Enhanced Cadaverine Production by Recombinant *Corynebacterium Glutamicum* with Response Regulator DR1558 at low pH Conditions

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Research

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Abstract

Background: *Corynebacterium glutamicum* is used industrially to produce various bio-based organic acids. However, it is often cultivated under abiotic stress conditions, such as low pH, which can reduce both cell growth and the yield of the target compound. Here, a response regulator from *Deinococcus radiodurans*, DR1558, was introduced into a recombinant *C. glutamicum* strain expressing lysine decarboxylase (*cadA*) to enhance cadaverine production at acidic pHs.

Results: During batch cultivation under acidic conditions, 6.4 g/L of cadaverine was produced by the recombinant *C. glutamicum* strain expressing *cadA* and *dr1558*; this yield was 1.7-fold higher than that produced by a recombinant *C. glutamicum* strain expressing only *cadA*. Transcriptional analysis revealed altered expression levels of stress defense- and cadaverine biosynthesis-related genes in the recombinant *C. glutamicum* strain expressing *dr1558*. During fed-batch cultivation, the recombinant *C. glutamicum* strain expressing *cadA* and *dr1558* showed a 2.4-fold increase in cadaverine production compared to that produced by the recombinant *C. glutamicum* strain expressing only *cadA*. The cell growth of *C. glutamicum* expressing both *cadA* and *dr1558* increased markedly during fed-batch cultivation at acidic pH.

Conclusion: These results indicated that the response regulator *dr1558* altered the expression of genes involved in metabolic pathways and stress defense mechanisms in *C. glutamicum*. Furthermore, *C. glutamicum* expressing the *D. radiodurans dr1558* can be used to produce bio-based organic acids by fermentation in processes requiring acidic conditions.

Background

Corynebacterium glutamicum is a generally-recognized-as-safe (GRAS) gram-positive bacterium that is primarily used for the industrial production of amino acids, especially, the flavor enhancer L-glutamate and the feed additive L-lysine [1]. *C. glutamicum* can also be used as a microbial cell factory to produce other commercially relevant chemicals, such as cadaverine, succinate, iso-butanol, and ethanol [2]. Therefore, from a metabolic perspective, *C. glutamicum* is a promising producer of bio-chemicals.

Cadaverine, which is synthesized from lysine, is an important industrial platform-associated chemical that has a variety of applications, including the production of polyamides, polyurethanes, chelating agents, and additives. Additionally, it is used as a precursor for bio-based nylon synthesis, and cadaverine can replace hexamethylene diamine to produce nylon-5,4, nylon-5,6, nylon-5,10, or nylon-5,12 [3]. Cadaverine is produced through an enzymatic reaction catalyzed by either constitutive (LdcC) or inducible (CadA) lysine decarboxylase [4, 5]. Expression of CadA is induced by external acidic pH, excess lysine, and low oxygen [6]. CadA prefers acidic conditions, has an optimum pH of 5.7, and displays higher thermal stability and enzyme activity than LdcC. Therefore, the biotransformation of lysine to cadaverine by CadA was investigated under acidic conditions.

When generating a target product through microbial cultivation, the microbial strain may be exposed to various stresses, depending on the requirements for target production, including high osmotic pressure, high temperature, and an unfavorable pH [7–9]. Among these stresses, low pH is a common factor that can reduce the yield of bio-based compounds produced by fermentation [10]. The acidic pH required for enzyme activity during the production of cadaverine using CadA leads to decreased cell growth and lower productivity. Recently, a response regulator from *Deinococcus radiodurans*, DR1558, was introduced into a recombinant *Escherichia coli* strain to minimize the effects of stress under acidic conditions [11]. *D. radiodurans* is highly resistant to abiotic stresses, including gamma radiation, reactive oxygen species (ROS), and oxidants [11–12], and *dr1558* is one of the genes responsible for its remarkable resistance. Introduction of *dr1558* improved the stress resistance of *E. coli* [11]. The *dr1558* gene has also been introduced into recombinant *E. coli* to improve the production of succinate, polyhydroxybutyrate, γ-aminobutyric acid, and 2,3-butanediol [13–16]. Since the expression of *dr1558* increased the tolerance of *E. coli* cells to low pH, it was expected that it would also increase the productivity of cadaverine, especially at an acidic pH.

In this study, a recombinant plasmid that expressed both *E. coli cadA* and *D. radiodurans dr1558* was constructed and transformed into *C. glutamicum*. Cadaverine biosynthesis by the *C. glutamicum* strain expressing *cadA* and *dr1558* and a strain expressing only *cadA* was compared. To investigate the metabolic changes induced by introducing *dr1558* into *C. glutamicum*, the changes in the transcriptome of recombinant *C. glutamicum* expressing *dr1558* was analyzed. Finally, cadaverine production by the recombinant *C. glutamicum* strain expressing *cadA* and *dr1558* was assessed in fed-batch cultivation at acidic pH.

Materials And Methods

Strains, plasmids, and culture media

All bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* XL1-Blue (Stratagene, La Jolla, CA, USA) was used for general cloning. *C. glutamicum* KCTC 1857 was obtained from the Korean Collection for Type Cultures (KCTC; Joengeup, Republic of Korea). The plasmids used for the expression of the *E. coli cadA* and *D. radiodurans dr1558* genes under the control of the synthetic H30 promoter were constructed as described below. Recombinant *C. glutamicum* KCTC 1857 strains were constructed to express *cadA* with or without *dr1558*.

Table 1
Strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Reference or source
Strain		
E. coli XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB lacI*^{q} *ZΔM15 Tn*10 (Tet^{R})]	Stratagene
C. glutamicum KCTC 1857	L-lysine-producing bacterium	KCTC
Plasmid		
pCES208H30GFP	pCES208 derivative; P_{H30}, eGFP, Km^{r}	[17]
pCES208H30dr1558	pCES208 derivative; P_{H30}, *D. radiodurans dr1558*, Km^{r}	[17]
pCES208H30cadA	pCES208 derivative; P_{H30}, *E. coli cadA*, Km^{r}	[18]
pCES208H30cadAdr1558	pCES208 derivative; P_{H30}, *E. coli cadA*, *D. radiodurans dr1558*, Km^{r}	This study

All DNA manipulations were performed according to standard procedures. A DNA fragment containing the *dr1558* gene under the H30 promoter was obtained from pCES208H30dr1558 [17] by digestion with the restriction endonuclease *Bam*HI. The fragment was inserted in plasmid pCES208H30cadA [18] at the *Bam*HI site to construct pCES208H30cadAdr1558. Each plasmid was transformed into *C. glutamicum* KCTC 1857 by electroporation. The transformed *C. glutamicum* KCTC 1857 strains were plated on medium containing kanamycin (Km) for selection.

E. coli XL1-Blue was cultured at 37°C in Lysogeny-broth medium (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl). *C. glutamicum* was grown in CG-50 medium (50 g/L glucose, 15 g/L yeast extract, 15 g/L $(NH_4)_2SO_4 \cdot 7H_2O$, 0.5 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.01 g/L $MnSO_4 \cdot H_2O$, and 0.01 g/L $FeSO_4 \cdot 7H_2O$, with 15 g/L $CaCO_3$ added for pH buffering). Km (30 mg/L) was added to the medium as needed.

Fermentation

Batch fermentations of recombinant *C. glutamicum* were carried out at 30°C and an initial agitation speed of 200 rpm in a 2.5-L jar fermenter (BioCNS, Daejeon, Republic of Korea) initially containing 500 mL of CG-100 medium (100 g/L glucose, 30 g/L yeast extract, 30 g/L $(NH_4)_2SO_4 \cdot 7H_2O$, 0.5 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.01 g/L $MnSO_4 \cdot H_2O$, 0.01 g/L $FeSO_4 \cdot 7H_2O$, 0.5 mg/L biotin, and 0.3 mg/L thiamine-HCl). When cultivating the recombinant strains, Km was added to the culture medium.

Fed-batch fermentations were carried out at 30°C and an initial agitation speed of 200 rpm in 2.5-L jar fermenters (BioCNS) initially containing 500 mL of CG-100 medium. The feeding solution contained (per liter): 400 g of glucose, 45 g of $(NH_4)_2SO_4 \cdot 7H_2O$, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $MnSO_4 \cdot H_2O$, and 0.01 g of $FeSO_4 \cdot 7H_2O$. The initial pH of the culture broth was 7.1, which was maintained for 8 h to promote cell growth. Then, the pH was changed to 5.7, which is the optimal pH for CadA activity, to promote the production of cadaverine. The fermentation pH was adjusted by automatic addition of 14% (v/v) NH_4OH and 1 M H_2SO_4. Foam formation was suppressed by adding Antifoam 204 (Sigma-Aldrich, St. Louis, MO, USA). The agitation speed was increased to maintain the dissolved oxygen (DO) level above 10%. Cell growth was monitored by measuring the optical density of the culture broth at 600 nm (OD_{600}).

Rna Extraction And Quantitative Real-time Pcr (Qrt-pcr)

A transcriptional analysis was performed to evaluate the gene expression changes in the *C. glutamicum* strain expressing *dr1558*. Cells were harvested by centrifugation (14,000 *g*, 10 min, 4°C) at 10 h during batch fermentation, and total RNA was extracted using the RNeasy Mini Kit (QIAGEN, Venlo, Netherlands), according to the manufacturer's protocol. The qRT-PCR analysis was performed using TB Green Premix ExTaq (TaKaRa Bio, Shiga, Japan) under the following cycling conditions: 40 cycles of 95°C for 10 s and 58°C for 30 s. The primers used are listed in Supplement Table 1. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method, and 16S rRNA was used as an internal control [19]. The experiment was performed in triplicate using an applied Eco™ Real-Time PCR System (Illumina, Inc., San Diego, CA, USA).

Analytical Procedures

The concentrations of organic acids and glucose were determined by high-performance liquid chromatography using an Infinity 1260 system (Agilent Technologies, Santa Clara, CA, USA). The glucose concentration was determined using an Aminex HPX-87H ion exclusion column (Bio-Rad, Hercules, CA, USA). The mobile phase was 5 mM H_2SO_4, the flow rate was 0.6 mL/min, and the column was maintained at 50°C. The concentrations of cadaverine and lysine were determined using an ZORBAX SB-C18 column (Agilent Technologies). The mobile phase was 25 mM sodium acetate buffer (pH 4) and 1 M acetonitrile, the flow rate was 1 mL/min, and the column was maintained at 35°C. The concentrations of cadaverine and lysine were measured after diethyl ethoxymethylenemalonate (DEEMM) derivatization [20].

Results And Discussion

Production of cadaverine by the recombinant C. glutamicum **strain expressing** cadA **and** dr1558 **during batch fermentation**

To investigate the effects of the *D. radiodurans* response regulatory gene *dr1558* on *C. glutamicum*, cell growth, glucose consumption, and lysine and cadaverine production were compared between the recombinant strain expressing both *cadA* and *dr1558* (Cg-cadA + dr1558) and a recombinant strain expressing only *cadA* (Cg-cadA; control) (Fig. 1) during batch cultivation.

The results of the batch fermentation confirmed that the recombinant strain Cg-cadA + dr1558 showed significantly increased rates of cell growth and glucose consumption in batch fermentation, compared to those of Cg-cadA. Even after the pH of the medium was adjusted to 5.7, the growth rate of Cg-cadA + dr1558 continued to increase, whereas the growth rate of Cg-cadA decreased. Additionally, despite the acidic conditions, Cg-cadA + dr1558 consumed 100 g/L of glucose within 20 h. In contrast, glucose remained detectable in the medium of the control fermentation at 24 h. The amounts of cadaverine produced in the fermentations were 3.29 g/L for Cg-cadA and 6.39 g/L for Cg-cadA + dr1558, representing a 1.9-fold increase in cadaverine production. From Fig. 1, the maximum specific growth rate and specific cadaverine productivity of Cg-cadA + dr1558 were 0.302 g cells/L/h and 2.697 mg cadaverine/g cells/L/h, respectively. However, the maximum specific growth rate and specific cadaverine productivity of Cg-cadA were decreased to 0.0653 g cells/L/h and 1.071 mg cadaverine/g cells/L/h, respectively. This indicates that the specific growth rate was greatly increased by the expression of *dr1558* at acidic pH conditions.

It was previously reported that *E. coli* expressing *dr1558* showed greater resistance to acidic conditions as well as the enhanced production of polyhydroxybutyrate and 2,3-butanediol due to the altered expression of genes in metabolic pathways [14, 15]. Based on these findings, we expected changes in the expression levels of genes related to cadaverine production and glucose consumption. To investigate the alterations in the expression of these genes in the recombinant *C. glutamicum* strain expressing *dr1558*, a transcriptional analysis was carried out.

Transcriptional analysis of the C. glutamicum **strain expressing** cadA **and** dr1558

A transcriptional analysis of Cg-cadA + dr1558 and Cg-cadA was performed to investigate the reason for the observed increases in cadaverine production, cell density, and glucose consumption rate under acidic conditions. The analysis included 37 metabolism-related genes and 25 genes related to acid stress resistance (Figs. 2 and 3).

In Cg-cadA + dr1558, upregulation of the glycolysis-related genes *pfkA*, *eno*, and *pyk* may improve the carbon flux of the phosphotransferase system (PTS), thereby increasing glucose uptake. In the TCA cycle, changes in the expression levels of genes involved in the biosynthesis of oxaloacetate were also observed; *pck* was upregulated by 2.71-fold and *pyc*, *ppc*, and *pyk* were upregulated by 2.70-, 2.26-, and 1.5-fold, respectively. Thus, in the recombinant strain expressing *dr1558*, the flux of oxaloacetate is also increased; ultimately, this enhanced the synthesis of lysine, which is a precursor of cadaverine.

In the terminal pathway, no significant changes were observed in the expression levels of *dapB*, *dapD*, *dapC*, *dapE*, *dapF*, and *ddh*, which are directly involved in cadaverine biosynthesis. However, the

expression of *cadA* was significantly higher (3.39-fold) than that in the control strain. The lysine-dependent acid resistance (LDAR) system, which consists of lysine and the inducible lysine decarboxylase CadA [21], operates most efficiently under mild acid stress conditions [22, 23]. The LDAR system is a proton consumption-dependent system. The *cadA* expression was upregulated in the presence of *dr1558*; this enhanced the acid resistance of the *dr1558*-expressing *C. glutamicum* strain and promoted the conversion of lysine to cadaverine. The function of *D. radiodurans dr1558* was investigated in *E. coli* [11]. It was reported that the foreign regulator DR1558 bound to the promoter regions of some sigma factors and modulated their expression levels. However, although the effect of *dr1558* expression in *Corynebacterium* has not been investigated, DR1558 could alter the expression levels of several regulators and may indirectly increase the expression of *cadA*.

We investigated the expression of genes involved in pH homeostasis, which enables *C. glutamicum* to respond to, and survive under, acidic pH conditions. Recent studies revealed the physiological and biochemical processes involved in the defense mechanism against low pH in *C. glutamicum* [24–26]. A previous comprehensive analysis of pH homeostasis in *C. glutamicum* demonstrated a functional link between the pH response, oxidative stress, iron homeostasis, and metabolic shift [27]. Therefore, the changes in the expression levels of key genes related to the intracellular defense against acidic conditions were investigated. The expression levels of 25 genes related to acid resistance were examined via transcriptome analysis (Fig. 3). The expression of DNA-binding Proteins from Starved cells (Dps) [9] and KatA (catalase) is cooperatively regulated by intracellular ROS scavenging, and these proteins are required for resistance to low pH stress in *C. glutamicum* [28]. qRT-PCR analysis of Cg-cadA + dr1558 confirmed that *dps* expression was increased by about 1.5-fold. Given that an external acidic environment can lead to an accumulation of ROS in cells, elimination of ROS is a promising way to confer acid resistance [27].

In *C. glutamicum*, mycothiol peroxidase (MPx), mycothiol disulfide reductase (Mtr), and mycothiol glycosyltransferase (MshA) have been shown to promote adaptation to acid stress by regulating ROS homeostasis [29, 30]. qRT-PCR analysis showed that expression of the *mtr* gene was upregulated by 1.2-fold. ROS accumulation in the cells induced by the acidic conditions was likely effectively reduced by the upregulation of *mtr*, and like the upregulated levels of *dps*, may contribute to the increased growth that was observed under acidic conditions.

The qRT-PCR analysis also revealed that the mRNA expression of *mcbR*, which encodes a TetR-type transcriptional inhibitor of sulfur metabolism, was approximately 1.1-fold higher in Cg-cadA + dr1558 than in Cg-cadA. The accumulation of certain sulfur-containing intermediates, such as cysteine, can disrupt intracellular thiol homeostasis and cause oxidative damage by driving the Fenton reaction [31]. Inhibition of the sulfur anabolic pathway by McbR has been shown to contribute to a reduction in L-cysteine accumulation and have a beneficial effect on cell growth under acidic pH conditions [27].

The iron storage protein ferritin, which is encoded by *ftn* [32], was upregulated in Cg-cadA + dr1558. To protect the reducing environment of the cells from unwanted Fe^{3+}/Fe^{2+} redox cycling, intracellular levels

of free Fe^{2+} are maintained by both limiting external iron absorption and enhancing intracellular iron storage [28]. Thus, this increase in *ftn* expression may help protect cells from iron-mediated oxidative stress.

Cg1328, which encodes a copper chaperone, has been implicated in copper metabolism and trafficking [33]. This cytoplasmic protein functions to specifically deliver copper to copper proteins in plant, bacterial, yeast, and animal cells. The *cg1328* gene also promotes cell survival under acid stress conditions, which is consistent with the interplay between acid stress and copper toxicity reported in some bacteria. In this study, qRT-PCR analysis confirmed that the expression of the *cg1328* gene was upregulated. Thus, intracellular acid resistance may also involve enhanced intracellular copper metabolism and transport. In addition, slight upregulation of *sucE*, a putative succinate exporter that has not yet been functionally characterized, was also observed. In addition, the expression of *cglK*, which was reported to encode a protein that is essential for pH homeostasis in the presence of acidic pHs in the absence of K^+, was downregulated. However, since potassium was added to the culture medium, the function of the putative channel protein CglK may not be important. Most researchers consider a $\log_2$ fold change of 2 in expression as the cutoff for a differentially expressed gene. However, to consider all the changes in gene expression to understand the mechanism underlying the enhanced cadaverine production and cell growth, a less strict condition, i.e., a $\log_2$ fold change of 1, was used for the analysis in this study.

These findings indicate that the expression of *dr1558* in *C. glutamicum* influences the expression of metabolic pathway-related genes and genes related to the defense against acidic stress. These changes in gene expression enhance pH homeostasis, leading to increases in the cell growth rate and cadaverine production.

Fed-batch fermentation for the production of cadaverine by recombinant C. glutamicum **expressing** dr1558 **and** cadA **at an acidic pH**

Cadaverine production by the recombinant *C. glutamicum* strain expressing *dr1558* was enhanced in batch fermentation. To further investigate the effect of DR1558 on the production of cadaverine, a fed-batch fermentation was carried out. When the glucose concentration in the broth decreased to below 1 g/L, an appropriate amount of feeding solution was added to adjust the glucose concentration to 50 g/L. The time profiles of cell growth and the concentrations of glucose, lysine, and cadaverine during the fed-batch fermentation of Cg-cadA + dr1558 and Cg-cadA are shown in Fig. 4.

During the culture of Cg-cadA + dr1558, the pH was adjusted from an initial value of 7.1 to 5.7 when the OD_{600} of the culture reached 50. Even at this acidic pH, additional glucose was consumed, and at the end of the fermentation (35 h), 10.3 g/L of cadaverine was produced (Fig. 4). Cell growth also continued for 35 h, even after the pH was adjusted to 5.7. In contrast, the control strain Cg-cadA, which did not express *dr1558*, displayed lower rates of glucose consumption and cell growth at the acidic pH (Fig. 4). The strain expressing *dr1558* and *cadA* showed a 1.5-fold increase in cadaverine production, compared to that of the control strain after 35 h.

Conclusions

In this study, enhanced cadaverine production was observed in a recombinant *C. glutamicum* strain co-expressing *dr1558* and *cadA*. The addition of the *dr1558* gene altered the expression levels of metabolism-related genes under acidic conditions. The metabolic changes induced in the recombinant *C. glutamicum* strain as a result of *dr1558* expression are summarized in Fig. 5. The expression levels of genes related to glycolysis, the TCA cycle, and terminal pathways were altered. Some genes involved in defense mechanisms, including *dps*, *mcbR*, *mtr*, *cg1328*, and *ftn*, were also upregulated during cultivation at acidic pH. These genes, which are related to mechanisms underlying the defense against low pH, may be associated with the positive effects on cell growth. The exact mechanisms underlying the upregulation of the *cadA* gene and other stress-related genes following overexpression of *dr1558* are still under investigation. However, *dr1558* might bind to some regulator genes and thus change the expression level of genes involved in cadaverine biosynthesis and acid tolerance. Furthermore, these results suggest the possible application of *dr1558* for the enhanced production of biochemicals under acidic conditions.

Declarations

Acknowledgments

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Authors' contributions

Conceptualization, J.C.; methodology, S.B.; software, S.B.; validation, S.B.; formal analysis, S.B.; investigation, S.B.; resources, J.C.; data curation, S.B.; writing—original draft preparation, S.B.; writing—review and editing, J.C.; visualization, S.B.; supervision, J.C.; project administration, J.C.; funding acquisition, J.C.. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials

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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interest.

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Figures

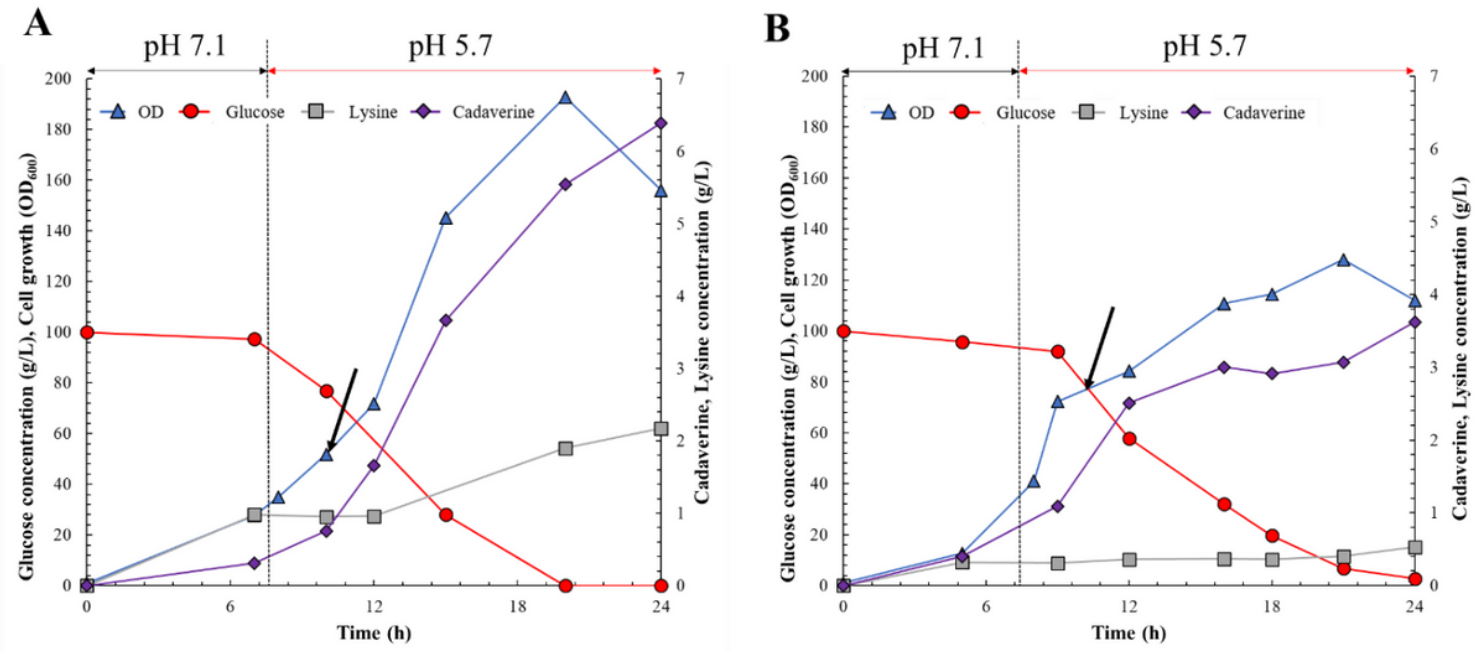

Figure 1

Time profiles of cell growth, the production of cadaverine and lysine, and glucose consumption during batch cultivation by (A) a recombinant C. glutamicum strain expressing cadA and dr1558 (Cg-cadA+dr1558) and (B) a recombinant C. glutamicum strain expressing cadA (Cg-cadA). The cells were harvested for mRNA preparation at 10 h (indicated by arrows).

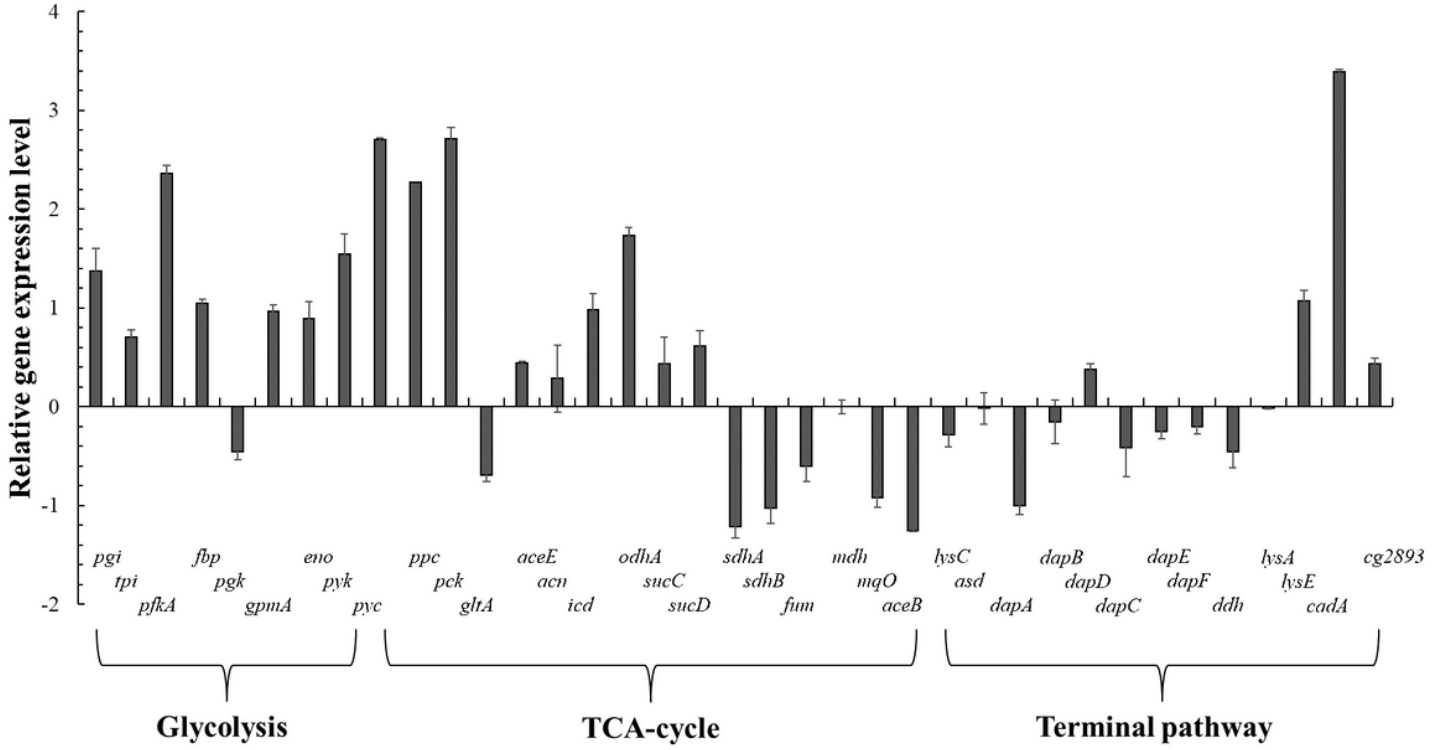

Figure 2

Relative expression levels of metabolic pathway-related genes in recombinant C. glutamicum expressing cadA and dr1558 compared to those in recombinant C. glutamicum expressing only cadA. Glycolytic pathway genes: pgi, tpi, pfkA, fbp, pgk, gpmA, eno, and pyk; TCA cycle genes: pyc, ppc, pck, gltA, aceE, acn, icd, odhA, sucC, sucD, sdhA, sdhB, fum, mdh, mqO, and aceB; and Central pathway genes: lysC, asd, dapA, dapB, dapC, dapE, dapF, ddh, lysE, cadA, and cg2893. Data were analyzed using the 2−ΔΔCt method. The histogram shows the mean of three biological replicates, and the error bars represent the standard deviations. The names of the genes are shown in the Supplementary Table.

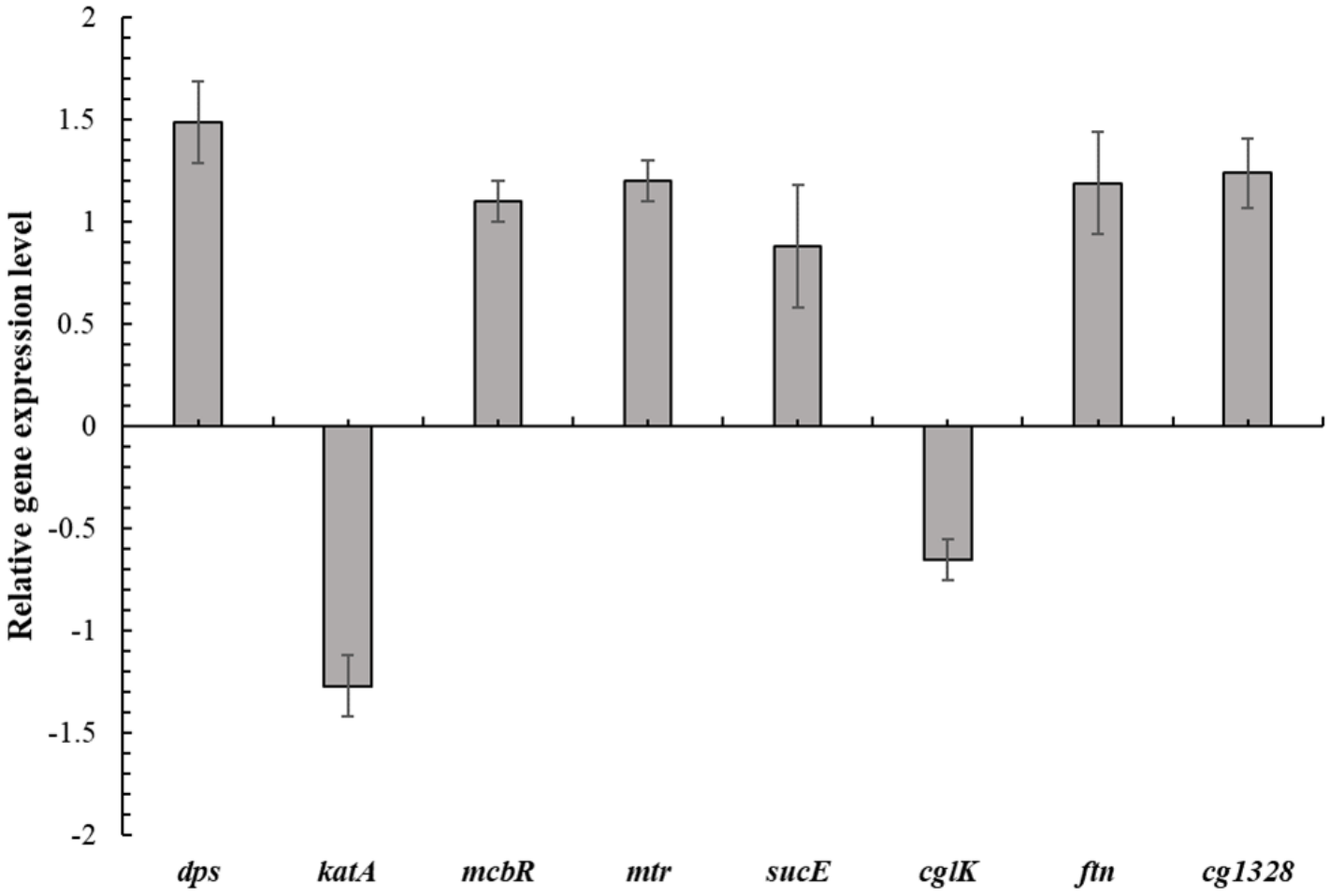

Figure 3

Relative expression levels of genes related to the acid resistance defense mechanism of recombinant C. glutamicum expressing cadA and dr1558 compared to those in recombinant C. glutamicum expressing only cadA. Data were analyzed using the 2−ΔΔCt method. The histogram shows the mean of three biological replicates, and the error bars represent the standard deviations.

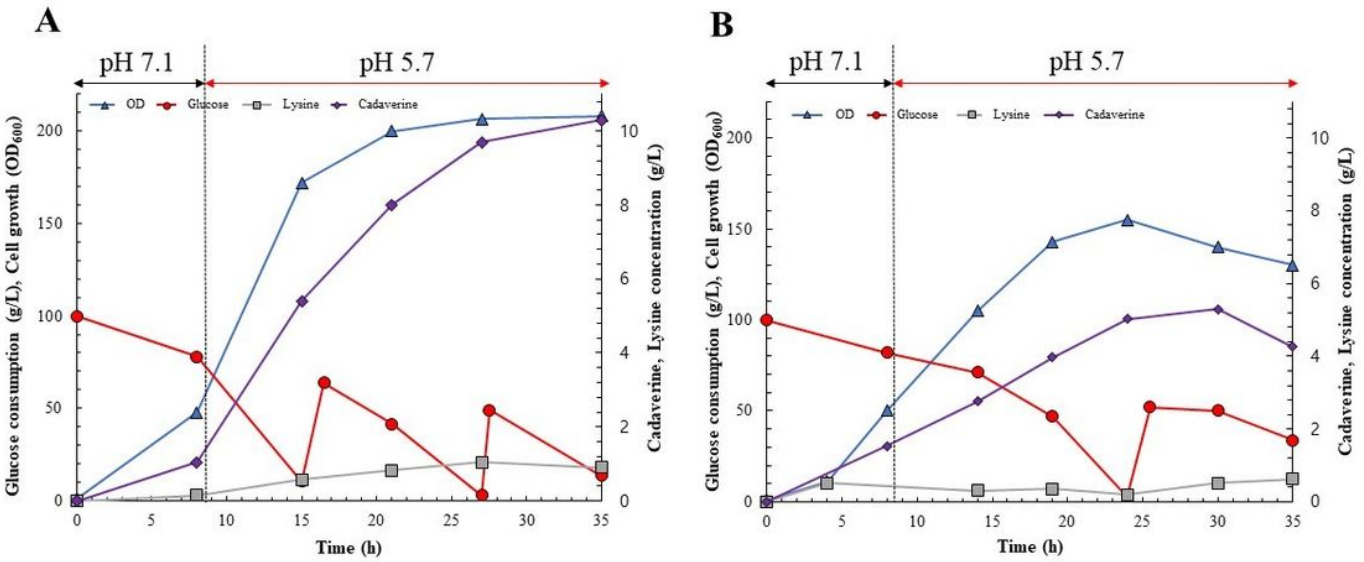

Figure 4

Time profiles of cell growth, the production of cadaverine and lysine, and glucose consumption during fed-batch cultivation by (A) recombinant C. glutamicum expressing cadA and dr1558 (Cg-cadA+dr1558) and (B) recombinant C. glutamicum expressing cadA (Cg-cadA).

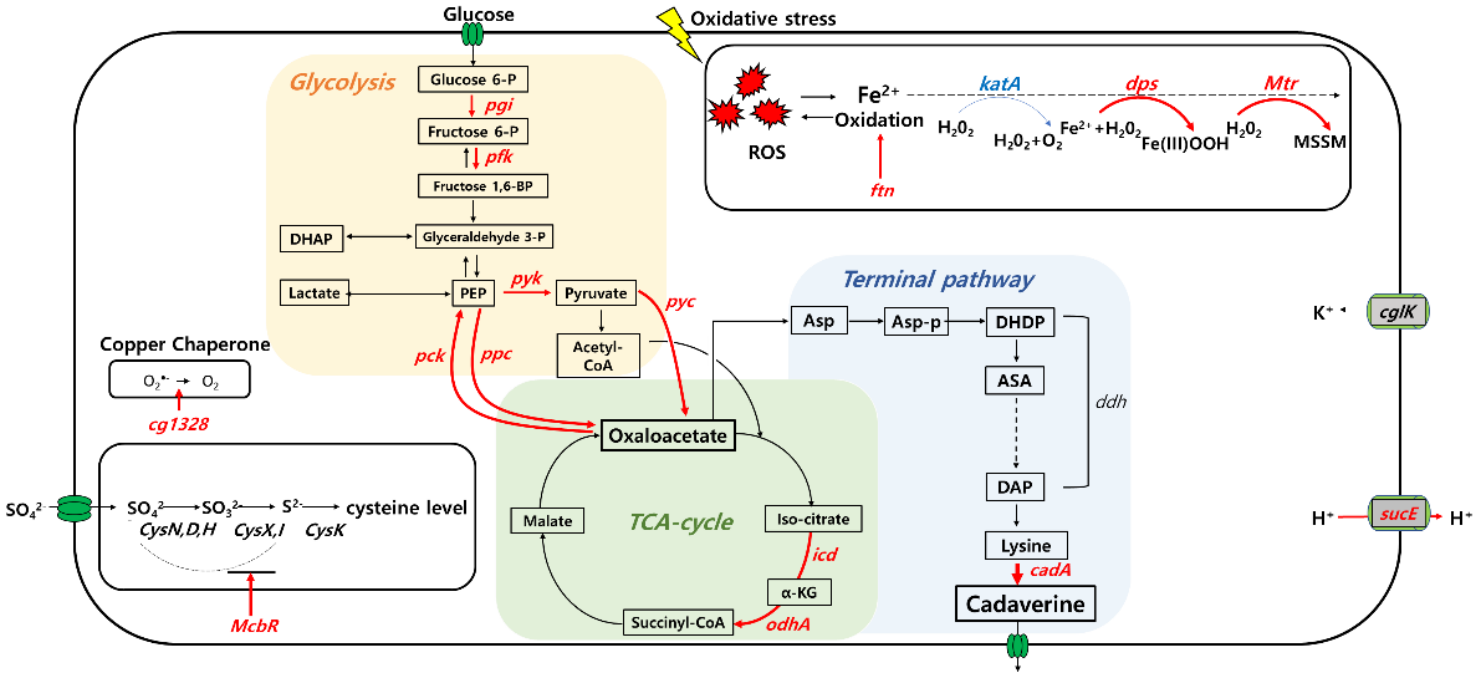

Figure 5

Schematic illustration of the changes in the expression levels of genes related to central metabolism and the acid resistance mechanism of recombinant C. glutamicum expressing dr1558. Genes with upregulated expression levels in the strain expressing dr1558 are shown in red.

Supplementary Files

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