J. Microbiol. Biotechnol. (2005), **15**(3), 477–483

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

© The Korean Society for Microbiology and Biotechnology

Thymidine Production by *Corynebacterium ammoniagenes* Mutants

SONG, KYUNG HWA[1], DO YOUNG KWON[1], SANG YONG KIM[2], JUNG KUL LEE[2], AND HYUNG HWAN HYUN[1]*

[1]*Department of Bioscience and Biotechnology, Hankuk University of Foreign Studies, San 89, Wangsan-Ri, Mohyun-Mmun, Yongin-Shi 449-791, Republic of Korea*
[2]*BioNgene Co., Ltd., 10-1, 1Ka, Myungryun-Dong, Jongro-Ku, Seoul 110-521, Republic of Korea*

Received: April 12, 2004
Accepted: July 11, 2004

Abstract *Corynebacterium ammoniagenes* ATCC 6872, which does not accumulate pyrimidine nucleoside or nucleotide, was metabolically engineered to secrete a large amount of thymidine. Characteristics of 5-fluorouracil resistance (FU^r), hydroxyurea resistance (HU^r), trimethoprim resistance (TM^r), thymidylate phosphorylase deficiency (*deo*A$^-$), inosine auxotrophy (ino$^-$), 5-fluorocytosine resistance (FC^r), thymidine kinase deficiency, and thymidine resistance (thym^r) were successively introduced into mutant strains KR3 and DY5T9-5, and shake-flask cultures were able to accumulate 408.1 mg/l and 428.2 mg/l of thymidine, respectively, as a major product. The mutant strains did not accumulate thymine at all and accumulated less than 10 mg/l of other pyrimidine nucleosides, such as cytosine, cytidine, and deoxycytidine, as byproducts.

Key words: *Corynebacterium ammoniagenes*, thymidine, pyrimidine, metabolic engineering

Pyrimidine deoxyribonucleosides, thymidine, and deoxyuridine are commercially useful starting compounds for producing antiviral compounds, such as azidothymidine and azidodeoxyuridine. They are currently produced by an organic synthetic method that involves multiple steps and is very costly. The high production cost of pyrimidine deoxyribonucleosides has contributed to the high cost of antiviral therapeutics like AZT. The development of cost-effective biological processes for thymidine or deoxyuridine production is, therefore, of a great interest.

Thymidine normally exists in cells as a mono-, di-, or triphosphate nucleotide, and functions primarily as a component of DNA. Generally, metabolic pathways of pyrimidine preclude the accumulation of end products such as UTP, CTP, dCTP, and TMP, due to tight biosynthetic regulation [3, 5]. The enzymatic steps in the *de novo* biosynthetic pathways of pyrimidine nucleotide are regulated *in vivo* by feedback inhibition of key enzymes, and by repression and/or attenuation of enzyme synthesis by the accumulation of end products or other metabolites [6, 7]. Hence, the accumulation of byproducts such as uridine, deoxyuridine, cytidine, deoxycytidine, and thymidine, which could theoretically be synthesized from the end products, is almost impossible. For example, dUMP, a precursor of the above byproduct, is converted directly to TMP, then to TDP and TTP.

If the concentration of thymidine is increased, either by metabolical engineering of its biosynthetic pathway or by supplying exogenous thymidine in the culture medium, cells degrade thymidine as a carbon source or use it to synthesize thymidine nucleotides, thereby preventing thymidine accumulation. For example, thymidine production by nonspecific phosphatase on TMP does not lead to thymidine accumulation, because any thymidine produced is either degraded rapidly to thymine and deoxyribose-1-phosphate by thymidine phosphorylase (a product of the *deo* A gene), or is rapidly consumed by thymidine kinase to synthesize TMP [3]. In addition, there are a number of branch points to synthesize precursors for RNA and DNA building blocks in the pyrimidine metabolic pathway, resulting in the accumulation of various byproducts other than major products [1, 13]. Therefore, to accumulate a large amount of thymidine, cells must be resistant to feedback regulation, and must lack thymidine phosphorylase and thymidine kinase enzyme activity.

In this study, we carried out metabolic engineering of *C. ammoniagenes* to develop mutants that accumulated recoverable amount of thymidine as a major product, by introducing resistant characteristics to substrate analogues and end product analogues, thymidine phosphorylase deficiency, and thymidine kinase deficiency.

*Corresponding author
Phone: 82-31-330-4279; Fax: 82-31-330-4566;
E-mail: hhhyun@san.hufs.ac.kr

MATERIALS AND METHODS

Bacterial Strains and Media

The *Corynebacterium ammoniagenes* strains used in this study are shown in Table 1. CM medium [10 g/l peptone, 10 g/l yeast extract, 5 g/l beef extract, and 2.5 g/l NaCl] was used for the routine cultivation of *C. ammoniagenes* ATCC 6872, and minimal medium [20 g/l glucose, 3 g/l $(NH_4)_2SO_4$, 1 g/l KH_2PO_4, 3 g/l K_2HPO_4, 0.3 g/l $MgSO_4 \cdot 7H_2O$, 10 mg/l $CaCl_2 \cdot 2H_2O$, 10 mg/l $FeSO_4 \cdot 7H_2O$, 1 mg/l $ZnSO_4 \cdot 7H_2O$, 5 mg/l thiamine-HCl, 10 mg/l Ca-D-pantothenate, and 30 μg/l D-biotin] was used to isolate and characterize mutants. Depending on the mutant characteristics, the minimal medium was supplemented with 50–150 mg/l adenine, 50–150 mg/l guanine, and 50 mg/l thymine or thymidine. To evaluate thymidine productivity, the cells were grown for 72 h in an Erlenmeyer flask (500 ml) containing 25 ml of production medium [50 g/l glucose, 3 g/l urea, 1 g/l KH_2PO_4, 3 g/l K_2HPO_4, 0.3 g/l $MgSO_4 \cdot 7H_2O$, 10 mg/l $CaCl_2 \cdot 2H_2O$, 10 mg/l $FeSO_4 \cdot 7H_2O$, 1 mg/l $ZnSO_4 \cdot 7H_2O$, 3.6 mg/l of $MnCl_2$, 20 mg/l L-cysteine-HCl, 2 g/l casamino acid, 5 mg/l thiamine-HCl, 10 mg/l Ca-D-pantothenate, and 30 μg/l D-biotin]. If needed, production medium was supplemented with 50 μg/l of adenine and 50 μg/l of guanine. The flask was shaken at 250 rpm and 30°C, and the thymidine content of the culture supernatant was determined by using HPLC.

Mutagenesis and Isolation of Mutants

To isolate 5-fluorouracil- (FU^r), 5-fluorocytosine- (FC^r), hydroxyurea- (HU^r), and trimethoprim (TM^r) resistant mutant strains, cells grown in CM medium to mid-logarithmic growth phase were centrifuged, suspended in fresh CM medium, and then treated with N-methyl-N'-nitro-N-nitrosoguanidine (30–90 mg-NTG/l) at 30°C for 1 h. The treated cells were washed three times with saline, cultivated in a CM medium for 2 h, transferred to minimal medium with appropriate amounts of 5-fluorouracil, 5-fluorocytosine, hydroxyurea, or trimethoprim, and then plated onto the same agar medium as above. Colonies that appeared on the agar plates were isolated as mutants which are resistant to the toxic chemicals or substrate analogues. Thymidine productivity was then evaluated, using shake-flask culture.

To isolate inosine auxotrophs (Ino^-), mutagenized cells were plated on minimal medium with 50 mg/l inosine, cultivated at 30°C for 72 h, and then in duplicate-plated on minimal medium without inosine. Colonies that grew in the presence of inosine and failed to grow in its absence were selected as inosine auxotrophs and characterized.

To isolate *deo*A-deficient mutants, mutagenized cells were plated on minimal medium and cultivated, and colonies that appeared were replica-plated, using a toothpick, onto minimal media with 50 mM 5-fluorodeoxyuridine and minimal medium with 50 mM 5-fluorodeoxyurdine and 50 mg/l thymine. Colonies that failed to grow on either of the media were selected as *deo*A-deficient mutants and further characterized.

To isolate thymidine kinase-deficient mutants, mutagenized cells were plated on minimal medium, and colonies that appeared were replica-plated, using a toothpick, on minimal medium with 50 mM 5-fluorodeoxyuridine and minimal medium with 50 mM 5-fluorodeoxyurdine and 50 mg/l

Table 1. Strains of *Corynebacterium ammoniagenes* used in this work and their pyrimidines productivities.

Strain	Characteristics	Thymine (mg/l)	Thymidine (mg/l)	Other pyrimidine nucleoside (mg/l)
Corynebacterium ammoniagenes ATCC 6872	–	0.00	0.00	0.00
XP-8	$5FU^r$	22.3	1.4	Uracil, 85.7; cytosine, 10
XL35-3	$5FU^r$, HU^r	12.5	16.1	Uracil, 145.2; cytosine, 10
X1-4	$5FU^r$, HU^r, TM^r	376.5	27.4	Uracil, 37.4; cytosine, 5
XF4-89	$5FU^r$, HU^r, TM^r, *deo A*$^-$	0.00	89.9	Uracil, 10; uridine, 10; deoxyuridine, 67.2; cytosine, 5
I22	$5FU^r$, HU^r, TM^r, *deo A*$^-$, ino^-	0.00	130.7	Uracil, 10; uridine, 10; deoxyuridine, 66.2; cytosine, 5
FC2-11	$5FU^r$, HU^r, TM^r, *deo A*$^-$, ino^-, $5FC^r$	0.00	196.5	Uracil, 10; uridine, 10; deoxyuridine,120.2; cytosine, 5
M3	$5FU^r$, HU^r, TM^r, *deo A*$^-$, ino^-, $5FC^r$, thymidine kinase deficience	0.00	224.8	Uracil, 10; uridine, 10; deoxyuridine, 143.2; cytosine, 5
KR3	$5FU^r$, HU^r, TM^r, *deo A*$^-$, ino^-, $5FC^r$, thymidine kinase deficience, $thym^r$	0.00	408.1	Uracil, 10; uridine, 10; deoxyuridine, 10; cytosine, 5
DY5T9-5	$5FU^r$, HU^r, TM^r, *deo A*$^-$, ino^-, $5FC^r$, thymidine kinase deficience, $thym^r$	0.00	428.2	Uracil, 10; uridine, 10; deoxyuridine, 10; cytosine, 5

Abbreviations: $5FU^r$, resistant to 5-fluorouracil; HU^r, resistant to hydroxyurea; TM^r, resistant to trimethoprim; *deo A*$^-$, thymidine phosphohydrolase deficient; Ino^-, inosine auxotroph; $5FC^r$, resistant to 5-fluorocytosine; $thym^r$, resistant to thymidine.

thymidine. Colonies that failed to grow on either of the media were selected as thymidine kinase-deficient mutants.

Preparation of Cell Extracts

To prepare cell extracts, organisms were cultured in an Erlenmeyer flask containing 50 ml of production medium. The cells were harvested at the late exponential phase by centrifugation at 10,000 ×*g* for 10 min. Cell suspensions were prepared by suspending the cell pellet in 2.5 volumes of TE buffer (0.01 M Tris-HCl, 2 mM EDTA, pH 7.2). The cells were disrupted by passage through a French press at 12,000 PSI. The supernatant was collected by centrifugation at 24,000 ×*g* for 1 h at 4°C, and used for enzyme assays.

Enzyme Assays

To measure thymidine phosphorylase activity, TE buffer (0.65 ml) and cell extract (0.2 ml) were mixed at 0°C, and 0.1 ml of potassium phosphate buffer (0.1 M, pH 7.2) was added to the mixture. The reaction was initiated by adding 0.05 ml of thymidine solution (0.1 M in potassium phosphate buffer), and carried out at 30°C for 2 min. At 5-min intervals, 0.3 ml samples were withdrawn and transferred into 0.7 ml of 0.5 N NaOH solution [10]. The amount of thymine produced was determined by measuring optical density at 300 nm. One unit of enzyme activity was defined as the amount of enzyme that cleaved 1 µmole of thymidine in 1 min at 30°C. Protein concentrations were determined by the Lowry method [4].

Thymidine kinase activity was assayed by HPLC measurement of the amount of TMP produced from the reaction between thymidine and ATP in cell extracts. The reaction was initiated by adding 25 µl of cell extract to 75 µl of the reaction mixture [0.7 ml 200 mM Tris buffer (pH 7.8), 0.4 ml of 50 mM sodium ATP, 0.4 ml of 50 mM $MgCl_2$, 0.6 mg of BSA, 0.5 ml of 5 mM thymidine, and 0.5 ml of 20 mM NaF], and carried out at 37°C. One unit of thymidine kinase was defined as the amount of enzyme to catalyze the formation of one µmole of deoxythymidine monophosphate per minute at 37°C.

HPLC Analysis

Pyrimidines and pyrimidine nucleotides were analyzed by HPLC (HP1100, UV-VIS detector), using a C18 reversed-phase column (Zorbax column Cat. No.; 990967.902), and a solution containing 50 mM phosphate buffer (pH 7.0) or 50 mM phosphate buffer (pH 2.0) with 2% acetonitrile.

RESULTS

Isolation of 5-Fluorouracil-Resistant Strains

As shown in Fig. 1, it is known that 5-fluorouracil inhibits the enzymes coded from *pyr* genes, such as carbamoyl phosphate synthase and aspartate transcarbamoylase, which are involved in the synthesis of orotate monophosphate. *C. ammoniagenes* ATCC 6872 does not typically accumulate thymidine, thymine, or other pyrimidine nucleosides in the medium, because of tight regulation of the enzymes by uridine and uridine derivatives. To isolate pyrimidine-accumulating strains, therefore, we isolated 5-fluorouracil-resistant mutant strain XP-8 by enriching the mutagenized cells in a minimal medium with 0.001–0.1% 5-fluorouracil, and evaluating the thymidine productivity of the isolated mutants by shake-flask culture. Table 1 shows the amounts of thymidine, thymine, and other pyrimidine nucleosides produced by the 5-fluorouracil-resistant mutants. Strain XP-8 produced 1.4 mg/l of thymidine, 22.3 mg/l of thymine, and 85.7 mg/l of uracil.

Isolation of Hydroxyurea-Resistant Mutants

Deoxyribonucleotides, the building blocks of DNA, are derived from the corresponding ribonucleotides with the action of ribonucleoside diphosphate reductase, an enzyme that catalyzes the reduction of ribonucleoside diphosphate to deoxyribonucleoside diphosphate (Fig. 1). Hydroxyurea, an inhibitor of ribonucleoside diphosphate reductase [12], was used to isolate mutant strains with increased ribonucleoside diphosphate reductase activity that accumulated more thymidine or thymine. Mutant strain XL35-3 was resistant to 10 mM hydroxyurea, and produced 10-fold more thymidine (16.1 mg/l) than its mother strain XP-8, while still accumulating 12.5 mg/l of thymine and 145.2 mg/l of uracil (Table 1).

Isolation of Trimethoprim-Resistant Mutants

TMP, a thymidine precursor, is synthesized from dUMP through the action of thymidylate synthase. In this reaction, a one-carbon unit is transferred from N^5,N^{10},-methylenetetrahydrofolate to dUMP, releasing dihydrofolate. Dihydrofolate is subsequently reduced to tetrahydrofolate by dihydrofolate reductase, an enzyme that is inhibited by trimethoprim (Fig. 1). To enhance dihydrofolate activity, we introduced trimethoprim resistance into strain XL35-3. Trimethoprim-resistant strain X1-4, isolated through mutagenesis with NTG, accumulated 376.5 mg/l of thymine, 27.4 mg/l of thymidine, and 37.4 mg/l of uracil (Table 1). The data indicate that this mutant accumulated 30-fold more thymine, but accumulated much less uracil than the mother strain, XL35-3.

Isolation of *deo*A-Deficient Mutants

Thymidylate phosphorylase is a *deo*A gene product that reversibly catalyzes the conversion of thymidine into thymine and deoxyribose-1-phosphate (Fig. 1). Prior to isolating *deo*A-deficient mutants, basic experiments were carried out to establish procedures for mutant selection and isolation to prevent catabolic degradation of thymidine to

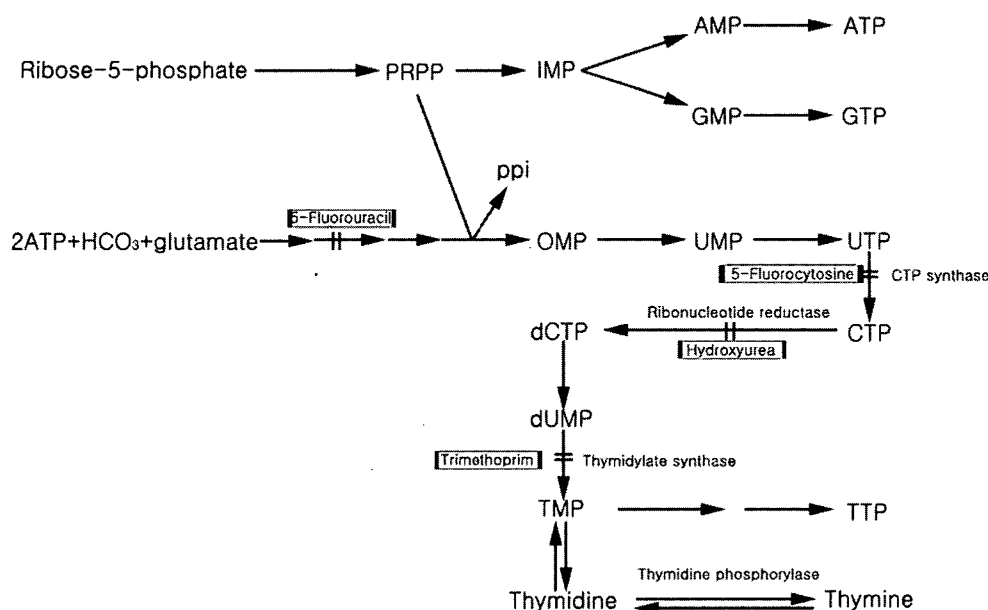

Fig. 1. Biosynthetic pathways of nucleosides and nucleotides and action sites of pyrimidine analogues in *Corynebacterium ammoniagenes*.

thymine and deoxyribose-1-phosphate. 5-Fluorodeoxyuridine is a potent inhibitor of thymidylate synthase, an enzyme that catalyzes the methylation of deoxyuridylate (dUMP) to TMP [8]. According to our experimental results (data not shown), *C. ammoniagenes* strain XL35-3 did not grow on minimal medium supplemented with 50 μM 5-fluorodeoxyuridine alone, but grew on minimal medium supplemented with 50 μM 5-fluorodeoxyuridine and 50 mg/l thymine. This indicated that, although *de novo* TMP biosynthesis was completely blocked by 5-fluorodeoxyuridine, the strain could synthesize TMP from thymine by using thymidine phosphorylase and thymidine kinase. Therefore, we selected mutants that could not grow on minimal medium supplemented with 50 μM 5-fluorodeoxyuridine, regardless of thymine addition. They were designated as *deo*A-deficient mutants, and their thymidine productivity and thymidine phosphorylase activity were measured. As shown in Table 2, the thymidine phosphorylase activity in crude cell extract preparations of *C. ammoniagenes* ATCC 6872, mother strain X1-4, and mutant strain XF4-89 was 1.42 U/mg-protein, 0.15 U/mg-protein, and 0.00 U/mg-protein,

Table 2. Comparison of thymidine phosphorylase activity in mutant strain XF4-89 and its mother strain X1-4.

Strain	Thymidylate phosphorylase activity (Units/mg-protein)
Corynebacterium ammoniagenes ATCC	1.49
Mother strain X1-4	0.15
Mutant strain XF4-89	0.00

respectively. The *deo*A-deficient mutant XF4-89 did not accumulate thymine at all, and secreted only 89.0 mg/l of thymidine (Table 1).

Isolation of Inosine-Auxotrophic Mutants

PRPP is used to synthesize both purine and pyrimidine nucleotides. To prevent PRPP from flowing into the purine biosynthetic pathway, inosine auxotrophy was introduced into strain X1-4. Inosine auxotrophs were selected that grew only on minimal medium supplemented with inosine or adenine and guanine, and their thymidine productivity was measured by shake-flask culture. Mutant strain I22 grew only when supplemented with inosine or adenine and guanine (Table 3), and produced about 1.5-fold more thymidine (130.7 mg/l) than the mother strain XF4-98 (Table 1).

Isolation of 5-Fluorocytosine-Resistant Mutants

5-Fluorocytosine, an analogue of cytosine, was used to screen mutants deficient in pyrimidine nucleotide biosynthesis regulation. 5-Fluorocytosine can be converted to 5-fluorocytidine and 5-fluorocytidine mono-, di-, and triphosphate within cells, and acts as an effector or inhibitor of enzymes involved in pyrimidine nucleotide biosynthesis (Fig. 1). Therefore, we introduced 5-fluorocytosine resistance into strain I22 to obtain strains that were not repressed and/or inhibited by pyrimidine nucleoside accumulation. The 5-fluorocytosine-resistant mutants were enriched and isolated by cultivating cells mutagenized with NTG in minimal medium containing 5-fluorocytosine and adenine/guanine. Mutant strain FC2-11, which was resistant to 0.1%

Table 3. Growth of mutant strains, XF4-89 and I22, in minimal medium containing inosine or adenine/guanine.

Strain	Growth (OD_{660})		
	Minimal medium	Minimal medium with inosine	Minimal medium with adenine and guanine
Mother strain XF4-89	12.0	12.0	13.0
Mutant strain I22	0.5	12.0	2.0

5-fluorocytosine, produced more thymidine (196.5 mg/l) than the mother strain I22 (Table 1).

Isolation of Thymidine Kinase-Deficient Mutants

Generally, excess thymidine in cells is converted to TMP by thymidine kinase and further metabolized into TTP via TDP. Therefore, to prevent the use of thymidine as a TTP synthesis precursor and achieve enhanced thymidine production, we tried to isolate thymidine kinase-deficient mutants. As with the *deo*A-deficient mutants, we used 5-fluorodeoxyuridine to isolate thymidine kinase deficient mutants. In the presence of 5-fluorodeoxyuridine, which inhibits thymidylate synthase, strains with active thymidine kinase grow on minimal medium containing thymidine, because they can synthesize TMP from thymidine. In contrast, thymidine kinase-deficient mutants cannot grow on the same medium. Colonies that failed to grow on minimal medium, containing 50 μM 5-fluorodeoxyuridine and 50 mg/l thymidine in addition to adenine and guanine, were selected as thymidine kinase-deficient mutants, and their thymidine productivity was evaluated by using shake-flask cultivation. Strain M3 did not grow on medium containing 5-fluorodeoxyuridine, 5-fluorouridine, or thymidine, and thymidine kinase activity was not detected in cell-free extracts of this mutant. However, enzyme activity in cell-free extracts of the wild-type and mother strain FC2-11 was 1.02 U/mg and 1.14 U/mg, respectively (Table 4). As shown in Table 1, thymidine production was slightly enhanced in mutant M3 (224.8 mg/l), as compared to the mother strain FC2-11 (196.5 mg/l).

Isolation of Thymidine-Resistant Mutants

We investigated the effect of exogenous thymidine on thymidine production and growth in strain M3, which could not use thymidine as a TMP precursor or as a thymidine phosphorylase substrate, and in its mother strain FC2-11, to establish whether these strains still had thymidine feedback regulation. As shown in Table 5, the growth of strain M3 was severely inhibited by exogenous thymidine, while the growth of strain FC2-11 growth was not. Therefore, we tried to isolate a thymidine-resistant strain that could grow in medium containing a large amount of exogenous thymidine (10 g/l). Mutant strains DY5T9-5 and KR3 grew in the presence of 10 g/l thymidine and produced 428.2 mg/l and 408.1 mg/l of thymidine, respectively. These values were almost 2-fold higher than those produced in the mother strain M3 (Tables 1 and 5).

Table 4. Comparison of thymidine kinase activity in mutant strain M3 and its mother strain FC2-11.

Strain	Thymidine kinase activity (Units/mg-protein)
Corynebacterium ammoniagenes ATCC	1.02
Mother strain FC2-11	1.14
Mutant strain M3	0.00

DISCUSSION

Thymidine is synthesized through a *de novo* pyrimidine pathway that comprises many branch points for the synthesis of RNA building blocks, such as UTP and CTP, and DNA building blocks, such as TTP. Although thymidine is an intermediate in pyrimidine biosynthetic pathways, it does not accumulate for the following reasons. The biosynthesis of pyrimidine nucleosides and nucleotides is tightly related to cell growth and is subjected to feedback regulation by end products [3]. TMP, a thymidine precursor, does not accumulate within cells, because it is converted into TTP via TDP as rapidly as it is synthesized [5]. In addition, thymidine is either degraded into thymine and ribose-1-phosphate by thymidine phosphorylase or converted into TMP by thymidine kinase, despite of being rapidly synthesized by metabolic changes [5].

Therefore, to develop *C. ammoniagenes* mutants that overproduce thymidine, we successively introduced a

Table 5. Effect of thymidine on the growth of thymidine nonmetabolizing strain M3.

Strain	Growth (OD_{660})	
	MAG* medium	MAG with thymidine (10 g/l)
FC2-11	10.0	9.0
Strain M3	13.0	0.2
KR3	12.0	10.0
DY5T9-5	11.0	11.0

*MAG, minimal medium supplemented with adenine (150 mg/l) and guanine (150 mg/l).
Cultivation was carried out after cells had been starved in MAG medium for 4 h.

variety of characteristics, including 5FU^r, HU^r, TM^r, *deo*A$^-$, ino$^-$, 5FC^r, thymidine kinase deficiency, and thym^r, into *C. ammoniagenes* ATCC 6872. As shown in Table 1, strain XP-8 and strain XL35-3, which were resistant to 5-fluorouracil and/or hydroxyurea, accumulated uracil as a major product. In general, the key regulatory enzymes of the *de novo* pyrimidine biosynthetic pathway are known to be carbamoyl phosphate synthase, aspartate transcarbamoylase, CTP synthase, and deoxycytidine triphosphate deaminase (Fig. 1). Among these, carbamoyl phosphate synthase and aspartate transcarbamoylase are regulated by uridine and uridine derivatives. The mutant strains, which acquired the resistance to 5-fluorouracil, might be relieved from regulation of the enzymes by uridine and uridine derivatives, but they accumulated a large amount of uracil in addition to thymidine. Also, as observed in mutant X1-4, the proportion of thymidine and thymine among the accumulated pyrimidine nucleosides was significantly increased by introduction of resistance to hydroxyurea, an inhibitor of ribonucleotide reductase [12], and resistance to trimethoprim, an inhibitor of thymidylate synthase [2]. These results partly coincide with those reported by Tsen [13]. He described the screening systems to isolate thymidine- or pyrimidine-secreting mutants by introducing 5-fluorouracil-, hydroxyurea-, and methotrexate-resistance into *E. coli*. The *E. coli* mutants isolated secreted only 10 mg/l of thymidine, and their major products were guanine and uracil other than thymidine. In our studies, however, introduction of trimethoprim resistance to strain XL35-3 dramatically reduced the proportion of uracil and secreted a large amount of thymine, being different from Tsen's results above. The strain XL1-4 with trimethoprim resistance accumulated 27.4 mg/l of thymidine, but its major product was still thymine (376.5 mg/l). This result implies that the thymidine produced was degraded into thymine and deoxyribose-1-phosphate by thymidine phosphorylase. Therefore, it was essential to isolate strains with defective thymidylate phosphorylase to enhance thymidine productivity. The *deo*A-deficient mutants were successfully isolated by using 5-fluorodeoxyuridine, a potent inhibitor of thymidylate synthase [8]. As long as 5-fluorodeoxyuridine blocks the synthesis of TMP from dUMP by thymidylate synthase, cells can not grow due to TTP depletion, unless exogenous thymine or thymidine is provided.

We then established an isolation system for thymidine phosphorylase-deficient mutants by using 5-fluorodeoxyuridine, which completely inhibited thymidylate synthase by acting as a competitive inhibitor of its substrate, dUMP. As long as 5-fluorodeoxyuridine blocks the synthesis of TMP from dUMP, TTP depletion prevents cell growth, unless exogenous thymine or thymidine is provided.

In general, thymine can be converted into TMP via thymidine through a salvage pathway. Thymidine phosphorylase reversibly catalyzes the reaction in which thymine is converted into thymidine. Therefore, although the *de novo* pyrimidine pathway is blocked, cells can grow on minimal medium containing both 5-fluorodeoxyuridine and thymine by using the salvage pathway. However, if the cells have defective thymidylate phosphorylase, they cannot grow. As shown in Table 1, mutant strain XF4-89 did not grow on minimal medium containing 5-fluorodeoxyuridine and thymine, and accumulated only thymidine, but not thymine. Also, we measured and compared the activities of thymidine phosphorylase in *C. ammoniagenes* ATCC 6872, strain Xl-4, and strain XF4-89 (Table 2). Enzyme activity in strain XF4-89 was not detectable, but strain Xl-4 had negligible thymidine phosphorylase activity, as compared to the wild-type strain. The fact that strain Xl-4 still accumulated thymine as a major product, instead of thymidine, suggests that complete thymidine phosphorylase knockout is essential for thymidine overproduction.

The data in Table 1 show that the strain with *deo*A$^-$ characteristics accumulated byproducts such as uridine and deoxyuridine in addition to thymidine. It was reported that *Bacillus stearothermophilus*, *Haemophilus influenzae*, and *Lactobacillus casei* express pyrimidine nucleoside phosphorylase (EC.2.4.22), an enzyme that acts on both thymidine and uridine [9, 11]. Therefore, the fact that the *deo*A$^-$ strains accumulated various pyrimidine nucleosides implies that *C. ammoniagenes* may also contain pyrimidine nucleoside phosphorylase, which acts on thymidine, uridine, and deoxyuridine.

The growth of strain M3, which could not metabolize thymidine, was severely inhibited by thymidine (Table 5), and the strain accumulated 143.2 mg/l of deoxyuridine. However, thymidine-resistant strains KR3 and DY5T9-5 accumulated less than 10 mg/l of deoxyuridine, implying that thymidine may strongly inhibit thymidylate synthase.

Further enhancement of thymidine production was achieved by isolating the mutant strains FC2-11 and M3 from strain XF4-89, which were inosine auxotrophs, 5-fluorocytosine-resistant, and thymidine kinase-deficient. It is highly significant to observe that strain M3 accumulated a large amount of deoxyuridine (143.2 mg/l). This implies that the synthesis of dTMP from dUMP by thymidylate synthase might be a rate-limiting step regulated by thymidine. Therefore, we investigated the effects of exogenous thymidine on the growth and thymidine production in strains FC2-11 and M3. As shown in Table 5, the growth of strain M3, which was thymidine kinase-deficient and could not utilize thymidine for the synthesis of TMP, was almost completely inhibited by thymidine, while strain FC2-11 with active thymidine kinase could grow in the medium with a large amount of thymidine. These results imply that some enzymes, including thymidylate synthase involved in the *de novo* biosynthetic pathway of TMP, are tightly regulated by thymidine, therefore, poor growth of strain M3 in the presence of thymidine was due to the

blocking of TMP synthesis from both thymidine and dUMP. On the basis of the result, the thymidine resistance was introduced into strain M3, and the isolated thymidine-resistant strains, KR3 and DY5T9-5, accumulated almost 2-fold more thymidine and less than 10 mg/l of deoxyuridine than strain M3.

In conclusion, fermentative thymidine production is of great interest because of its low production cost and ease of purification. Investigations into enhancing thymidine yields by further strain improvement and development of fermentation processes are in progress.

Acknowledgment

This work was supported by a grant of the Korea Research Foundation Grant (KRF-1998-001-E01401).

REFERENCES

1. Asahi, S. and Y. Tsueni. 1989. Method for production of cytidine and/or deoxycytidine. US patent 4,839,285.
2. Freisheim J. H., C. C. Smith, and P. M. Guzy. 1972. Dihydrofolate reductase and thymidylate synthetase in strains of *Streptococcus faecium* resistant to pyrimethamine, chlorguanide triazine, trimethoprim, and amethopterin. *Arch. Biochem. Biophys*. **148:** 1–9.
3. Hammer, J. K. 1983. Nucleotide catabolism, pp. 203–258 *In* Munch-Petersen, A. (ed.), *Metabolism of Nucleotides, Nucleosides and Nucleobases in Microorganism*. Academic Press, London.
4. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem*. **193:** 265–275.
5. Neuhard, J. 1983. Utilization of preformed pyrimidine bases and nucleoside, pp. 95–148. *In* Munch-Petersen, A. (ed.) *Metabolism of Nucleotides, Nucleosides and Nucleobases in Microorganism*. Academic Press, London.
6. Roland, K. L., F. E. Powell, and C. L. Jr. Turnbough. 1985. Role of translation and attenuation in the control of pyrBI operon expression in *Escherichia coli* K-12. *J. Bacteriol.* **163:** 991–999.
7. Potvin, B. W., R. J. Jr. Kelleher, and H. Gooder. 1975. Pyrimidine biosynthetic pathway of *Bacillus subtilis*. *J. Bacteriol*. **123:** 604–615.
8. Santi, D. V. and C. S. McHenry. 1972. 5-Fluoro-2'-deoxyuridylate: Covalent complex with thymidylate synthetase. *Proc. Natl. Acad. Sci. USA* **69:** 1855–1857
9. Saunders P. P., B. A. Wilson, and G. F. Saunders. 1969. Purification and comparative properties of a pyrimidine nucleoside phosphorylase from *Bacillus stearothermophilus*. *J. Biol. Chem*. **244:** 3691–3697.
10. Schwartz, M. 1976. Thymidine phosphorylase from *Escherichia coli*, pp. 442–443. *In* A. H. Patricia and E. J. Mary (eds.). *Methods in Enzymology*, vol. **51**, Academic Press, Avenue, New York, U.S.A.
11. Scocca, J. J. 1971. Purification and substrate specificity of pyrimidine nucleoside phosphorylase from *Haemophilus influenzae*. *J. Biol. Chem*. **246:** 6606–6610.
12. Timson J. 1975. Hydroxyurea. *Mutat. Res*. **32:** 115–132.
13. Tsen, S. D. 1994. Chemostat selection of *Escherichia coli* mutants secreting thymidine, cytosine, uracil, guanine, and thymine. *Appl. Microbiol. Biotechnol*. **41:** 232–238.