Biotechnology Letters **26:** 265–268, 2004.

Overproduction of thymidine by recombinant *Brevibacterium helvolum* amplified with thymidine monophosphate phosphohydrolase gene from bacteriophage PBS2

Hyeon Cheol Lee, Jung Mi Ahn, Sang Nam Lee & Jung Hoe Kim*
Department of Biological Sciences, Korea Advanced Institute of Science and Technology, 373-1 Guseong-dong, Yuseong-gu, Daejeon 305-701, Republic of Korea
**Author for correspondence (Fax: +82-42-869-2610; E-mail: kimjh@kaist.ac.kr)*

Received 2 June 2003; Revisions requested 11 June 2003/29 August 2003; Revisions received 15 August 2003/8 December 2003; Accepted 9 December 2003

Key words: PBS2 bacteriophage, thymidine, TMP phosphohydrolase

Abstract

A microbial fermentation process could be used to produce thymidine biologically but many of the enzymes related to nucleotide biosynthesis are highly regulated. To overcome the complex regulation steps, an analogue mutant of *Brevibacterium helvolum* resistant to fluorouracil, hydroxyurea, and trimethoprim was constructed. This mutant accumulated 380 mg thymidine l^{-1} in 16 h in shake-flask culture. However, the accumulation of thymidine monophosphate (TMP) inside the cells suggested a low activity of nucleotidase which degrades TMP to thymidine. This limitation was overcome by cloning the TMP phosphohydrolase (TMPase) gene of the unusual bacteriophage, PBS2. As a result, TMP in recombinant cells decreased from 230 μmol g^{-1} cell to 20 μmol g^{-1} cell with accumulation of 500 mg thymidine l^{-1} in the medium.

Introduction

Thymidine is an intermediate in the chemical synthesis of azidothymidine, the active ingredient in a formulation for the treatment of AIDS. Although the fermentative production of a polysaccharide containing thymidine in a mutant strain of *B. subtilis* was suggested in Japanese Patent Publication No. 39-16345 (1964), at present thymidine is produced chemically. The chemical synthesis of thymidine from anhydrous glucose requires numerous chemicals and a process of about 14 reaction steps. As a result, multi-step chemical synthesis process makes the production of thymidine very expensive.

Compared to chemical synthesis, biological production employing a microorganism would be low-cost and relatively efficient. However, thymidine levels inside cells are very low and are highly controlled because thymidine is involved only in DNA synthesis (Lerner *et al.* 1987, Jensen *et al.* 1982). There are consequently few fermentation processes available for thymidine production.

The degradation of intracellular TMP to thymidine by TMP phosphohydrolase (TMPase) plays an important role in the fermentative production of thymidine. Bacteriophage PBS2 contains uracil instead of thymine (Hemphill *et al.* 1975) and can infect *B. subtilis* strains, expressing certain enzymes, such as TMPase and dCMP deamidase, after infection. The TMPase of phage-infected *B. subtilis* is then able to catalyze the hydrolysis of TMP to thymidine (Roscoe *et al.* 1966).

In these present studies, firstly, the flux of thymidine biosynthesis was metabolically increased in analogue mutants that were constructed by selecting for resistance to the metabolic analogues, fluorouracil, hydroxyurea, and trimethoprim. Secondly, PBS2 TMPase was expressed in analogue mutants in order to further increase the degradation rate of intracellular TMP to thymidine. This paper shows the experimental

results on the fermentative production of thymidine by the recombinant *Brevibacterium* constructed for the first time.

Materials and methods

Bacterial strains, bacteriophage, and plasmids

Bacteriophage PBS2 was from the Bacillus Genetic Stock Center (The Ohio State University, Dept. of Biochemistry, OH). *Bacillus subtilis* SB19 was host strain for the amplification of bacteriophage. *E. coli* JM109 was used as the host strain for gene manipulations. *Brevibacterium helvolum* and all mutant strains were grown with shaking at 30 °C in nutrient broth and semi-defined medium [20 g glucose l^{-1}, 10 g $(NH_4)_2SO_4$ l^{-1}, 3 g urea l^{-1}, 1 g yeast extract l^{-1}, 0.4 g $MgSO_4 \cdot 7H_2O$ l^{-1}, 2 g Casamino acids l^{-1}, 10 mg pantotheate l^{-1}, 10 mg $CaCl_2$ l^{-1}, 20 mg aspartic acid l^{-1}, and trace elements].

The *Corynebacterium* expression vector, pEKEx1, was used to express viral TMPase in *B. helvolum*. The plasmid pEKEx1 includes the origin of pBL1, a kanamycin resistance gene, and an expression cassette.

Screening for the thymidine-producing strain

Nucleotide analogues (fluorouracil, hydroxyurea, trimethoprim) were used to construct analogue mutants derived from *deoA* mutant strain. For the screening of thymidine-producing strain from an analogue mutant library, a thymidine auxotroph, *E. coli* KCTC 2319 (*thyA*$^-$, *lacZ*) was used. Using a honeycomb plate, the pre-cultured indicator *E. coli* KCTC 2319 was inoculated to mutant colonies on minimal media containing IPTG/X-gal, cultures were incubated for 12 h, and blue colonies were selected.

Cloning and expression of TMPase

PCR was used to amplify the TMPase gene of PBS2 with flanking primers containing restriction sites for *Eco*RI and *Bam*HI to facilitate the subcloning of TMPase. The amplified open reading frame was 726 bp long and was verified by DNA sequencing. For the expression of TMPase, PBS2 TMPase gene was ligated into pEKEx1, a *Corynebacterium* vector and transformed into *B. helvolum*. The transformed cells were induced by adding 1 mM IPTG to the culture medium.

Enzyme assay and HPLC analysis

Transformed cells were harvested by centrifugation and washed with 10 mM Tris/HCl (pH 7.4) containing protein inhibitor, Complete (Amersham Phamacia). The TMPase activity was assayed by quantifying the inorganic phosphate released. One unit of enzyme catalyzed the hydrolysis of 1 μmol TMP per min at 37 °C (Petersen 1968). The protein concentration was determined by the Lowry method.

The quantitative determination of bases and nucleosides was carried out by HPLC. The operating conditions were: column, Primesphere 5 C18-HC (3.2 × 250 mm) (Phenomenex Co., Ltd., USA); solvent, 4% (v/v) acetonitrile in water containing 0.05% (v/v) trifluoroacetic acid; flow rate, 1 ml min^{-1}; detection at 260 nm.

Results

Thymidine production by genetically modified B. helvolum

Thymidine is converted to thymine by thymidine phosphorylase, the product of the inducible *deoA* gene. Mutants were generated by EMS to select the *deoA*-knockout mutant. A few mutants, which were unable to utilize thymidine as a sole carbon source, were firstly screened by taking the poorly growing colonies on thymidine minimal media. Secondly, the identities of *deoA* mutant strains isolated during this screening were confirmed by selecting mutants unable to metabolize thymine in the presence of thymine and fluorouracil, which blocks the *de novo* biosynthesis of thymidine. Thymidine phosphorylase activity of the selected strain was 0.34 U mg^{-1} protein, only 21% that of wild type (1.61 U mg^{-1} protein), yet no accumulation of thymidine was detected in the culture medium. Even though the activity of thymidine phosphorylase decreased, the degradation rate of thymidine still exceeded its synthesis rate.

The possibility of increasing the rate of thymidine synthesis by enhancing metabolic flux of critical pathways was investigated by constructing sequentially the metabolic analogue mutants; FU3, resistant to 2 mM fluorouracil, FH3-5, resistant to 100 mM hydroxyurea, and FHT3-5-10, resistant to 12 mM trimethoprim, respectively. Additionally, FHT^{10}3-5-10, resistant to high concentrations of thymidine (10 g l^{-1}), was screened using a thymidine-producer indicating sys-

Table 1. Enhancement of thymidine productivity by introduction of metabolic analogues resistance into the strain.

Strain	Cell growth ($g\ l^{-1}$)	Thymine ($mg\ l^{-1}$)	Thymidine ($mg\ l^{-1}$)	Specific thymidine productivity ($mg\ l^{-1}\ h^{-1}$)
deoA[a]	2.8	ND	ND	ND
FU3[b]	3	12	1	0.1
FH3-5[c]	3.2	10	20	1
FHT3-5-10[d]	3.2	23	300	12
FHT^{10}3-5-10[e]	3.3	63	380	24

These data were obtained from flask cultures for 16 h.
[a]*deoA* – *deoA* activity-reduced mutant.
[b]FU3 – Mutant resistant to 2 mM fluorouracil derived from *deoA*.
[c]FH3-5 – Mutant resistant to 100 mM hydroxyurea derived from FU3.
[d]FHT3-5-10 – Mutant resistant to 12 mM trimethoprim derived from FH3-5.
[e]FHT^{10}3-5-10 – Mutant resistant to 10 g thymidine l^{-1} derived from FHT3-5-10.
ND – Not detected.

tem with a thymidine auxotroph in screening media containing IPTG/X-gal.

The production of thymidine by analogue resistant mutants constructed with a semi-defined media in flask culture was investigated (Table 1). We found that the metabolic flux of the thymidine biosynthetic pathway was enhanced by introducing analogues resistance, but the degradation step of TMP still remained to be manipulated.

Cloning and expression of PBS2 TMPase

PCR amplification was carried out under appropriate conditions using a primer set that amplified the full ORF, and a strong band of around 700–800 bp was obtained. That fragment was cloned into a vector and sequenced. It was confirmed that the PBS2 TMPase gene was composed of 726 bp, and the coded protein appeared to be 28 kDa.

The expression vector containing PBS2 TMPase was constructed. The TMPase expression vector, designated pEKEx::TMPase, was transformed into the *B. helvolum* analogue mutant, FHT^{10}3-5-10. These recombinant cells permit transcription from the *tac* promoter in the presence of IPTG as an inducer. The expression of TMPase was confirmed by a TMPase activity assay. TMPase activity in the recombinant cells, FHT^{10}3-5-10 harboring pEKEx::TMPase was about 6 U μg^{-1} proteins and the activity remained for 4 h after infection.

The effect of PBS2 TMPase on thymidine production

The production of thymidine is unidirectional (Potvin *et al.* 1975). After TMP is synthesized, it is either degraded to thymidine or converted to DNA. The *B. helvolum* mutant FHT^{10}3-5-10 significantly accumulated TMP inside cells due to low TMPase activity and feedback regulation. However, in the recombinant cells, FHT^{10}3-5-10 harboring pEKEx::TMPase, the accumulation of TMP inside cells was a tenth of that in FHT^{10}3-5-10 (Table 2). This means that TMP degrades rapidly to thymidine due to high TMPase activity.

The recombinant cells accumulated 500 mg thymidine l^{-1} in the medium after 16 h, which is about 40% more than with the mutant FHT^{10}3-5-10 (Table 2). Thus, the expression of TMPase in analogue resistant mutants enhances the productivity of thymidine and reduces the accumulation of TMP inside cells.

Discussion

Thymidine production by the expression of TMPase is metabolically unfavorable. The expression of PBS2 TMPase in unmodified wild type *B. helvolum* inhibits its growth due to the retardation of nucleotide input. Ahmad *et al.* (1998) reviewed that thymine-less death (TLD) occurred in this case. However, the expression of PBS2 TMPase did not cause TLD and had a little inhibition on growth in *B. helvolum* mutants with enhanced thymidine metabolism. The metabolic burden for TMP consumption in FHT^{10}3-5-10 is smaller than in wild type *B. helvolum* because of the increased flux into thymidine. The thymidine-producing mutants used in this study are relatively free of the complex regulation of metabolites, so the increased flux

Table 2. Production of thymidine in recombinant *B. helvolum* harboring pEKEx::TMPase.

	Cell growth ($g\ l^{-1}$)	Thymidine ($mg\ l^{-1}$)	Intracellular TMP ($\mu mol\ g^{-1}$ cell)
FHT^{10}3-5-10 mutant	3.3	380	230
Recombinant cells harboring pEKEx::TMPase	2	500	20

These data were obtained from flask cultures for 16 h. FHT^{10}3-5-10 mutant–mutant resistant to 10 g thymidine l^{-1} derived from FHT3-5-10. pEKEx::TMPase – Expression vector containing TMPase gene.

into TMP brought about the accumulation of TMP in cells before transformation with pEKEx::TMPase, as presented. From this work, a high activity of TMPase is not required for thymidine production though some activity is needed to overcome TMP phosphorylation by thymidine kinase. Therefore, if the inducible promoter used in this study is substituted with its own constitutive promoter so as not to inhibit cell growth, then thymidine production by mutants that integrate TMPase into their chromosome and express it constitutively may be more effective (Mateos *et al.* 1996).

The expression of PBS2 TMPase represents an advance in the deregulation of nucleotides. Another PBS2 gene expressed in infected *B. subtilis*, dCMP deamidase, is also capable of enhancing the production of thymidine (Weiss *et al.* 1994). Although it may require some minor trimming, the construction of an artificial operon that includes dCMP deamidase and TMPase under the control of its own constitutive promoter may be effective in the production of thymidine.

References

Ahmad SI, Kirk SH, Eisenstark A (1998) Thymine metabolism and thymineless death in prokaryotes and eukaryotes. *Annu. Rev. Microbiol*. **52**: 591–625.

Hemphill E (1975) Bacteriophages of *Bacillus subtilis*. *Bacteriol. Rev*. **39**: 257–315.

Jensen F, Neuhard J, Schack L (1982) RNA polymerase involvement in the regulation of expression of *Salmonella typhimurium pyr* genes. Isolation and characterization of a fluorouracil-resistant mutant with high constitutive expression of the *pyrB* and *pyrE* genes due to a mutation in *rpoBC*. *EMBO J*. **1**: 69–74.

Lerner G, Stephenson BT, Switzer RL (1987) Structure of the *Bacillus subtilis* pyrimidine biosynthetic (*pyr*) gene cluster. *J. Bacteriol*. **169**: 2202–2206.

Mateos M, Schafer A, Kalinowski J, Martin JF, Puhler A (1996) Integration of narrow-host-range vectors from *Escherichia coli* into the genomes of amino acid-producing *Corynebacteria* after intergeneric conjugation. *J. Bacteriol.* **178**: 5768–5775.

Petersen M (1968) On the catabolism of deoxyribonucleosides in cells and cell extracts of *Escherichia coli*. *Eur. J. Biochem* **6**: 432–442.

Potvin W, Kelleher Jr RJ, Gooder H (1975) Pyrimidine biosynthetic pathway of *Bacillus subtilis*. *J. Bacteriol*. **123**: 604–615.

Roscoe H, Tucker RG (1966) The biosynthesis of 5-hydroxymethyl deoxyuridylic acid in bacteriophage-infected *Bacillus subtilis*. *Virology* **29**: 157–166.

Weiss, Wang L (1994) *De novo* synthesis of thymidylate via deoxycytidine in *dcd* (dCTP deaminase) mutants of *Escherichia coli*. *J. Bacteriol.* **176**: 2194–2199.