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Enzymatic Manufacture of Deoxythymidine-5′-Triphosphate with Permeable Intact Cells of *E. coli* Coexpressing Thymidylate Kinase and Acetate Kinase

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A one-pot process of enzymatic synthesis of deoxythymidine-5′-triphosphate (5′-dTTP) employing whole cells of recombinant *Escherichia coli* coexpressing thymidylate kinase (TMKase) and acetate kinase (ACKase) was developed. Genes *tmk* and *ack* from *E. coli* were cloned and inserted into pET28a(+), and then transduced into *E. coli* BL21 (DE3) to form recombinant strain pTA in which TMKase and ACKase were simultaneously overexpressed. It was found that the relative residual specific activities of TMKase and ACKase, in pTA pretreated with 20 mM ethylene diamine tetraacetic acid (EDTA) at 25°C for 30 min, were 94% and 96%, respectively. The yield of 5′-dTTP reached above 94% from 5 mM deoxythymidine 5′-monophosphate (5′-dTMP) and 15 mM acetyl phosphate catalyzed with intact cells of pTA pretreated with EDTA. The process was so effective that only 0.125 mM adenosine-5′-triphosphate was sufficient to deliver the phosphate group from acetyl phosphate to dTMP and dTDP.

Keywords: Deoxythymidine-5′-triphosphate, thymidylate kinase, coexpression, permeabilization, ATP regeneration

Introduction

Deoxythymidine-5′-triphosphate (5′-dTTP) is known as one of the raw materials that are used to build DNA. It is also required in modern molecular biological research as the essential precursor for the artificial synthesis of DNA, PCRs, and other PCR-based applications. Moreover, 5′-dTTP is an important intermediate in the biosynthesis of some saccharides. Several dTDP-sugars, which are the precursors of polysaccharides, were synthesized from 5′-dTTP [7]. Owing to the increasing demand of PCR applications and the emerging fields of DNA biosynthesis and sugar chemistry research, the requirement of 5′-dTTP continues to increase steadily [11, 27].

Deoxynucleoside-5′-triphosphates (dNTPs) have been traditionally produced *via* a chemical method. In this process, deoxynucleoside-5′-monophosphates (dNMPs) were used as the starting materials, and the reaction reactants pyrophosphoric acid and dicyclo-hexylcarbodiimide (DCC) as the phosphorylating agents [4, 32]. However, the yield of dNTP is low owing to the low reaction speed. Otherwise, the separation of dNTPs from the reaction solution is a little complex. Many unreacted reactants, such as dNMP, dNDP, pyrophosphate, and DCC, must be separated, in addition to the by-products orthophosphoric acid and deoxynucleosides. Finally, the solvents, such as the pyridine or *N,N*-dimethylformamide used in the process, should be recovered and recycled to reduce the production cost and environmental pollution [36].

To overcome these limitations of the chemical process, the biosynthesis of dNTPs from related dNMPs is promising. Several enzymatic synthesis processes have been developed to produce dNTPs [20, 21]. Oh *et al.* [26] have already reported the pathway for 5′-dTTP synthesis, using thymidylate kinase (TMKase; E.C. 2.7.4.9) and acetate kinase (ACKase; E.C. 2.7.2.1). However, at least two types of strains, expressing TMKase and ACKase respectively, must be prepared. In addition, as nucleotides cannot freely

enter and go out of cells owing to the barrier of the cell's envelop, enzymes in the cells should be released to the reaction solution. This process is complex and unsuitable for large-scale production, as several enzymes are manufactured and used in one process [1]. Therefore, we constructed a coexpression recombinant strain that would only need to be cultured once. Otherwise, whole cells pretreated with reagents were used as biocatalysts, which eliminate the tedious and expensive procedures required to isolate and purify enzymes.

Many studies have reported the coexpression of two or more enzyme proteins in one cell [10, 16, 22, 30]. The proximity of two or more enzymes creates a microenvironment for the reaction system, which overcomes the issue of transferring intercellular mass and reduces the diffusion time of the substrate to the second enzyme [18, 28, 31]. There are also many studies about benefits of exploiting intact bacterial cells as biocatalysts [3, 5, 12]. However, the cell membrane often retards the movement of substrates into or out of the cell. Efforts have been proposed to address the permeability barrier imposed by the cell envelope [5]. Permeabilization may be an effective method. The permeability of the cell can be increased by permeabilizers [3, 12, 33], such as toluene [6, 17, 19], chelating agents, EDTA [5], detergents, Triton X-100 [14, 17, 23], and Tween [15, 37]. Furthermore, other methods, such as salt stress [5], sonication, and freeze-thawing [5, 8], have also been reported to increase cell permeability.

Here, we demonstrated an economical enzymatic production system for 5′-dTTP from deoxythymidine-5′-monophosphate (5′-dTMP), using intact pretreated recombinant *E. coli* that coexpressed ACKase and TMKase. According to many studies about reagents for the modification of the permeability layer [5, 38], EDTA and Triton X-100 are commonly used and have higher efficiency than others such as Tween 80 and PEG1000. Furthermore, chemical permeabilizing reagents often cause extensive damage to the membrane system, even cell lysis [13]. This makes the reuse of cells or cofactor regeneration impossible. EDTA, toluene, and Triton X-100 were chosen to overcome the permeability barrier of the cell envelope and their concentration and time duration were optimized in our reaction system. 5′-dTMP was first phosphorylated into 5′-dTDP with TMKase, coupled with ATP regeneration by ACKase. Then, 5′-dTDP was phosphorylated into 5′-dTTP with ACKase (Fig. 1). Acetyl phosphate (ACP) was substituted for ATP as the donor of a phosphate group, but ATP must exist in very small amounts in the reaction.

Materials and Methods

Strains, Plasmids, and Chemical Reagents

The pET-28a(+) used as the expression vector was purchased from Invitrogen (Shanghai, China). Competent cells of *E. coli* DH5α and *E. coli* BL21 (DE3) were purchased from TianGen Biotech. Inc. (Beijing, China). DNA purification kits, plasmid mini kits, and Cycle-Pure kits were purchased from Omega Bio-Tek (Shanghai, China). All of the restriction endonucleases were purchased from TaKaRa Biotechnology Co. Ltd. (Dalian, China). Nucleosides and nucleotides were purchased from Biocaxis Chemicals Co., Ltd. Isopropyl-β-D-thiogalactoside (IPTG), ethylene diamine tetraacetic acid (EDTA), toluene, Triton X-100, and other chemical reagents were commercially available. Disodium acetyl phosphate (ACP-Na_2) was synthesized in our laboratory according to the method of Crans [9].

Construction of Recombinant Plasmids

The target genes were amplified from *E. coli* K12 using the following synthetic primers: 5′-CTAGCTAGCATGCGCAGTAAG

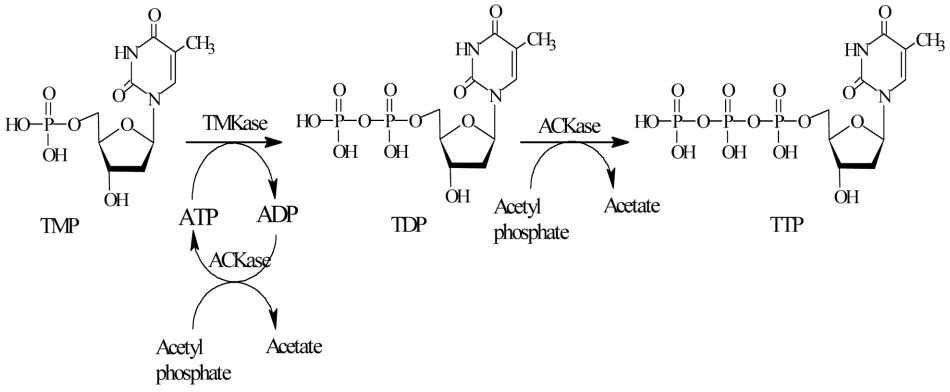

Fig. 1. Biosynthesis of 5′-dTTP.
TMKase: thymidylate kinase; ACKase: acetate kinase.

TATATCGTC-3′ (*Nhe*I) and 5′-CGGGATCCTCATGCGTCCAACT-3′ (*Bam*HI) for *tmk*; 5′-CGCCATATGATGTCGAGTAAGTTAGTA CTGGT-3′ (*Nde*I) and 5′- CGGGATCCTCAGGCAGTCAGGC-3′ (*Bam*HI) for *ack*. The PCR products containing the *tmk* and *ack* genes were digested using the appropriate restriction endonucleases and then cloned into vector pET-28a, yielding pET-28a-tmk and pET-28a-ack, respectively. The recombinant plasmids correctly sequenced were then transformed into *E. coli* DH5α for amplification.

Construction of Recombinant *E. coli* Hosting Coexpression Plasmid

The two coexpression systems with different inserts order of TMKase and ACKase were constructed as outlined in Fig. 2. Isocaudarners of *Bam*HI and *Bgl*II were used to reduce the PCR process. The fragment from pET-28a-ack digested with endonucleases *Bgl*II/*Eco*RI was linked with linear pET-28a-tmk, which was predigested with *Bam*HI/*Eco*RI to form pET-28a-tmk-ack plasmids. Similarly, pET-28a-ack-tmk was constructed after the fragment hosting gene *tmk* was inserted downstream of the *ack* gene in pET-28a-ack. The pET-28a-tmk-ack and pET-28a-ack-tmk plasmids were transformed into competent *E. coli* BL21 (DE3) to form recombinant pTA or pAT, respectively.

Coexpression of TMKase and ACKase

One loop of pTA or pAT from the related slope was inoculated in 3 ml of LB broth with 50 μg/ml kanamycin and cultured overnight. Three hundred microliters of the culture was then diluted in 30 ml of LB broth (kanamycin: 50 μg/ml) and cultured at 200 rpm and 37°C until the optical cell density at 600 nm (OD_{600}) reached 0.6. The growth of pTA continued at 16°C overnight after IPTG (0.05 mM) was added, and likewise, the growth of pAT continued at 37°C for another 6 h after IPTG (0.5 mM) was added. The culture broth was centrifuged (13,000 ×*g*, 2 min, 4°C), and the cell pellets were washed twice with Tris-HCl buffer (100 mM, pH 7.5) and then stored at −20°C until use.

SDS-PAGE of Recombinant Strains

The cells harvested, as described above, from 1 ml of culture broth were fully dispersed in 1 ml of Tris-HCl buffer (100 mM, pH 7.5) and then subjected to sonication. The lysate was centrifuged at 13,000 ×*g* for 2 min at 4°C, and the supernatant was used as the raw enzyme solution for SDS-PAGE according to the published method [34]. The protein content was determined *via* the Bradford method [2].

Permeabilization of Recombinant *E. coli*

The pTA or pAT cells were treated with different concentrations of EDTA, toluene, or Triton X-100 at room temperature for various amounts of time, and then harvested by centrifuging at 13,000 ×*g*. The cell pellets were washed twice with Tris-HCl buffer (100 mM, pH 7.5). The pretreated intact cells (harvested from the 1 ml culture broth) were dispersed in 1 ml of phosphate buffer (50 mM, pH 7.5) and used as biocatalysts.

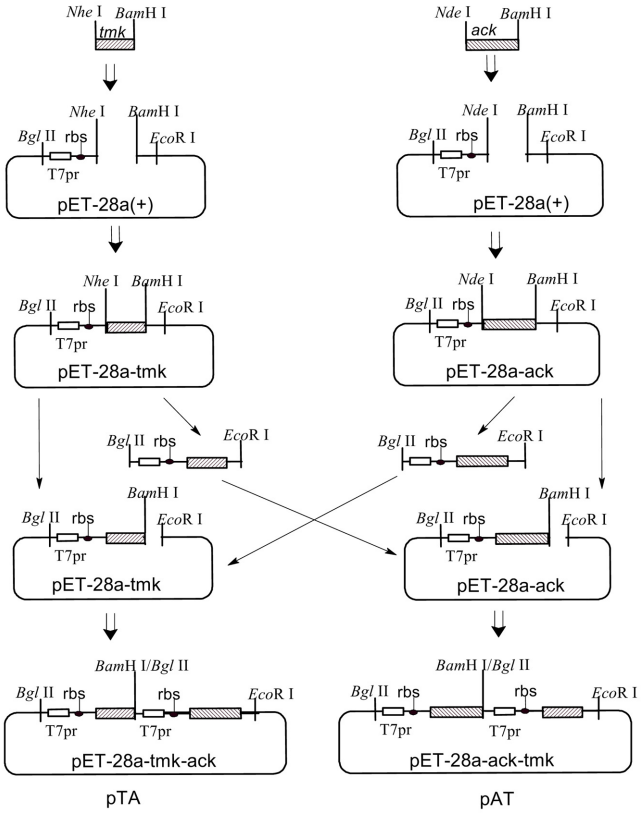

Fig. 2. Construction of multi-promoter vectors with the pET28a-derived BioBrick base vector.
T7pr: T7 promoter. rbs: ribosome binding site.

Activity Assay of TMKase and ACKase

The TMKase activity assay was performed as previously described [29] but with some modification. ACKase activity was assayed as previously reported [35]. TMKase activity in the intact cells of pAT or pTA treated with EDTA, toluene, and Triton X-100 was determined by measuring the amount of dTDP formed at 37°C in a reaction system of 1 ml containing 5 mM 5′-dTMP, 5 mM ATP, 50 mM (pH 7.5) potassium phosphate buffer, 10 μl of pAT, and 5 mM Mg^{2+}. The mixture was heated in boiling water for 2 min to stop the reaction and then diluted with double-distilled water (1:50) to detect the synthesis of dTDP. The ACKase activity was analyzed according to the amount of ATP produced at 37°C in a reaction system of 1 ml containing 5 mM ADP, 15 mM ACP, 50 mM (pH 7.5) potassium phosphate buffer, 0.25 μl of pAT, and 5 mM Mg^{2+}.

One unit (U) of TMKase or ACKase activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of product per minute under the above conditions. The specific activity was defined as U/mg dry cell weight (DCW) cells. Relative specific activity (%) = specific activity of TMKase or ACKase of pAT or pTA treated with EDTA, toluene, or Triton X-

100 (U/mg)/specific activity of TMKase or ACKase under sonication.

Biosynthesis of 5′-dTTP

One milliliter of reaction solution, which contained 5 mM 5′-dTMP, 0.125 mM ATP, 15 mM ACP, 10 mM Mg^{2+}, 20 µl of pAT, and 50 mM (pH 7.5) potassium phosphate buffer, was submerged in a water bath at 37°C for 10 h. The reaction was stopped by heating the mixture in boiling water for 2 min. The products were detected using high-performance liquid chromatography (HPLC) after the reaction solution was diluted 50 times with double-distilled water.

Analytical Method

Nucleotides were analyzed *via* HPLC (Agilent 1200) with an ultraviolet detector at 254 nm. The column was Hypersail SAX (5 µm, 4.6 × 250 mm), and the mobile phase was 30 mM $NH_4H_2PO_4$ (pH 4.5). The flow rate was 1 ml/min. The retention times for dTMP, dTDP, and dTTP were 3.6, 4.9, and 6.8 min, respectively.

Results

Coexpression of TMKase and ACKase by pTA or pAT

ACKase exhibited good expression in both pTA and pAT, as shown in Fig. 3, when it was induced at 37°C and 0.5 mM IPTG was used as an inducer. Unlike ACKase, TMKase exhibited different expression profiles in pTA and pAT. In pAT, no inclusion bodies of TMKase were found, but the amount of protein expression was not the same as that of ACKase. However, under the same conditions, pTA produced a large number of inclusion bodies of TMKase, although the total expression level of TMKase was slightly higher than that of ACKase.

To improve the soluble expression of TMKase in pTA, we lowered the culture temperature and reduced the inducer concentration. At 26°C, the amount of inclusion bodies was similar to that at 37°C, although the IPTG concentration was lowered. TMKase exhibited some soluble expression at 16°C, but part of the expression was still inclusion bodies. After lowering the IPTG concentration at 16°C, TMKase exhibited higher soluble expression; the result is shown in Fig. 4. For 0.05 mM IPTG (below this concentration, no expression was found), very few inclusion bodies were observed in pTA. Owing to the harsh inducing conditions, pTA was not a good candidate for the coexpression of TMKase and ACKase. Thus, only pAT was used in the following experiments

Permeabilization of pAT

Owing to the barrier of the cell envelope, some substrates containing phosphate groups, such as nucleotides, cannot enter or exit freely. Unless ACKase and TMKase are released from the cells, they cannot enter the cells to react with dTMP and ATP, which are used to biosynthesize 5′-dTTP. Therefore, the cells must be destroyed, and intact cells are not used as biocatalysts, which presents great difficulty for large-scale production.

To improve the permeability of pAT, EDTA, toluene, and

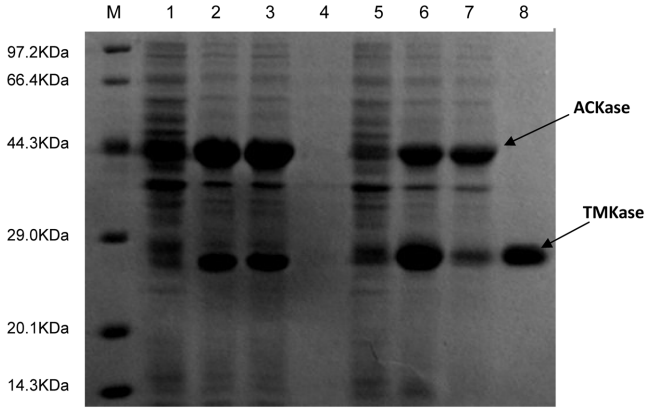

Fig. 3. SDS-PAGE analysis of target proteins.
pAT and pTA were cultured for 6 h after induction with IPTG (0.5 mM) in LB medium at 37°C. Lane M: molecular weight marker; lane 1: recombined bacteria pAT without IPTG; lanes 2, 3, and 4: intact cells, sonicated supernatant, and precipitate of pAT after induction; lane 5: pTA strain without IPTG; lanes 6, 7, and 8: intact cells, supernatant, and precipitation of pTA.

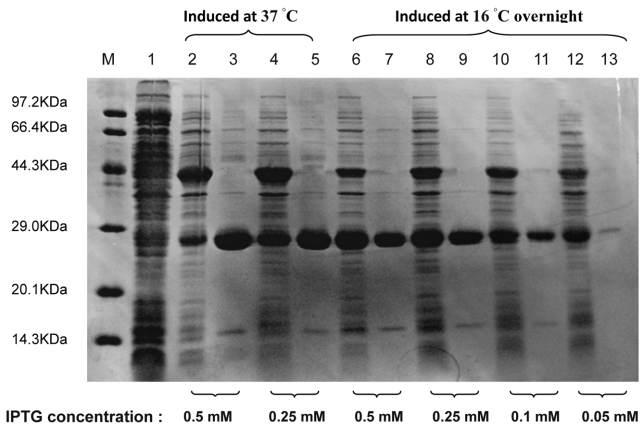

Fig. 4. Recombined bacteria pTA induced at 37ºC and 16ºC overnight with different concentrations of IPTG.
Lane M: molecular weight marker; lane 1: pTA strain without induction; lanes 2, 4, 6, 8, 10, and 12: supernatants of bacteria pTA after induction; lanes 3, 5, 7, 9, 11, and 13: precipitates of pTA after induction.

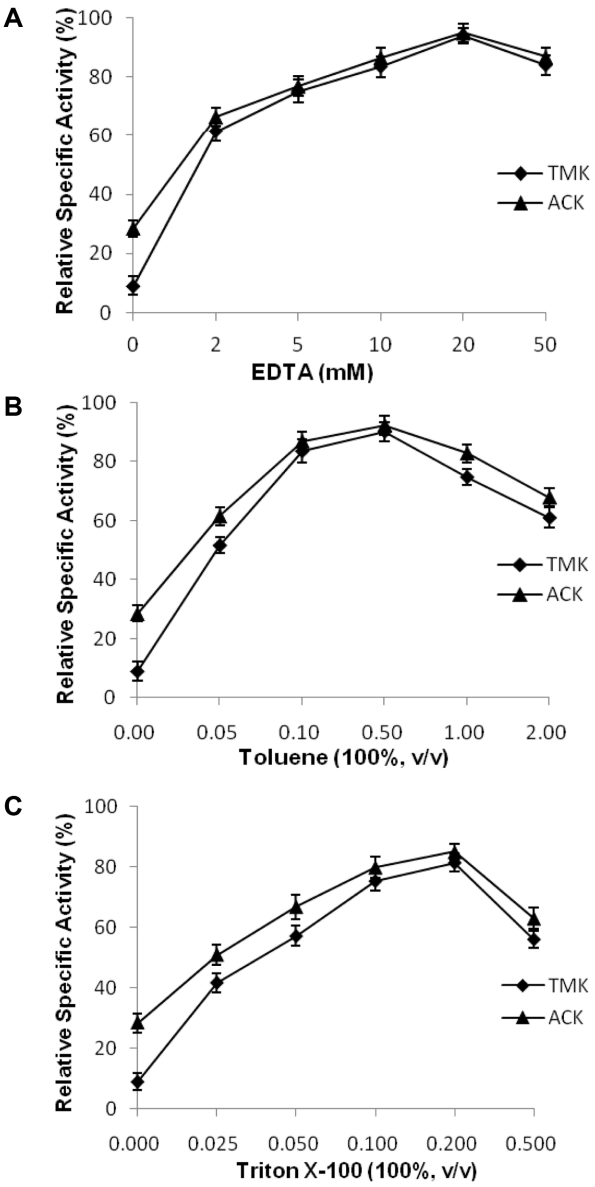

Fig. 5. Relative residual specific activities of TMKase and ACKase in pAT treated with different concentrations of reagents for 30 min.
(**A**) Treated with EDTA. (**B**) Treated with toluene. (**C**) Treated with Triton X-100. A percentage of 100% represents the specific activity of TMKase and ACKase in the ultrasonication supernatant of pAT. All measurements were performed in triplicate.

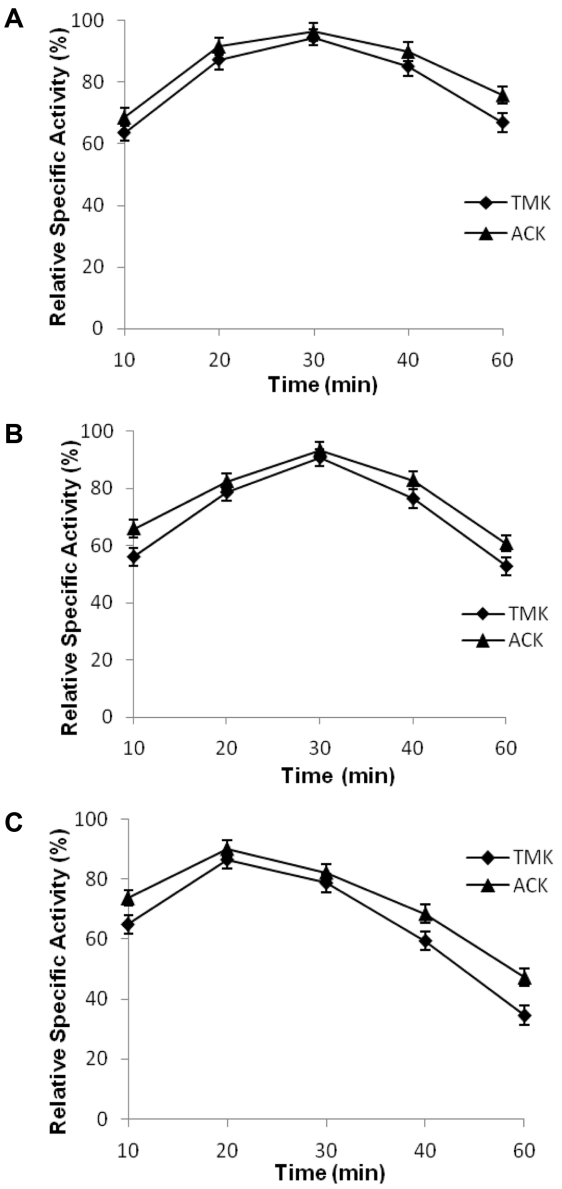

Fig. 6. Relative residual specific activities of TMKase and ACKase in pAT treated with permeation reagents for various amounts of time.
(**A**) Treated with 20 mM EDTA. (**B**) Treated with 0.50% toluene. (**C**) Treated with 0.20% Triton X-100. All measurements were performed in triplicate.

Triton X-100 were chosen to overcome the permeability barrier of the cell envelope. All of these approaches had little effect on the activity of ACKase and TMKase and greatly increased the permeability of pAT. The concentration and time duration of the treatment are important parameters for optimization. As shown in Figs. 5 and 6, after pAT was treated with 20 mM EDTA at 25°C for 30 min, the relative residual specific activities of ACKase and TMKase in the intact cells reached 96.4% and 94.5%, respectively. There was no detectable activity in the supernatant prepared from the treated cells, which indicated that the enzyme protein did not leak from the cells during permeabilization.

The relative specific activities of ACKase and TMKase in the cells treated with 0.5% toluene for 30 min were 93.3% and 90.7%, respectively, and the relative specific activities of ACKase and TMKase in the cells treated with 0.2% Triton X-100 for 20 min were 90.2% and 86.3%, respectively.

Biosynthesis of 5′-dTTP by Intact Cells of Permeable pAT

The biosynthesis of 5′-dTTP catalyzed by whole cells of pAT treated with different permeabilization reagents was highly effective. When equivalent amounts of different permeabilized pAT (calculated as the equivalent activity of TMKase) were added to the reaction mixture at a final volume of 1 ml, the yield of 5′-dTTP was very high (Fig. 7). Among the reactions catalyzed by pretreated pAT, the yield of 5′-dTTP obtained from pAT treated with 20 mM EDTA was 94.1%, closer to the yield obtained from the crude enzyme solution under ultrasonication. The cells treated with EDTA were more stable in the reaction and easy to recover from the reaction solution than those treated with Triton X-100 and toluene. Thus, EDTA was considered to be the better permeation reagent in our study.

Effect of Enzyme Concentration on the Conversion Yields of 5′-dTTP

In our previous study [39], we showed that deoxynucleoside kinase plays a key role in the synthesis of deoxynucleoside monophosphate when it is accompanied by ACKase. Here, we showed that TMKase was a critical enzyme for catalyzing the phosphorylation of 5′-dTMP to the corresponding 5′-dTTP. As the ratio of TMKase to ACKase is fixed in intact pAT cells, the amount of intact pAT cells added to the reaction solution was regulated by the activity of TMKase, and the activity of ACKase subsequently changed.

As shown in Fig. 8, for a TMKase activity equal to or greater than 0.358 U, the yield of 5′-dTTP reached 94% after 4 h. Higher activities of TMKase did not produce higher yields but contributed to the reaction speed. For a TMKase activity of 0.179 U, the yield of 5′-dTTP reached only 82% after 6 h. The product of 5′-dTTP was highly stable in the reaction, and no significant degradation was detected even after the reaction was maintained for 24 h. Thus, this method is very convenient for the synthesis of 5′-dTTP.

Effects of Phosphate Donors and Concentration on the Production of 5′-dTTP

In the process, two phosphate groups were required to convert dTMP to 5′-dTTP, catalyzed by TMKase and ACKase. Acetyl phosphate, which is much cheaper than ATP and exerts little effect on separation, was used to indirectly replace NTP as the phosphate donor. The direct phosphate donor was still NTP. When 0.357 U of TMK and 40.2 U of ACK were added to our reaction system, ATP was the most efficient phosphate donor with the yield of 5′-dTTP of 94.8% (Fig. 9A). If ATP was not added to the reaction solution, 5′-dTTP could not be formed, although intact cells treated with EDTA were used as the catalyst. UTP, CTP, and GTP were also used as direct phosphate donors, but the reaction proceeded slowly, and the yields of 5′-dTTP were only 24.0%, 11.3%, and 6.2%, respectively.

In the reaction system, when 0.063 mM ATP (1/80 initial concentration of dTMP) was added to the reaction, the

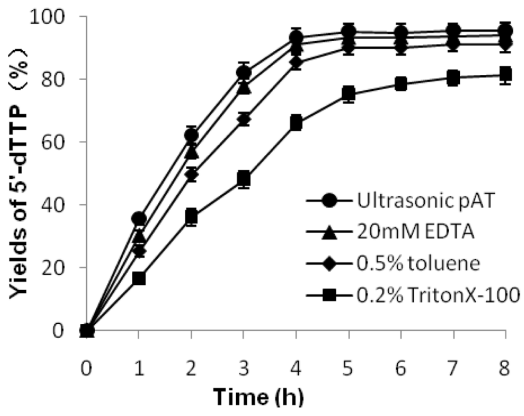

Fig. 7. Production of 5′-dTTP catalyzed by intact pAT cells that underwent various pretreatments.
The cell amount added to reaction was calculated as the activity of TMKase. All measurements were performed in triplicate.

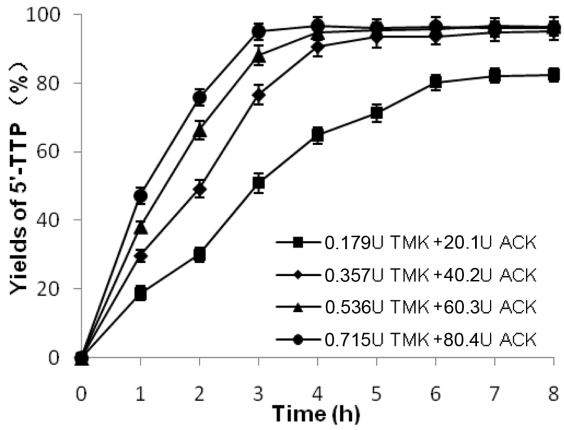

Fig. 8. Effects of varying amounts of intact pAT cells pretreated with 20 mM EDTA on the production of 5′-dTTP.
The amount of cells added to the reaction was calculated as the activity of TMKase. All measurements were performed in triplicate.

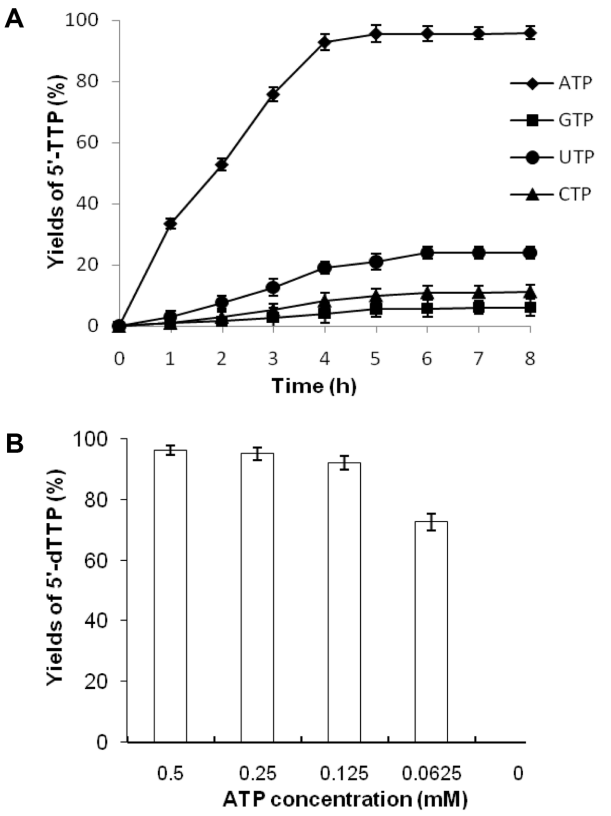

Fig. 9. Effects of phosphate donors on the production of 5′-dTTP.

(**A**) The synthesis of 5′-dTTPs for different phosphate transfers (ATP, GTP, UTP, or CTP). NTP was added to a final concentration of 0.25 mM. (**B**) The effect of ATP concentration on the production of 5′-dTTP. All measurements were performed in triplicate.

yield of 5′-dTTP was 86% after 7 h, and the reaction speed was slow (Fig. 9B). However, when 0.125 mM (1/40 of the initial concentration of dTMP) ATP was added to the reaction, the yield of 5′-dTTP was 94% after 4 h, which is similar to that achieved when 0.25 mM (1/20 initial concentration of dTMP) or 0.5 mM (1/10 initial concentration of dTMP) ATP were used. Therefore, ATP regeneration was highly efficient, as a very small amount of ATP was sufficient for the synthesis of 5′-dTTP in our reaction system.

Discussion

In this study, we proposed an efficient one-pot process for the production of 5′-dTTP using pretreated intact cells that could coexpress high activities and high amounts of TMKase and ACKase. The yield of 5′-dTTP reached 94%, and no significant degradation was detected over the course of the reaction. The process was highly convenient because only one type of strain was needed to be cultured, which reduces the cost of organism fermentation. Moreover, whole cells used as biocatalysts were easy to recycle and elimated the complicated procedures to isolate and purify. Otherwise, no nucleotides intermediates were required to be separated from the reaction solution.

TMKase and ACKase were used as biocatalysts. TMKase can catalyze the phosphorylation of dTMP to form dTDP in both *de novo* and salvage pathways of 5′-dTTP [29]. ACKase is widely used to efficiently regenerate NTP in various biosynthetic processes [9, 24]. In this study, ACKase phosphorylated dTDP to dTTP, and therefore a nucleoside diphosphate kinase was not required in the reaction. To construct a strain that simultaneously coexpressed TMKase and ACKase, *tmk* and *ack* genes from *E. coli* K12 were cloned into a single pET-28a(+) vector.

Although each gene had its own promoter, their expression levels differed. When *tmk* was located downstream of *ack*, both enzymes were expressed in solution form despite the differing amounts of expression. However, when *ack* was located downstream of *tmk*, TMKase was primarily expressed as inclusion bodies. Lower temperatures and concentrations of IPTG yielded some improvement, but the induction conditions were very rigorous, the reason for which remains unclear. The occurrence of this phenomenon is rare, as in most previous coexpression studies, the location of the gene had little effect on expression.

Intact cells are more convenient for synthesizing nucleotides compared with crude enzyme solutions. However, nucleotides cannot freely enter or exit cells owing to the cell wall and cell membrane barriers. Many reagents are reported to relieve this barrier [38]. Among them, we chose EDTA, Triton X-100, and toluene as permeation reagents, which are commonly used in the laboratory. EDTA was found to be the better reagent, although Triton X-100 and toluene also exhibited some potential. The cells treated with 20 mM EDTA for 30 min were more stable in the reaction. The yield of 5′-dTTP in the reaction catalyzed by pAT treated with EDTA was similar to the yield of the reaction catalyzed by ultrasonicated crude enzyme solution.

In our reaction system, ATP was used as the direct phosphate donor and must take part in the transfer of phosphate groups for the formation of 5′-dTTP. Because ATP is unstable and expensive, it can be regenerated from ADP and ACP, catalyzed by ACKase. Only a small amount of ATP (1/40 of the original concentration of thymidylate) was required to satisfy the reaction. Although several types of NTP regeneration have been reported [25, 26], ACKase is

considered to be the best. The phosphate donor ACP can be synthesized according to the method of ATP regeneration at low cost. In addition, the use of small amounts of ATP is beneficial in that there is little interference in the separation of 5′-dTTP. In conclusion, 5′-dTTP was efficiently produced from deoxythymidine-5′-monophosphoric acid catalyzed by permeabilized pAT that coexpressed TMKase and ACKase. Our study identified a high-efficiency and one-pot biosynthesis method for 5′-dTTP that is both convenient and economic.

Acknowledgments

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