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The Thermostable Enzyme Genes of the dTDP-L-Rhamnose Synthesis Pathway (*rmlBCD*) from a Thermophilic Archaeon

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1. Introduction

The biosynthesis of saccharides is important because these diverse molecules mediate various functions from structure and storage to signaling. The biosynthesis of oligosaccharides, polysaccharides and glycoconjugates involves glycosyltransferases (EC 2.4), which transfer a sugar moiety from a nucleotide-activated donor sugar onto acceptors (Breton et al., 2006).

L-rhamnose is found widely in bacteria and plants (Giraud & Naismith, 2000). It is a common component of the cell wall and the capsule of many pathogenic bacteria, and has been indicated to play an essential role in many pathogenic bacteria (Giraud & Naismith, 2000). L-rhamnose is also found in the cytoplasmic membrane of archaea (Sprott et al., 1983), while pathogenic archaea have not been identified (Eckburg et al., 2003).

The nucleotide-activated L-rhamnose, dTDP-L-rhamnose, is synthesized from dTTP and glucose-1-phosphate (G-1-P) by a conserved four-step reaction. In the first reaction, RmlA (G-1-P thymidylyltransferase, EC 2.7.7.24) transfers the thymidylmonophosphate nucleotide to G-1-P. RmlB (dTDP-D-glucose 4,6-dehydratase, EC 4.2.1.46) then catalyzes oxidation of the C4 hydroxyl group of the sugar, followed by dehydration. Third, RmlC (dTDP-4-dehydrorhamnose 3,5-epimerase, EC 5.1.3.13) catalyzes an unusual double epimerization reaction at positions C3 and C5. Finally, RmlD (dTDP-4-dehydrorhamnose reductase, EC 1.1.1.133) reduces the C4 keto group to generate the final product, dTDP-L-rhamnose (Giraud & Naismith, 2000).

Thermal instability of the Rml enzymes has been raised as an issue (Graninger et al., 1999, 2002). Thus far, the highest reported temperature for the dTDP-L-rhamnose synthesis reaction is around 50 °C using thermophilic bacterial enzymes (Graninger et al., 2002; Novotny et al., 2004). The presence of dTDP-L-rhamnose has not been reported in archaea.

However, putative *rmlABCD* genes have been identified in the genomes of thermophilic archaea, including *Pyrococcus horikoshii* (Kawarabayasi et al., 1998), *Archaeoglobus fulgidus* (Klenk et al., 1997), *Sulfolobus solfataricus* (She et al., 2001) and *Sulfolobus tokodaii* (Kawarabayasi et al., 2001). Their *rml* genes possibly encode thermostable enzymes, and could thus greatly contribute to the thermostability of the Rml enzymes by *in vitro* protein evolution techniques such as family shuffling (Kikuchi et al., 2000). *S. tokodaii* 7 grows optimally at 80°C (Suzuki et al., 2002), and thus its putative *rmlABCD* genes were functionally and biochemically analyzed in this study.

2. Materials and methods

2.1 Sequence analysis

Sequences were compared to those in the SWISS-PROT protein sequence database (Bairoch & Apweiler, 2000) using BLAST (Altschul et al., 1990).

2.2 Vector construction

The four genes *rmlCDAB* (genes ST1969 to ST1972; DDBJ accession number BA000023) were PCR-amplified with each of the following sets of primers designed to introduce an *Nde*I site (overlapping the initiating ATG codon) and a downstream *Hind*III or *Xho*I site (the restriction sites are underlined): 5′-GGAATTCCATATGCCTTTTGAATTCGAAAATCTGGG-3′ (forward; contains the *Nde*I site) and 5′-GCGGCCGCAAGCTTTTAATCAAAGACTTCAGCCTTTTC-3′ (reverse; contains the *Hind*III site) for *rmlC* (gene ST1969); 5′-GGAATTCCATATGCGAACACTAATAACTGGTGC-3′ (forward; contains the *Nde*I site) and 5′-GGTGCTCGAGCACCACCATACCGTCTAGATCC-3′ (reverse; contains the *Xho*I site) for *rmlD* (gene ST1970) (His tag at the C terminal); 5′-GGAATTCCATATGGAGGCGGTAATTTTACAC-3′ (forward; contains the *Nde*I site) and 5′-GGTGCTCGAGTCATAATATCACCGAAGAATTCTC-3′ (reverse; contains the *Xho*I site) for *rmlA* (gene ST1971); 5′-GGAATTCCATATGATAATTATTGGTGGTGCTGG-3′ (forward; contains the *Nde*I site) and 5′-GGTGCTCGAGTTTACTAACTTTTACTTTCCAAGGC-3′ (reverse; contains the *Xho*I site) for *rmlB* (gene ST1972) (His tag at the C terminal). PCR was performed in 50 µl of standard PCR mixture with 1 unit of KOD-plus DNA polymerase (Toyobo, Japan), 10 ng of *S. tokodaii* 7 genomic DNA (NITE Biological Resource Center, Chiba, Japan) and 100 pmol of each primer. The amplification program was as follows: (i) 94°C for 2 min; (ii) 25 cycles of 94°C for 30 sec, X°C for 30 sec (X = 55 for *rmlCDB*; X = 58 for *rmlA*) and 68°C for 1 min. PCR products were digested at the restriction sites in the primers described above and ligated into the same restriction sites of the pET21(b) expression vector (Novagen). Nucleotide sequences of the resultant plasmids were confirmed after transformation of *Escherichia coli* DH5α (Takara, Japan) with the resultant plasmids and purification of the plasmids from the *E. coli* (QIAprep Spin Miniprep kit; Qiagen). The resultant plasmids to express *rmlC*, *rmlD*, *rmlA* and *rmlB* were designated pSTC, pSTD, pSTA and pSTB, respectively.

2.3 Preparation of supernatants containing the expressed proteins

E. coli BL21-Codon Plus (DE3)-RIL cells (Stratagene) were transformed with either pSTC, pSTD, pSTA or pSTB. The transformed *E. coli* was cultured at 37°C in 600 ml LB medium supplemented with 100 µg ml^{-1} ampicillin and 20 µg ml^{-1} chloramphenicol to an OD_{600} of X

(X = 0.49 for RmlC; X = 0.56 for RmlD; X = 0.12 for RmlA; X = 0.60 for RmlB). Isopropyl β-D-thiogalactopyranoside was then added to the culture at a final concentration of 0.1 mM, and the culture was grown at 16°C overnight. The culture was then harvested by centrifugation, washed twice with 50 mM Tris-HCl buffer (pH 7.5), and resuspended in 2.5 ml of the 50 mM Tris-HCl buffer. The suspension was sonicated on ice for 4 min with a UD-201 ultrasonic disruptor (output, 6; duty, 50%; Tomy Seiko, Japan), heated at 80°C for 20 min, and centrifuged at 20,000 g for 10 min at 4°C. The resultant supernatant (2.5 ml) was desalted through a PD10 Sephadex G-25 column (GE Healthcare) with the 50 mM Tris-HCl buffer as the elution buffer. The resultant supernatant (3.5 ml) containing the expressed protein was stored at 4°C.

2.4 SDS-PAGE

Eight μl of each supernatant was analyzed on an E-R12.5L polyacrylamide gel (ATTO, Japan) at 30 mA constant current together with 10 μl of the broad range molecular weight standards (Bio-rad), and visualized with Coomassie Brilliant Blue R-250. The gel was photographed with a luminescent image analyzer Las-4000 mini (Ver 2.1; Fuji film, Japan). The amount of expressed protein was determined using MultiGauge software (Ver 3.2; Fuji film) in comparison to the molecular weight standards.

2.5 Reaction of the expressed protein

The reaction to detect G-1-P thymidylyltransferase activity of RmlA was performed at 80°C for 3 h in 50 mM Tris-HCl buffer (pH 7.5), 2 mM $MgCl_2$, 10 mM dTTP, 10 mM UTP and 10 mM G-1-P with the RmlA supernatant (40 μl of the supernatant, which contained 4 μg RmlA, was added to the reaction mixture for a total volume of 100 μl). UTP was also used as a substrate candidate because a product of a *rmlA* homolog (ST0452) from *S. tokodaii* 7 shows the G-1-P thymidylyltransferase activity (RmlA activity) as well as G-1-P uridylyltransferase activity (Zhang et al., 2005).

The reaction to determine substrate specificity of RmlB was performed at 80°C for 3 h in 50 mM Tris-HCl buffer (pH 7.5), 2 mM $MgCl_2$, 10 mM dTDP-D-glucose, and 10 mM UDP-D-glucose with the RmlB supernatant (40 μl of the supernatant, which contained 4 μg RmlB, was added to the reaction mixture for a total volume of 100 μl). To serve as controls, this reaction was performed with the RmlC supernatant or the RmlD supernatant (40 μl of the supernatant, which contained 20 μg of RmlC or RmlD, was added to the reaction mixture for a total volume of 100 μl) instead of the RmlB supernatant. Optimal reaction temperature for RmlB was determined in the same way as just described above except that UDP-D-glucose was not included in the reaction mixture and the reaction was preformed at the indicated temperature for 2 h. The reaction to characterize the thermostability of RmlB was carried out in the same way as described above for determining the optimal temperature except that the reaction was performed at 80°C for the indicated time period.

The dTDP-L-rhamnose synthetic reaction was performed at 80°C for 3 h in 50 mM Tris-HCl buffer (pH 7.5), 2 mM $MgCl_2$, 10 mM dTDP-D-glucose, 5 mM NADPH, and 5 mM NADH with a combination of the supernatants (4 μgs each of RmlB, RmlC and/or RmlD were added to the reaction mixture for a total volume of 100 μl). Each reaction was stopped by adding ten times the reaction volume of 500 mM KH_2PO_4.

2.6 HPLC of the reaction

A 50-µl aliquot of the reaction solution mixed with 500 mM KH_2PO_4 was subjected to HPLC using a LaChrom ELITE system with a L-2420 UV-VIS detector and a L-2130 pump (Hitachi) equipped with a Wakosil 5C18-200 column (4.6 x 250 mm; Wako, Japan). The 500 mM KH_2PO_4 was run as the elution buffer at a constant flow rate of 1 ml min^{-1}. The substrates and products were detected at 254 nm, and the peak area was used for calculation of the amount.

2.7 Mass spectrometry (MS) of the reaction products

The reaction samples for MS were prepared with a smaller amount of the substrate dTDP-D-glucose (2 mM) and thus with 2 mM NADPH and 2 mM NADH to completely consume the dTDP-D-glucose (this reaction was otherwise performed in the same way as described above), as a standard sample dTDP-L-rhamnose (the predicted product; Genechem Inc., Taejon, Korea) was unable to be separated from dTDP-D-glucose by HPLC used for MS (described below).

Reaction samples, to which 500 mM KH_2PO_4 was not added, were separated by HPLC (LC-10AT, Shimadzu, Japan) performed on a Wakosil 5C18-200 column using a water/methanol/acetic acid mixture (98.9/1.0/0.1, v/v/v) as the elution buffer at a flow rate of 1 ml min^{-1}. Mass spectra of the peaks detected by the HPLC were then measured by an electrospray ionization/ion trapped mass spectrometer (LCQ^{DECA}, ThermoQuest, USA) connected to the HPLC under the following instrumental conditions: detection mode, negative; mass range, *m/z* 100–1500; spray voltage, 5 kV; capillary temperature, 350°C; capillary voltage, -10 V; sheath gas flow, 100 units; auxiliary gas flow, 20 units (1 unit is roughly 0.025 L min^{-1}).

3. Results and discussion

3.1 Putative dTDP-L-rhamnose synthesis gene cluster (*rmlABCD*) from the thermophilic archaeon *S. tokodaii* 7

The *rml* genes from *S. tokodaii* 7 are clustered in the order of *rmlCDAB*, and the *rmlC* is oriented in a different direction from the others *rmlDAB* (Kawarabayasi et al., 2001). The RmlBCD were most similar to the dTDP-L-rhamnose synthesis enzymes (Table 1). However, the most similar homolog of the RmlA was UDP-N-acetylglucosamine pyrophosphorylase/glucosamine-1-phosphate N-acetyltransferase from *Methanocaldococcus jannaschii* (39% identity and 79% similarity; Q58501; Namboori & Graham, 2008), and its second closest homolog was RmlA from *Streptococcus mutans* (29% identity and 71% similarity; P95778; Tsukioka et al., 1997).

The RmlA from *S. tokodaii* possessed a signature sequence for recognition of nucleoside triphosphate, $GXGTRX_8PK$, and that of G-1-P, LVEKP (Thorson et al., 1994). RmlB contained motifs characteristic of dTDP-D-glucose 4,6-dehydratase, PSSPYSASKA and GGAGFIG (Allard et al., 2001). A region covering approximately 150 residues and conserved in dTDP-4-dehydrorhamnose 3,5-epimerase for substrate binding (Giraud et al., 2000) was found in RmlC. RmlD possessed the $PX_3YX_3KX_3E$ motif, which is characteristic of a reductase/epimerase/dehydrogenase/dehydratase superfamily for L-rhamnose synthesis from G-1-P, and another motif, STDYVF, unique in RmlD sequences (Graninger et al., 1999). However, the conserved NAD(P) binding motif (Wierenga et al., 1985), $GXGX_2G$, was not found in RmlD.

Product	Deduced MW (kDa)	Homolog	% Identity /% similarity	Accession no.
RmlB	37.0	dTDP-D-glucose 4,6-dehydratase (RmlB)		
		from *Salmonella typhimurium*	41/73	P26391
		from *Escherichia coli*	41/79	P55293
RmlC	20.5	dTDP-4-dehydrorhamnose 3,5-epimerase (RmlC)		
		from *Shigella flexneri*	45/75	P37780
		from *Salmonella typhimurium*	40/77	P26394
RmlD	31.0	dTDP-4-dehydrorhamnose reductase (RmlD)		
		from *Salmonella typhimurium*	28/63	P26392
		from *Shigella flexneri*	27/62	P37778

Table 1. The *rmlBCD* products from *Sulfolobus tokodaii* 7 and their homologs

3.2 Activity of RmlA

RmlA was expressed as a 35-kDa protein (data not shown), in agreement with the molecular weight (MW) of 38 kDa deduced from its nucleotide sequence. The predicted G-1-P thymidylyltransferase (RmlA) activity was assayed at 80°C with dTTP and G-1-P, but was not detected. RmlA may thus function as the UDP-N-acetylglucosamine pyrophosphorylase/glucosamine-1-phosphate N-acetyltransferase as deduced from its sequence similarity. This deserves further investigation. A product from another *rmlA* homolog, located away from the *rmlABCD* cluster in *S. tokodaii* 7, shows RmlA activity (Zhang et al., 2005). This is consistent with the reports that *rmlABCD* genes are not always found together (Cole et al., 1998; Giraud & Naismith, 2000).

3.3 RmlB as a thermophilic dTDP-D-glucose 4,6-dehydratase

RmlB was expressed as a 35-kDa protein (Fig. 1), which corresponded to the deduced MW (Table 1). The predicted activity (Table 1) was assayed at 80°C with dTDP-D-glucose and UDP-D-glucose (Fig. 2). UDP-D-glucose was also used as a substrate candidate because UDP-D-glucose as well as dTDP-D-glucose can be produced by the product from the another *rmlA* homolog (Zhang et al., 2005). As shown in Fig. 2, dTDP-D-glucose was used as a substrate by RmlB, whereas UDP-D-glucose was not. As controls, RmlC (Fig. 2) and RmlD (data not shown) did not use dTDP-D-glucose and UDP-D-glucose as the substrate. RmlB (+B) produced a broad peak in the mass chromatogram by the selected ion monitoring of the *m/z* 545 peak (data not shown; this broad peak was not obvious in Fig. 2, +B, 3h), which corresponded to the deprotonated molecule $(M-H)^-$ of the dTDP-D-glucose 4,6-dehydratase (RmlB) product, dTDP-4-dehydro-6-deoxy-D-glucose (MW = 546). Consistent with this result, dTDP-4-dehydro-6-deoxy-D-glucose has previously been eluted as a broad peak from a C18 column (Nakano et al., 2000; Watt et al., 2004). Together with the sequence homology, these data indicate that RmlB from *S. tokodaii* was thermostable dTDP-D-glucose 4,6-dehydratase (RmlB). Peaks 1 and 2, which became prominent by the addition of RmlB (Fig. 2), were indicated by MS to be from TMP and TDP, respectively (data not shown). It is unclear if RmlB degraded dTDP-D-glucose to TDP and TMP.

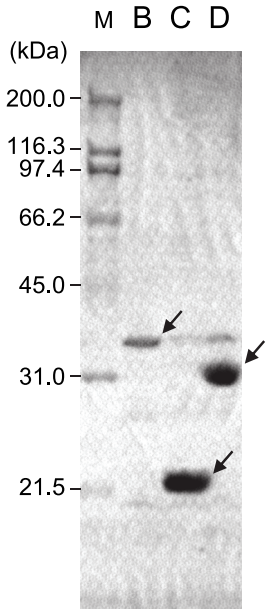

Fig. 1. SDS-PAGE analysis of RmlBCD. Eight µl of each cell-free supernatant from *E. coli* cells expressing *rmlB* (lane B), *rmlC* (lane C) or *rmlD* (lane D) was analyzed, and contained 0.8, 4.0 or 4.0 µg, respectively, of the deduced product (indicated by arrows). M, molecular weight markers.

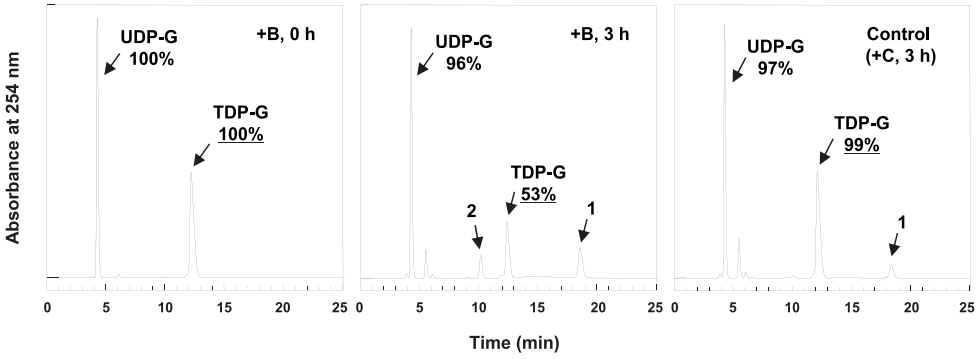

Fig. 2. Substrate specificity of RmlB shown by HPLC. RmlB-containing supernatant was incubated with dTDP-D-glucose (TDP-G) and UDP-D-glucose (UDP-G) at 80°C for the indicated period (+B). RmlC-containing supernatant was also incubated in the same way instead of the RmlB supernatant as a control (+C). Relative amounts of TDP-G and UDP-G are indicated compared to the amounts of TDP-G and UDP-G in the +B sample at 0 h, respectively. Peaks 1 and 2 of the reaction products were indicated by MS to be from TMP and TDP, respectively.

Temperature range for the dTDP-D-glucose-utilizing activity of RmlB was measured from 60 to 99°C, and the optimal temperature was shown to be 80°C (Fig. 3A). Therefore, RmlB

from *S. tokodaii* was thermophilic; its optimal temperature for the activity coincided with the optimal growth temperature of 80˚C for its host *S. tokodaii* 7 (Suzuki et al., 2002). The activity of RmlB gradually diminished at 80°C over hours (Fig. 3B). Specific dTDP-D-glucose-utilizing activity of RmlB was calculated to be 4.2 U/mg protein based on the data from the first 1 h of Fig. 3B. On the other hand, *E. coli* RmlB shows a high activity of approx. 3700 U/mg protein (Marolda & Valvano, 1995).

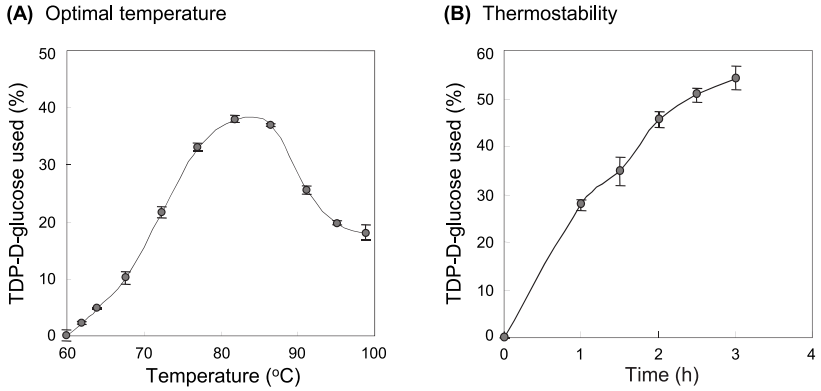

Fig. 3. Thermophilic TDP-D-glucose-utilizing activity of RmlB. (A) Optimal temperature of the activity. RmlB supernatant was incubated with TDP-D-glucose for 2 h at the indicated temperature. The amount of TDP-D-glucose used was shown as a percentage of the amount of TDP-D-glucose in the control sample incubated for 2 h at 80°C without supernatant (100% remained). (B) Thermostability of the activity at the optimal temperature of 80°C. RmlB supernatant was incubated with TDP-D-glucose at 80°C for the indicated period. The amount of TDP-D-glucose used was expressed as a percentage of the amount of TDP-D-glucose in the sample at 0 h (100% remained). Each value is the mean ± standard error from two independent experiments.

3.4 The dTDP-L-rhamnose synthesis reaction from dTDP-D-glucose at 80°C catalyzed by RmlBCD

RmlC and RmlD were expressed as a 22-kDa protein and a 30-kDa protein, respectively (Fig. 1), which corresponded to their respective deduced MWs (Table 1). The dTDP-L-rhamnose synthesis reaction catalyzed by RmlBCD, suspected based on their homology (Table 1), was analyzed at 80°C using dTDP-D-glucose and NAD(P)H as substrates (Fig. 4). A combination of RmlB plus RmlD produced peak 3 (+BCD and +BD in Fig. 4); the retention time and MS spectrum of this peak were identical to those of the standard sample dTDP-L-rhamnose. RmlB(C)D from *S. tokodaii* were thus shown to synthesize dTDP-L-rhamnose from dTDP-D-glucose at 80°C (discussed in the next paragraph). Without NAD(P)H, peak 3 was not produced in the reaction (data not shown). RmlB plus RmlC (+BC in Fig. 4) did not yield peak 3. The broad *m/z* 545 peak produced by RmlB disappeared with the addition of RmlD (+BD and +BCD in Fig. 4; MS data not shown). Together with the results indicating that RmlB from *S. tokodaii* was dTDP-D-glucose 4,6-dehydratase (RmlB) and with the sequence homology, the results strongly suggest that RmlD from *S. tokodaii* was thermostable dTDP-4-dehydrorhamnose reductase (RmlD).

It is possible that peak 3 produced by the combination of RmlB plus RmlD (+BD) could have been an epimer of dTDP-L-rhamnose produced without the possible epimerase RmlC (Table 1). Addition of RmlC showed no effect on the broad *m/z* 545 peak produced by RmlB (+BC in Fig. 4; MS data not shown), which is consistent with the previous observation using a C18 column (Watt et al., 2004). Therefore, unfortunately, the dTDP-4-dehydrorhamnose 3,5-epimerase (RmlC) activity, predicted activity of RmlC from *S. tokodaii*, was unable to be detected with the system used.

The concentrations of peak 3 (indicated from dTDP-L-rhamnose) and dTDP-D-glucose in the +BCD sample (Fig. 4) were determined to be 2.4 and 4.8 mM, respectively, showing that 52% of the added dTDP-D-glucose was used and that 46% of the dTDP-D-glucose used was converted to dTDP-L-rhamnose in the reaction. Consequently, RmlB and RmlD were estimated to show their respective activities of at least 0.33 U/mg protein.

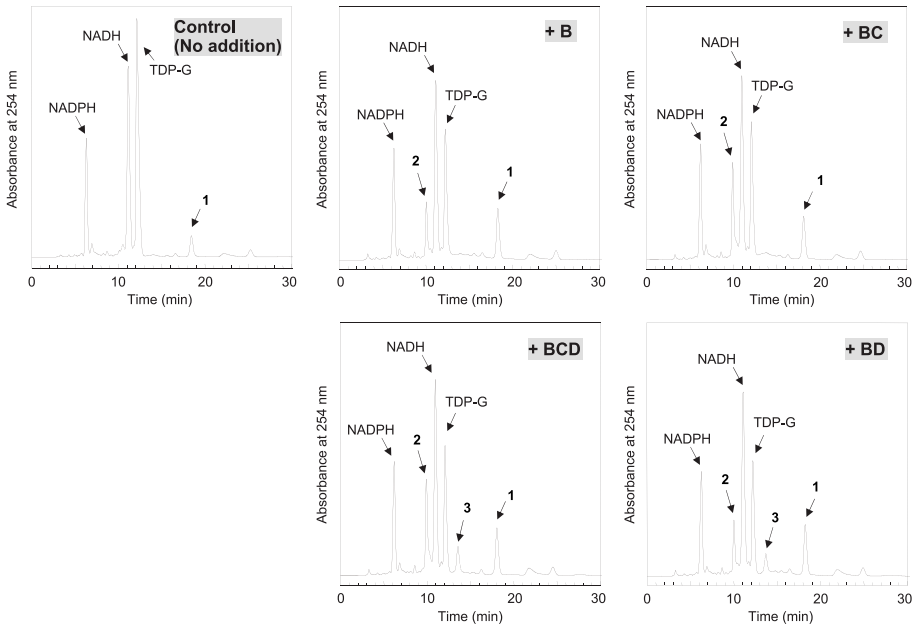

Fig. 4. The dTDP-L-rhamnose synthesis reaction from TDP-D-glucose by RmlBCD shown by HPLC. Combinations of RmlB (B), RmlC (C) and RmlD (D) supernatants were incubated with TDP-D-glucose (TDP-G), NADPH and NADH for 3 h at 80°C. The sample treated in the same way without the supernatants is also shown as a control. Peaks 1, 2 and 3 of the reaction products were indicated to be from TMP, TDP and dTDP-L-rhamnose, respectively, by MS.

4. Conclusions

Genes for thermostable RmlB and RmlD of the dTDP-L-rhamnose synthesis pathway were functionally identified from a thermophilic archaeon *S. tokodaii* 7. S. *tokodaii* Rml enzymes were suggested to be functionally identical to the bacterial counterparts, and exhibited superior thermostability. The temperature level of 80°C that was tested in this study is the

highest value yet reported for the dTDP-L-rhamnose synthesis reaction from dTDP-D-glucose. Therefore, *S. tokodaii rml* genes could confer thermostability on the high-activity Rml enzymes, including the *E. coli* RmlB (Marolda & Valvano, 1995), by *in vitro* protein evolution techniques such as family shuffling (Kikuchi et al., 2000), and are useful for a broad field of potential applications requiring Rml enzyme including production of rhamnose-containing antigens as vaccines (Hsu et al., 2006; Prakobphol & Linzer, 1980).

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Biotechnology is the scientific field of studying and applying the most efficient methods and techniques to get useful end-products for the human society by using viable micro-organisms, cells, and tissues of plants or animals, or even certain functional components of their organisms, that are grown in fully controlled conditions to maximize their specific metabolism inside fully automatic bioreactors. It is very important to make the specific difference between biotechnology as a distinct science of getting valuable products from molecules, cells or tissues of viable organisms, and any other applications of bioprocesses that are based on using the whole living plants or animals in different fields of human activities such as bioremediation, environmental protection, organic agriculture, or industrial exploitation of natural resources. The volume Advances in Applied Biotechnology is a scientific book containing recent advances of selected research works that are ongoing in certain biotechnological applications. Fourteen chapters divided in four sections related to the newest biotechnological achievements in environmental protection, medicine and health care, biopharmaceutical producing, molecular genetics, and tissue engineering are presented.

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