ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Metabolic Engineering of *Escherichia coli* for the Production of Cadaverine: A Five Carbon Diamine

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Received 10 June 2010; revision received 16 August 2010; accepted 17 August 2010

Published online 1 September 2010 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/bit.22918

ABSTRACT: A five carbon linear chain diamine, cadaverine (1,5-diaminopentane), is an important platform chemical having many applications in chemical industry. Bio-based production of cadaverine from renewable feedstock is a promising and sustainable alternative to the petroleum-based chemical synthesis. Here, we report development of a metabolically engineered strain of *Escherichia coli* that overproduces cadaverine in glucose mineral salts medium. First, cadaverine degradation and utilization pathways were inactivated. Next, L-lysine decarboxylase, which converts L-lysine directly to cadaverine, was amplified by plasmid-based overexpression of the *cadA* gene under the strong *tac* promoter. Furthermore, the L-lysine biosynthetic pool was increased by the overexpression of the *dapA* gene encoding dihydrodipicolinate synthase through the replacement of the native promoter with the strong *trc* promoter in the genome. The final engineered strain was able to produce 9.61 g L^{-1} of cadaverine with a productivity of 0.32 g L^{-1} h^{-1} by fed-batch cultivation. The strategy reported here should be useful for the bio-based production of cadaverine from renewable resources.

Biotechnol. Bioeng. 2011;108: 93–103.

KEYWORDS: metabolic engineering; *Escherichia coli*; diamine; cadaverine; 1,5-diaminopentane

Correspondence to: Sang Yup Lee

Contract grant sponsor: Korean Systems Biology Research Project of the Ministry of Education, Science and Technology through National Research Foundation

Contract grant sponsor: World Class University Program of the Ministry of Education, Science and Technology

Contract grant sponsor: KAIST Institute for the BioCentury

Contract grant number: 20100002164; R32-2009-000-10142-0

Additional Supporting Information may be found in the online version of this article.

Introduction

Cadaverine, also known as 1,5-diaminopentane, is an important platform chemical with many industrial applications. Similar to putrescine (1,4-diaminobutane), cadaverine serves as a component of polymers such as polyamides and polyurethane, chelating agents, and other additives. In particular, polyamide 54 is made by polycondensation of cadaverine and succinic acid. Polyamide 54 is expected to become a bio-based alternative to conventional petroleum-based polyamides that have an annual global market of 3.5 million tons (Kind et al., 2010; Mimitsuka et al., 2007).

Cadaverine is a minor polyamine found only in a few microorganisms (Tabor and Tabor, 1985). In the Gram-negative bacterium *Escherichia coli*, cadaverine is biosynthesized directly from L-lysine by L-lysine decarboxylase (Fig. 1). There are two forms of L-lysine decarboxylase: one is a constitutive one encoded by the *ldcC* gene, and the other is an inducible one at low pH, which is encoded by the *cadA* gene. The level of cadaverine in *E. coli* is regulated by biosynthesis, degradation, uptake, and export (Soksawatmaekhin et al., 2004). It has been shown that cadaverine is not detectable within wild-type *E. coli*, and only trace amounts are present in mutants that are defective in the biosynthesis of other polyamines (Hafner et al., 1979). However, microorganisms can tolerate a higher concentration of cadaverine. For example, a wild-type *Corynebacterium glutamicum* strain was able to grow even in the presence of ~0.3 M cadaverine (Mimitsuka et al., 2007), although this bacterium does not synthesize cadaverine naturally. The high tolerance implies that microorganisms might potentially be metabolically engineered to overproduce cadaverine to industrially useful levels.

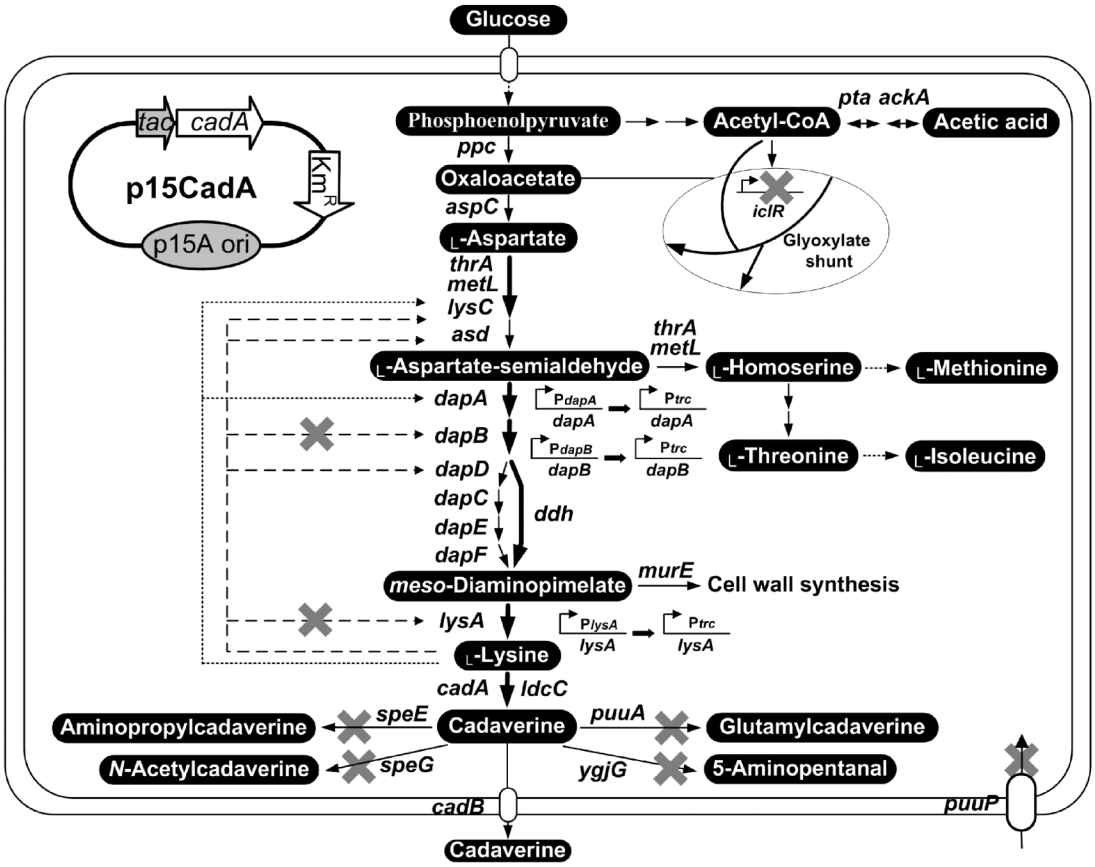

Figure 1. The pathways and regulatory circuits involved in cadaverine metabolism in *E. coli* and the strategies for metabolic engineering for cadaverine overproduction. The gray X's indicate that the genes are knocked out or the repressions are removed. Thick arrows indicate increased flux or activity by directly overexpressing the corresponding genes. Dashed lines indicate repression, and dotted lines indicate feedback inhibition. Enzymes encoded by the genes shown are: *ackA*, acetate kinase; *asd*, aspartate semialdehyde dehydrogenase; *aspC*, PLP-dependent L-aspartate aminotransferase, *cadA*, inducible L-lysine decarboxylase; *cadB*, cadaverine-lysine antiporter; *dapA*, dihydrodipicolinate synthase; *dapB*, dihydrodipicolinate reductase; *dapC*, *N*-succinyldiaminopimelate-aminotransferase; *dapD*, tetrahydrodipicolinate succinylase; *dapE*, *N*-succinyl-L-diaminopimelate desuccinylase; *dapF*, diaminopimelate epimerase; *ddh*, *meso*-diaminopimelate dehydrogenase from *C. glutamicum*; *iclR*, isocitrate lyase regulator; *ldcC*, constitutive L-lysine decarboxylase; *lysA*, diaminopimelate decarboxylase; *lysC*, aspartate kinase III; *metL*, aspartate kinase II/homoserine dehydrogenase II; *murE*, UDP-*N*-acetylmuramoylalanyl-D-glutamate 2,6-diaminopimelate ligase; *ppc*, phosphoenolpyruvate carboxylase; *pta*, phosphate acetyltransferase; *puuA*, glutamate-putrescine/glutamate-cadaverine ligase; *puuP*, putrescine importer; *speE*, putrescine/cadaverine aminopropyltransferase; *speG*, spermidine acetyltransferase; *thrA*, aspartate kinase I/homoserine dehydrogenase I; *ygjG*, putrescine/cadaverine aminotransferase.

Attempts have thus been made for the bio-based production of cadaverine from renewable resources. One approach is decarboxylation of L-lysine either chemically or enzymatically (Hashimoto et al., 1986; Nishi et al., 2007). Alternatively, microorganisms have been employed to produce cadaverine from cheaper carbon sources such as glucose and starch. For example, *C. glutamicum* has attracted much attention because the industrial bacterium produces more than 1,000,000 tons of L-lysine per year (Kind et al., 2010) with a price of about 2,000$ per ton. In a pioneering study, the wild-type *C. glutamicum* strain ATCC 13032 was genetically engineered to replace the chromosomal homoserine dehydrogenase gene with the *E. coli cadA* gene (Mimitsuka et al., 2007). Although the engineered strain produced 2.6 g L^{-1} of cadaverine from 50 g L^{-1} of glucose, comparable level of L-lysine was unfavorably excreted into the medium. Recently, a *C. glutamicum* strain coexpressing α-amylase and CadA produced 2.39 g L^{-1} of cadaverine in the medium from 50 g L^{-1} of soluble starch (Tateno et al., 2009). More recently, the cadaverine yield could be increased by metabolic engineering of the upstream L-lysine biosynthetic pathway and central metabolic pathways (Kind et al., 2010). However, substantial formation of a cadaverine derivative, *N*-acetylcadaverine, was found in the engineered *C. glutamicum* strains. This indicates that unidentified enzymes might be involved in cadaverine degradation or utilization in *C. glutamicum*, within which cadaverine metabolism is largely unclear.

E. coli is an attractive bacterium due to the merits such as fast growth in inexpensive media, well-demonstrated scale-up processes, clear genetic background, and metabolism, and well-established metabolic engineering tools. In particular, this bacterium has recently been metabolically engineered to produce biomass-derived biofuels and

chemicals (Atsumi et al., 2008; Lee et al., 2007; Park and Lee, 2008; Park et al., 2007; Qian et al., 2009; Steen et al., 2010; Tyo et al., 2009). Here, we report the development of a cadaverine overproducing *E. coli* strain by combined engineering of the cadaverine biosynthesis, degradation, and utilization pathways. The metabolically engineered *E. coli* strain was able to constitutively produce cadaverine to a high concentration in glucose–mineral salts medium by fed-batch cultivation.

Materials and Methods

Strains and Growth Condition

E. coli strains used in this study are listed in Table I. *E. coli* TOP10 was used for general gene cloning studies. WL3110, a *lacI* mutant of K12 W3110 (Park et al., 2007), was used as a starting strain to develop cadaverine overproduction strains so that constitutive expression of genes under the *tac* or *trc* promoter can be achieved. For the construction of plasmids and strains, cells were grown in Luria–Bertani (LB) broth or on LB plates (1.5 wt% agar) containing appropriate antibiotics at the following concentrations: 25 $\mu g\,mL^{-1}$ of kanamycin (Km), 100 $\mu g\,mL^{-1}$ of ampicillin (Ap), 35 $\mu g\,mL^{-1}$ of chloramphenicol (Cm), and 34 $\mu g\,mL^{-1}$ of streptomycin (Str).

Cadaverine Tolerance Test With *E. coli* K12 W3110

Cadaverine tolerance was studied for *E. coli* K12 wild-type strain W3110 at 37°C in the chemically defined R/2 medium supplemented with 10 $g\,L^{-1}$ of glucose and 3 $g\,L^{-1}$ of $(NH_4)_2SO_4$. The R/2 medium (pH 6.80) contains ($g\,L^{-1}$): $(NH_4)_2HPO_4$, 2; KH_2PO_4, 6.75; citric acid, 0.85; and $MgSO_4{\cdot}7H_2O$, 0.7. In addition, 5 mL L^{-1} of a trace metal stock solution (Lee and Chang, 1993) was added. Briefly, a small aliquot of cell glycerol stock was inoculated into a test tube containing 10 mL of LB medium and cultured overnight at 37°C and 250 rpm in a shaking incubator. This seed culture was used to inoculate the test tubes containing 5 mL of the R/2 medium. When the OD_{600} reached ~0.4, cells were exposed to a final concentration of 0.2, 0.3, 0.4, or 0.5 M cadaverine dihydrochloride (Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan) using a 2.5 M stock solution, which was prepared by dissolving cadaverine dihydrochloride in fresh R/2 medium and adjusted to pH 6.80 with 5 M NaOH. The control cultures received the same volume of fresh R/2 medium instead of cadaverine dihydrochloride solution. Samples were taken periodically for the measurement of OD_{600}. The experiments were carried out in duplicates, and the data are presented as mean values with standard deviation.

Plasmids

Plasmids used in this study are listed in Table I. Polymerase chain reaction (PCR) primers for plasmid construction and genome engineering are listed in Table II. DNA manipulations were performed according to standard protocols. *Pfu* DNA polymerase and the restriction enzymes were purchased from Solgent (Daejeon, Korea) and New England Biolabs (Ipswich, MA), respectively. T4 DNA ligase was purchased from Roche (Mannheim, Germany). The DNA sequences of the constructs containing the cloned fragments were confirmed by sequencing with an automatic DNA sequencer (ABI Prism Model 377, Perkin Elmer Applied Biosystems, Foster City, CA).

Table I. *Escherichia coli* strains and plasmids used in this study.

Strain/plasmid	Description[a]	Source or Refs.
Strains		
W3110	*Coli* Genetic Stock Center strain (CGSC) No. 4474	CGSC[b]
TOP10	F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ¢80*lacZ*Δ M15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara–leu*)*7697 gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G	Invitrogen[c]
WL3110	W3110 Δ*lacI*	Park et al. (2007)
XQ27	W3110 Δ*lacI* Δ*speE* Δ*speG* Δ*ygjG* Δ*puuPA*	This study
XQ56	XQ27 P*dapA*::P*trc*	This study
XQ60	XQ27 P*dapA*::P*trc* P*lysA*::P*trc* P*dapB*::P*trc*	This study
XQ64	XQ27 P*dapA*::P*trc* P*lysA*::P*trc* P*dapB*::P*trc iclR*::*ddh*	This study
Plasmids		
pKD46	Ap^R, λ Red recombinase under arabinose-inducible *araBAD* promoter, ts origin, 6.3 kb	Datsenko and Wanner (2000)
pECmulox	Ap^R, Cm^R, lox66-Cm^R-lox71, 3.5 kb	Kim et al. (2008)
pmuloxDdh	Ap^R, Cm^R, *C. glutamicum ddh* gene in *Kpn*I–*Spe*I site of pECmulox, 4.6 kb	This study
pJW168	Ap^R, Cre-recombinase under IPTG-inducible *lacUV5* promoter, ts origin, 5.5 kb	Palmeros et al. (2000)
pTac15K	Km^R, *tac* promoter, p15A ori, 4.0 kb	Lab stock
p15CadA	Km^R, *E. coli cadA* gene in *Eco*RI–*Sac*I site of pTac15K, 5 nt (GCGTC) inserted upstream of the *Eco*RI–*cadA*–*Sac*I cassette, 6.1 kb	This study

[a]Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Str, streptomycin; R, resistance; ts, temperature sensitive.
[b]Coli Genetic Stock Center, New Haven, CT.
[c]Invitrogen, Corp., Carlsbad, CA.

Table II. PCR primers used in this study.

Primer	Sequence (5'–3')[a]	Source or Refs.
FcadAEco	CGTCGAATTCATGAACGTTATTGCAATATTG	This study
RcadASac	GCTCGAGCTCTTATTTTTTGCTTTCTTCTTTC	This study
FddhKpn	CATGGTACCTGATGAAAGAGATGTCCCT	This study
RddhSpe	CCTACTAGTGACACCCTCTGAAAAGGCTAA	This study
FygjGKO	CTGCAATACTTAAATCGGTATCATGTGATACGCGAGCCTCCGGAGCATATGACACTATAGAACGCGGCCG	This study
RygjGKO	CGTCGTATCGCCATCCGATTTGATATTACGCTTCTTCGACACTTACTCGCCCGCATAGGCCACTAGTGGA	This study
FiclRKO	TGAAAATGATTTCCACGATACAGAAAAAAGAGACTGTCATGGTCGCACCCGACACTATAGAACGCGGCCGC	This study
RiclRKO	GGAAATAGAAATTGCGGCAAACGGTTCACGGTGCTCATCGAAAATACACGGCCGCATAGGCCACTAGT	This study
FPdapA1	GGTGAGTTGTTCTTAAGGAAAGCATAAAAAAAACATGCATACAACAATCAGAACGGGACACTATAGAACGCGGCCG	This study
RPargE1	TATCCGCTCACAATTCCACACATTATACGAGCCGGATGATTAATTGTCAACAGCTCCGCATAGGCCACTAGTGGA	Qian et al. (2009)
FPdapA2	TCACCAGATAATGTTGCGATGACAGTGTCAAACTGGTTATTCCTTTAAGGGGTGAGTTGTTCTTAAGGAAAG	This study
RPdapA2	GTAACAATCGCGACAATACTTCCCGTGAACATGGTCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACA	This study
FPdapB1	GTCATTCATCGACTCATGCCTTTCACTGATATCCCTCCCTGTTTGACACTATAGAACGCGGCCG	This study
FPdapB2	TGGCTCTGGCGTCGTAACCTGTCACATGTTATTGGCATGCAGTCATTCATCGACTCATGCC	This study
RPdapB2	GGCAACGCGGATGTTTGCATCATGCATGGTCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACA	This study
FPdapB3	GATGTGAAAGGCTTCCAGCAGTGGGTGGCTGAGGTGCTGGCTCTGGCGTCGTAACCT	This study
RPdapB3	TGAATCAACTGGCGGCCCATACGCCCCCCGGCTCCCGCGATGGCAACGCGGATGTTTGCAT	This study
FPlysA1	TAAGTTAACGGCGGCCATTAGCGCTCTCTCGCAATCCGGTAATCCATATCATTGACACTATAGAACGCGGCCG	This study
FPlysA2	CTCAGTCAGGCTTCCGGCGGTCATTACCGCATGAAAAATTTCAATATGACGTAAGTTAACGGCGGCCATTA	This study
RPlysA2	GATCGGTATCGGTGCTGAACAGTGAATGTGGCATGGTCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACA	This study

[a]Restriction sites are underlined.

For the overexpression of L-lysine decarboxylase, the *cadA* gene was amplified from the genomic DNA of *E. coli* W3110 using *Pfu* polymerase and PCR primers FcadAEco and RcadASac (Table II). The PCR product was digested completely with *Sac*I and partially with *Eco*RI, and ligated with *Eco*RI–*Sac*I digested plasmid pTac15K (about 15 copies per cell; Qian et al., 2009). Notably, attempts to find the designed plasmid failed in spite of repeated trials; instead, we obtained a plasmid variant with 5-nucleotide insertion (GCGTC) upstream of the *Eco*RI–*cadA*–*Sac*I cassette (see Supplementary Fig. 1 for plasmid map). The plasmid variant was named p15CadA, which allows constitutive expression of the *cadA* gene under the strong *tac* promoter in *lacI*-deficient *E. coli* strain (see below for the details).

Deletion of Chromosomal Genes

The chromosomal *speE*, *speG*, and *puuPA* genes were deleted as described earlier (Qian et al., 2009). Deletion of the *ygjG* gene was conducted essentially in the same manner, using the one-step inactivation method (Datsenko and Wanner, 2000). The lox71-Cm^R-lox66 cassette was PCR-amplified from pECmulox (Kim et al., 2008), using primers with 50-nucleotide extensions homologous to sequences upstream and downstream of the *ygjG* gene (Table II). The PCR products were purified and eletroporated into *E. coli* strains harboring λ-Red recombinase (pKD46; Datsenko and Wanner, 2000). Colonies were selected on LB agar plates containing Cm, and successful gene replacement with the lox71-Cm^R-lox66 cassette was confirmed by direct colony PCR. The Cm^R marker was subsequently eliminated by Cre recombinase (pJW168; Palmeros et al., 2000). The loss of Cm^R was further verified by PCR.

Promoter Replacement

Substitution of the native promoter of the *dapA* gene by the *trc* promoter was performed by PCR-mediated λ-Red recombination (Yuan et al., 2006). The PCR fragment required for the promoter replacement was amplified in two steps. A 1,253-bp fused lox71-Cm^R-lox66 DNA fragment was obtained by the first PCR reaction using pECmulox as a template, and FPdapA1 and RPargE1 as primers (Table II). Primer RPargE1 contains the *trc* promoter sequence. The second PCR reaction was performed with the primers FPdapA2 and RPdapA2 using the first PCR product as a template. The final PCR product (1,359 bp) was electroporated into *E. coli* cells carrying λ-Red recombinase (pKD46); cells with double crossover homologous recombination were selected on the LB agar plate containing Cm, and confirmed by direct colony PCR. The Cm^R marker was subsequently eliminated by the helper plasmid pJW168.

The native promoter of the *lysA* gene was substituted by the *trc* promoter in the same manner. Similarly, the native promoter of the *dapB* gene was substituted by the *trc* promoter, except that the final PCR product for electroporation was amplified in three steps. Replacement of the endogenous promoters by the *trc* promoter in the chromosome was confirmed by DNA sequence analysis. Notably, the *dapA*, *dapB*, and *lysA* genes encode the metabolic enzymes catalyzing the first, second, and last steps of the *meso*-diaminopimelate (DAP)-lysine biosynthetic

branch. The promoter replacements increased the expression levels of the enzymes by eliminating original transcriptional repression (see Results below for details).

Integration of the *C. glutamicum ddh* Gene Into the *E. coli* Chromosome

The *C. glutamicum ddh* gene was integrated into the *E. coli* chromosome, using the one-step inactivation method (Datsenko and Wanner, 2000). Briefly, the *ddh* gene with its own promoter and transcription terminator was amplified from the genomic DNA of *C. glutamicum* ATCC 13032 using *Pfu* polymerase and PCR primers FddhKpn and RddhSpe (Table II). After digestion with *Kpn*I and *Spe*I, the PCR product was cloned into plasmid pECmulox to construct pmuloxDdh, which contains the *C. glutamicum ddh* gene downstream of the lox71-Cm^R-lox66 cassette. Then, the lox71-Cm^R-lox66-*ddh* fragment was PCR-amplified with primers FiclRKO and RiclRKO, each having 50-nucleotide extension homologous to sequences upstream and downstream of the *E. coli iclR* gene (Table II). The PCR products were purified and eletroporated into *E. coli* strains harboring λ-Red recombinase. Colonies were selected on LB agar plates containing Cm, and successful gene replacement was confirmed by direct colony PCR. The Cm^R marker was subsequently eliminated by Cre recombinase.

Batch and Fed-Batch Cultivation

Batch cultures were carried out at 37°C in a 6.6-L jar fermentor (Bioflo 3000; New Brunswick Scientific Co., Edison, NJ) containing 2 L of R/2 medium supplemented with 10 g L^{-1} of glucose and 3 g L^{-1} of $(NH_4)_2SO_4$. One milliliter of overnight culture was transferred into a 350-mL Erlenmeyer flask containing 50 mL of R/2 medium at 37°C and 220 rpm in a shaking incubator. The fermentor was inoculated with the seed culture (200 mL) to obtain an initial OD_{600} of ~0.2. The culture pH was kept at 6.80 by adding 6 M KOH. The dissolved oxygen concentration was kept at 20% of air saturation by automatically increasing the agitation speed.

Fed-batch cultivation started with batch culture described above. A nutrient feeding solution was added by using the pH-stat feeding strategy (Lee, 1996; Suzuki et al., 1990). The culture pH was kept at 6.80 by adding 10 M KOH except for the short periods of pH increase due to glucose depletion. When the pH rose to 6.81, an appropriate volume of a feeding solution was automatically added into the fermentor to increase the glucose concentration to ~3 g L^{-1} (Lee, 1996; Suzuki et al., 1990). The feeding solution contained (g L^{-1}): glucose, 577; $MgSO_4 \cdot 7H_2O$, 8; and $(NH_4)_2SO_4$, 115. Samples were periodically taken for the measurements of OD_{600} and biomass concentration. After centrifugation at 13,200*g* and room temperature for 5 min, the resulting supernatant was used for the determination of glucose and cadaverine concentrations. Fermentation experiments were carried out in duplicates.

Analytical Methods

Cell growth was monitored by measuring the absorbance at 600 nm (OD_{600}) with an Ultrospec 3000 spectrophotometer (Amersham Biosciences, Uppsala, Sweden). To determine dry cell concentration (gDCW L^{-1}), 2–4 mL of cell suspension was centrifuged in a preweighed tube for 10 min at 3,300*g* and dried for 24 h at 85°C. Glucose concentration was determined using a glucose analyzer (model 2700 STAT; Yellow Springs Instrument, Yellow Springs, OH). Cadaverine concentration was determined by precolumn *o*-phthaldialdehyde derivatization coupled with reverse-phase HPLC and UV detection, which was essentially the same as the protocol described previously for determining putrescine concentration (Qian et al., 2009). Cadaverine dihydrochloride (Tokyo Kasei Kogyo Co., Ltd) was used as a standard. The derivatized cadaverine was detected by a variable wavelength detector (G1314A; Agilent Technologies, Palo Alto, CA) at 230 nm. When necessary, samples were diluted to give cadaverine concentration of less than 50 mg L^{-1} for accurate measurement.

Results

Cadaverine Tolerance Test

Tolerance of *E. coli* to cadaverine was first studied before carrying out metabolic engineering studies for cadaverine overproduction. The test was performed with *E. coli* K12 wild-type strain W3110 at 37°C in a chemically defined medium (modified R/2 medium with glucose). The exponentially growing cells of W3110 were exposed to different concentrations of cadaverine dihydrochloride (Fig. 2). After exposure to 0.2 M cadaverine dihydrochloride (equivalent to 20.4 g L^{-1} of cadaverine), cells grew well although the growth rate was 35% lower than that of the control (without exposure to cadaverine dihydrochloride). Cells still grew in the presence of up to 0.5 M cadaverine dihydrochloride (51.1 g L^{-1} of cadaverine). However, partial cell lysis was observed at 8 h after exposure to 0.3–0.5 M cadaverine dihydrochloride. These results suggested that *E. coli* K12 is able to tolerate at least 0.2 M cadaverine. *E. coli* W3110 was able to tolerate 0.5 M putrescine under the same test condition (Qian et al., 2009), which indicates that *E. coli* K12 is less tolerable to cadaverine than to putrescine. It seems that the C5 diamine (cadaverine) is more toxic to living organisms than the C4 diamine (putrescine). The toxicity of an amine compound depends on its aliphatic chain length and the number of free amino groups as shown in bacteria and animals (Bachrach and Weinstein, 1970; Smith et al., 1996). Further study is needed to understand the exact mechanism of the toxicity of diamine to the cell.

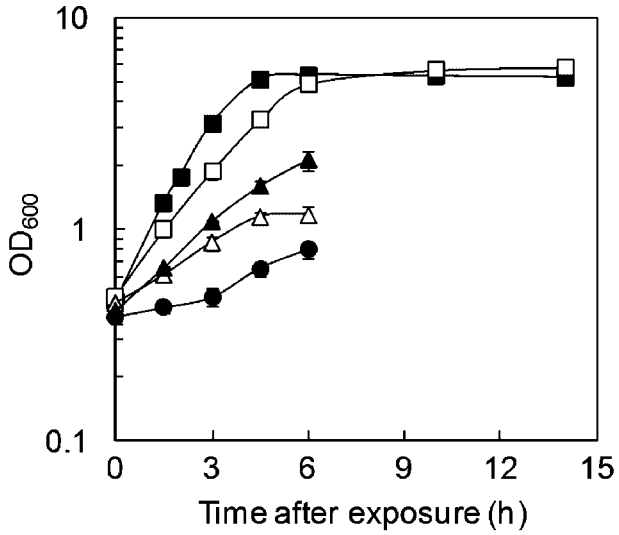

Figure 2. Time profiles of *E. coli* W3110 cultures treated with cadaverine dihydrochloride at various concentrations. Cells were grown at 37°C in a glucose minimal salts medium, and exposed to cadaverine dihydrochloride at the exponential growth phase (OD_{600} of ca. 0.4). Data shown are the averages of two independent experiments with the standard deviations. Cadaverine dihydrochloride concentrations are: filled rectangle, control; open rectangle, 0.2 M; filled triangle, 0.3 M; open triangle, 0.4 M; filled circle, 0.5 M.

Construction of a Base Strain for Cadaverine Production

E. coli WL3110, a *lacI*-mutant of W3110, was used as the starting strain to make a cadaverine-producing base strain. Since the cadaverine utilization and degradation pathways most likely reduce cadaverine titer, these pathways are targeted for disruption (Fig. 1). The cadaverine utilization pathway was first eliminated by deleting the *speE* gene that encodes putrescine/cadaverine aminopropyltransferase, and the *speG* gene that encodes spermidine acetyltransferase, a putative diamine acetyltransferase (Haywood and Large, 1985). Next, the YgjG-YdcW and the Puu pathways, both of which are initially reported to be responsible for catabolizing putrescine, were disrupted because they might degrade cadaverine, which is similar to putrescine. For example, the first enzyme of the YgjG-YdcW pathway, YgjG, has been shown to be able to transaminate cadaverine (Samsonova et al., 2003). Also, the first enzyme of the Puu pathway, PuuA, catalyzes the γ-glutamylation of cadaverine (Kurihara et al., 2008). In addition, a dominant putrescine importer (PuuP), was inactivated because it might import cadaverine as well due to the similar structure. The WL3110 (*ΔspeE ΔspeG ΔygjG ΔpuuPA*) strain constructed this way was named XQ27.

To increase the conversion of L-lysine to cadaverine, the *cadA* gene encoding the inducible L-lysine decarboxylase was overexpressed from a multicopy plasmid p15CadA. This plasmid allowed constitutive overexpression of L-lysine decarboxylase to ca. 5% of total cellular proteins in WL3110. Batch culture of the base strain XQ27 harboring p15CadA was carried out in R/2 medium supplemented with 10 g L^{-1} of glucose and 3 g L^{-1} of $(NH_4)_2SO_4$ (Fig. 3). The strain showed a maximum specific growth rate (μ_{max}) of 0.41 h^{-1}, and excreted 1.19 g L^{-1} of cadaverine into the culture medium (a yield of 0.119 g per gram of glucose). When compared with the recombinant strain XQ27 (p15CadA), the control strain WL3110 (p15CadA) exhibited 46% higher growth rate, but excreted only 0.79 g L^{-1} of cadaverine (a yield of 0.079 g per gram of glucose). These results

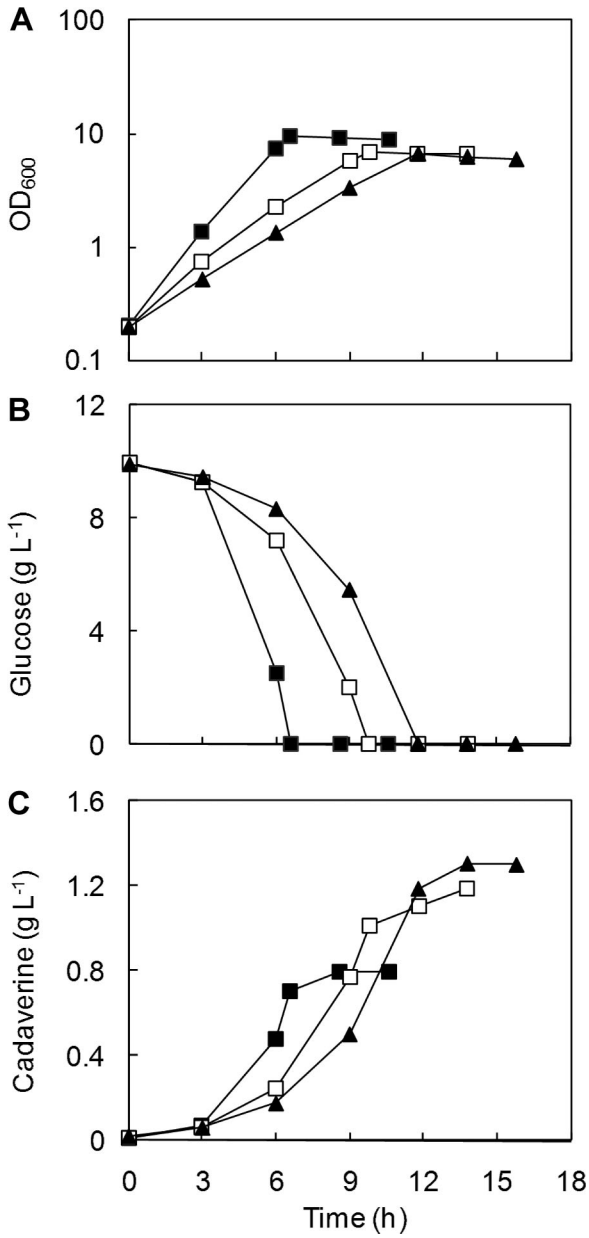

Figure 3. Batch cultures of *E. coli* WL3110 harboring p15CadA (filled rectangle), XQ27 harboring p15CadA (open rectangle), and XQ56 harboring p15CadA (filled triangle). Cells were grown in a 6.6-L jar fermentor containing R/2 medium supplemented with 3 g L^{-1} of $(NH_4)_2SO_4$ and 10 g L^{-1} of glucose at 37°C. (**A**) Cell growth, (**B**) glucose concentration, and (**C**) cadaverine concentration.

demonstrate the significance of removing the cadaverine utilization and degradation pathways for cadaverine overproduction.

Effect of Increasing Metabolic Flux to L-Lysine Formation

It is reasoned that cadaverine production might be enhanced by increasing the metabolic flux to L-lysine, a direct precursor of cadaverine. In *E. coli*, lysine biosynthesis involves nine successive enzymatic reactions from L-aspartate. The very first step, phosphorylation of aspartate, is performed by three isoenzymes: aspartokinase III (*lysC*) is specific to the *meso*-diaminopimelate (DAP)-lysine biosynthetic branch, whereas aspartokinase I (*thrA*) and II (*metL*) are bifunctional enzymes that also convert the branch point intermediate L-aspartate-semialdehyde to L-homoserine for the biosynthesis of L-methionine, L-threonine, and L-isoleucine. Directing L-aspartate-semialdehyde to the DAP-lysine branch is catalyzed by dihydrodipicolinate synthase (*dapA*). DAP, the ultimate lysine precursor and an essential constituent of cell wall peptidoglycan, is another branch point intermediate.

Since the DAP-lysine branch competes with the L-homoserine branch for L-aspartate-semialdehyde, overexpression of the first enzyme of the former branch, DapA, might increase the metabolic flux for cadaverine biosynthesis. To overexpress the *dapA* gene, we first increased the gene copy number by cloning the gene into plasmid p15CadA. Unexpectedly, cell growth and cadaverine production were severely retarded by the plasmid-based *dapA* overexpression (data not shown), suggesting possible metabolic imbalance in partitioning of L-aspartate-semialdehyde. Alternatively, *dapA* overexpression can be achieved by replacing the native *dapA* promoter with the strong *trc* promoter directly on the chromosome. It has been known that the native *dapA* promoter is repressed by intracellular DAP and its direct precursor, L,L-diaminopimelate (Acord and Masters, 2004). Substitution of the native *dapA* promoter with the *trc* promoter can eliminate the repression and increase the expression level of DapA. The base strain XQ27 was subjected to the promoter substitution, which resulted in strain XQ56. As shown in Figure 3, batch culture of XQ56 (p15CadA) allowed production of $1.31\,g\,L^{-1}$ of cadaverine, which is 10% higher than that obtained with XQ27 (p15CadA). The yield of cadaverine obtained with strain XQ56 (p15CadA) was 0.131 g per gram of glucose.

After chromosome-based overexpression of DapA, we attempted to overexpress the remaining enzymes of the DAP-lysine branch. The second and third steps encoded by the *dapB* and *dapD* genes as well as the last one encoded by the *lysA* gene are regulated by L-lysine through transcriptional repression. DapD and the following three steps are employed to synthesize DAP from tetrahydrodipicolinate involving succinylated intermediates. It should be noted that tetrahydrodipicolinate can be converted to DAP in a single step by DAP dehydrogenase, which is encoded by the *ddh* gene in *C. glutamicum*.

Here, the DAP-lysine branch was strengthened by overexpression of the *dapB* and *lysA* genes as well as the *C. glutamicum ddh* gene. Starting from strain XQ56, the native promoters of the *dapB* and *lysA* genes were chromosomally replaced with the *trc* promoter to construct strain XQ60. Furthermore, the *ddh* gene was integrated into the chromosome of strain XQ60 at the locus of isocitrate lyase regulator gene (*iclR*), which resulted in strain XQ64 with the *iclR* gene disrupted. It should be noted that IclR negatively regulates the expression of the *aceBA* genes encoding isocitrate lyase and malate synthase (Yamamoto and Ishihama, 2003). Disrupting the *iclR* gene increased expression of the two enzymes in glyoxylate shunt and thus the supply of oxaloacetate for enhanced production of an aspartate-derived amino acid (Lee et al., 2007); this was why the *iclR* locus was chosen for the integration of the *C. glutamicum ddh* gene.

Batch cultures of XQ60 and XQ64 strains harboring p15CadA were carried out (Fig. 4). The two recombinant strains excreted cadaverine at the levels comparable to XQ56 (p15CadA) strain. These results suggest that the second to the last steps of DAP-lysine branch might not be limiting the cadaverine production in the XQ56 strain. In addition, an earlier step, aspartokinase III, does not limit cadaverine production either because overexpression of either wild-type *lysC* or its lysine feedback resistant variant did not increase cadaverine production (data not shown). At this point, it seems likely that the supply of oxaloacetate is a potential bottleneck and a target for metabolic engineering. As the key tricarboxylic acid cycle intermediate and the major building block of L-aspartate family amino acids, oxaloacetate plays a critical role in central metabolism of microorganisms. Recently, an increased supply of oxaloacetate has been shown to be beneficial for elevating the production of cadaverine in *C. glutamicum* (Kind et al., 2010).

Cadaverine Production by Fed-Batch Cultivation

Fed-batch culture of recombinant XQ56 (p15CadA) was carried out using the pH-stat feeding strategy. As shown in Figure 5, the recombinant strain can be grown to a cell density of $16.55\,g\,L^{-1}$. The maximum cadaverine concentration obtained was $9.61\,g\,L^{-1}$ at 30 h (a yield of ~0.12 g per gram of glucose) resulting in the cadaverine productivity of $0.32\,g\,L^{-1}\,h^{-1}$. It was noted that much acetic acid was formed and excreted in the late stage of fed-batch cultivation. To examine the possibility of reducing the formation of acetic acid, we further disrupted the *pta* gene encoding phosphate acetyltransferase, which is involved in the production of acetic acid from acetyl-CoA (Fig. 1). Although extracellular concentration of acetic acid was dramatically decreased in the resulting strain, cadaveine

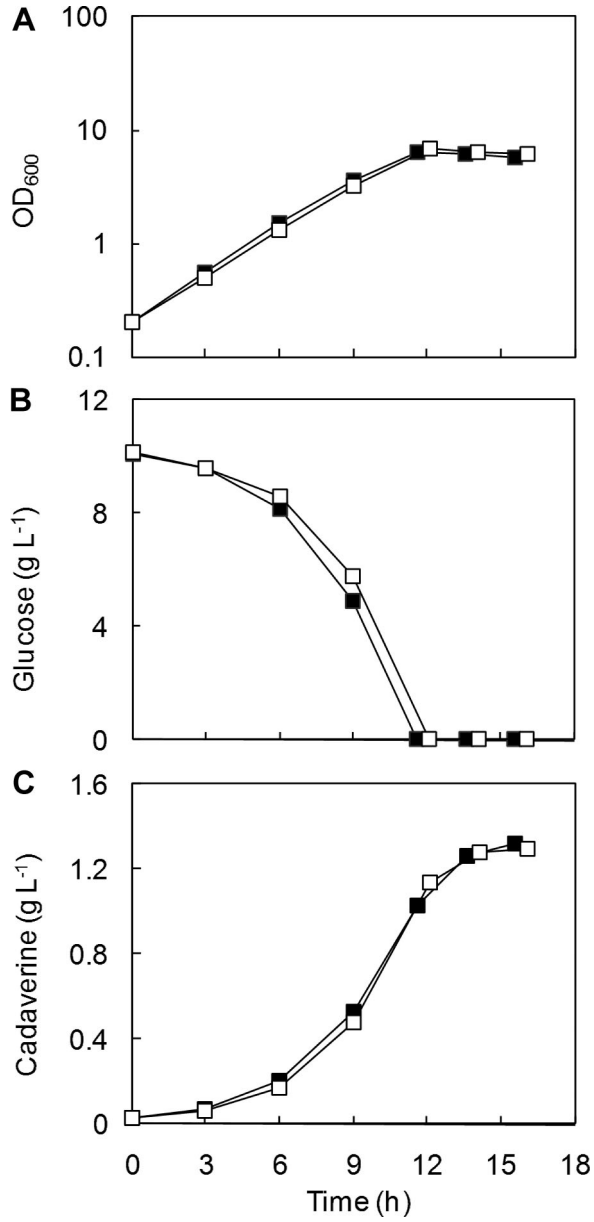

Figure 4. Batch cultures of *E. coli* XQ60 harboring p15CadA (filled rectangle) and XQ64 harboring p15CadA (open rectangle). Cells were cultured under the same condition as described in Figure 3. (**A**) Cell growth, (**B**) glucose concentration, and (**C**) cadaverine concentration.

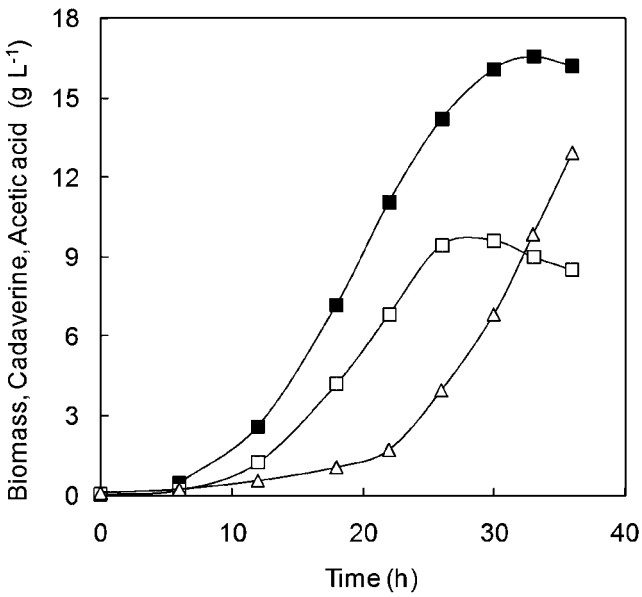

Figure 5. Fed-batch culture of *E. coli* XQ56 harboring p15CadA. Cells were cultured at 37°C in a 6.6-L jar fermentor containing 2 L of R/2 medium supplemented with 10 g L^{-1} of glucose and 3 g L^{-1} of $(NH_4)_2SO_4$. An appropriate volume of a feeding solution (577 g L^{-1} of glucose, 8 g L^{-1} of $MgSO_4{\cdot}7H_2O$, and 115 g L^{-1} of $(NH_4)_2SO_4$) was automatically added into the fermentor by the pH-stat strategy to increase the glucose concentration to 3 g L^{-1} per feeding. Symbols are: filled rectangle, biomass concentration (g DCW L^{-1}); open rectangle, cadaverine concentration; open triangle, acetic acid concentration.

production was also reduced (Supplementary Fig. 2). Further study on the formation and reutilization of acetic acid in this engineered strain is required to solve this problem.

Discussion

Production of chemicals from renewable biomass has recently received increased attention due to the concerns on limited nature of fossil resources and environmental problems. Cadaverine is an attractive platform chemical, particularly as a building block to synthesize polyamide 54 by polycondensation with succinic acid. Since microbial fermentative production of succinic acid has been well developed (Hong et al., 2004; Jantama et al., 2008; Kim et al., 2007), bio-based production of cadaverine is highly desirable towards renewable production of polyamide 54. In this paper, we reported the development of a metabolically engineered *E. coli* strain that efficiently produces cadaverine.

Theoretically, *E. coli* is a native cadaverine producer because it possesses a biosynthesis pathway converting L-lysine to cadaverine by L-lysine decarboxylase. Previous studies attempted to develop a cadaverine producer by engineering the industrial L-lysine producer, *C. glutamicum* by the heterologous overexpression of the *E. coli* L-lysine decarboxylase. However, the engineered *C. glutamicum* strains usually excrete L-lysine and transform significant amount of cadaverine into its acetylated variant by unidentified enzymes, which are not desirable. On the contrary, the physiological role of cadaverine and its degradation and utilization pathways are relatively well studied in *E. coli*. Thus, *E. coli* was chosen as the host strain in this study to develop a cadaverine overproducer using the metabolic and regulatory information available in the literature.

The metabolic pathways involved in cadaverine degradation and utilization were first targeted for inactivation. Those pathways, which were initially found to catabolize putrescine, showed relaxed substrate specificity on the putrescine structural analog, cadaverine. Disruption of these pathways resulted in the cadaverine-producing base strain showing superior performance over the control strain.

L-Lysine decarboxylase is believed to be a key enzyme to be overexpressed due to several reasons. First, less optimal overexpression of the enzyme would result in a bottleneck in cadaverine biosynthesis. Second, efficient in vivo conversion of L-lysine might decrease its intracellular concentration, which would alleviate L-lysine repression and feedback inhibition and thus pull the metabolic flux through the L-lysine biosynthesis pathway. Furthermore, under such a scenario, L-lysine excretion might be completely prevented. For high-level expression of L-lysine decarboxylase, we initially attempted to clone either the constitutive *ldcC* or the inducible *cadA* gene in plasmid pTac15K (about 15 copies per cell) under the strong *tac* promoter. However, the effort to obtain the designed constructs was not successful. Even though the reason is not clear, the expression of the cloned gene(s) from the designed constructs seems to be toxic to the host cell. Instead, we obtained a unique variant plasmid for *cadA* overexpression, termed p15CadA. As described in the Materials and Methods Section, a 5-nucleotide insertion (GCGTC) was found upstream of the *Eco*RI–*cadA*–*Sac*I cassette in p15CadA (Supplementary Fig. 1). To examine the effect of GCGTC insertion on *cadA* expression, we compared the mRNA features upstream of the translation start site (ATG) relevant to translation for the initially designed and the variant plasmids. As revealed by mfold (http://mfold.bioinfo.rpi.edu/; Zuker, 2003), the 5-nucleotide insertion results in a hairpin structure (Fig. 6); such stable secondary structure can lead to reduced translation initiation of the *cadA* gene from the variant plasmid. This might be the reason why we could obtain the variant plasmid p15CadA by repeated attempts. It is believed that p15CadA permitted desirable expression of the *cadA* gene (~5% of total cellular proteins) because the recombinant strains harboring p15CadA excreted considerable amount of cadaverine without extracellular accumulation of L-lysine.

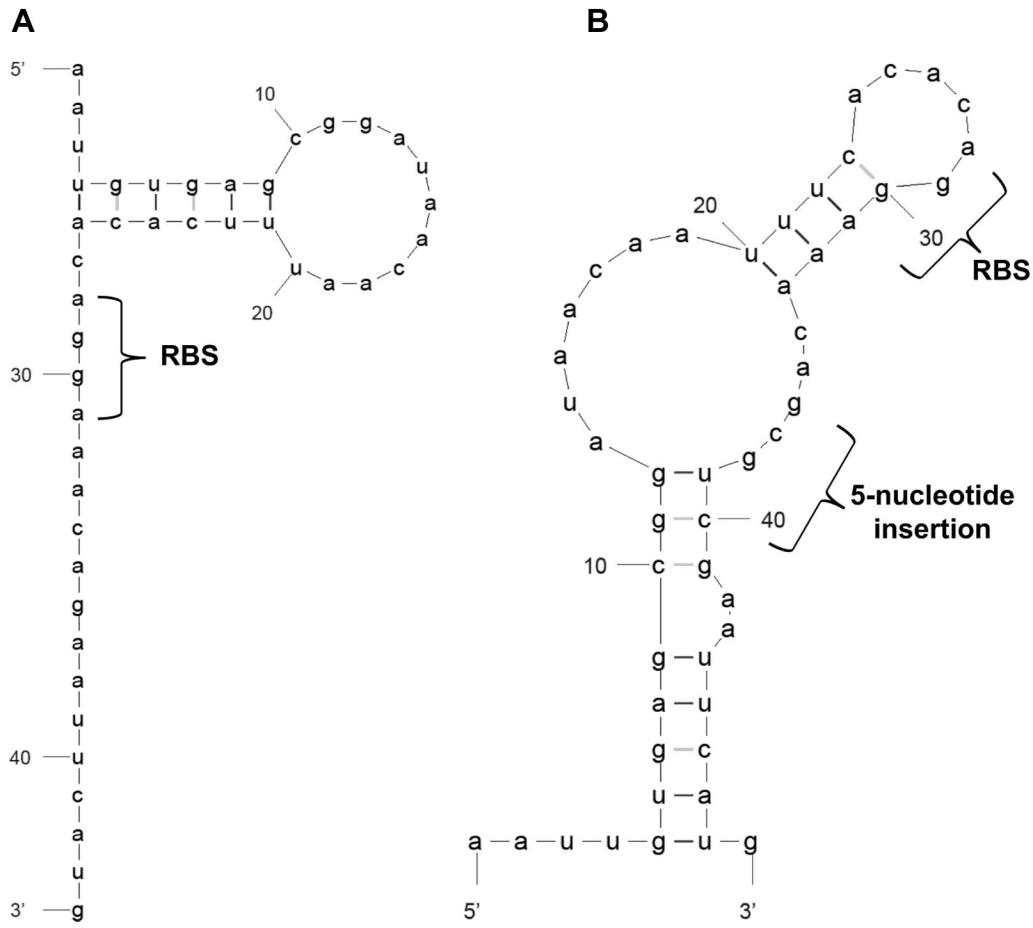

Figure 6. Secondary structures of the *cadA* transcripts from initially designed (**A**) and the variant plasmid p15cadA (**B**). The sequences from the transcription start site (5′) to the start codon of the *cadA* gene (3′) were analyzed using mfold (version 3.2) web server (http://mfold.bioinfo.rpi.edu/; Zuker, 2003). The ribosome binding sites (RBS) are indicated. The 5-nucleotide insertion results in hairpin formation that can lead to reduced translation initiation of the *cadA* gene from the variant plasmid.

The cadaverine-lysine antiporter CadB is the only known protein showing cadaverine excretion activity (Meng and Bennett, 1992), although cadaverine uptake activity was also reported for this protein (Soksawatmaekhin et al., 2004). To test whether *cadB* overexpression would improve the strain performance, the *cadBA* operon containing the *cadB* and *cadA* genes was cloned into plasmid pTac15K under the control of the *tac* promoter. When the resulting plasmid was transformed into the base strain XQ27, the recombinant strain showed substantially retarded cell growth and reduced cadaverine production titer (data not shown). This might be due to that the overexpression of the inner membrane protein CadB interferes with the membrane integrity and negatively affects cell viability.

There exist diverse L-lysine biosynthetic pathways among different species. Yeasts and higher fungi employ the α-aminoadipate pathway to synthesize L-lysine from α-ketoglutarate (Xu et al., 2006). All known bacteria derive L-lysine from L-aspartate by the DAP pathway. In different bacterial species, the first two steps (*dapA* and *dapB*) and the last one (*lysA*) are common to synthesize L-lysine from L-aspartate semialdehyde. There are three pathways in these steps: the succinylase variant involving succinylated intermediates, the acetylase variant, and the dehydrogenase (*ddh*) variant. *C. glutamicum* has both the succinylase and dehydrogenase pathways, whereas *E. coli* possesses the former pathway only. Integration of the more efficient dehydrogenase pathway into *E. coli* did not increase cadaverine production in the engineered strain, which was unexpected. This might be due to that the supply of an upstream precursor, oxaloacetate, might be limiting the metabolic flux through the L-lysine biosynthetic pathway. Further metabolic engineering of central metabolic pathways might solve the problem.

In summary, we reported the development of metabolically engineered *E. coli* strain capable of efficiently producing cadaverine by removing the metabolic pathways for cadaverine degradation and utilization, and by amplifying the key enzymes CadA and DapA. Even though the engineered strain excreted 9.61 g L^{-1} of cadaverine by fed-batch cultivation, further strain improvement will be pursued to make this biotechnological process more competitive. Removal of acetic acid formation will be attempted for obvious reason. Genome-scale identification of knock-out and overexpression targets (Park et al., 2008) as well as improvement of the strain tolerance to cadaverine are expected to further increase the cadaverine titer and productivity.

We thank B.L. Wanner for providing us with plasmid pKD46. This work was performed in part as a new fusion research initiative supported by the KAIST Institute for the BioCentury.

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