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Deoxycytidine Production by Metabolically Engineered *Corynebacterium ammoniagenes*

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Corynebacterium ammoniagenes **N424 was metabolically modified to isolate overproducers of deoxycytidine. Inosine auxotrophy (ino$^-$) was initially introduced to prevent the flow of PRPP (phosphoribosyl pyrophosphate) into the purine biosynthetic pathway by random mutagenesis using N-methyl-N'-nitro-N-nitrosoguanidine. Following that, mutants possessing hydroxyurea resistance (HU^r) were isolated to increase the activity of ribonucleoside diphosphate reductase, which catalyzes the reduction of ribonucleoside diphosphate to deoxyribonucleoside diphosphate. Then, in order to block the flow of dCTP into the TMP biosynthetic pathway via dUTP, thymine auxotrophy (thy$^-$) was introduced into the mutant IH30 with ino$^-$ and HU^r. The resulting mutant IM7, possessing the characteristics of ino$^-$, HU^r, and thy$^-$, was deficient in dCTP deaminase and produced significantly higher amounts of deoxycytidine (81.3 mg/L) compared to its mother strain IH30 (6.2 mg/L). Deoxycytidine productivity was further enhanced by isolating the mutant IU19, which was resistant to 5-fluorouracil, an inhibitor of carbamoyl phosphate synthase. This enzyme catalyzed the synthesis of carbamoyl phosphate from glutamine, HCO_3^-, and ATP. 5-Fluorouracil also inhibited aspartate transcarbamoylase, catalyzeing the condensation of carbamoyl phosphate and aspartate. Finally, 5-fluorocytosine resistance (FC^r) was introduced into the mutant strain IU19 to relieve the repression caused by accumulation of pyrimidine nucleosides. The mutant strain IC14-C6 possessing all the five characteristics described above produced 226.3 mg/L of deoxycytidine, which was at least 2,000 fold higher compared to the wild type, and accumulated only a negligible amount of other pyrimidines under shake flask fermentation.**

Keywords: *Corynebacterium ammoniagenes*, deoxycytidine, mutation, metabolic engineering, NTG, dCTP deaminase

Pyrimidine deoxyribonucleosides such as deoxycytidine, deoxyuridine and thymidine are industrially useful as precursors for the production of antiviral compounds including dideoxycytidine (ddC), 2'-deoxy-3'-thiacytidine (3TC), azidodeoxyuridine, and 3'-azido-2',3'-dideoxythymidine (AZT) (Asahi and Tsunemi, 1989; De Clercq, 1992; Schinazi *et al*., 1992; Prasad and Wengel, 1996). Microbial production of deoxycytidine has recently been of great interest because of its cost effectiveness in comparison with chemical production processes.

Deoxycytidine can be made from dCMP by dephosphorylation and also converted via dTDP to dCTP which is used for the synthesis of DNA in cells. In general, the enzymatic steps in the *de novo* biosynthetic pathways of pyrimidine nucleotides such as UTP, CTP, dCTP, and TTP 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 (Potvin *et al*., 1975; Roland *et al*., 1985). Accordingly, pyrimidine nucleosides such as deoxycytidine, cytidine, uridine, deoxyuridine, and thymidine, which could theoretically be synthesized from the end products (pyrimidine nucleotides), are almost impossible to secrete out of cells and accumulate in media. In addition, the pyrimidine metabolic pathway is branched to synthesize various precursors for DNA and RNA building blocks, and therefore, undesirable byproducts can accumulate due to metabolic or regulational change (Tsen, 1994). Hence, to accumulate a large amount of deoxycytidine, cells must be resistant to feedback regulation and metabolically modified.

Previously, we reported on the production of thymidine by mutant strains of *Corynebacterium ammoniagenes* ATCC 6872 (Song *et al*., 2005). Corynebacterial strains have been used for the industrial production of nucleotides such as inosine monophosphate (IMP) and guanosine monophosphate (GMP), and amino acids such as lysine and glutamate because the biosynthetic regulations of nucleotides or amino acids in those strains were relatively simple and they could accumulate those metabolites in a medium in large quantities (Eggeling and Bott, 2004).

C. ammoniagenes KFCC10614, which was adenine and guanine auxotrophic, (ade$^-$, gua$^-$) and could excrete a large amount of IMP into media without manganese limitation, was developed and used for the industrial production of IMP (Hyun *et al*., 1990). For the overproduction of deoxycytidine, it was necessary to isolate inosine auxotrophs in order to shift the flow of IMP synthesis to the synthesis of pyrimidine nucleotides. Therefore, we decided to use *C. ammoniagenes* N424, which was a revertant strain (ade$^+$, gua$^+$) derived from *C. ammoniagenes* KFCC10614 because the adenine and guanine auxotrophic strains could not grow in minimal medium supplemented with inosine. In this study, we carried out metabolic engineering of the *C. ammoniagenes* N424 to develop mutant

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Table 1. Strains of *C. ammoniagenes* and *E. coli* used and isolated in this study and their deoxycytidine productivity. Abbreviations: ino^-, inosine auxotroph; HU^r, resistant to hydroxyurea; thi^-, thymine auxotroph; $5FU^r$, resistant to 5-fluorouracil; $5FC^r$, resistant to 5-fluorouracil

Strain	Characteristics	Deoxycytidine (mg/L)	Thymine (mg/L)
C. ammoniagenes			
N424	-	0.0	0.0
I31	ino^-	0.5	12.7
IH30	ino^-, HU^r	6.2	82.0
IM7	ino^-, HU^r, thy^-	81.3	0.0
IU19	ino^-, HU^r, thy^-, $5FU^r$	113.4	0.0
IC14-C6	ino^-, HU^r, thy^-, $5FU^r$, $5FC^r$	226.3	0.0

strains that can accumulate a considerable amount of deoxycytidine.

Materials and Methods

Bacterial strains and media

The *C. ammoniagenes* strains developed and used in this study are shown in Table 1. *C. ammoniagenes* was routinely cultivated in a complex medium (CM) consisting of 2.5 g of NaCl, 5 g of beef extract, 5 g of yeast extract, and 10 g of peptone per L. The minimal medium (MM) used for the isolation and characterization of mutants consisted of 20 g of glucose, 3 g of $(NH_4)_2SO_4$, 1 g of KH_2PO_4, 0.3 g of $MgSO_4 \cdot 7H_2O$, 10 mg of $CaCl_2 \cdot H_2O$, 10 mg of $FeSO_4$, 1 mg of $ZnSO_4 \cdot 7H_2O$, 5 mg of thiamine·HCl, 10 mg of Ca-D-pantothenate, and 30 mg of D-biotin per L. MM was supplemented with 100 mg of inosine or 50 mg of adenine and 50 mg of guanine, and/or 100 mg of thymine per L, depending on the mutant characteristics. The shake flask fermentations were carried out in an Erlenmeyer flask (500 ml) containing 25 ml of MM supplemented with the appropriate growth factors, and shaken at 250 rpm at 30°C. During the fermentation process, samples were taken at appropriate time intervals for the determination of growth and deoxycytidine content.

Mutagenesis and isolation of mutants

To isolate inosine (ino^-) or thymine auxotrophs (thy^-), cells grown on CM medium till the mid logarithmic growth phase were centrifuged at 10,000×g for 10 min, suspended in fresh CM, and then treated with N-methyl-N'-nitro-N-nitrosoguanidine (30-90 μg-NTG/ml) at 30°C for 1 h. The treated cells were washed three times with saline, cultivated in CM for 2 h, diluted appropriately, and plated on MM agar with 100 mg/L of inosine or 50 mg/L of thymine. After incubating the plates at 30°C for 72 h, they were replica-plated on MM agar. Colonies that grew in the presence of inosine or thymine and not in their absence were selected as inosine or thymine auxotrophs, respectively.

To isolate hydroxyurea-(HU^r), 5-fluorouracil-(FU^r), and 5-fluorocytosine-(FC^r) resistant mutant strains, mutagenized cells were plated onto MM agar medium with appropriate amounts of hydroxyurea, 5-fluorouracil or 5-fluorocytosine. If needed, the MM medium was supplemented with inosine (100 mg/L) or thymine (50 mg/L). Colonies that appeared on the agar plates were isolated as resistant mutants. Deoxycytidine productivity was then evaluated by shake-flask fermentation.

Preparation of cell extracts

To generate cell extracts, cell suspensions were initially prepared by cultivating organisms in an Erlenmeyer flask containing 50 ml CM, harvesting the cells at the late exponential phase by centrifugation at 10,000×g for 10 min, and suspending the cell paste in 2.5 vol of TE buffer (0.01 M Tris-HCl, 2 mM EDTA, pH 7.2). The cells were disrupted by passage through a French pressure cell at 12,000 PSI. The supernatants (cell extracts) were harvested and used for the dCTP deaminase assay.

dCTP deaminase assay

dCTP deaminase activity was determined by measuring the amount of dUTP generated from dCTP by enzymatic conversion. The reaction was initiated by adding 0.1 ml of cell extract to 0.4 ml of reaction mixture consisting of 50 mM potassium phosphate buffer (pH 6.8), 10 mM dCTP, 50 mM $MgCl_2$, and 2 mM 2-mercaptoethanol, and stopped by adding 0.9 ml of 0.5 M perchloric acid to the mixture. The reaction was carried out at 37°C, and 1 unit of dCTP deaminase was defined as the amount of enzyme that converted 1 μmole of dCTP into dUTP per minute at 37°C. Total protein was determined by the Lowry method (Lowry *et al*., 1951).

Analytical methods

To determine turbidity, culture broths were appropriately diluted with distilled water and optical densities were measured at 660 nm using a spectrophotometer (Genesys 20, ThermoSpectronic, USA). Pyrimidine and pyrimidine nucleotides were analyzed by HPLC (HP1100, Agilent, USA) using a C_{18} reversed-phase column (Zorbax XDB-C18, Agilent), and an isocratic solution of 50 mM phosphate buffer (pH 7.0) or 50 mM phosphate buffer (pH 2.0) and 2% acetonitrile (97:3 ratio).

Results

Isolation of inosine-auxotrophic mutants

C. ammoniagenes N424 does not accumulate deoxycytidine, cytosine or other pyrimidine nucleosides in media. PRPP (phosphoribosyl pyrophsphate) is used for the synthesis of both purine and pyrimidine nucleotides and therefore, in order to prevent PRPP from being incorporated into the purine biosynthetic pathway, inosine auxotrophy was introduced into *C. ammoniagenes* N424. Inosine auxotrophs which only grew on MM supplemented with inosine were selected. The mutant strain I31 grew only when supplemented with inosine or with adenine and guanine (Table 2), implying that the enzyme in the biosynthetic pathway prior to IMP might be deficient. This strain accumulated 0.5 mg/L of deoxycytidine and 12.7 mg/L of thymine (Table 1).

Isolation of hydroxyurea-resistant mutants

Deoxyribonucleotides, the building blocks of DNA, are synthesized from the corresponding ribonucleotides by the enzyme

Table 2. Growth of mutant strains, N424 and I31 in the minimal medium containing inosine or adenine/guanine

Strain	Growth (OD_{660})[a]		
	Minimal medium (MM)	MM with inosine (100 mg/L)	MM with adenine and guanine (50 mg/L each)
C. ammoniagenes			
Strain N424	12.0	12.0	13.0
Mutant strain I31	0.5	12.0	12.0

[a] Growth was measured at appropriate time intervals and the peak value of cell concentration was chosen and expressed here.
Table 3. Growth of mutant strains, N424, IH30, and IM7 in the minimal medium containing inosine or inosine/thymine.

ribonucleoside diphosphate reductase, which catalyzes the reduction of ribonucleoside diphosphate to deoxyribonucleoside diphosphate in *C. ammoniagenes* (Fig. 1). Hydroxyurea, an established inhibitor of ribonucleoside diphosphate reductase (Timson, 1975), was used to isolate mutant strains that might have increased ribonucleoside diphosphate reductase activity and accumulate more pyrimidine. The mutant strain IH30 which was resistant to 15 mM hydroxyurea was isolated, but this strain accumulated 6.2 mg/L of deoxycytidine and 82.0 mg/L of thymine (Table 1). The data indicates that it is necessary to block the biosynthetic pathway from dCTP to TMP via dUTP for the overproduction of deoxycytidine.

Isolation of thymine auxotrophic mutants

To block the flow of dCTP into dUTP and convert it into deoxycytidine (Fig. 1), thymine auxotrophy was introduced into the strain IH30 (ino^-, HU^r) via mutagenesis with NTG. The isolated mutant IM7 could only grow in the presence of 100 mg/L of thymine (Table 3) and accumulated 81.3 mg/L of deoxycytidine but no thymine (Table 1). In order to elucidate the mutation site in the mutant IM7, dCTP deaminase activity was measured in crude cell extract preparations of *C. ammoniagenes* N424 as well as the mutant strains (Table 4). The thymine auxotrophic mutant IM7 revealed no dCTP deaminase activity (0.00 units/mg-protein) whereas the strains N424 and IH30 exhibited 1.02 units/mg-protein and 1.07 units/mg-protein of activity, respectively, indicating that thymine auxotrophy was attributed to the deficiency of dCTP deaminase.

Isolation of 5-fluorouracil-resistant strains

In *C. ammoniagenes*, the synthesis of pyrimidine nucleosides and nucleotides is tightly regulated by uridine or uridine de-

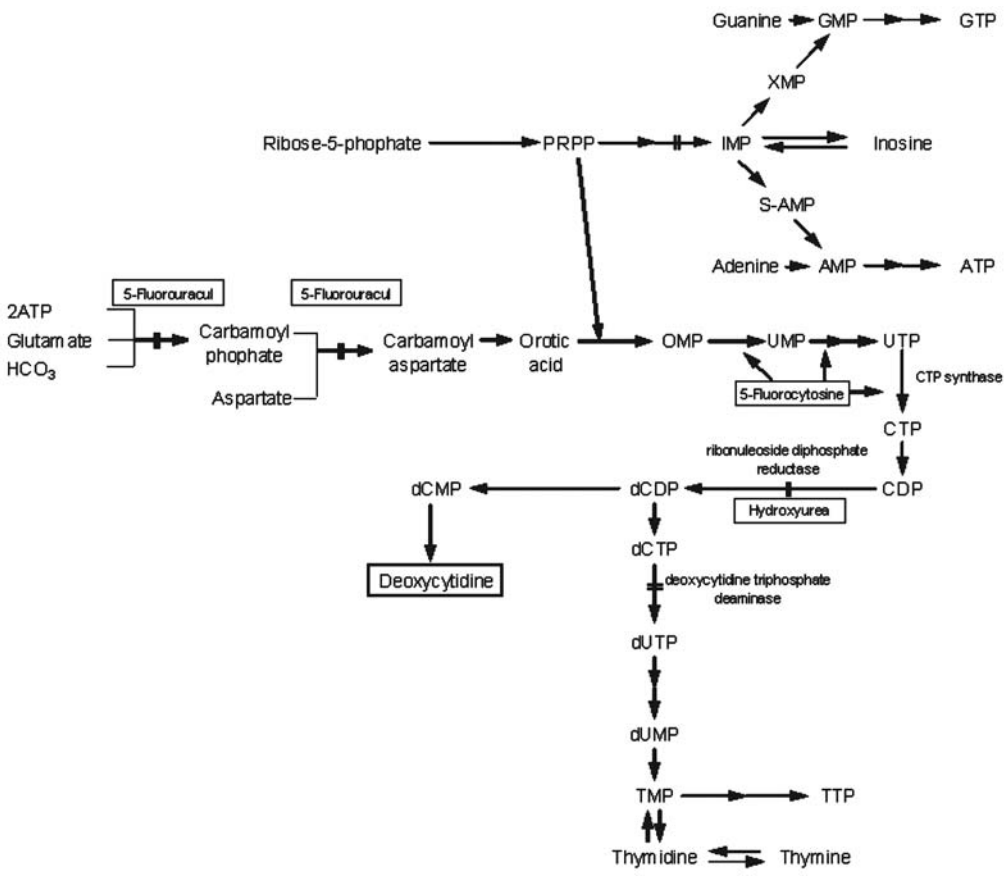

Fig. 1. Biosynthetic pathway of deoxycytidine and thymidine.

Table 3. Growth of mutant strains, N424, IH30, and IM7 in the minimal medium containing inosine or inosine/thymine

Strain	Growth (OD_{660})[a]		
	Minimal medium (MM)	MM with inosine (100 mg/L)	MM with adenine and guanine (50 mg/L each)
C. ammoniagenes			
Strain N424	12.0	12.0	13.0
Mutant IH30	0.5	12.0	12.0
Mutant IM7	0.5	0.5	12.0

[a] Growth was measured at appropriate time intervals and the peak value of cell concentration was chosen and expressed here.

rivatives, and therefore, deoxycytidine or thymidine is not accumulated in the media. As shown in Fig. 1, 5-fluorouracil inhibits carbamoyl phosphate synthase which catalyzes the synthesis of carbamoyl phosphate from glutamine, HCO_3^- and ATP, and aspartate transcarbamoylase which catalyzes the condensation of carbamoyl phosphate and aspartate, which are the initial steps in the biosynthetic pathway of pyrimidine nucleotides. In order to isolate mutant strains which acquired resistance against 5-fluorouracil by being relieved from inhibition, *C. ammoniagenes* IM7 cells were mutagenized with NTG and enriched in MM with 0.05% 5-fluorouracil. Deoxycytidine production in the isolated mutants was evaluated by shake-flask culture, and was measured at 113.4 mg/L in the resulting mutant strain IU19 (Table 1).

Isolation of 5-fluorocytosine-resistant mutants

The analogue of cytosine, 5-fluorocytosine, can be converted into 5-fluorocytidine or 5-fluorocytidine mono-, di-, and triphosphate within cells, and act as an inhibitor for enzymes involved in pyrimidine nucleotide biosynthesis (Fig. 1). Therefore, in order to isolate strains that were not repressed and/or inhibited by accumulation of pyrimidine nucleosides, we introduced 5-fluorocytosine resistance into the strain IU19. The isolation of 5-fluorocytosine-resistant mutants was achieved by enriching and isolating the cells mutagenized with NTG using MM containing 5-flourocytosine in addition to thymine and inosine. The isolated mutant strain IC14-C6 produced higher amounts of deoxycytidine (226.3 mg/L) compared to the mother strain IU19 (Table 1).

Discussion

Corynebacterial strains have been widely used for the industrial production of amino acids and nucleotides, and IMP and GMP especially have been used as food additives (Eggeling and Bott, 2004). We decided to use an IMP-producing corynebacterial strain as the mother strain to develop deoxycytidine overproducers, assuming that the overproducers of IMP might be able to excrete deoxycytidine and accumulate it in the medium. In *C. ammoniagenes*, deoxycytidine is synthesized through a *de novo* pyrimidine pathway that is comprised of many branch points for the synthesis of RNA building blocks such as UTP and CTP, and DNA building blocks such as dCTP and TTP. This organism does not accumulate deoxycytidine because the biosynthesis of pyrimidine nucleosides and nucleotides is tightly related to cell growth and is subjected to feedback regulation by the end products (Hammer, 1983).

In order to develop *C. ammoniagenes* mutant strains that overproduce deoxycytidine, it was necessary to isolate inosine auxotrophs where the synthesis of IMP was blocked. The IMP producer strains are definitely adenine and guanine double-auxotrophic and the inosine auxotrophic strains derived from them could not grow in a minimal medium supplemented with inosine. Therefore, we isolated the inosine-auxotrophic mutant strain I31 from the *C. ammoniagenes* strain N424, a revertant strain (ade^+, gua^+) which was derived from *C. ammoniagenes* KFCC10614, a producer strain (ade^-, gua^-) of IMP (Hyun *et al*., 1990). The strain N424 did not produce deoxycytidine but the mutant strain 131 produced a small amount (Table 1), possibly by switching the flow of PRPP from the purine biosynthetic pathway partly to the pyrimidine biosynthetic pathway. We then introduced hydroxyurea resistance to, which is an inhibitor of ribonucleoside diphosphate reductase, into the mutant strain I31. As shown in Table 1, strain IH30, which was resistant to hydroxyurea and inosine-auxotrophic, accumulated higher levels of deoxycytidine (6.2 mg/L) compared to its mother strain I31 but still accumulated thymine (82.0 mg/L) as a major product. Therefore, we obtained mutant strains for which the metabolic flow from dCTP to thymine, thymidine or TTP was blocked (Fig. 1). The strain IM7, a thymidine auxotroph, produced 81.3 mg/L of deoxycytidine whereas it did not produce thymine at all in shake-flask fermentations. In subsequent studies, it was evident that this mutant was deficient in dCTP deaminase, an enzyme catalyzing the conversion of dCTP into dUTP (Table 4). If nucleoside diphosphate kinase, which catalyzes the phosphorylation of dCDP to dCTP, is knocked out to block the thymidine synthesis pathway, such cells will not be able to grow due to their inability to synthesize dCTP, even though thymine or thymidne is supplied. Therefore, a strain with a defective dCTP deaminase will be desirable for the production of deoxycytidine.

In general, carbamoyl phosphate synthase, aspartate transcarbamoylase, and CTP synthase are known to be key regulatory enzymes of the *de novo* pyrimidine biosynthetic pathway (Fig. 1). Carbamoyl phosphate synthase and aspartate transcarbamoylase were regulated by uridine and uridine derivatives. The fact that the mutant strain IU19, which acquired resist-

Table 4. Comparison of dCTP deaminase activity in mutant strain IM7 and its mother strain IH30

Strain	dCTP deaminase activity (units/mg-protein)
C. ammoniagenes	
Strain N424	1.02
Mutant IH30	1.07
Mutant IM7	0.00

ance to 5-fluorouracil, revealed a significant increase in deoxycytidine productivity (Table 1) shows that this strain might be relieved from enzyme regulation by uridine and uridine derivatives. Asahi *et al.* (1989) also claimed that the production of deoxycytidine by *Bacillus subtilis* was successfully improved by introducing resistance to 5-fluorouracil, 6-azauracil, 2-thiouracil, or 5-fluoroorotic acid. Further enhancement of deoxycytidine productivity was achieved by isolating the mutant strain IC14-C6 which was resistant to 5-fluorocytosine. This mutant accumulated approximately two fold higher deoxycytidine than its mother strain IU19. CTP synthase is known to be regulated by cytosine and cytosine derivatives, and 5-fluorocytosine, an analogue of cytosine, can act as an inhibitor of CTP synthase. CTP synthase is one of the enzymes involved in pyrimidine nucleotide biosynthesis, after being converted to 5-fluorocytidine and 5-fluorocytidine mono-, di, and triphosphate within a cell (Fig. 1). Therefore, the strain IC14-C6 might be relieved from this enzyme regulation by pyrimidine nucleoside accumulation. Asahi *et al*. (1989) isolated overproducers of deoxycytidine in *Bacillus subtilis* by introducing cytidine deaminase deficiency, which catalyzes the conversion of deoxycytidine into deoxyuridine. However, the accumulation of deoxyuridine generated by degradation of deoxycytidine was negligible in *C. ammoniagenes* IC14-C6 (data not shown), implying that cytidine deaminase might not be highly active in this strain.

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

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