7.48 ± 0.35 |
| UDP-N-acetyl-D-glucosamine | 16.34 ± 0.47 |
| GDP-α-glucose         | ND | ND |
which was 36.72 ± 2.01 nmol/ml/mg protein. The fact that the truncated enzyme possessed residual RmlA activity promoted the next question, which was whether the radical structural change induced by heat treatment affected the enzymatic activity. To determine this, N-acetyl-D-glucosamine-1-phosphate uridylyltransferase activity remaining after 5 min of treatment of the truncated enzyme at different temperatures was measured. As shown in Fig. 6, N-acetyl-D-glucosamine-1-phosphate uridylyltransferase activity remained in the truncated enzyme after 5 min of heating at 65 °C but was completely removed by treatment over 70 °C. These results indicated that the additional C-terminal domain of ST0452 plays an important role in both the thermostability and nucleotidylyltransferase activity of this enzyme.

**DISCUSSION**

RmlA is an important enzyme in prokaryotic microorganisms, because it is capable of biosynthesizing dTDP-D-glucose, an important precursor for a large number of modified sugars included in cell-surface structures (17). Among these, l-rhamnose, which is synthesized from dTDP-D-glucose in a three-step reaction, has been detected in bacterial cell wall and capsular antigens as a component involved in modulating virulence and adhesion to host tissues. In previous studies, bacterial RmlA enzymes have been purified and well characterized, including cloning of the encoding genes and analysis of enzymatic activity (12, 15). Moreover, it has been demonstrated that the adenosine 5’-diphosphate-glucose pyrophosphorylase from potato, a similar enzyme to RmlA, was stable to −70 °C (20). However, there have been no previous reports of thermostability of RmlA enzymatic activity or isolation of this enzyme from thermophilic Archaea.

In this study, we have successfully expressed recombinant protein in *E. coli* harboring a heterologous expression vector containing the ST0452 gene. The ST0452 ORF was identified as a homologue of RmlA by a similarity search of the *S. tokodaii* strain 7 genome, for which the entire sequence is available. The purified recombinant enzyme possesses strict thermostability as indicated by Figs. 2 and 4C. The 50% of maximum activity remaining after 60 min of heating at 95 °C and after 180 min of treatment at 80 °C indicates that the recombinant protein encoded by ST0452 is highly thermostable and is not expected to be degraded at room temperature or lower. This property is highly desirable for potential utilization of this enzyme in industrial applications. Maximum enzymatic activity was detected between 80 and 100 °C, but 50% of activity still remained at 65 °C, indicating that the enzyme is capable of functioning over a broad temperature range. This is also desirable from the point of view of industrial use, because the enzyme could be used at different reaction temperatures as required by certain materials or conditions. It is noteworthy that this enzyme is the first extremely thermostable enzyme exhibiting RmlA activity to be identified from Archaea.

Comparison of the effect of different metal ions on the activity of ST0452 indicated that Co²⁺ and Mn²⁺ were 2.4 and 1.6 times as effective, respectively, as Mg²⁺. Enzymatic activity with Zn²⁺ was 71% of that with Mg²⁺. Previous studies indicated that Mg²⁺ is essential for RmlA activity, which was confirmed by structural analysis (21). The residues Lys²⁸⁹, Asp³⁰⁵, and Asp³⁲⁶ correspond to the Mg²⁺-binding residues Lys³⁰⁵, Asp³¹¹, and Asp³²⁶ in the *E. coli* RmlA and therefore are completely conserved between these two enzymes. Thus, the preference for different types of metal ion reflects the different overall structure surrounding these three residues in the ST0452 protein.

The activity of the ST0452 protein was found to be pH-dependent with its maximum at approximately neutral pH. However, the original host cell, *S. tokodaii* strain 7, grows optimally at pH values between 2 and 4. This supports the hypothesis that the intracellular environment is maintained at neutral pH through the use of proton pumps to transport hydrogen ions to the extracellular environment.

As shown in Fig. 1, the additional C-terminal domain of the ST0452-encoded protein is not found in the other known RmLAs. To determine the function of this domain, a deletion mutant lacking the C-terminal region from the 232nd residue, was expressed in *E. coli* and purified using the histidine tag. Solubility analysis of this truncated enzyme indicated that the maximum temperature at which the correct structure of the enzyme was maintained was reduced to 60 °C, as shown in Fig. 5. This indicates that the structure of the truncated protein was drastically changed between 60 and 70 °C. To identify any change in activity, the RmlA activity of the truncated enzyme was assayed at 37 °C, where it still possessed normal conformation. At 37 °C, the activity of the truncated enzyme was 23-fold lower than that of native ST0452 enzyme measured at 37 °C. This indicates that the additional C-terminal domain plays a role in both the thermostability and the activity of this enzyme. Following on, activity of the truncated enzyme was assayed at 37 °C after 5 min of treatment between 37 and 80 °C. Fig. 6 indicates that the activity was not significantly

**Fig. 5.** SDS-PAGE analysis of the truncated enzyme treated at different temperatures. Truncated enzyme at 1 mg/ml in 50 mM Tris-HCl (pH 7.5), 2 mM MgCl₂ was exposed to the temperature indicated for 5 min. After temperature treatment, the solution was centrifuged at 20,000 × g for 10 min at 4 °C. The protein remaining in the solution was separated by 0.1% SDS, 12% polyacrylamide gel electrophoresis and detected by staining with Coomassie Brilliant Blue R-250. Lane C indicates non-heat-treated protein as a control.

**Fig. 6.** Residual activity of the truncated enzyme after heat treatment. Analyses of residual activity were performed in 30 μl of reaction mixture containing 50 mM Tris-HCl (pH 7.5), 2 mM MgCl₂, 1 mM UTP, and 10 μM N-acetyl-D-glucosamine-1-phosphate at 37 °C for 5 min. The truncated enzyme used in this assay was exposed to the temperature indicated for 5 min, and then 1 μg of chilled protein was added to the reaction mixture. The UDP-N-acetyl-D-glucosamine produced was calculated by HPLC according to the protocol under "Experimental Procedures." Relative activity is expressed as a percentage of the activity detected on the protein exposed at 50 °C. The experiments were repeated four times.
altered in the truncated enzyme heated at 60 °C or below, but treatment at 65 °C reduced the activity to 40% of the maximum value. Furthermore, no activity was detected after treatment at 70 or 80 °C. This result reveals that the changes in conformation correlated with the loss of the residual activity, and therefore, the correct structure is necessary for activity. Based on the fact that the three-dimensional structures determined for other RmlA proteins are dimer-dimer (tetramer), with the region necessary for dimerization located at the C terminus, it is thought that the additional C-terminal domain of the ST0452 protein may also be important for dimerization. However, the truncated protein still contains all of the amino acids identified as important or essential for activity. This indicates that dimerization may be important or essential for thermostability and enzymatic activity. Such speculation is based on the fact that the temperature for maximum activity, 95 °C, is 15 °C higher than the optimal temperature for growth of this archaeon. It is thought that this difference in optimal temperatures may be due to increased thermostability and enzymatic activity conferred by dimerization.

Kinetic constants for RmlA activity of the ST0452 protein are shown in Table II. In comparison with the RmlA of S. enterica LT2 (12), the $K_v$ values for the four substrates are very similar. However, the $V_{max}$ values for both the forward and reverse reactions are ~30 times lower than those of the S. enterica LT2 RmlA. This indicates that the ST0452 enzyme has a similar substrate binding affinity to the S. enterica LT2 enzyme, but the rate at which it catalyzes conversion of substrate to product is much lower than that of the S. enterica LT2 RmlA. Three-dimensional structures have been solved for three RmlA enzymes from P. aeruginosa (16), E. coli (17, 21), and S. enterica LT2 (22). Examination of the structure around the active site identified residues that directly interact with substrates and conserved across these three enzymes, and corresponding residues were identified on the ST0452 protein. Lys25, Asp99, and Asp208, which are important for binding of Mg$^{2+}$, are conserved in the ST0452 protein, as are Ser10, Gly11, Arg13, Glu146, Lys147, Arg179, and Asp186, which are important for binding of substrates. However, the His117 and Pro96 residues (numbered according to the E. coli RmlA), which are conserved across the bacterial RmlAs, are absent in the ST0452 protein. This indicates that the comparatively low $V_{max}$ value of the ST0452 enzyme was a result of differences between this enzyme and other RmlA proteins in the residues surrounding the active site.

Differential use of substrate compared with other RmlA proteins is also due to the differences in the residues surrounding the active site. As shown in Table III, the ST0452 enzyme is not capable of utilizing α-D-glucosamine-1-phosphate as a substrate, although RmlA enzymes from other microorganisms readily utilize this molecule as a substrate. Conversely, whereas the other known RmlA enzymes cannot use N-acetyl-α-D-glucosamine-1-phosphate as a substrate, the ST0452 enzyme is capable of catalyzing the formation of the nucleotide N-acetyl-α-D-glucosamine. The E. coli GlmU enzyme, which also catalyzes this reaction, can only use UTP as a nucleotide substrate (19). However, the ST0452 enzyme is capable of utilizing both UTP and dTTP as the nucleotide substrate. Thus, the ST0452 enzyme possesses a activity, comprising the activities of both the RmlA and GlmU enzymes. The differences in substrate utilization between the ST0452 enzyme and other RmlA or GlmU enzymes is likely to be a consequence of differences in residues surrounding the active sites. Glu83 and Glu88 (numbered according to the E. coli RmlA), which are necessary for binding of the base moiety of TTP, are absent or substituted in the ST0452 protein. Elucidation of the three-dimensional structure of the enzyme is necessary for more detailed understanding of the mechanism of differential substrate utilization between ST0452 and other RmlA and GlmU enzymes.

Three RmlA-like ORFs, ST0452, ST1971, and ST2352, are present in the S. tokodaii genome. These three homologous ORFs have different sizes; the ST0452 protein contains 401 residues, the ST1971 protein contains 344 residues, and the ST2352 protein contains 258 residues. RmlA enzymes from E. coli and P. aeruginosa contain 293 residues. Because the ST1971 ORF is located in the ORF cluster containing RmlA to RmID homologues, it is thought to be the actual RmlA enzyme of S. tokodaii. Functional analysis of ST1971-encoded protein will clarify this. Genes similar to ST0452 have also been identified in the genomes of other archaea, including Methanococcus jannaschii DSM 2661 (GenBank accession number AAB99104), Methanococcus maripaludis S2 (GenBank accession number CAF30632), Methanopyrus kandleri AV19 (GenBank accession number AAM02105), P. horikoshii OT3 (GenBank accession number BAA31052), Pyrococcus abyssi (GenBank accession number CAB50605), and S. solfataricus P2 (GenBank accession number AAK41400). These homologues are slightly smaller than the E. coli GlmU enzyme (19), which is 456 residues long. Therefore, ST0452 protein is midway in size between the RmlA and GlmU enzymes from E. coli and possesses an activity consisting of the activities of both RmlA and GlmU, indicating that ST0452 represents an intermediate in the evolution of RmlA to GlmU. In E. coli, these two genes are completely separate, and the activities of their protein products do not overlap. It can be speculated that the ST0452 ORF arose from duplication of the original RmlA gene of S. tokodaii followed by mutation into its present form.

Detection of enzymatic activity producing dTDP-glucose from glucose-1-phosphate and dTTP revealed that the pathways for biosynthesis of L-rhamnose and other modified sugars should be present in the archaeon, S. tokodaii. Other ORFs included in the cluster, consisting of ST1969 to ST1972, should be expressed and utilized in functional analyses. Such analyses should clarify the issue of saccharide metabolism in archaea. In addition, the ST0452 enzyme with its high stability may be useful for industrial synthesis of sugar nucleotides, and this potential application should be explored in the near future.

REFERENCES

1. Roberts, I. S. (1996) Annu. Rev. Microbiol. 50, 285–315
2. Keppler, R., Wang, G., Hottes, B., Priefler, U. B., and Pühler, A. (1993) J. Bacteriol. 175, 7786–7792
3. McNeil, M., Duffe, M., and Brennan, P. J. (1990) J. Biol. Chem. 265, 18200–18206
4. Deng, L., Mikusova, K., Bobuch, K. G., Scherman, M., Brennan, P. J., and McNeil, M. R. (1995) Antimicrob. Agents Chemother. 39, 694–701
5. Nienitz, R., Karcher, U., Kandler, O., Tindall, B. J., and König, H. (1997) Eur. J. Biochem. 249, 955–911
6. Kawarabayasi, Y., Sawada, M., Horikawa, H., Haikawa, Y., Hino, Y., Yamamoto, S., Sekine, M., Baba, S., Kosugi, H., Hosoyama, A., Nagai, Y., Sakai, M., Ogura, K., Otsuka, R., Nakazawa, H., Takamiyi, M., Ohfuji, Y., Fuchikoshi, T., Tanaka, H., Yoshioka, Y., Yamashita, K., Hida, N., Oguchi, A., Aoki, K., and Kikuchi, H. (1998) DNA Res. 5, 147–155
7. Klein, H. P., Clayton, R. A., Tomb, J. F., White, O., Nelson, K. E., Ketchum, K., Dodson, R. J., Gwinn, M., Hickey, E. R., Peterson, J. D., Richardson, D. L., Kerlavage, A. R., Graham, D. E., Kyrpides, N. C., Fleischmann, R. D., Quackenbush, J., Lee, N. H., Sutton, G. G., Gill, S., Kirkness, E. F., Dougherty, B. A., McKenney, K., Adams, M. D., Loftus, B., Peterson, S., Rech, C. I., McNeil, L. K., Badger, J. H., Godelke, A., Zhou, L., Overbeck, B., Gocayne, J. D., Weidman, J. F., McDonald L., Utterback, T., Coton, M. D., Spriggs, T., Artiach, P., Kaine, B. P., Sykes, S. M., Sadow, P. W., D’Andrea, K. J., Bowman, C., Fujii, C., Garland, S. A., Mason, T. M., Olsen, G. J., Fraser, C. M., Smith, H. O., Woeser, C. E., and Venter, J. C. (1997) Nature 390, 364–370
8. She, Q., Singh, R. K., Confalonieri, F., Zivonov, Y., Allard, G., Aways, M. J., Chan-Weifer, C. C., Clausen, I. G., Curtis, B. A., De Moores, A., Erauso, G., Fischer, C., Gordon, P. M., Heikamp-de Jong, I., Jeffries, A. C., Kozera, C. J., Medina, N., Peng, X., Thi-Ngoc, H. F., Redder, P., Schenk, M. E., Theriault, C., Tolstrup, N., Charlebois, R. L., Doll, T., Duguet, M., Gaasterland, T., Garrett, R. A., Ragan, M. A., Sensen, C. W., and Van der Oost, J. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 7835–7840
9. Kawarabayasi, Y., Hino, Y., Horikawa, H., Jin-no, K., Takahashi, M., Sekine,
A Thermostable Sugar-1-phosphate Nucleotidylyltransferase

M., Baba, S., Ankai, A., Kosugi, H., Hosoyama, A., Fukui, S., Nagai, Y., Nishijima, K., Otenka, R., Nakazawa, H., Takamiya, M., Kato, Y., Yoshizawa, T., Tanaka, T., Kudoh, Y., Yamazaki, J., Kushida, N., Oguchi, A., Aoki, K., Masuda, S., Yanagii, M., Nishimura, M., Yamagishi, A., Oshima, T., and Kikuchi, H. (2001) *DNA Res.* **8**, 123–140

11. Melo, A., and Glaser, L. (1965) *J. Biol. Chem.* **240**, 398–405

12. Lindquist, L., Kaiser, R., Reeves, P. R., and Lindberg, A. A. (1993) *Eur. J. Biochem.* **211**, 763–770

13. Li, Q., and Reeves, P. R. (2000) *Microbiology* **146**, 2291–2307

14. Macpherson, D. F, Manning, P. A., and Morona, R. (1994) *Mol. Microbiol.,* **11**, 281–292

15. Ma, Y., Mills, J. A., Belisle, J. T, Vissa, V., Howell, M., Bowlin, K., Scherman, M. S., and McNeil, M. (1997) *Microbiology* **143**, 937–945

16. Blankenfeldt, W., Asuncion, M., Lam, J. S., and Naismith, J.H. (2000) *EMBO J.* **19**, 6652–6663

17. Zuccotti, S., Zanardi, D., Rosano, C., Sturla, L., Tonetti, M., and Bolognesi, M. (2001) *J. Mol. Biol.* **313**, 831–843

18. Thorson, J. S., Kelly, T. M., and Liu, H. W. (1994) *J. Bacteriol.* **176**, 1840–1849

19. Brown, K., Pompeo, F., Dixon, S., Mengin-Lecreulx, D., Cambillau, C., and Bourne, Y. (1999) *EMBO J.* **18**, 4096–4107

20. Ballicora, M. A., Laughlin, M. J., Fu, Y., Okita, T. W., Barry, G. F., and Preiss, J. (1995) *Plant Physiol.* **109**, 245–251

21. Sivaraman, J., Sauve, V., Matte, A., and Cygler, M. (2002) *J. Biol. Chem.* **277**, 44214–44219

22. Barton, W. A., Lesniak, J., Biggins, J. B., Jeffrey, P. D., Jiang, J., Rajashankar, K. R., Thorson, J. S., and Nikolov, D. B. (2001) *Nat. Struct. Biol.* **8**, 545–551
Identification of an Extremely Thermostable Enzyme with Dual Sugar-1-phosphate Nucleotidylyltransferase Activities from an Acidothermophilic Archaeon, *Sulfolobus tokodaii* strain 7

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