

Fermentative Production of Thymidine by a Metabolically Engineered *Escherichia coli* Strain[∇]

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Thymidine is an important precursor in the production of various antiviral drugs, including azidothymidine for the treatment of AIDS. Since thymidine-containing nucleotides are synthesized only by the de novo pathway during DNA synthesis, it is not easy to produce a large amount of thymidine biologically. In order to develop a host strain to produce thymidine, thymidine phosphorylase, thymidine kinase, and uridine phosphorylase genes were deleted from an *Escherichia coli* BL21 strain to develop BLdtu. Since the genes coding for the enzymes related to the nucleotide salvage pathway were disrupted, BLdtu was unable to utilize thymidine or thymine, and thymidine degradation activity was completely abrogated. We additionally expressed T4 thymidylate synthase, T4 nucleotide diphosphate reductase, bacteriophage PBS2 TMP phosphohydrolyase, *E. coli* dCTP deaminase, and *E. coli* uridine kinase in the BLdtu strain to develop a thymidine-producing strain (BLdtu24). BLdtu24 produced 649.3 mg liter⁻¹ of thymidine in a 7-liter batch fermenter for 24 h, and neither thymine nor uridine was detected. However, the dUTP/dTTP ratio was increased in BLdtu24, which could lead to increased double-strand breakages and eventually to cell deaths during fermentation. To enhance thymidine production and to prevent cell deaths during fermentation, we disrupted a gene (encoding uracil-DNA *N*-glycosylase) involved in DNA excision repair to suppress the consumption of dTTP and developed BLdtug24. Compared with the thymidine production in BLdtu24, the thymidine production in BLdtug24 was increased by ~1.2-fold (740.3 mg liter⁻¹). Here, we show that a thymidine-producing strain with a relatively high yield can be developed using a metabolic engineering approach.

Thymidine, which is composed of 2-deoxyribose and a thymine base, is a commercially useful precursor in the chemical synthesis of various antiviral drugs, including stavudine and zidovudine (azidothymidine), the active ingredient in a formulation for the treatment of AIDS (18, 19). Because thymidine is required only in DNA synthesis, intracellular thymidine levels are very low and are tightly controlled (40). For the production of precursors for antiviral drugs, thymidine is either biologically produced in a low yield by a few modified microorganisms or chemically synthesized through a very costly process (17, 33, 48, 49). Thus, there is a need for developing a more efficient strain for thymidine production on a large scale.

In nature, there are two distinct pathways for dTTP synthesis, the salvage and de novo pathways. The salvage pathway enables the cells to utilize preformed nucleobases and nucleosides for nucleotide synthesis, using thymidine phosphorylase (*deoA*), uridine phosphorylase (*udp*), and thymidine kinase (*tdk*) (Fig. 1) (40).

As the name indicates, the de novo pathway enables the cells to synthesize nucleobases de novo. The de novo pathway leading to thymidine biosynthesis starts with the condensation of aspartate and carbamoylphosphate, synthesized by carbamoylphosphate synthase (*pyrA*) (41). This condensation reaction is catalyzed by aspartate-carbamoyl transferase (*pyrBI*) to produce carbamoyl aspartate, which undergoes several re-

actions to produce UMP, the common precursor for the synthesis of the pyrimidine ribonucleoside and deoxynucleosides (Fig. 1) (39–41). For thymidine biosynthesis, UMP is converted to UDP in a reaction catalyzed by UMP kinase (*pyrH*), and UDP is converted to dUDP by ribonucleoside diphosphate reductase (*nrdAB*), which is regulated by NTP effectors through binding to specific allosteric sites on ribonucleotide diphosphate reductase (*nrdA*). *Escherichia coli* can synthesize dUMP from both dCDP and dUDP. The major pathway involves phosphorylation of dCDP to dCTP, deamination of dCTP to dUTP, and hydrolysis of dUTP to dUMP. Only 20 to 30% of the cellular dUMP is supplied by hydrolysis of dUTP (29, 37). The deamination of dCTP (*dcd*) is located at a branch point in the pyrimidine metabolic pathway. Because of its importance, *dcd* is regulated by a positive homotropic cooperativity toward dCTP and by a feedback inhibition by dTTP (29, 31, 40).

Deoxyuridine triphosphatase (dUTPase [*dut*]) is a pyrophosphatase that contains zinc ions (42). dUTPase catalyzes the hydrolysis of dUTP to PP_i and dUMP, a substrate for thymidylate synthase (*thyA*). Generally, the intracellular concentration of dUTP is <10 nmol per 1 g dry cell weight (DCW), and that of dTTP exceeds 500 nmol per 1 g DCW (5, 39, 52). The intracellular dUTP-to-dTTP ratio is increased in *dut*-deficient mutants, leading to an increased frequency of misincorporation of uracil for thymine in DNA (34). This incorporation is transient only because uracil is removed from DNA via a subsequent excision repair initiated by uracil-DNA *N*-glycosylase, which is encoded by *ung* (15, 50). Attempted repair of deoxyuridine residues from DNA without adequate dTTP available to complete the repair reaction can result in multiple single-strand breaks, eventually leading to double-strand

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TABLE 1. Strains and plasmids

Strain or plasmid	Description	Source or reference
Strains		
BL21 Star(DE3)	F ⁻ <i>ompT hsdS_B(r_B⁻ m_B⁻) gal dcm rne131</i> (DE3)	Invitrogen
XL1-Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> [F' <i>proAB lacI^qΔM15 Tn10</i> (Tet ^r)]	Stratagene
BW25141	<i>lacI^q rrrB_{T14} ΔlacZ_{WJ16} ΔphoBR580 hsdR514 ΔaraBAD_{AH33} ΔrhaBAD_{LD78} galU95 endA_{BT333} uidA(ΔMluI)::pir⁺ recA1</i>	22
BLd	BL21 <i>ΔdeoA</i>	This study
BLdt	BL21 <i>ΔdeoA Δtdk</i>	This study
BLdtu	BL21 <i>ΔdeoA Δtdk Δudp</i>	This study
BLdtu1	BLdtu harboring pETD::Td	This study
BLdtu2	BLdtu harboring pETD::TdNr	This study
BLdtu23	BLdtu2 harboring pACD::DU	This study
BLdtu24	BLdtu2 harboring pACD::DUTm	This study
BLdtu24 (<i>dut</i>)	BLdtu2 harboring pACD::DUTmUt	This study
BLdtug24	BLdtu24 <i>Δung</i>	This study
Plasmids		
pETDuet	ColE1 replicon, <i>bla</i>	Novagen
pACYCDuet	P15A replicon, <i>cat</i>	Novagen
pKD3	Template plasmid, derivative of pANTS _γ , FRT-flanked <i>cat</i>	13
pKD20	λ Red helper plasmid, derivative of pINT-ts, <i>araC-P_{araB}</i> and γ β <i>exo</i> DNA fragments	13
pCP20	<i>bla</i> and <i>cat</i> , <i>ori</i> (Ts), thermal inducible FRT recombinase	13
pETD::Td	pETDuet T4 TDΔI expression under <i>tac</i> promoter, <i>bla</i>	This study
pETD::TdNr	pETDuet T4 TDΔI, T4 <i>nrdCAB</i> operon expression under <i>tac</i> promoter, <i>bla</i>	This study
pACD::DU	pACYCDuet <i>udk-dcd</i> operon expression under <i>tac</i> promoter, <i>cat</i>	This study
pACD::DUTm	pACYCDuet <i>udk-dcd</i> operon, PBS2 TMPase expression under <i>tac</i> promoter, <i>cat</i>	This study
pACD::DUTmUt	pACYCDuet <i>udk-dcd</i> operon, <i>dut</i> , PBS2 TMPase expression under <i>tac</i> promoter, <i>cat</i>	This study

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and plasmids. *E. coli* strains and plasmids used in this study are listed in Table 1. *E. coli* BL21 Star (Invitrogen, Groningen, The Netherlands) was used as the host for gene disruption and expression of foreign genes. *E. coli* XL1-Blue (Stratagene, La Jolla, CA) was used as the cloning host in construction of expression vectors and was grown in Luria-Bertani (LB) medium (5 g liter⁻¹ yeast extract, 10 g liter⁻¹ Bacto tryptone, and 10 g liter⁻¹ NaCl). Conditional replicative *oriRγ* plasmids were maintained in the *pir⁺* *E. coli* BW25141 host strain (22). Bacteriophage PBS2 was obtained from the *Bacillus* Genetic Stock Center (Department of Biochemistry, The Ohio State University). *Bacillus subtilis* SB19 (ATCC 23856) and bacteriophage T4 (ATCC 11303-B4) were used for the propagation of PBS2 and the amplification of genes, respectively.

The template plasmid pKD3 is a derivative of pANTS_γ that contains an FLP recombinase target (FRT) flanking chloramphenicol resistance gene (13, 43). The λ Red helper plasmid, pKD20, is a derivative of pINT-ts which contains *araC-P_{araB}* and γ β *exo* DNA fragments that were PCR generated by using pBAD18 and λ DNA as templates, respectively (24). pCP20 is an ampicillin- and chloramphenicol-resistant plasmid that shows temperature-sensitive replication and thermal induction of FLP synthesis (9).

The *E. coli* expression vectors pETDuet (Novagen, Madison, WI) and pACYCDuet (Novagen) were used to express foreign genes in *E. coli*. The pETDuet derivatives contain the origin of ColE1, an ampicillin resistance gene,

and foreign genes. The pACYCDuet derivatives contain the origin of P15A, a chloramphenicol resistance gene, and foreign genes.

PCR-mediated gene disruption. PCR-mediated disruptions of genes were done with slight modifications to the method previously reported (13, 38). PCR fragments which contain a selectable marker flanked by 70-nucleotide (nt)-long oligonucleotides that were comprised of 50-nt-long homology extensions and 20-nt priming sequences for the template, pKD3, were generated. Oligonucleotides used for the generation of gene disruption fragments are shown in Table 2. PCR mixtures containing 1 U thermostable DNA polymerase (Roche Molecular Biomedicals, Basel, Switzerland), 20 mM Tris (pH 8.4), 1.5 mM MgCl₂, 1 μM gene disruption primers, 0.2 mM deoxynucleoside triphosphates, and 0.5 μg ml⁻¹ of plasmid pKD3 were incubated at 94°C for 5 min, followed by 30 cycles at 94°C (45 s), 55°C (45 s), and 72°C (1 min), and followed by a final extension time of 10 min at 72°C.

Transformants carrying a λ Red helper plasmid (pKD20) were grown in 5 ml SOB medium (5 g liter⁻¹ yeast extract, 20 g liter⁻¹ Bacto tryptone, 0.5 g liter⁻¹ NaCl, 0.19 g liter⁻¹ KCl, and 0.95 g liter⁻¹ MgCl₂ [pH 7.0]) with 50 mg liter⁻¹ ampicillin and 100 g liter⁻¹ L-arabinose at 30°C to an optical density at 600 nm (OD₆₀₀) of approximately 0.6 and then were made electrocompetent by concentrating 100-fold and washing three times with ice-cold 10% glycerol. The respective PCR products were purified, digested with DpnI, and then transformed into electrocompetent *E. coli* BL21 Star carrying a λ Red helper plasmid. Electroporation was done by using a MicroPulser (Bio-Rad Laboratories, Inc., Hercules,

TABLE 2. Oligonucleotides for gene disruption

Gene	Direction	Sequence ^a
<i>ΔdeoA</i>	Forward	<u>GTTTCAAACCGGGCGGGCGCGTACTGCGGAAGATGCGCAGAAATATGTGTAGGCTGGAGCTGCTTC</u>
	Reverse	<u>GACGGGTCAGATTTGGCAGATTGAGCGGGCCCTTACGACCGTTATCAGCTCATATGAATATCCTCCTTAG</u>
<i>Δtdk</i>	Forward	<u>ATGGCACAGCTATATTTCTACTATTCCGCAATGAATGCGGGTAAAGTCTACGCTGAGGCTGGAGCTGCTTC</u>
	Reverse	<u>TTAAGCGTGGCGATGCCTTTCCTGAATAGCCGTTAATGAGTTCGACTTGTACATATGAATATCCTCCTTAG</u>
<i>Δudp</i>	Forward	<u>TGATGTTTTTCATCTCGGCCCTCACTAAAAACGATTTACAAGGGGCTACGCGTGTAGGCTGGAGCTGCTTC</u>
	Reverse	<u>GAATTACAGCAGACGACGCGCCGCTTCCACCACGATTTTACCAGCATGGCCATATGAATATCCTCCTTAG</u>
<i>Δung</i>	Forward	<u>TGATGTTTTTCATCTCGGCCCTCACTAAAAACGATTTACAAGGGGCTACGCGTGTAGGCTGGAGCTGCTTC</u>
	Reverse	<u>GAATTACAGCAGACGACGCGCCGCTTCCACCACGATTTTACCAGCATGGCCATATGAATATCCTCCTTAG</u>

^a Underlined sequences are priming site sequences for template pKD3.

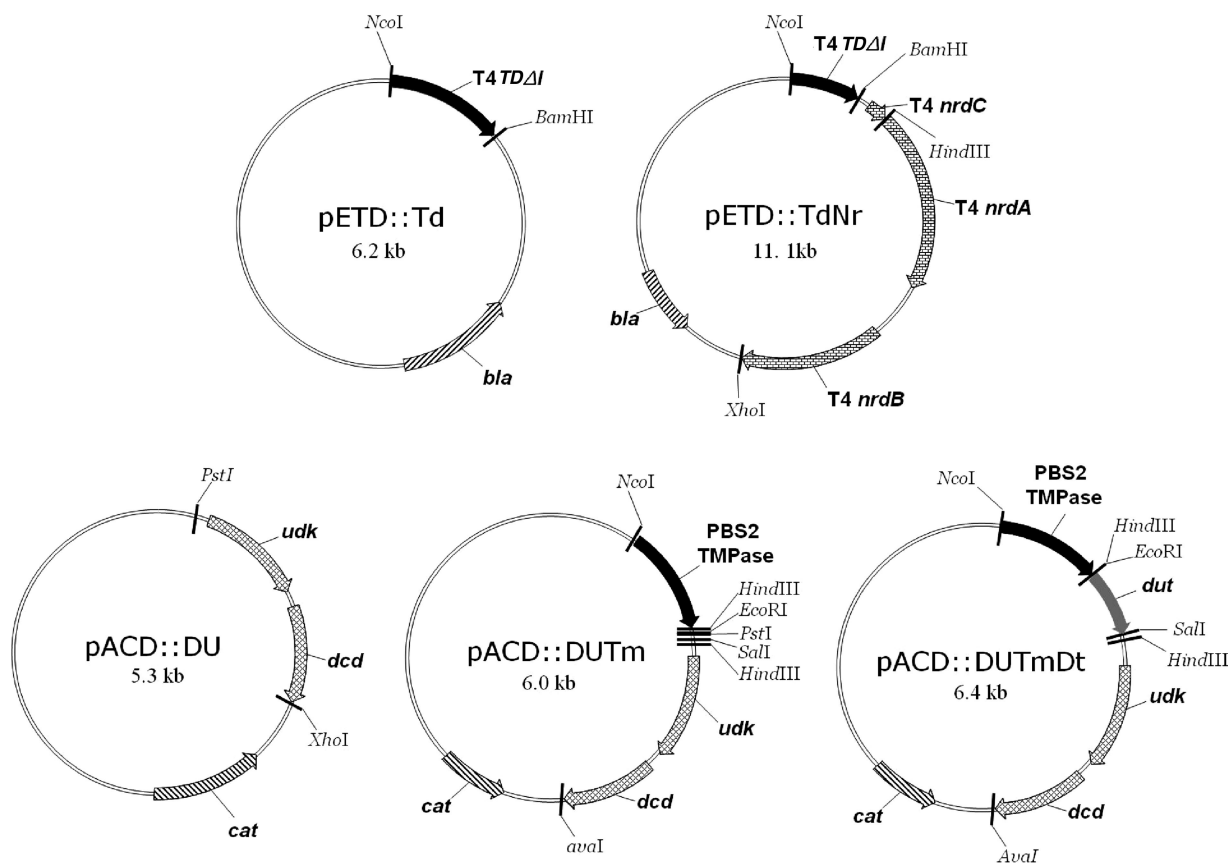


FIG. 2. Maps of plasmids. Plasmid pETDuet was used for the expressions of T4 TDΔI and the T4 *nrdCAB* artificial operon. Plasmid pACYCDuet was used for the expressions of the *udk-dcd* operon, the PBS2 TMPase gene, and *dut*.

CA) with a voltage booster and 0.2-cm chambers using 50 μ l of cells and 10 to 100 ng of PCR product, according to the manufacturer's instructions. After electroporation, the cells were grown in 1 ml SOC medium (SOB medium containing 20 mM glucose) to recover the phenotype for 2 h at 37°C, and then one-half was spread onto LB agar plates with 5 μ g ml⁻¹ chloramphenicol to select chloramphenicol-resistant transformants. After primary selection, mutants were maintained on medium without antibiotics. To remove helper plasmid, individual colonies were purified nonselectively at 37°C.

Elimination of the antibiotic resistance gene. Chloramphenicol-resistant mutants selected on plates were transformed with pCP20, and ampicillin-resistant transformants were selected at 30°C, after which a few colonies were nonselectively purified at 43°C and then investigated for loss of all antibiotic resistances. The majority lost the FRT-flanked resistance gene and the FLP helper plasmid, simultaneously (13). Gene disruption and marker elimination were confirmed by using the appropriate antibiotic markers and PCR analysis.

Expression vector construction. Standard procedures were used for plasmid preparation, restriction enzyme digestions, ligations, transformations, and agarose gel electrophoresis. The T4 thymidylate synthase gene (TD gene) was amplified from the T4 phage by PCR (1,878 bp), and then TDΔI was prepared with deletion of the intron (550 bp to 1,566 bp region) by a gap deletion method for obtaining an active enzyme, since the T4 TD gene is transcribed as preform mRNA and then the intron is deleted by self-splicing (4, 8, 12). The T4 *nrdC* gene and the T4 *nrdA-nrdB* operon were amplified by PCR with the flanking primers including 6-nt restriction site extensions for easy cloning and ligated to each other with a ribosome binding site to construct an artificial operon (*nrdC-nrdA-nrdB*). TDΔI and the *nrdC-nrdA-nrdB* operon were sequentially ligated to the pETDuet expression vector, according to the standard protocol. The PBS2 TMPase gene, *dut*, and the *udk-dcd* operon were amplified by PCR with the flanking primers from each source, and they were sequentially ligated to the pACYCDuet expression vector (Fig. 2). The plasmids pETD::Td, pETD::TdNr, pACD::DU, pACD::DUTm, and pACD::DUTmDt were sequenced by the dideoxy chain termination method with an automatic DNA sequencer to confirm proper cloning.

Each expression vector derivative was transformed into BLdty by the electroporation method, as described in Materials and Methods. These recombinant strains permit transcription from the *tac* promoter in the presence of IPTG (isopropyl- β -D-thiogalactopyranoside).

Thymidine degradation assay and TMP phosphohydrolase assay. Equal amounts of transformed cells were harvested by centrifugation and washed with 10 mM Tris (pH 7.4) containing a protease inhibitor mixture (Complete; Amersham Pharmacia, Uppsala, Sweden). Each enzyme solution was prepared by sonication, and its protein concentration was determined by the Lowry assay. For the thymidine degradation assay, 100 μ l of 1 mM thymidine was mixed with 100 μ l of enzyme solution (1.2 mg ml⁻¹), and the reaction mixture was incubated at 30°C for 1 h, 4 h, and 18 h. The enzyme reaction was subsequently stopped by filtration with polyvinylidene difluoride, and the residual thymidine concentration was measured in the reaction mixture.

For the TMPase assay, the inorganic phosphate released from dTMP was measured by colorimetric assay, as previously described (44). One unit of enzyme catalyzed the hydrolysis of 1 μ mol of TMP per minute at 37°C.

RNA isolation and reverse transcription-PCR (RT-PCR). Total cellular RNA was extracted from mid-log-phase cells with a Qiagen RNeasy mini kit (Qiagen, Hilden, Germany) as described by the manufacturer. RNase-free DNase I (Takara Bio, Shiga, Japan) was treated during the isolation procedure to eliminate possible DNA contamination. Absence of DNA was verified by control PCRs, using the RNA as a template. RNA preparation was diluted to a final concentration of 1 μ g μ l⁻¹, and reverse transcription was performed with an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA), using random primer mix, according to the manufacturer's instructions. The sequences of the genes studied were obtained from GenBank, and the primers were designed with the aid of the OLIGO software (version 5.0; Molecular Biology Insights). The PCR was performed under the appropriate condition for each target gene. Relative transcript ratios were quantified using *rpoD* as an internal control. A negative control was included in all PCR assays.

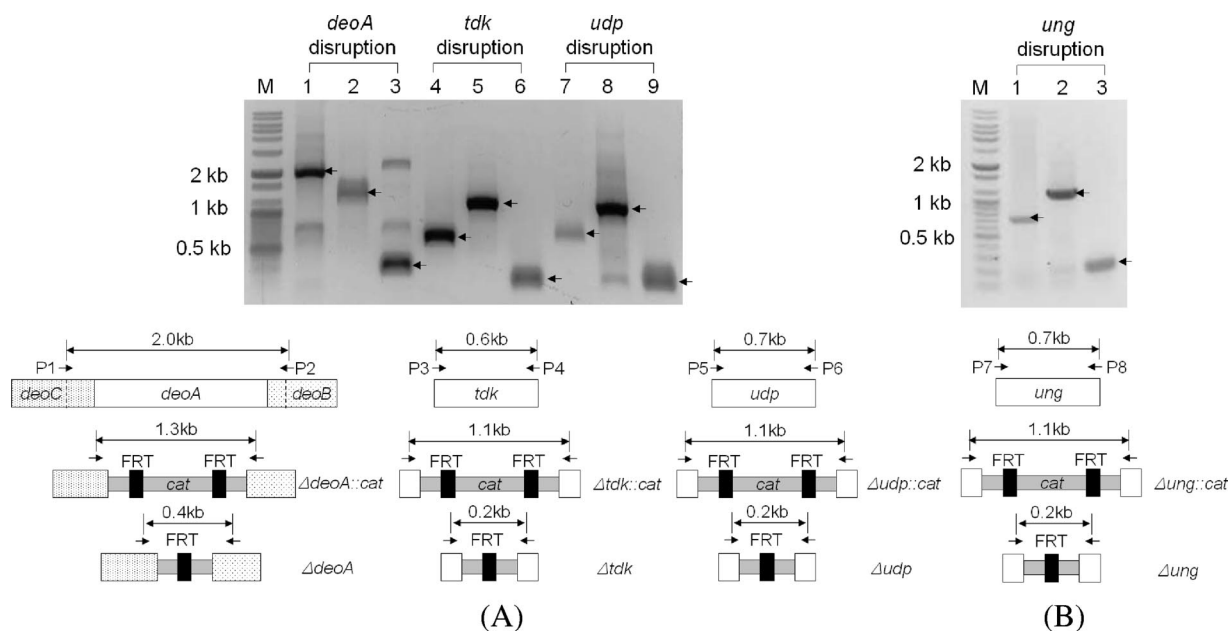


FIG. 3. PCR analysis of three disruption mutants. P1 to P8 refer to priming sites. PCR amplification for identifying the deletion of each target gene was performed using each *E. coli* BL21 mutant genomic DNA as a template. (A) Salvage pathway gene disruptions. M, size marker; lane 1, *deoC* and *deoB* region including *deoA* (2.0 kb); lane 2, $\Delta deoC::cat::\Delta deoB$ (1.3 kb); lane 3, $\Delta(deoC-deoB)$ (0.4 kb), represented to $\Delta deoA$; lane 4, *tdk* (0.6 kb); lane 5, $\Delta tdk::cat$ (1.1 kb); lane 6, Δtdk (0.2 kb); lane 7, *udp* (0.7 kb); lane 8, $\Delta udp::cat$ (1.1 kb); lane 9, Δudp (0.2 kb). The FRT (black bar)-flanked chloramphenicol-resistant gene was amplified by PCR. The linear disruption PCR fragment (gray bar) was transformed into a strain expressing the λ Red recombinase, and then chloramphenicol-resistant transformants were selected. The selective marker was eliminated by FLP recombinase system. (B) *ung* disruption. Lane 1, *ung* (0.7 kb); lane 2, $\Delta ung::cat$ (1.1 kb); lane 3, Δung (0.2 kb).

Culture condition for thymidine production. A suspension of cells was inoculated into a 250-ml flask containing 50 ml of LB medium and incubated at 37°C and 250 rpm for 8 h. For flask cultures, a 5-ml aliquot of culture broth was transferred to a 500-ml baffled flask containing 50 ml of production medium (60 g liter⁻¹ glycerol as carbon source, 10 g liter⁻¹ CaCO₃, 10 g liter⁻¹ yeast extract, 0.4 g liter⁻¹ MgSO₄ · 7H₂O, 14.84 g liter⁻¹ soytone, 0.24 g liter⁻¹ phenol red, 50 μg ml⁻¹ chloramphenicol, 50 μg ml⁻¹ ampicillin, and trace elements) and 0.5 mM IPTG for induction and was then incubated at 37°C and 250 rpm for 24 h.

For jar fermenter culture, cells grown in regular LB media in exponential phase were inoculated to the production medium to a final concentration of 10%. All cultivations were performed in a 7-liter jar fermenter (KoBioTech Co., Ltd., Incheon, South Korea) with a volume of 3.5 liters and a stirring rate of 500 rpm for 2 h that was then increased to 800 rpm (two six-blade Rushton-type impellers) at 37°C. The pH was controlled at a minimum of 7.5 with 3 M NH₄OH. Thymidine concentration in the medium was determined from the supernatant of cultivation samples. Dissolved oxygen and pH levels were measured with a dissolved-oxygen analyzer (KoBioTech Co., Ltd., Incheon, South Korea) and a pH meter (Mettler Toledo Co., Ltd.).

Analytical methods. Biomass measured by the OD₆₀₀ was converted to DCW by using a standard curve (1.0 OD₆₀₀ = 0.45 g DCW liter⁻¹). The quantitation of bases and nucleosides was analyzed by high-pressure liquid chromatography (Waters 2690; Waters Co., Milford, MA) using an octyldecyl silane MG 5-μm column (4.6 by 250 mm; Shiseido Co., Ltd., Tokyo, Japan) and a UV detector (Waters 2487; Waters Co.) (33). Samples were eluted isocratically with 4% (vol/vol) acetonitrile containing 0.05% (vol/vol) trifluoroacetic acid at a flow rate of 1 ml min⁻¹ and detected at 260 nm. Intracellular dUTP and dTTP levels were measured by high-pressure liquid chromatography as previously reported (20).

RESULTS

Disruption of *deoA*, *tdk*, and *udp* encoding salvage enzymes.

Disruption of *deoA* was performed using a linear PCR fragment with 50 nt of homology extensions. Using a 1.3-kb fragment, including the 3' homologous region of *deoC*, the 5' homologous region of *deoB*, and the *cat* gene, *deoA* was suc-

cessfully deleted by gene replacement. Marker elimination was performed using FRT recombinase induced by a temperature shift from 30°C to 42°C in the absence of antibiotic selection. The removal of antibiotic marker was confirmed by PCR amplification of the flanking region of the knocked-out gene. Both *tdk* and *udp* were disrupted sequentially by the same method, and the knockout of genes was confirmed by the same method (Fig. 3A).

The salvage pathway gene-disrupted strains of *E. coli*, namely, BLΔ ($\Delta deoA$), BLΔt ($\Delta deoA \Delta tdk$), and BLΔtu ($\Delta deoA \Delta tdk \Delta udp$), were prepared and analyzed by in vitro thymidine degradation assays. The thymidine in the wild-type *E. coli* strain BL21 Star was almost completely degraded to thymine after 4 h (Fig. 4). On the other hand, single disruption of the *deoA* gene reduced the rate of degradation of thymidine. The residual thymidine concentrations in the BLΔ strain decreased slowly for 4 h and remained constant at approximately 70% thereafter. The degradation rate of the BLΔt strain was almost the same as that of BLΔ, which is consistent with the fact that Tdk does not participate in thymidine degradation. As shown in Fig. 4, the disruption of *deoA* alone was not enough to prevent the degradation of thymidine. Since *deoA* and *udp* have enough activities to degrade thymidine in *E. coli* (40), we constructed another strain (BLΔtu), in which two genes encoding major enzymes involved in thymidine degradation were disrupted. The result showed that thymidine degradation was completely prevented, not by *deoA* disruption only but by disruption of both *deoA* and *udp*. DeoA and Udp were found to contribute to approximately 60 to 70% and approximately 30

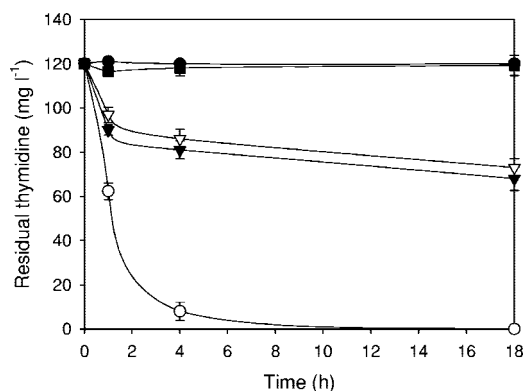


FIG. 4. Thymidine degradation assay. Negative control with thymidine (●), BL21 Star (○), BLd (▼), BLdt (▽), and BLdtu (■). Values are means \pm standard deviations of the results from triplicate experiments.

to 40% of thymidine degradation activity after incubation for 18 h, respectively.

Expression of de novo thymidine biosynthetic enzymes in a strain with the salvage pathway-deficient background. Knocking out three genes (*deoA*, *tdk*, *udp*) involved in the salvage pathway completely abolished thymidine degradation activities, which led to the production of a negligible or small amount of thymidine (Fig. 5). Even though a very small amount of thymidine was produced in the salvage pathway-deficient strain (BLdtu), the inactivation of this pathway can prevent thymidine degradation, so it may be helpful to increase thymidine production, combined with overexpressing foreign genes that are involved in thymidine biosynthesis.

Since ThyA is known to be the committed enzyme to the pyrimidine de novo pathway in *E. coli*, and since NrdAB is known to be a highly regulated enzyme, increasing the activities of both enzymes could be an effective method to increase thymidine production. Therefore, our strategy to enhance thymidine production involves a combinatorial expression of de novo thymidine biosynthetic pathway genes in a salvage pathway-deficient BLdtu strain. In order to enhance enzyme activities efficiently, the genes encoding intron-deleted thymidylate

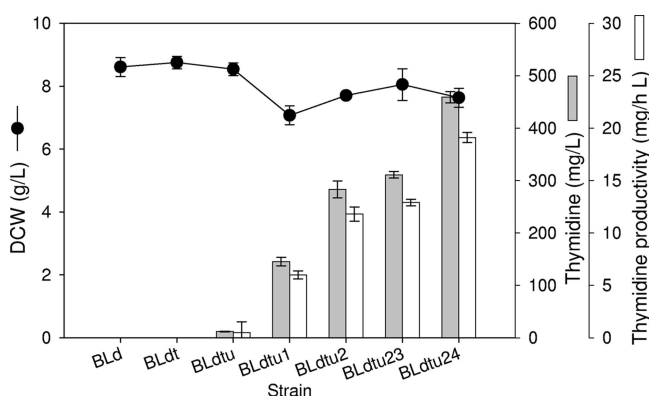


FIG. 5. Thymidine production by recombinant *E. coli* strains. Values are means \pm standard deviations of the results from triplicate experiments. Thymidine productivity was defined as the produced thymidine concentration per 1 h.

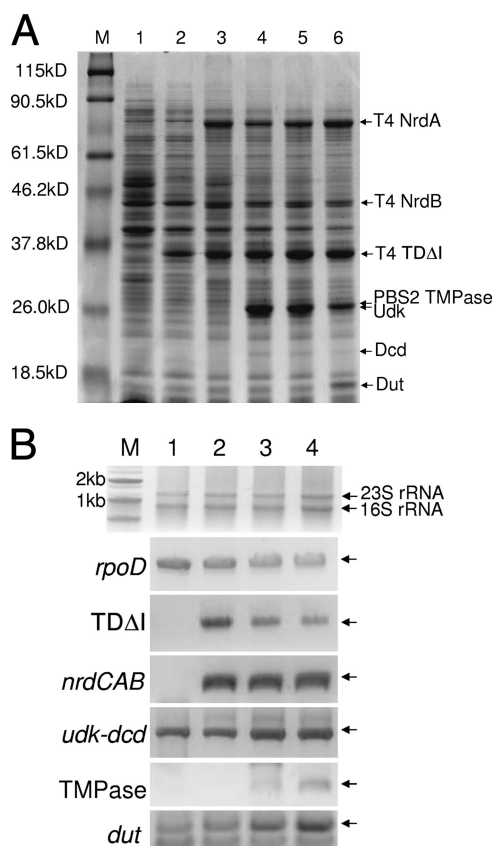


FIG. 6. Expression of genes for thymidine de novo biosynthesis in recombinant *E. coli* strains. (A) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of overexpressing proteins. M, molecular-weight size marker; lane 1, BLdtu; lane 2, BLdtu1; lane 3, BLdtu2; lane 4, BLdtu23; lane 5, BLdtu24; lane 6, BLdtu24 (*dut*). T4 NrdA, T4 nucleotide diphosphate reductase subunit α ; T4 NrdB, T4 nucleotide diphosphate reductase subunit β ; T4 TD Δ I, T4 thymidylate synthase (intron deleted); PBS2 TMPase, PBS2 TMP phosphohydrolyase; Udk, *E. coli* uridine kinase; Dcd, *E. coli* dCTP deaminase; Dut, *E. coli* deoxyribonucleotide triphosphatase. (B) RT-PCR analysis of mRNA transcription. M, size marker; lane 1, BLdtu; lane 2, BLdtu2; lane 3, BLdtu24; lane 4, BLdtu24 (*dut*).

synthase (TD Δ I), ribonucleotide diphosphate reductase (NrdAB), and thioredoxin (NrdC) from T4 phage were cloned and expressed in BLdtu under the control of the *tac* promoter. The expression of each enzyme was identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 6A). In order to verify whether introduced genes were being reliably expressed, RT-PCR was performed to identify the presence of mRNA transcripts (Fig. 6B).

The expression of T4 TD Δ I (BLdtu1) alone triggered extracellular accumulation of a significant amount of thymidine (Fig. 5). In addition, the coexpression of the T4 TD Δ I and T4 *nrdCAB* artificial operons brought about an almost twofold increase in the thymidine titer (BLdtu2), compared with that of BLdtu1. The thymidine concentration of BLdtu2 was further increased by additional expression of T4 *nrdCAB*, confirming the contribution of *nrdCAB* to thymidine biosynthesis.

As shown in Fig. 1, the intracellular level of dUMP had a direct effect on thymidine production. In order to increase the influx of dUMP, the *udk-dcd* operon was cloned from *E. coli*

TABLE 3. Effects on the expression of *dut* and the disruption of *ung*

Strain	Cell growth (g/liter) ^a	Thymidine (mg/liter) ^a	Thymidine production per cell (mg/g DCW)	dUTP (nmol/g DCW) ^b	dTTP (nmol/g DCW) ^b	dUTP/dTTP ratio
BL21	12.5 ± 0.3 ^c	ND ^d	0	3.1 ± 0.9	630.2 ± 10.2	0.005
BLdtu24	10.9 ± 0.3	459.0 ± 10.8	42.1	21.1 ± 3.2	621.3 ± 22.9	0.034
BLdtu24 (<i>dut</i>)	6.5 ± 1.5	376.2 ± 9.2	57.8	9.0 ± 0.2	1,317.8 ± 16.9	0.007
BLdtug24	9.5 ± 0.5	550.7 ± 6.2	58.0	18.5 ± 2.3	441.4 ± 2.1	0.042

^a These data were obtained from flask cultures for 24 h.

^b Cultures were harvested at a mid-log phase.

^c The standard deviations were calculated by using standard propagation of error methods.

^d ND, not detected.

BL21 and overexpressed in a BLdtu23 strain (Fig. 6A). However, the coexpression of the *udk-dcd* operon in this strain (BLdtu23) increased the thymidine titer only by approximately 30 mg liter⁻¹, suggesting that there can be other rate-limiting factors (Fig. 5).

To increase the efficiency of thymidine synthesis *in vivo*, one possible approach is to elevate the activity for TMP phosphohydrolyzation to thymidine by expressing a TMP hydrolysis enzyme (TMPase). Since the hydrolysis of dTMP to thymidine did not seem to occur efficiently (1.3 pmol mg⁻¹ min⁻¹), TMPase from phage PBS2 was cloned and coexpressed in a BLdtu24 strain with other *de novo* pyrimidine biosynthetic genes (Fig. 6). Corresponding to the elevation in TMP hydrolysis activity by TMPase overexpression (from 1.3 [BLdtu23] to 16.6 pmol mg⁻¹ min⁻¹ [BLdtu24]), the amount of thymidine accumulated using BLdtu24 was 1.5-fold greater than that accumulated using BLdtu23 (Fig. 5). This result clearly indicated the importance of TMP phosphohydrolyzation in thymidine production.

Expression of *dut* and disruption of *ung*. As described in Fig. 5, the coexpression of *de novo* biosynthetic genes and PBS2 TMPase in BLdtu24 resulted in elevated thymidine levels. However, in BLdtu24, the intracellular concentration of dUTP was increased by sevenfold, whereas dTTP pool concentrations were similar, compared with those of BL21 Star (Table 3). The elevated dUTP/dTTP ratio of BLdtu24 (from 0.005 [BL21] to 0.034 [BLdtu24]) may lead to an increased frequency of misincorporation of uracil for thymine in DNA. The resulting excision repair events may result in the consumption of dTTP, thereby reducing thymidine production.

To determine whether decreasing the dUTP/dTTP ratio in BLdtu24 can boost thymidine production further, dUTPase (*dut*) from *E. coli* BL21 was overexpressed in this strain. The transcription level of *dut* in BLdtu24 (*dut*) was increased by threefold, compared with that in BLdtu24 (Fig. 6B). Therefore, the intracellular dUTP level was decreased to 43% due to an increase in *dut* activity, compared with that in BLdtu24. As a result, the dUTP/dTTP ratio of BLdtu24 (*dut*) decreased from 0.034 to 0.007, and dUTP levels also decreased. At this point, the increase in the *Dut* expression level was expected to cause an increase in thymidine production, due to the subsequent high activity of TMPase (12.8-fold increase). Even though the overexpression of *dut* increased thymidine production by 1.4-fold per cell by decreasing intracellular dUTP levels, intracellular dTTP levels were increased, and overall thy-

midine production was decreased, due to growth retardation (Table 3).

Since the overexpression of *dut* was not advantageous in overall thymidine production, we tried to enhance thymidine production by preventing cells from consuming additive thymidine nucleotides by suppressing subsequent excision repair. In this approach, *ung* in BLdtu24 was disrupted without expressing *dut* (BLdtug24) (Fig. 3B). The disruption of *ung* brought about an almost 1.2-fold increase in the overall thymidine titer, compared with that in BLdtu24, even though cell growth rate was decreased to some extent (Table 3).

Production of thymidine using BLdtu24 and BLdtug24. To compare the levels of thymidine production in the BLdtu24 and BLdtug24 strains, aerobic batch cultivation was performed in a 7-liter jar fermenter with thymidine production medium (Fig. 7). In the fermentation of BLdtu24, the carbon source (glycerol) was consumed within 20 h, and the amount of biomass did not increase further after approximately 24 h. The rates of glycerol consumption and cell growth in BLdtug24 were lower than those in BLdtu24. After 30 h of fermentation, BLdtu24 (Fig. 7A) and BLdtug24 (Fig. 7B) reached final biomass concentrations of 28.1 g DCW liter⁻¹ and 25.1 g DCW liter⁻¹, corresponding to final thymidine concentrations of 649.3 mg liter⁻¹ and 740.3 mg liter⁻¹, respectively. Overall thymidine yields for BLdtu24 and BLdtug24 were 10.8 and 12.3 mg of thymidine per 1 g of glycerol, respectively. Neither thymine nor uracil was produced in either strain. These results showed that BLdtug24 is a more effective strain for achieving the overproduction of thymidine than is BLdtu24, even though the growth rate of BLdtug24 was lower than that of BLdtu24.

DISCUSSION

Generally, *de novo* biosynthesis of pyrimidine nucleotides is regulated by intracellular concentrations of various nucleotides through feedback inhibition. The intracellular dUMP level is tightly controlled by intracellular dTTP levels through the feedback inhibition of dCTP deaminase and the control of ribonucleotide diphosphate reductase (28, 29, 32). Previously, other workers found that the incorporation rate of exogenously supplied thymine through the salvage pathway during DNA synthesis is very poor in wild-type cells and that the dTTP concentration is maintained at a normal level and increases only during DNA replication (25, 37). In *thyA*-defective cells, low-dTTP pools promote dUMP accumulation to increase de-

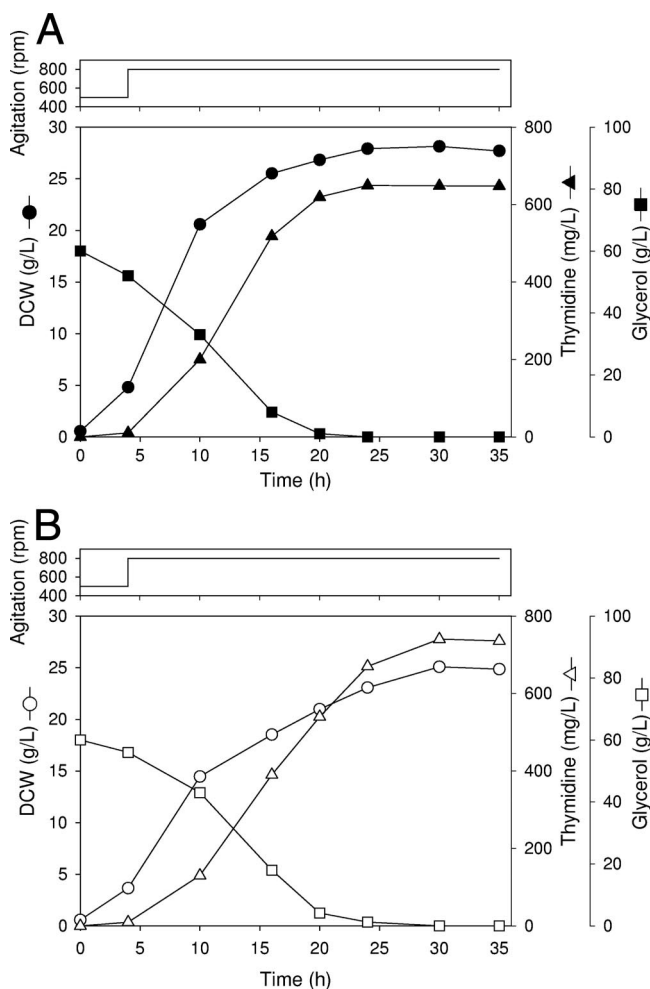


FIG. 7. Thymidine production in a 7-liter batch fermenter. (A) Fermentation profile using BLdtu24. (B) Fermentation profile using BLdtug24. ● and ○, cell growth; △ and ▲, thymidine; ■ and □, glycerol; and solid line, agitation speed.

oxyribose-1-phosphate production through the degradation of excess dUMP, which in turn stimulates the thymidine phosphorylase reaction in the direction of thymidine synthesis (37). On the other hand, thymidine synthesis through the salvage pathway did not occur in a thymidine-overproducing strain (BLdtug24), due to disruptions of *deoA* and *udp* (Fig. 4). Because the expression of PBS2 TMPase (44, 45) in this strain may cause the inhibition of DNA biosynthesis from DNA precursor (dTTP) depletion, it can result in decreased feedback inhibitions of dCTP deaminase and ribonucleotide diphosphate reductase. It has been reported that the response of T4 ribonucleotide diphosphate reductase to allosteric effectors such as dATP is different from that of *E. coli* ribonucleotide diphosphate reductase, although the complete mechanism is not currently known (23, 26, 31). It seems that the expression of T4 nucleotide diphosphate reductase in our system contributed to the enhancement of thymidine production under complex regulatory conditions (Fig. 5), suggesting that the reduction of UDP to dUDP by T4 nucleotide diphosphate reductase may be less inhibited by allosteric regulators.

In this study, increasing the transcriptional levels of *E. coli* dCTP deaminase fourfold resulted only in an approximately 1.1-fold increase in thymidine production, although a higher yield was expected (Fig. 5). There are several explanations for this behavior. One possibility is that the amount of dCTP deaminase originally present in our system was sufficient to carry out the deamination of dCTP to dUTP, so that increasing the copy number did not greatly increase thymidine production, due to the presence of other limiting factors. A second possibility is that a fourfold overexpression was not sufficient to increase thymidine production and that further increasing the copy number of dCTP deaminase may have a more positive effect on enhancing thymidine production. A third possibility is that *E. coli* dCTP deaminase is not a good candidate to increase thymidine production. Therefore, expressing foreign deaminase enzymes could potentially be used to increase the thymidine yield. So far, dCTP deaminase is known to be present exclusively in prokaryotes (28, 29, 51, 53). Both bacteria (such as *E. coli* and *Salmonella enterica* serovar Typhimurium) and archaea (such as *Methanocaldococcus jannaschii*) have been shown to contain this enzyme, which is responsible for delivering up to 70 to 80% of the total dUMP needed for dTTP synthesis (29, 40). In most gram-positive bacteria (such as *Bacillus subtilis*), bacteriophages, and all eukaryotes, dCMP deaminase plays a similar role in the conversion of cytosine nucleotide into uridine nucleotide as that of the above-mentioned dCTP deaminase (1, 6, 14, 36, 46). Both enzyme reactions are key regulatory points of deoxyribonucleotide metabolism and represent the major pathways for dTTP synthesis. It seems that the regulation of dTTP synthesis by dCMP and dCTP deaminases is a part of the overall regulation of deoxynucleoside triphosphate synthesis, along with the complex regulation of ribonucleotide reductase (29, 32). *E. coli* dCTP deaminase that was used in this study is a monofunctional enzyme that carries out only the deamination of dCTP to dUTP, which is then hydrolyzed to dUMP by dUTPase. Unlike monofunctional dCTP deaminase, the archaeal enzyme sequentially deaminates dCTP and hydrolyzes dUTP to directly yield dUMP and, thus, is bifunctional (7, 27). Therefore, expressing other enzymes that increase the influx of dUTP or dUMP, such as bifunctional dCMP deaminase or dCTP deaminase from other organisms, is a possibility for further enhancing thymidine production in our system.

dUTPase is known to be highly specific for dUTP so that it does not interact with dUDP, UTP, CTP, dCTP, or dTTP, and nonspecific nucleoside diphosphate kinase is known to be a highly active enzyme (21). Thus, it seems reasonable to assume that the dUMP for thymidine synthase is provided via dUTP from dUDP through combined actions of both enzymes. At this point, we do not know exactly why the expression of *dut* in BLdtu24 was not effective in thymidine production and caused growth rate retardation. One possible explanation could be that intracellular dUTPase activity in BLdtu24 was already too high to show a positive effect on thymidine production and the cells were burdened with the overexpression of *dut*.

In BLdtu24, the intracellular concentration of dUTP was increased sevenfold, whereas the concentration of the dTTP pool was not altered, resulting in an approximately sevenfold increase in the dUTP/dTTP ratio, compared with that of the parental BL21 strain (Table 3). The increase in the dUTP/

dTTP ratio, which was caused by an increase in the dUTP level, may induce incorporation of uracil into its DNA. Therefore, it is possible that an increase in excision repair could have resulted in a decrease in thymidine production by draining the dTTP pool for DNA repair in BLdtu24. *ung* was disrupted to prevent the loss of dTTP which is consumed in frequent DNA repair, and thymidine production was increased by 1.2-fold compared with that in BLdtu24 (Fig. 7). However, the disruption of *ung* can cause a build-up of mutations in the BLdtu24 strain, which will eventually lead to an increase in genetic instability. Therefore, it will be desirable to find an approach that increases thymidine production while maintaining genetic stability.

In this study, the expression of several viral genes (encoding T4 thymidylate synthase, T4 deoxyribonucleotide diphosphate reductase, and PBS2 TMPase) significantly enhanced thymidine production (Fig. 5). More often than not, viral enzymes are under different regulations compared with *E. coli* enzymes. Therefore, expressing other viral enzymes can also be considered possible candidates for enhancing thymidine production. For instance, T4 dihydrofolate reductase is known to be more active with NADH than with NADPH, whereas the opposite is true for *E. coli* enzymes (35). This T4 enzyme is known to have a different stability and response to inhibitors (31). Thus, the expression of T4 dihydrofolate reductase may have beneficial effects on thymidine overproduction.

Previously, we found that more reduced carbon sources, such as glycerol or sorbitol, were better substrates than were all other tested carbon sources (glucose, fructose, lactose, maltose, galactose, or acetate) (unpublished data). In the EMP pathway, two molecules of glycerol are metabolized to two molecules of glyceraldehyde 3-phosphate while producing two molecules of NADH, whereas one glucose molecule is metabolized to two glyceraldehyde 3-phosphate without producing any reducing equivalent (40). In *E. coli*, there are two nicotinamide nucleotide transhydrogenases (PntAB and UdhA) with a redox-balancing function that can potentially transfer electrons directly from NADH to NADP⁺ and vice versa (47). In the thymidine biosynthetic pathway, NADPH is known to be essential for the reduction of nucleotides, and the lack of NADPH may inhibit thymidine overproduction by lowering the activities of ribonucleotide diphosphate reductase and dihydrofolate reductase (37, 40). Therefore, growth on glycerol can increase the level of NADH, which in turn can be converted to NADPH by nicotinamide nucleotide transhydrogenases, and thus can be more advantageous to thymidine production.

In this study, several recombinant strains have been engineered to improve thymidine production (Fig. 5). We found that thymidine production using BLdtug24 was significantly improved compared with that using other strains used in previous studies, while suppressing the formation of undesirable by-products to prevent thymidine loss (33, 48, 49). Foreign de novo biosynthetic genes were expressed to enhance thymidine production, while both the salvage pathway genes and *ung* were disrupted. We found that the overexpression of viral enzymes enhanced thymidine production (Fig. 5). When T4 TD and T4 NrdCAB were overexpressed in a BLdtu strain (BLdtu2), thymidine production was increased approximately 25-fold (from 12 mg liter⁻¹ to 283 mg liter⁻¹) in a flask culture, compared

with that using BLdtu. Moreover, overexpressing PBS2 TMPase in BLdtu24 increased thymidine production approximately twofold, compared with that in BLdtu23, in a flask culture. In addition, blocking the drain of dTTP to cells by disrupting *ung* in BLdtug24 also enhanced thymidine production, although this disruption can potentially lead to genetic instability. As a consequence of these alterations, it was possible to produce thymidine with considerable productivity by simple batch fermentation (7-liter jar) in BLdtug24 (649.3 to 740.3 mg liter⁻¹). This could in principle be easily improved by fed-batch fermentation to further enhance thymidine production and cell growth.

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REFERENCES

- Bannwarth, H., and H. G. Schweiger. 1983. The influence of the nucleus on the regulation of the dCMP deaminase in acetabularia. *Cell. Biol. Int. Rep.* 7:859–868.
- Belfort, M., G. F. Maley, J. Pedersen-Lane, and F. Maley. 1983. Primary structure of the *Escherichia coli thyA* gene and its thymidylate synthase product. *Proc. Natl. Acad. Sci. USA* 80:4914–4918.
- Belfort, M., G. F. Maley, and F. Maley. 1983. Characterization of the *Escherichia coli thyA* gene and its amplified thymidylate synthetase product. *Proc. Natl. Acad. Sci. USA* 80:1858–1861.
- Belfort, M., J. Pedersen-Lane, D. West, K. Ehrenman, G. Maley, F. Chu, and F. Maley. 1985. Processing of the intron-containing thymidylate synthase (*td*) gene of phage T4 is at the RNA level. *Cell* 41:375–382.
- Bertani, L. E., A. Haeggmark, and P. Reichard. 1963. Enzymatic synthesis of deoxyribonucleotides. II. Formation and interconversion of deoxyuridine phosphates. *J. Biol. Chem.* 238:3407–3413.
- Bianchi, V., E. Pontis, and P. Reichard. 1987. Regulation of pyrimidine deoxyribonucleotide metabolism by substrate cycles in dCMP deaminase-deficient V79 hamster cells. *Mol. Cell. Biol.* 7:4218–4224.
- Björnberg, O., J. Neuhard, and P. O. Nyman. 2003. A bifunctional dCTP deaminase-dUTP nucleotidohydrolase from the hyperthermophilic archaeon *Methanocaldococcus jannaschii*. *J. Biol. Chem.* 278:20667–20672.
- Capco, G. R., J. R. Krupp, and C. K. Mathews. 1973. Bacteriophage-coded thymidylate synthetase: characteristics of the T4 and T5 enzymes. *Arch. Biochem. Biophys.* 158:726–735.
- Cherepanov, P. P., and W. Wackernagel. 1995. Gene disruption in *Escherichia coli*: Tc^R and Km^R cassettes with the option of FLP-catalyzed excision of the antibiotic-resistance determinant. *Gene* 158:9–14.
- Reference deleted.
- Reference deleted.
- Chiu, J., P. E. March, R. Lee, and D. Tillett. 2004. Site-directed, ligase-independent mutagenesis (SLIM): a single-tube methodology approaching 100% efficiency in 4 h. *Nucleic Acids Res.* 32:e174.
- Datsenko, K. A., and B. L. Wanner. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. USA* 97:6640–6645.
- de Saint Vincent, B. R., M. Dechamps, and G. Buttin. 1980. The modulation of the thymidine triphosphate pool of Chinese hamster cells by dCMP deaminase and UDP reductase. Thymidine auxotrophy induced by CTP in dCMP deaminase-deficient lines. *J. Biol. Chem.* 255:162–167.
- Duncan, B. K., P. A. Rockstroh, and H. R. Warner. 1978. *Escherichia coli* K-12 mutants deficient in uracil-DNA glycosylase. *J. Bacteriol.* 134:1039–1045.
- el-Hajj, H. H., L. Wang, and B. Weiss. 1992. Multiple mutants of *Escherichia coli* synthesizing virtually thymineless DNA during limited growth. *J. Bacteriol.* 174:4450–4456.
- Freskos, J. N., and K. P. A. Senaratne. March 1988. Synthesis of betathymidine. U.S. patent 4,914,233.
- Furman, P. A., J. A. Fyfe, M. H. St. Clair, K. Weinhold, J. L. Rideout, G. A. Freeman, S. N. Lehrman, D. P. Bolognesi, S. Broder, H. Mitsuya, et al. 1986. Phosphorylation of 3'-azido-3'-deoxythymidine and selective interaction of the 5'-triphosphate with human immunodeficiency virus reverse transcriptase. *Proc. Natl. Acad. Sci. USA* 83:8333–8337.
- Gallo, R. C., S. Z. Salahuddin, M. Popovic, G. M. Shearer, M. Kaplan, B. F. Haynes, T. J. Palker, R. Redfield, J. Oleske, B. Safai, et al. 1984. Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. *Science* 224:500–503.
- Garrett, C., and D. V. Santi. 1979. A rapid and sensitive high pressure liquid chromatography assay for deoxyribonucleoside triphosphates in cell extracts. *Anal. Biochem.* 99:268–273.

21. Greenberg, G. R., and R. L. Somerville. 1962. Deoxyuridylylase kinase activity and deoxyuridylylase activity in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **48**:247–257.
22. Haldimann, A., L. L. Daniels, and B. L. Wanner. 1998. Use of new methods for construction of tightly regulated arabinose and rhamnose promoter fusions in studies of the *Escherichia coli* phosphate regulon. J. Bacteriol. **180**:1277–1286.
23. Hanson, E., and C. K. Mathews. 1994. Allosteric effectors are required for subunit association in T4 phage ribonucleotide reductase. J. Biol. Chem. **269**:30999–31005.
24. Hasan, N., M. Koob, and W. Szybalski. 1994. *Escherichia coli* genome targeting. I. Cre-lox-mediated *in vitro* generation of *ori* – plasmids and their *in vivo* chromosomal integration and retrieval. Gene **150**:51–56.
25. Jensen, K. F., J. C. Leer, and P. Nygaard. 1973. Thymine utilization in *Escherichia coli* K12 on the role of deoxyribose 1-phosphate and thymidine phosphorylase. Eur. J. Biochem. **40**:345–354.
26. Ji, J. P., R. G. Sargent, and C. K. Mathews. 1991. T4 phage ribonucleotide reductase. Allosteric regulation *in vivo* by thymidine triphosphate. J. Biol. Chem. **266**:16289–16292.
27. Johansson, E., O. Bjornberg, P. O. Nyman, and S. Larsen. 2003. Structure of the bifunctional dCTP deaminase-dUTPase from *Methanocaldococcus jannaschii* and its relation to other homotrimeric dUTPases. J. Biol. Chem. **278**:27916–27922.
28. Johansson, E., M. Fano, J. H. Bynck, J. Neuhard, S. Larsen, B. W. Sigurskjold, U. Christensen, and M. Willemoes. 2005. Structures of dCTP deaminase from *Escherichia coli* with bound substrate and product: reaction mechanism and determinants of mono- and bifunctionality for a family of enzymes. J. Biol. Chem. **280**:3051–3059.
29. Johansson, E., M. Thymark, J. H. Bynck, M. Fano, S. Larsen, and M. Willemoes. 2007. Regulation of dCTP deaminase from *Escherichia coli* by non allosteric dTTP binding to an inactive form of the enzyme. FEBS J. **274**:4188–4198.
30. Kalirai, S. K., B. Scanlon, S. C. Taylor, and S. I. Ahmad. 2000. Screening of microbes, isolation, genetic manipulation, and physiological optimization of *Brevibacterium helvolum* to produce and excrete thymidine and deoxyuridine in high concentrations. J. Gen. Appl. Microbiol. **46**:217–224.
31. Koerner, J. F. 1970. Enzymes of nucleic acid metabolism. Annu. Rev. Biochem. **39**:291–322.
32. Larsson, A., and P. Reichard. 1966. Enzymatic synthesis of deoxyribonucleotides. IX. Allosteric effects in the reduction of pyrimidine ribonucleotides by the ribonucleoside diphosphate reductase system of *Escherichia coli*. J. Biol. Chem. **241**:2533–2539.
33. Lee, H. C., J. M. Ahn, S. N. Lee, and J. H. Kim. 2004. Overproduction of thymidine by recombinant *Brevibacterium helvolum* amplified with thymidine monophosphate phosphohydrolase gene from bacteriophage PBS2. Biotechnol. Lett. **26**:265–268.
34. Lindahl, T. 1979. DNA glycosylases, endonucleases for apurinic/apyrimidinic sites, and base excision-repair. Prog. Nucleic Acid Res. Mol. Biol. **22**:135–192.
35. Mathews, C. K. 1967. Evidence that bacteriophage-induced dihydrofolate reductase is a viral gene product. J. Biol. Chem. **242**:4083–4086.
36. McIntosh, E. M., and R. H. Haynes. 1986. Sequence and expression of the dCMP deaminase gene (DCD1) of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **6**:1711–1721.
37. Møllgaard, H., and J. Neuhard. 1983. Biosynthesis of deoxythymidine triphosphate, p. 149–198. In A. Munch-Petersen (ed.), Metabolism of nucleotides, nucleosides, and nucleobases in microorganisms. Academic Press, London, United Kingdom.
38. Murphy, K. C., K. G. Campellone, and A. R. Poteete. 2000. PCR-mediated gene replacement in *Escherichia coli*. Gene **246**:321–330.
39. Neuhard, J. 1966. Studies on the acid-soluble nucleotide pool in thymine-requiring mutants of *Escherichia coli* during thymine starvation. 3. On the regulation of the deoxyadenosine triphosphate and deoxycytidine triphosphate pools of *Escherichia coli*. Biochim. Biophys. Acta **129**:104–115.
40. Neuhard, J., and P. Nygaard. 1987. Purines and pyrimidines, p. 446–473. In F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*. Cellular and molecular biology, vol. 1. American Society of Microbiology, Washington, DC.
41. O'Donovan, G. A., and J. Neuhard. 1970. Pyrimidine metabolism in microorganisms. Bacteriol. Rev. **34**:278–343.
42. Palmén, L. G., K. Becker, L. Bulow, and J. O. Kvassman. 2008. A double role for a strictly conserved serine: further insights into the dUTPase catalytic mechanism. Biochemistry **47**:7863–7874.
43. Pósfai, G., M. Koob, Z. Hradecna, N. Hasan, M. Filutowicz, and W. Szybalski. 1994. *In vivo* excision and amplification of large segments of the *Escherichia coli* genome. Nucleic Acids Res. **22**:2392–2398.
44. Price, A. R. 1978. Deoxythymidylate phosphohydrolase from PBS2 phage-infected *Bacillus subtilis*. Methods Enzymol. **51**:285–290.
45. Price, A. R., and S. M. Fogt. 1973. Deoxythymidylate phosphohydrolase induced by bacteriophage PBS2 during infection of *Bacillus subtilis*. J. Biol. Chem. **248**:1372–1380.
46. Sargent, R. G., and C. K. Mathews. 1987. Imbalanced deoxyribonucleoside triphosphate pools and spontaneous mutation rates determined during dCMP deaminase-defective bacteriophage T4 infections. J. Biol. Chem. **262**:5546–5553.
47. Sauer, U., F. Canonaco, S. Heri, A. Perrenoud, and E. Fischer. 2004. The soluble and membrane-bound transhydrogenases UdhA and PntAB have divergent functions in NADPH metabolism of *Escherichia coli*. J. Biol. Chem. **279**:6613–6619.
48. Song, K. H., D. Y. Kwon, S. Y. Kim, J. K. Lee, and H. H. Hyun. 2005. Thymidine production by *Corynebacterium ammoniagenes* mutants. J. Microbiol. Biotechnol. **15**:477–483.
49. Tsen, S. 1994. Chemostat selection of *Escherichia coli* mutants secreting thymidine, cytosine, uracil, guanine, and thymine. Appl. Microbiol. Biotechnol. **41**:233–238.
50. Tye, B. K., P. O. Nyman, I. R. Lehman, S. Hochhauser, and B. Weiss. 1977. Transient accumulation of Okazaki fragments as a result of uracil incorporation into nascent DNA. Proc. Natl. Acad. Sci. USA **74**:154–157.
51. Wang, L., and B. Weiss. 1992. *dcd* (dCTP deaminase) gene of *Escherichia coli*: mapping, cloning, sequencing, and identification as a locus of suppressors of lethal *dut* (dUTPase) mutations. J. Bacteriol. **174**:5647–5653.
52. Warner, H. R., B. K. Duncan, C. Garrett, and J. Neuhard. 1981. Synthesis and metabolism of uracil-containing deoxyribonucleic acid in *Escherichia coli*. J. Bacteriol. **145**:687–695.
53. Weiss, B., and L. Wang. 1994. De novo synthesis of thymidylate via deoxycytidine in *dcd* (dCTP deaminase) mutants of *Escherichia coli*. J. Bacteriol. **176**:2194–2199.