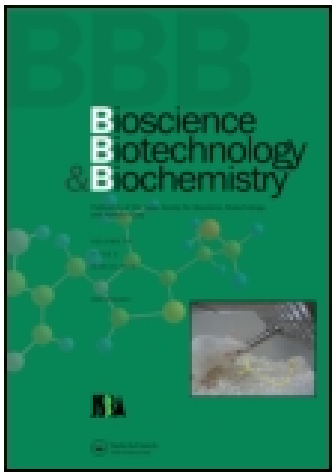

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Construction of a Plasmid Carrying both CTP Synthetase and a Fused Gene Formed from Cholinephosphate Cytidylyltransferase and Choline Kinase Genes and Its Application to Industrial CDP-Choline Production: Enzymatic Production of CDP-Choline from Orotic Acid (Part II)

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Construction of a Plasmid Carrying both CTP Synthetase and a Fused Gene Formed from Cholinephosphate Cytidylyltransferase and Choline Kinase Genes and Its Application to Industrial CDP-Choline Production: Enzymatic Production of CDP-Choline from Orotic Acid (Part II)

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A new method for enzymatic production of cytidine diphosphate choline (CDP-choline) from orotic acid and choline chloride was developed. To establish an industrial manufacturing process, we constructed a plasmid, pCKG55, which simultaneously expressed in *Escherichia coli* the three following enzymes; CTP synthetase (encoded by the *pyrG* gene from *E. coli*), cholinephosphate cytidylyltransferase (encoded by the *CCT* gene from *Saccharomyces cerevisiae*), and choline kinase (encoded by the *CKI* gene from *S. cerevisiae*). *CCT* and *CKI* genes on pCKG55 were designed to be expressed as a single CCT/CKI fused protein. This CCT/CKI fused protein retained both activities and the thermal stability of its cholinephosphate cytidylyltransferase activity was nearly the same as the native CCT enzyme. *Corynebacterium ammoniagenes* KY13505 and *E. coli* MM294/pCKG55 were cultured in 5-liter jar fermentor independently. Equal volumes of each broth were mixed in a 2-liter jar fermentor, and then the enzymatic reaction was done using 47 mM orotic acid and 60 mM choline chloride as substrates. After 23 h of the reaction at 32°C, 21.5 mM (11 g/liter) of CDP-choline was accumulated.

Key words: CDP-choline; orotic acid; enzymatic production; ATP regeneration; PRPP supply

Cytidine diphosphate choline (CDP-choline) is a biosynthetic intermediate of phosphatidyl choline (lecithin)[1,2] and a useful drug for various kinds of brain injuries.[3] Several studies have been done for the enzymatic production of CDP-choline, which used cytidine nucleotide or nucleoside such as CMP or cytidine as the substrate and used microorganism cells such as yeast cells as the enzyme source.[4,5] Orotic acid is a biosynthetic intermediate for pyrimidine nucleotides and is obtained at relatively high yield by culturing mutant of *Corynebacterium glutamicum*.[6] Therefore, we attempted to develop the new enzymatic process for CDP-choline production from orotic acid instead of cytidine derivatives.

In our previous paper,[7] we reported the possibility of enzymatic accumulation of CDP-choline from orotic acid and choline chloride in which *C. ammoniagenes* mutant cells and three recombinant *E. coli* cells were used as the enzyme sources. These three recombinant *E. coli* strains expressed respectively three following enzymes required for CDP-choline synthesis; CTP synthetase (originated from *E. coli*), cholinephosphate cytidylyltransferase (originated from *S. cerevisiae*), and choline kinase (originated from *S. cerevisiae*). It was seen that *C. ammoniagenes* cells could produce UTP from orotic acid. *C. ammoniagenes* cells were also shown to regenerate ATP well enough[8] to drive the enzymatic chain reactions from UTP to CDP-choline occurred in recombinant *E. coli* cells.

However, this reaction system seemed impractical on an industrial scale, because it required four different harvested cells. It is difficult and complicated to operate four kinds of strains and to use harvested cells.

In this paper, we describe the construction of plasmid pCKG55, which simultaneously expressed three kinds of enzymes, CTP synthetase, cholinephosphate cytidylyltransferase, and choline kinase. We also describe CDP-choline production in the reaction using two separately cultured broths of *C. ammoniagenes* KY13505 and *E. coli* MM294/pCKG55.

Materials and Methods

Bacterial strains and plasmids. A mutant of *C. ammoniagenes* KY13505 (decoyinine-resistant, nucleotidase-weak, and temperature-sensitive for growth) was used as both an ATP regeneration activity donor and efficient UTP synthesizing activity donor from orotic acid. *E. coli* strain MM294 (F^-, *sup*E44, *thi*1, *end*A1, *hsd*R17)[9] was used as a host strain. Plasmid pMW6 was provided by Howard Zalkin *et al.*, Purdue University.[10] Plasmids pCC41 and pCK1D were donated by Satoshi Yamashita *et al.*, Gunma University.[11,12] Constructions of plasmids pMW6 and pCC41 were described in our previous paper.[7] Plasmid pCK1D was constructed by ligating the 2.7-kb *Pst*I–*Hin*dIII fragment of the *S. cerevisiae* CKI gene encoding choline kinase into the YEpM4 yeast-*E. coli* shuttle vector, which was composed of the 2-μm replication origin, the LEU2 selectable marker and a part of the pUC18 sequence containing multicloning sites.[13]

Media and cultivation. LB medium (1% bactotryptone, 0.5% yeast extract, 0.5% NaCl, adjusted to pH 7.2 with NaOH) was used to cultivate *E. coli*. A loopful cells of *E. coli* was inoculated into 10 ml of LB medium containing 50 mg/liter ampicillin in a test tube (20 cm × 2 cm), and then cultured at 25°C for 18 h on a reciprocal shaker (300 rpm). One-tenth ml of the seed culture was inoculated into 10 ml of LB medium containing 50 mg/liter ampicillin in a test tube and cultured at 33°C for 10 h with shaking. A half ml of the culture was centrifuged, with the supernatant discarded afterwards. The harvested cells were suspended in 0.5 ml of 20 mM potassium phosphate buffer (pH 7.0), followed by the addition of 5 μl of xylene and stirred at 30°C for 10 min. Thus the xylene-treated *E. coli* cell suspension was used as a crude enzyme solution the activities of which

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were determined as described in the following paragraph.

The cultivation of *E. coli* in a 5-liter jar fermentor was done as follows. A loopful cells of *E. coli* MM294/pCKG55 was inoculated into 10 ml of LB medium containing 50 mg/liter ampicillin in a test tube and then cultured with reciprocal shaking at 25°C for 24 h. Twenty ml of the resulting culture was inoculated into 400 ml of LB medium containing 50 mg/liter ampicillin in a 2-liter Erlenmeyer flask with baffles and then incubated at 25°C for 16 h on a rotary shaker (190 rpm). A portion of the culture (125 ml) was transferred to a 5-liter jar fermentor containing 2.5 liter of the medium (without pH adjustment) composed of 5 g/liter glucose (separately sterilized), 5 g/liter peptone, 6 g/liter Na_2HPO_4, 3 g/liter KH_2PO_4, 5 g/liter NaCl, 250 mg/liter $MgSO_4 \cdot 7H_2O$ (separately sterilized), and 4 mg/liter vitamin $B_1 \cdot HCl$ (separately sterilized). Cultivation was done in two stages; the first at 25°C for 11 h and the second at 32°C for 13 h both with stirring (600 rpm) and aeration (2.5 liter/min). The pH was maintained at around 7.0 with 14% NH_4OH throughout the two-staged cultivation. After 11 h of cultivation a mixed solution of 167 g/liter glucose and 167 g/liter peptone was started and continued for 13 h by a Perista pump at a rate of 30 ml/h.

C. ammoniagenes KY13505 was cultivated in a 5-liter jar fermentor as described in our previous paper.[7]

Enzyme assays. CTP synthetase activity was measured by the method of Zalkin[14] with a slight modification. The reaction mixture (2 ml) was as follows: 40 mM Tris-HCl (pH 7.1), 10 mM $MgCl_2$, 1 mM ATP, 1 mM UTP, 0.2 mM GTP, 2 mM glutamine, 8 mM phosphoenolpyryvate, and xylene-treated *E. coli* cells. The reaction mixture was shaken in a test-tube at 38°C for 60 min. Intermittently, 0.2-ml portions were sampled and centrifuged at $1600 \times g$ for 10 min after mixing with 1.8 ml of 3.5% perchloric acid. The absorbancy at 291 nm of the supernatant was measured. Under this acidic condition, the absorbancy at 291 nm increases with the formation of CTP. Under these conditions, one unit of the enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of CTP per min.

Cholinephosphate cytidylyltransferase (CCTase) activity was assayed by modifications of the methods of Hosaka *et al.*[15] and Nikawa *et al.*[16] The reaction mixture (0.5 ml) was as follows: 150 mM potassium phosphate buffer (pH 7.5), 25 mM $MgCl_2$, 5 mM CTP, 5 mM phosphorylcholine, and xylene-treated *E. coli* cells. The reaction mixture was incubated in a 1.5-ml microfuge tube at 30°C for 2 h. During the reaction, 50-μl portions were intermittently removed, and added to microfuge tubes containing 50 μl of 0.2 mM acetic acid, and heated at 100°C for 2 min to stop the reaction. The resulting mixture was centrifuged at $13,000 \times g$ for 1 min, and its supernatant was diluted five-fold with distilled water. The CDP-choline formed was measured by high performance liquid chromatography (HPLC) under the conditions described previously.[7] Under these conditions, one unit of the activity is defined as the amount of enzyme required for the formation of 1 μmol of CDP-choline per min.

Genetic techniques. DNA manipulations were done essentially according to the protocols.[17] The nucleotide sequence of N-terminal region of the gene encoding the CCT/CKI fused protein on pCK55 was analyzed by the dideoxy method of Sanger *et al.*[18]

Enzymatic reaction. An equal volume (360 ml) of the cultured broths of *E. coli* MM294/pCKG55 and *C. ammoniagenes* KY13505 were mixed in a 2-liter jar fermentor, followed by addition of 100 g/liter glucose, 10 g/liter (47 mM) orotic acid, 8.4 g/liter (60 mM) choline chloride, 5 g/liter $MgSO_4 \cdot 7H_2O$, and 20 ml/liter xylene. Thus the reaction mixture was adjusted to 800 ml with distilled water before the reaction. The enzymatic reaction was done at 32°C with stirring (800 rpm) and aeration (0.8 liter/min). The pH was maintained at 7.2 with 10 N NaOH, and KH_2PO_4 concentration of the mixture was kept at the range between 1 to 5 g/liter (by discontinuous feeding).

Analyses. CDP-choline and other related intermediates were measured by HPLC. Glucose, inorganic phosphate, and magnesium ion were measured by three assay kits; GlucoseB-Test Wako, PhosphorB-Test Wako, and MagnesiumB-Test Wako, all purchased from Wako Pure Chemical Industry Ltd. (Osaka).

Chemicals. Orotic acid used for the reaction was the product of Kyowa Hakko Kogyo Co., Ltd. All other chemicals used here were commercially available and of analytical grade.

Results and Discussion

Construction of plasmid pCK1

CCT gene and *CKI* gene have already been cloned from *S. cerevisiae* and their nucleotide sequences have been analyzed by Tsukagoshi *et al.*[19] and Hosaka *et al.*[12] Based on the nucleotide sequence data, it was expected that CCT/CKI fused protein could be expressed by in-frame ligation at *Hpa*I sites located in the C-terminal region of the *CCT* gene and the N-terminal region of the *CKI* gene. In the plasmid pCC41, 24 amino acids coding a sequence of the N-terminal region of the *CCT* gene were deleted at the *Dra*I site, and replaced by an 11-amino-acid coding sequence from multicloning sites of pUC18. Therefore, the plasmid pCC41 expressed the *CCT* gene consisted of 411 amino acids. On the other hand, the plamid pCKD1 expressed full length (582 amino acids) of the *CKI* gene. By digestion with *Hpa*I, 14 amino acids of the C-terminal region of CCTase and 31 amino acids of the N-terminal region of CKIase were deleted respectively. The *CCT/CKI* fused gene on the plasmid pCK1 encodes a fused protein consisted of 948 amino acids.

Figure 1 shows the construction of pCK1 including the *CCT/CKI* fused gene. The CCTase activity of strain MM294/pCK1 was measured by the method described in Materials and Methods. Two hundred μl of xylene-treated *E. coli* MM294/pCK1 cells which were concentrated fourfold from the cultured broth using a test tube, were added to the reaction mixture (0.5 ml). After 4 h of reaction, 4.2 mM of CDP-choline was formed from 5 mM CTP and 5 mM phosphorylcholine, indicating pCK1 expressed the CCTase activity. Under the same reaction conditions, approximately an equal amount (4.4 mM) of CDP-choline was formed when 5 mM phosphorylcholine was replaced by 5 mM choline chloride, in the presence of 5 mM ATP. These results indicated that the *E. coli* strain carrying pCK1 had both the CCTase and CKIase activities. It was an unexpected result that such a large fused protein retained these two enzyme activities.

The thermal stability of the CCT/CKI fused protein was investigated as to CCTase activity. Comparing before and after protein fusion, we found that the CCT/CKI fused protein (expressed by plasmid pCK1) held similar thermal stability (Fig. 2) to the single CCT protein expressed by plasmid pCC41. We tried to measure CKIase activities of the CCT/CKI fused protein and the single CKI protein, but obtained data that was low in reliability. Therefore, we do not mention the data for CKIase activity in this report.

Deletion of N-terminal region of the CCT/CKI fused protein

To increase the expression degree of the CCT/CKI fused protein, we tried to delete part of the N-terminal sequence of this gene, because we considered that the N-terminal sequence might be important for the gene expression. Plasmid pCK1 was digested with *Kpn*I, treated with *Bal*31 nuclease for a short time, and ligated with T4 DNA ligase. *E. coli* MM294 strain was transformed with this ligation mixture. The ampicillin-resistant transformants obtained were cultivated and examined for the CCTase activity. Some of the transformants had higher CCTase activity than the strain MM294/pCK1 did. The plasmid extracted from the strain with the highest CCTase activity was named pCK55. The enzyme activity of the strain MM294/pCK55 will be

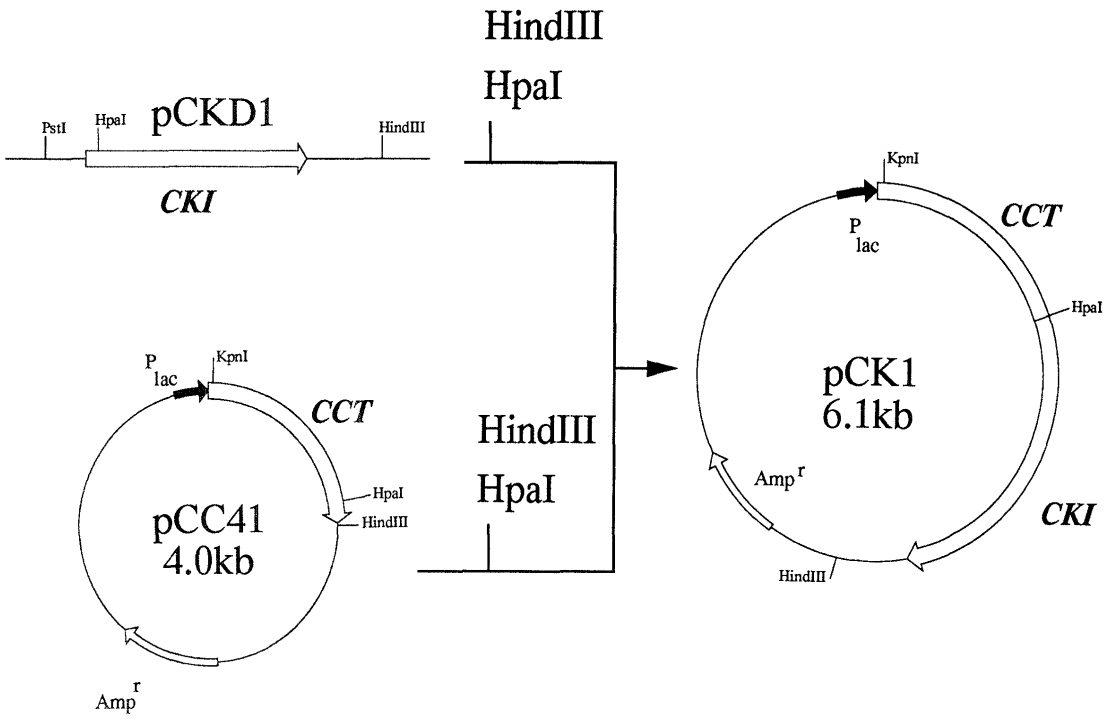

Fig. 1. Construction of Plasmid pCH1.

CCT and *CKI* genes are shown by the thick open arrows. The ampicillin resistance genes (Amp^r) are indicated by the thin open arrows. Thin black arrows represent the lactose promoter (Plac).

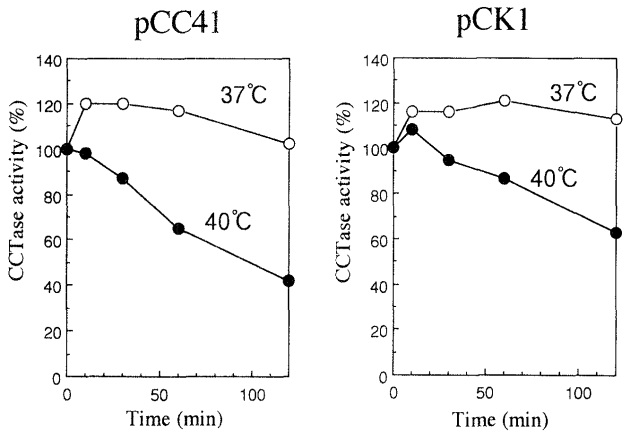

Fig. 2. Thermal Stability of the *CCT/CKI* Fused Protein.

Twenty ml of cultured broths of *E. coli* MM294/pCC41 and MM294/pCK1 using LB medium were centrifuged. Cells were suspended in 4 ml of 100 mM potassium phosphate buffer (pH 7.5) and sonicated to obtain crude cell extract. To each 50 μl of these cell extracts, 100 μl of 500 mM potassium phosphate buffer (pH 7.5), 25 μl of 250 mM $MgCl_2$, and 25 μl of distilled water were added. These mixtures were heat treated at 37°C or 40°C for 0–120 min. After treatment, 25 μl of 250 mM CTP and 25 μl of 200 mM phosphoryl choline were added and the reaction was done for 4 h at 30°C.

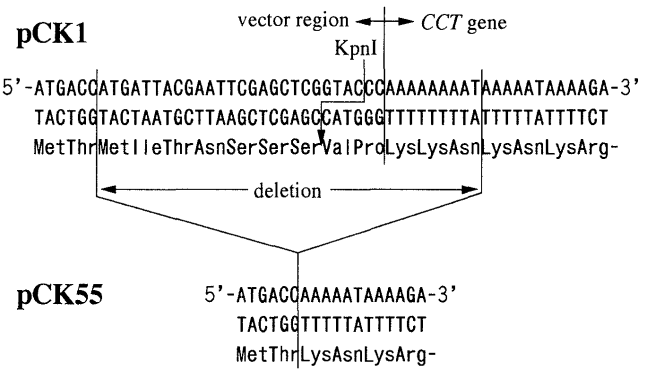

Fig. 3. Nucleotide Sequence of N-Terminal Region of *CCT/CKI* Fused Gene.

N-Terminal region of plasmid pCK1 was deleted with Bal 31 nuclease from the *Kpn*I site, and the deleted plasmid pCK55 had 36 bp deleted (horizontal arrow).

described after (Table I).

After analyzing the nucleotide sequence of N-terminal region of the *CCT/CKI* fused gene on pCK55, 12 amino acids at the N-terminal region, 9 from *E. coli* vector pUC18 and 3 from the *CCT* gene, were found to be deleted from the sequence of original pCK1 (Fig. 3). Thus the deleted fused protein is consisted of 936 amino acids. We speculate that the deleted sequence thus obtained is suitable for the gene expression because an AT-rich sequence is connected just downstream of the initiation codon.

Construction of the plasmid pCKG55

Plasmid pMW6 containing the pyrG gene was digested with *Mlu*I and treated with T4 DNA polymerase to make the *Mlu*I cleavage site into a blunt end. The treated DNA was digested with *Hin*dIII, and its larger fragment was isolated by agarose gel electrophoresis. Meanwhile, plasmid pCK55 was cleaved with *Hin*dIII and *Pvu*II, and its larger fragment was isolated.

Both of these fragments were ligated with T4 DNA ligase. *E. coli* MM294 strain was transformed with this ligation mixture, and ampicillin-resistant transformants were selected. Among them, a strain was selected that carried a plasmid with its expected length of 8.3 kb, and this plasmid was named pCKG55 (Fig. 4).

Enzyme activities of the recombinant E. coli strains

To test whether such both activities were sustained at the same level as the original pCC41 and pMW1, the strain carrying the different plasmids were compared for CCTase and CTP synthetase activities. As shown in the Table,

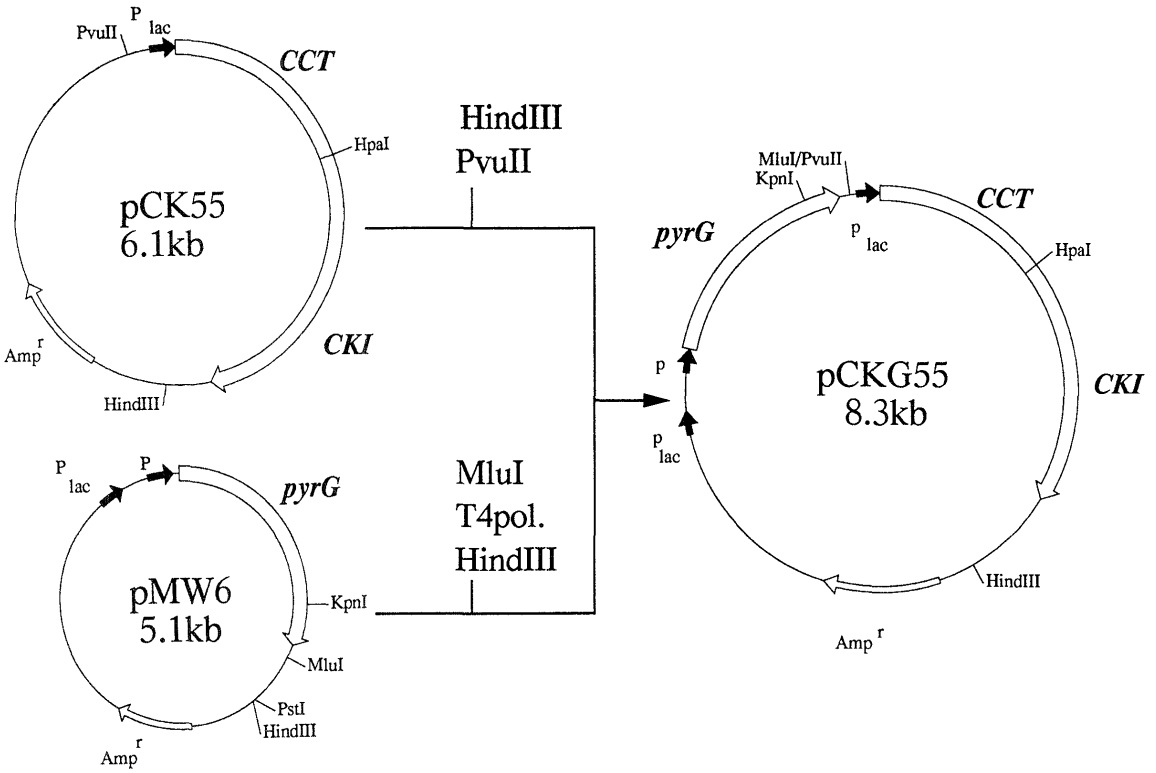

Fig. 4. Construction of Plasmid pCKG55.

CCT, *CKI*, and *pyrG* genes are shown by the thick open arrows. The ampicillin resistant genes (Amp^r) are indicated by the thin open arrows. Thin black arrows represent the lactose promoter (Plac) and the *pyrG* own promoter (P).

Table Comparison of Enzyme Activities of the Recombinant *E. coli* Strain[a]

Plasmid	Genes	CCTase activity (U/ml)	CTP synthetase activity (U/ml)
No	—	0	trace
pCC41	*CCT*	0.052	NM[b]
pMW6	*pyrG*	NM	0.27
pCK1	*CCT/CKI*	0.035	NM
pCK55	*CCT/CKI*	0.053	NM
pCKG55	*CCT/CKI, pyrG*	0.048	0.21

[a] *E. coli* MM294 strain was used as a host.
[b] Not measured.

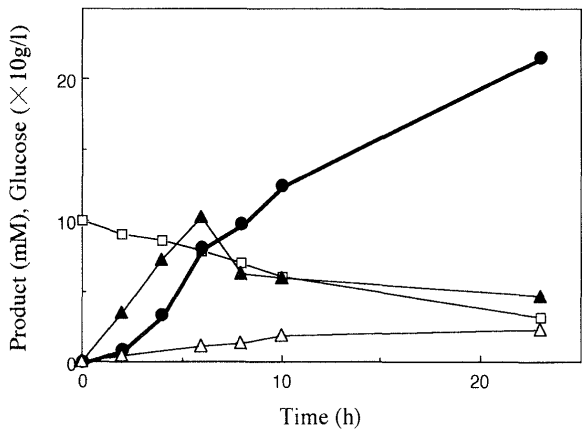

Fig. 5. Time Course of CDP-Choline Production.
Symbols: ●, CDP-choline; △, UTP; ▲, UMP; □, glucose.

plasmid pCK1 had lower CCTase activity than plasmid pCC41, but pCK55 expressed almost the same level of CCTase activity compared to plasmid pCC41. Plasmid pCKG55 showed nearly the same levels of both CCTase and CTP synthetase activities as pCK55 and pMW6 showed respectively. All plasmids obtained in this study expressed *pyrG*, *CCT*, and/or *CKI* gene under the control of lac promoter, therefore we examined the effect of inducer, IPTG (isopropyl-1-thio-β-D-galactopyranoside), in the cultivation of recombinant *E. coli* strains, but the enzyme activities were almost the same level in all cases (data not shown). Therefore, it was considered that each gene was expressed under the conditions of derepression.

Enzymatic production of CDP-choline

Enzymatic reaction using the cultured broths of *E. coli* MM294/pCKG55 and *C. ammoniagenes* KY13505 was done as described in Materials and Methods. Figure 5 shows a typical time course of the reaction in a 2-liter jar fermentor. After 23 h of reaction, 11 g/liter (21.5 mM) of CDP-choline was accumulated from 47 mM of orotic acid and 60 mM of choline chloride. UMP and UTP were co-produced in a small amount, while CTP was co-produced at a lower level (less than 1 mM, data not shown). These results show that CTP was fully converted to CDP-choline in contrast to the lower conversion of UTP to CTP due to weak CTP synthetase activity. In this reaction, CTP synthetase activity was considered rate-limiting and the increase of this activity might increase the yield of CDP-choline. Asahi *et al.* reported that 14.2 g/liter of cytidine was accumulated by a mutant of *Bacillus subtilis*.[20] In this report, it was important to increase cytidine productivity by releasing the CTP synthetase of this mutant strain from regulation by CTP.

In this study, we used enough of the substrates in the enzymatic reactions. If all the reaction conditions including concentration levels of the substrates were optimized, the co-production of UMP, UTP, and CTP or any other intermediates should be repressed at lower levels, resulting in a high conversion rate of CDP-choline.

This new process, which only requires two cultured broths, allows CDP-choline production from orotic acid and choline chloride on industrial scale. It should also be noted that this new process achieved seven steps of enzyme chain reaction, ATP regeneration, and PRPP supply in a one-batch process. In summary, we consider that this process provides a cheaper product, CDP-choline, compared to the other processes, because of using cheap substrates, orotic acid and choline chloride. We suppose that this process is the first example of an enzymatically produce valuable product using such a long pathway.

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