n-Butylamine production from glucose using a transaminase-mediated synthetic pathway in Escherichia coli

Matsumoto, Takuya
Mori, Yuki
Tanaka, Tsutomu
Kondo, Akihiko

(Citation)
Journal of Bioscience and Bioengineering,129(1):99-103

(Issue Date)
2020-01

(Resource Type)
journal article

(Version)
Accepted Manuscript

n-Butylamine production from glucose using a transaminase-mediated synthetic pathway in *Escherichia coli*

Takuya Matsumoto,[1] Yuki Mori,[2] Tsutomu Tanaka,[2,] and Akihiko Kondo[3]*

[1] Department of Chemical Engineering, Osaka Prefecture University, 1-1 Gakuen-cho, Naka-ku, Sakai, Osaka, 599-8531, Japan.

[2] Department of Chemical Science and Engineering, Graduate School of Engineering, Kobe University, 1-1 Rokkodaicho, Nada, Kobe 657-8501, Japan

[3] Graduate School of Science, Technology and Innovation, Kobe University, 1-1 Rokkodaicho, Nada, Kobe 657-8501, Japan

[*]Corresponding author;

e-mail: tanaka@kitty.kobe-u.ac.jp

tel/fax:+81-78-803-6202

16 **Running title**

17 • *n*-Butylamine production from glucose in *E. coli*

18

19 **Highlights**

20 •*n*-Butanol was converted to *n*-butylamine by a three enzyme-mediated cascade.

21 •The cascade was combined with part of the *n*-butanol-producing pathway.

22 •*n*-Butylamine was produced in *Escherichia coli* from glucose as a carbon source.

23

ABSTRACT

Bioamination methods using microorganisms have attracted much attention because of the increasing demand for environmentally friendly bioprocesses. *n*-Butylamine production from glucose in *Escherichia coli* was demonstrated in this study, which has never been reported because of the absence of *n*-butylamine-producing pathway in nature. We focused on a transaminase-mediated cascade for bioamination from an alcohol or aldehyde. The cascade can convert an alcohol or an aldehyde to the corresponding amine with L-alanine as an amine donor. Here, *n*-butyraldehyde, which is a metabolic intermediate in the *n*-butanol producing pathway, is a potential intermediate for producing *n*-butylamine using this cascade. Hence, the *n*-butanol-producing pathway and the transaminase-mediated cascade were combined into a synthetic metabolic pathway for producing *n*-butylamine from glucose. Firstly, we demonstrated the conversion of *n*-butanol to *n*-butylamine using a three enzyme-mediated cascade. *n*-Butanol was successfully converted to *n*-butylamine in 92% yield in the presence of L-alanine and ammonium chloride. Then, the *n*-butanol-producing pathway and transaminase-mediated cascade were introduced into *E. coli*. Using this system, *n*-butylamine was successfully produced from glucose as a carbon source at a concentration of 53.2 mg L^{-1} after 96 h cultivation using a *ppc* (phosphoenolpyruvate carboxylase)-deficient strain. To the best of our knowledge, this is the first report of the direct production of *n*-butylamine from glucose, and may provide a starting point for the development of microbial methods to produce other bioamines.

Keywords: transaminase, cascade, *n*-butylamine, metabolic engineering, *Escherichia coli*

Introduction

Microbial bioproduction has gained increasing attention because of environmental and energy concerns. Microbial bioproduction is performed under mild conditions contrary to chemical methods, which are often performed at relatively high pressure and temperature using an organic solvent. Microorganisms such as *Escherichia coli* and *Saccharomyces cerevisiae*, which use biomass as a carbon source and produce a variety of chemicals, have been employed for bioproduction (1-3). Recent progress in genome engineering tools has facilitated the development of microbial bioproduction at an accelerated rate (4, 5). Amines are one of the essential building blocks in the chemical, pharmaceutical and agrochemical industries. While the conversion of amines from carbonyl compounds is usually catalyzed by a metal catalyst (6-9), bioamination processes using microorganisms have attracted much attention because of the increasing demand for environmentally friendly bioprocesses.

Three enzyme-mediated cascades have been previously used to synthesize amine compounds using alcohol as the substrate (10-12). Firstly, the alcohol is oxidized to an aldehyde by alcohol dehydrogenase (ADH), and then the transamination between the aldehyde and L-alanine, the amine donor, is catalyzed by transaminase (TA). At the same time, the NAD^+ consumed by alcohol dehydrogenase is regenerated to NADH by alanine dehydrogenase (ALADH), resulting in a cascade that is redox-neutral (Fig. 1). Therefore, only the supplementation of ammonium sources for the regeneration of L-alanine from pyruvate is required for the cascade. Although a variety of amines, including alkylamine, diamine and aromatic amines, have been synthesized using three enzyme-mediated cascades or whole-cell biocatalysts (10-12), only alcohols, aldehydes or alkanes are used as substrates for bioamination and these substrates are often toxic to enzymes or cells thus limiting the effectiveness of bioamination. Therefore, we

focused on bioamination using other carbohydrates as the carbon source, such as glucose, which can be assimilated. Several diamines or aminocarboxylic acids have been produced from glucose using *E. coli*, such as cadaverine (13), putrescine (14), gamma-amino butyric acid (15), and 5-aminovalerate (16). Although *E. coli* can be engineered to produce these amines by genetically introducing naturally occurring exogenous metabolic pathways, it is usually challenging to engineer *E. coli* to produce other amines using metabolic pathways that are not present in nature.

Herein, we demonstrate alkylamine production from glucose based on a synthetic metabolic pathway inspired from an enzyme cascade. To produce alkylamine with a synthetic pathway, alkylaldehyde is required as a metabolic intermediate. We focused on *n*-butylamine as the target product, which can be converted from *n*-butanol using a transaminase-mediated cascade. The production of *n*-butylamine from glucose by metabolically engineered microorganisms has not been reported because a pathway for the direct metabolism of glucose to *n*-butylamine does not exist in nature. *n*-Butyraldehyde is one potential substrate that can be converted into *n*-butylamine by transaminase. *n*-Butanol production has been achieved by metabolically engineered *E. coli* to contain an exogenous metabolic pathway that converts acetyl-CoA into *n*-butanol through *n*-butyraldehyde as a metabolic intermediate (17, 18). In this study, *n*-butanol producing pathway and transaminase-mediated cascade were combined in *E. coli* for direct production of *n*-butylamine from glucose. This study is the first report of direct *n*-butylamine production from glucose in *E. coli*.

Materials and methods

Bacterial Strains and Growth Conditions

The bacterial strains used in this study are listed in Table 1. *E. coli* NovaBlue cells (Novagen Inc., Madison, WI, USA) were used for DNA manipulations. *E. coli* BL21(DE3) cells and MG1655 (National BioResource Project) cells were used as the base strain for *n*-butylamine production. Cells were precultivated with 100 μg mL^{-1} of ampicillin and 20 μg mL^{-1} of kanamycin containing 5 mL of Luria–Bertani (LB) medium in a test tube overnight. For the *n*-butylamine production from *n*-butanol, cells were grown in 5 mL of LB medium at 37 °C, 220 rpm in test tubes (initial optical density at 600 nm: 0.1). After 3 h of cultivation, protein expression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG; final concentration = 0.5 mM). Then, *n*-butanol and NH_4Cl or L-alanine (final concentration = 20 mM, 40 mM or 20 mM) were also added and then the cultivation temperature was decreased to 30 °C. The reaction was carried out for 24 h.

For the production of *n*-butylamine from glucose, cells were grown in 5 mL of 20 g L^{-1} glucose containing M9Y medium (5 g L^{-1} yeast extract containing M9 minimal medium) at 37 °C and 220 rpm in test tubes (initial optical density at 600 nm: 0.1). After 3 h of cultivation, protein expression was induced by the addition of IPTG (final concentration = 0.5 mM). Then NH_4Cl or L-alanine (final concentration = 40 mM or 20 mM) was also added and the cultivation temperature was decreased to 30°C.

Plasmid Construction and Gene Disruption

The plasmids used in this study are listed in Table 1, the primers used in this study are described in Table S1, and the sequence of synthetic genes are shown in Fig.S1-6. The polymerase chain reaction (PCR) was performed with KOD FX polymerase (TOYOBO CO., Ltd., Osaka, Japan). Vectors and inserts were ligated with the In-Fusion HD Cloning Kit following the manufacturer's

protocol (TAKARA BIO INC., Shiga, Japan). Primer 1-12 were used for amplifying fragments including *atoB* from *E. coli*, *ter* from *Treponema denticola* (optimized for *E. coli*), *phaB* from *Ralstonia eutropha* and *phaJ* from *Aeromonas caviae*. The amplified fragment by using primer 1, 2 and the amplified fragment by using primer 3, 4 were conjugated with overlap extension PCR. Then, the conjugated fragment was inserted into *Nco*I/*Not*I site of the pETDuet-1 vector and the resulting plasmid was named *atoBter*-pETd. The amplified fragment by using primer 5, 6 and the amplified fragment by using primer 7, 8 were also conjugated with overlap extension PCR. Then the conjugated fragment was inserted into *Nde*I/*Avr*II site of the *atoBter*-pETd and the resulting plasmid was named *atoBter-phaBphaJ*-pETd. The amplified fragment by using primer 13~15 with pET32-Gly5-RFP (19) as a template was inserted into the *Xho*I/*Xba*I site of the pZE12-luc or pZA23-MCS vector (Expressys). The resulting plasmids were named pMR1 or pMR2, which include a red fluorescent protein under the M1-93 promoter in pZE12-luc or pZA23-MCS (20). Then, the fragments amplified by using primer 18, 19 or primer 20, 21 with *atoBter-phaBphaJ*-pETd as a template were inserted into the *Kpn*I/*Xba*I site of pMR1 vector and the resulting plasmid was named *atoBter*-pMR1 or *phaBphaJ*-pMR1. The amplified fragment by using primer 26, 27 with *phaBphaJ*-pMR1 as a template was inserted into *Avr*II site of *atoBter*-pMR1 and the resulting plasmid was named *atoBter-phaBphaJ*-pMR1. Finally, ATPP-pZE12 was constructed by using primer 28-31, *atoBter-phaBphaJ*-pMR1 as a template and the pZE12-MCS vector. Primer 32-35 was used for amplifying fragments including *csbld* from *Clostridium saccharoperbutylacetonicum* and *cvta* from *Chromobacterium violaceum*. The amplified fragment was inserted into the *Kpn*I/*Xba*I site of pMR2 vector and the resulting plasmid was named *csbldcvta*-pMR2. The amplified fragment by using primer 36, 37 with *csbldcvta*-pMR2 as a template and the amplified fragment by using primer 38, 39 were conjugated with overlap extension PCR and inserted into

136 the *Kpn*I/*Hin*dIII site of pZA23-MCS. The resulting plasmid was named BCA-pZA23. Similarly,
137 primer 40-45, pET-22b(+) and pCOLA Duet-1 were used for constructing pET22-*bsadh* and
138 pCOLA-*bsaladh-cvta*. The deletion of endogenous genes (phosphoenolpyruvate carboxylase (*ppc*)
139 and malate synthase G (*glcB*)) was performed by the gRNA-containing plasmid (pTargetT-*ppc*
140 and pTargetT-*glcB*, respectively) and pCas. The gRNA-containing plasmids were prepared with
141 pTartgetF and primer 46-57. The *E. coli* MG1655 genes, *ppc* and *glcB,* were deleted using the
142 CRISPR-Cas9 system as described in a previous report (21). Confirmation of gene deletions was
143 carried out by colony PCR. The resulting strains are shown in Table 1.

144 **Metabolite Analysis**

145 The concentration of glucose was determined by high-performance liquid
146 chromatography (Shimadzu Co., Kyoto, Japan; solvent delivery system, LC-20AB; column,
147 Shodex SUGAR KS-801; column temperature, 50 °C; detector, RID-10A). Ultra-pure water was
148 used as the mobile phase. Chromatography was carried out at a flow rate of 0.8 mL min^{-1}. The
149 concentration of *n*-butylamine was determined using a gas chromatograph equipped with a flame
150 ionization detector (Shimzadzu Co., Kyoto, Japan; gas chromatography, GC-2025; auto-injector,
151 AOC-20i/s; column, SH-Stabiliwax). The culture (50 μL) was dissolved in 500 μL benzene, and
152 then 0.05 M of trimethylamine containing benzene (100 μL) was added. Then heptafluorobutyric
153 acid anhydride (Merck KGaA, Darmstadt, Germany) (5 μL) was added to the sample and
154 incubated at 50 °C for 15 min. Ice-cooled 5% aqueous ammonia (500 μL) was added and mixed.
155 The sample (1 μL) from the upper layer was injected at a split ratio of 1:50. The initial oven
156 temperature was 40 °C for 1 min and then was increased at a rate of 10 °C/min to 150 °C and at a
157 rate of 35 °C/min until 220 °C, where it was held for 2 min. Helium was used as the carrier gas at
158 a linear velocity of 39.2 cm/sec. The detector was maintained at 250 °C.

Results and discussion

n-Butylamine production from *n*-butanol using a three enzyme-mediated cascade

A three enzyme-mediated cascade was examined for *n*-butylamine conversion from *n*-butanol. Sattler *et al.* showed that the use of ADH from *Bacillus stearothermophilus* (bsADH), TA from *Chromobacterium violaceum* (cvTA) and ALADH from *Bacillus subtilis* (bsALADH) converted primary alcohols, such as 1-hexanol and 1-octanol, to their corresponding amines with high efficiency. Although elongating the chain length of the substrate alcohols led to lower conversion, the addition of a co-solvent partly improved the yield (10). In this study, we examined the conversion of *n*-butanol to *n*-butylamine using bsADH, cvTA, and bsALADH. A BL21(DE3) and pET system were used for the substrate feeding experiments. A total of 5.92 mM of *n*-butylamine was synthesized from 20 mM *n*-butanol using the cascade (the yield of the conversion was about 30%). This result indicates that the cascade was capable of converting *n*-butanol to *n*-butylamine. As shown in Fig. 1, the cascade is totally redox-neutral, requiring only a supply of ammonium. However, there have been several reports that the residual addition of L-alanine or ammonium salt as an amine donor facilitated the conversion (10-12). Hence the influence of the addition of L-alanine or ammonium chloride on the cascade was evaluated. The conversion was clearly improved when either L-alanine or ammonium chloride was added, and moreover, the addition of both L-alanine and ammonium chloride was most effective contributing to 92% of the yield in the substrate-feeding experiments (Fig. 2). These results imply that the transamination by cvTA was the rate-limiting step and improvement of this step by the addition of NH_4^+ source directly increased the conversion. In addition, the elementary reactions on transaminase-mediated

cascade are reversible and concentration-driven. The addition of L-alanine probably was effective because the delta-G value of *n*-butylamine production was smaller than the value of *n*-butyraldehyde and pyruvate. Therefore, the supplementation of both L-alanine and ammonium chloride was determined to be optimal in this study.

Direct *n*-butylamine production from glucose through the synthetic metabolic pathway

To the best of our knowledge, there have been no reports demonstrating direct *n*-butylamine production from glucose because of the absence of *n*-butylamine-producing pathway in nature. By harnessing the cascade in this study, *n*-butylamine was successfully produced from *n*-butanol as a substrate. Here, we focused on *n*-butyraldehyde, which was an intermediate in the reaction. *n*-Butanol production from glucose was demonstrated in previous reports (17-18), some of which show that *n*-butyraldehyde is a metabolic intermediate in the synthetic pathway (17). In this study, part of the *n*-butanol-producing pathway and the enzyme-mediated cascade were combined to directly produce *n*-butylamine from glucose (Fig. 3). Glucose was converted into *n*-butyraldehyde through 2 acetyl-CoA, and subsequently, *n*-butyraldehyde was aminated by transaminase using L-alanine as the amine donor. The synthetic pathway is partly redox-neutral because residual NADH was used for the regeneration of L-alanine from pyruvate. The MG1655 strain was used for producing *n*-butylamine from glucose. Only MG1655 harboring ATPP-pZE and BCA-pZA produced *n*-butylamine from glucose (3.55 mg L^{-1}), whereas the other strains which were lack of n-butanol-producing pathway or transaminase-mediated cascade did not produce *n*-butylamine (Fig. 4A), indicating that the synthetic combined pathway was functional. Additionally, the production of *n*-butylamine was decreased when the reaction was not supplemented with L-alanine or ammonium chloride (Fig. 4B). This implies that the supplementation of both L-alanine and ammonium chloride was important for producing *n*-butylamine in the synthetic pathway.

The effect of knocking out a gene for accumulating metabolites

To enhance the production of *n*-butylamine, pyruvate needs to accumulate for the regeneration of alanine by bsALADH. However, pyruvate is usually a catabolite in the endogenous metabolic pathway in *E. coli*. Blocking pyruvate-catabolizing pathways has been shown as an effective approach for amination in a whole-cell biocatalyst (11). Therefore, one of the pyruvate catabolizing pathways in MG1655 was blocked by the deletion of the relevant gene (ppc) using the CRISPR-Cas9 system. The deletion of the ppc gene is known to result in the accumulation of pyruvate (22). As predicted, the production of *n*-butylamine in a ppc-deficient strain was clearly increased compared with wild-type strain at 53.2 mg L^{-1} after 96 h cultivation (Fig. 5). Additionally, the effect of deletion of the *glcB* gene, which is involved in acetyl-CoA catabolism in the glyoxylate shunt, was also determined as the intracellular acetyl-CoA level is critical for enhancing the production of *n*-butanol from glucose (18). As shown in Fig. 5, the production of *n*-butylamine by the *glcB*-deficient strain was also increased compared with wild-type strain at 34.8 mg L^{-1} after 72 h of cultivation. The deletion of both *ppc* and *glcB* resulted in a slightly lower production of *n*-butylamine (32.0 mg L^{-1} after 96 h cultivation) compared with the *ppc*-deficient strain. These results indicate that blocking the pyruvate- or acetyl-CoA-catabolizing pathway is one effective approach for producing *n*-butylamine. In addition, produced n-butylamine was decreased in several strains maybe because the transamination by cvTA step was reversible. Although most of the synthetic pathways used in this study are identical with the *n*-butanol-producing pathway, the amount of *n*-butylamine produced was relatively less than the *n*-butanol previously generated (17, 18). This is probably because the *n*-butanol-producing pathway employed herein was not fully optimized because *n*-butyraldehyde was not detected in culture as a byproduct. In addition, *n*-butanol-producing pathway in this study was employed *phaB* for the conversion of acetoacetyl-

CoA to 3-hydroxybutyryl-CoA. PhaB only utilizes NADPH as a cofactor, thereby occurring redox unbalance in the synthetic pathway. Therefore, further improvement of the strain, such as deleting competing endogenous pathways or enhancing the production pathway for accumulating intracellular acetyl-CoA or L-alanine, should increase the production of *n*-butylamine.

In summary, we demonstrated direct *n*-butylamine production from glucose using a transaminase synthetic pathway in *E. coli*. The conversion of *n*-butanol from *n*-butylamine using a three-enzyme cascade was successfully demonstrated as shown by the high reaction rate (92% under the optimized condition). After combining the cascade with part of the *n*-butanol-producing pathway, the resulting strains directly produced *n*-butylamine from glucose. To the best of our knowledge, this study is the first report of *n*-butylamine production from glucose and may provide inspiration for producing other bioamines, such as short-chain or long-chain alkylamines and aromatic amines.

ACKNOWLEDGMENTS

This work was supported by the Special Coordination Funds for Promoting Science and Technology provided by the Creation of Innovation Centers for Advanced Interdisciplinary Research Areas (Innovative Bioproduction Kobe), MEXT, Japan. We thank Renee Mosi, PhD, from Edanz Group for editing a draft of this manuscript.

REFERENCES

1. **Matsumoto, T., Tanaka, T., and Kondo, A.:** Engineering metabolic pathways in *Escherichia coli* for constructing a "microbial chassis" for biochemical production, Bioresour. Technol., **245**, 1362-1368 (2017).

2. **Hong. K.K., and Nielsen. J.:** Metabolic engineering of *Saccharomyces cerevisiae*: a key cell factory platform for future biorefineries, Cell Mol. Life Sci., **69**, 2671-2690 (2012).

3. **Liao, J.C., Mi, L., Pontrelli, S., and Luo, S.:** Fuelling the future: microbial engineering for the production of sustainable biofuels, Nat. Rev. Microbiol., **14**, 288-304 (2016).

4. **Lian, J., Mishra, S., and Zhao, H.:** Recent advances in metabolic engineering of *Saccharomyces cerevisiae*: New tools and their applications, Metab. Eng., **50**, 85-108 (2018).

5. **Jakočiūnas, T., Jensen, M.K., and Keasling, J.D.:** CRISPR/Cas9 advances engineering of microbial cell factories, Metab. Eng., **34**, 44-59 (2016).

6. **Imm, S., Bähn, S., Neubert, L., Neumann, H., and Beller, M.:** An efficient and general synthesis of primary amines by ruthenium-catalyzed amination of secondary alcohols with ammonia, Angew. Chem. Int. Ed. Engl., **49**, 8126-8129 (2010).

7. **Pingen, D., Müller, C., and Vogt, D.:** Direct amination of secondary alcohols using ammonia, Angew. Chem. Int. Ed. Engl., **49**, 8130-8133 (2010).

8. **Imm S1, Bähn S, Zhang M, Neubert L, Neumann H, Klasovsky F, Pfeffer J, Haas T, and Beller M. :** Improved ruthenium-catalyzed amination of alcohols with ammonia: synthesis of diamines and amino esters, Angew. Chem. Int. Ed. Engl., **50**, 7599-7603 (2011).

9. **Lafrance, M., Roggen, M., and Carreira, E.M.:** Direct, enantioselective iridium-catalyzed allylic amination of racemic allylic alcohols, Angew. Chem. Int. Ed. Engl., **51**, 3470-3473 (2012).

10. **Sattler, J.H., Fuchs, M., Tauber, K., Mutti, F.G., Faber, K., Pfeffer, J., Haas, T., and Kroutil, W.:** Redox self-sufficient biocatalyst network for the amination of primary alcohols, Angew. Chem. Int. Ed. Engl., **51**, 9156-9159 (2012).

11. **Klatte, S., and Wendisch, V.F.:** Role of L-alanine for redox self-sufficient amination of alcohols, Microb. Cell. Fact., **14**, 9 (2015)

12. **Ladkau, N., Assmann, M., Schrewe, M., Julsing, M.K., Schmid, A., and Bühler, B.:** Efficient production of the Nylon 12 monomer ω-aminododecanoic acid methyl ester from renewable dodecanoic acid methyl ester with engineered *Escherichia coli*, Metab. Eng., **36**, 1-9. (2016)

13. **Qian, Z.G., Xia, X.X., Lee, S.Y.:** Metabolic engineering of *Escherichia coli* for the production of cadaverine: a five carbon diamine, Biotechnol. Bioeng., **108**, 93-103 (2011).

14. **Qian, Z.G., Xia, X.X., Lee, S.Y.:** Metabolic engineering of *Escherichia coli* for the production of putrescine: a four carbon diamine, Biotechnol. Bioeng., **104**, 651-662 (2009).

15. **Dung Pham, V., Somasundaram, S., Lee, S.H., Park, S.J., and Hong, S.H.:** Efficient production of gamma-aminobutyric acid using *Escherichia coli* by co-localization of glutamate synthase, glutamate decarboxylase, and GABA transporter, J. Ind. Microbiol. Biotechnol., **43**, 79-86 (2016).

16. **Park, S.J., Kim, E.Y., Noh, W., Park, H.M., Oh, Y.H., Lee, S.H., Song, B.K., Jegal, J., and Lee, S.Y.:** Metabolic engineering of *Escherichia coli* for the production of 5-aminovalerate and glutarate as C5 platform chemicals, Metab. Eng., **16**, 42-47 (2013).

17. **Kataoka, N., Vangnai, A.S., Pongtharangkul, T., Tajima, T., Yakushi, T., Matsushita, K., Kato, J.:** Construction of CoA-dependent 1-butanol synthetic pathway functions under aerobic conditions in *Escherichia coli*, J. Biotechnol., **204**, 25-32 (2015).

18. **Atsumi, S., Cann, A.F., Connor, M.R., Shen, C.R., Smith, K.M., Brynildsen, M.P., Chou, K.J., Hanai, T., and Liao, J.C.:** Metabolic engineering of *Escherichia coli* for 1-butanol production, Metab. Eng., **10**, 305-311. (2008).

19. **Matsumoto, T., Tanaka, T., Kondo, A.:** Sortase A-catalyzed site-specific coimmobilization on microparticles via streptavidin., Langmuir, **28**, 3553-3557 (2012).

20. **Lu, J., Tang, J., Liu, Y., Zhu, X., Zhang, T., Zhang, X.:** Combinatorial modulation of *galP* and *glk* gene expression for improved alternative glucose utilization., Appl. Microbiol. Biotechnol., **93**, 2455-2462 (2012).

21. **Jiang, Y., Chen, B., Duan, C., Sun, B., Yang, J., Yang, S.:** Multigene editing in the *Escherichia coli* genome via the CRISPR-Cas9 system, Appl. Environ. Microbiol., **81**, 2506-2514 (2015).

22. **Zhu, J., Shimizu, K.:** Effect of a single-gene knockout on the metabolic regulation in *Escherichia coli* for D-lactate production under microaerobic condition, Metab. Eng., **7**, 104-115 (2005).

306 **Figure legends**

307 **Figure 1.** A three enzyme-mediated cascade for the conversion of *n*-butanol to *n*-butylamine.
308 Abbreviations: bsADH, alcohol dehydrogenase from *Bacillus stearothermophilus*; bsALADH,
309 alanine dehydrogenase from *Bacillus subtilis* subsp. *subtilis str.168*; cvTA, ω-transaminase from
310 *Chromobacterium violaceum*.

311 **Figure 2.** *n*-Butylamine production in the presence of various additives in BL21(DE3) harboring
312 pET22-*bsadh* and pCOLA-*bsaladh-cvta*. Control (1), NH_4Cl (2), L-alanine (3), NH_4Cl and L-
313 alanine (4). Data are presented as the average of three independent experiments and error bars
314 represent the standard deviation.

315 **Figure 3.** Metabolic pathways used in this study. Gene abbreviations: atoB, acetyl-CoA acetyl
316 transferase; phaB, Acetoacetyl-CoA reductase; phaJ, (R)-specific enoyl-CoA hydratase; ter, trans-
317 enoyl-CoA reductase; bld, butyraldehyde dehydrogenase; bsaladh, alanine dehydrogenase; cvta,
318 ω-transaminase. phaB, phaJ, ter, bld, bsaladh and cvta are derived from *R. eutropha*, *A. caviae*, *T.*
319 *denticola*, *C. saccharoperbutylacetonicum*, *B. subtilis* and *B. stearothermophilus*, respectively.

320 **Figure 4.** (A) *n*-Butylamine production by MG1655 harboring different combinations of plasmids.
321 pZE12-MCS and pZA23-MCS (1), pZE12-MCS and BCA-pZA (2), ATPP-pZE and pZA23-MCS
322 (3), ATPP-pZE and BCA-pZA (4). (B) *n*-Butylamine production of MG1655 harboring ATPP-
323 pZE and BCA-pZA in the presence of various additives. Control (1), NH_4Cl (2), L-alanine (3),
324 NH_4Cl and L-alanine (4). Data are presented as the average of three independent experiments and
325 error bars represent the standard deviation.

Figure 5. The production of *n*-butylamine (A) and the consumption of glucose (B) in various strains. Symbols represent MG1655Δ*ppc* circle; MG1655Δ*glcB*, triangle; and MG1655Δ*ppc*Δ*glcB*, square, harboring ATPP-pZE12 and BCA-pZA23. Data are presented as the average of three independent experiments and error bars represent the standard deviation.

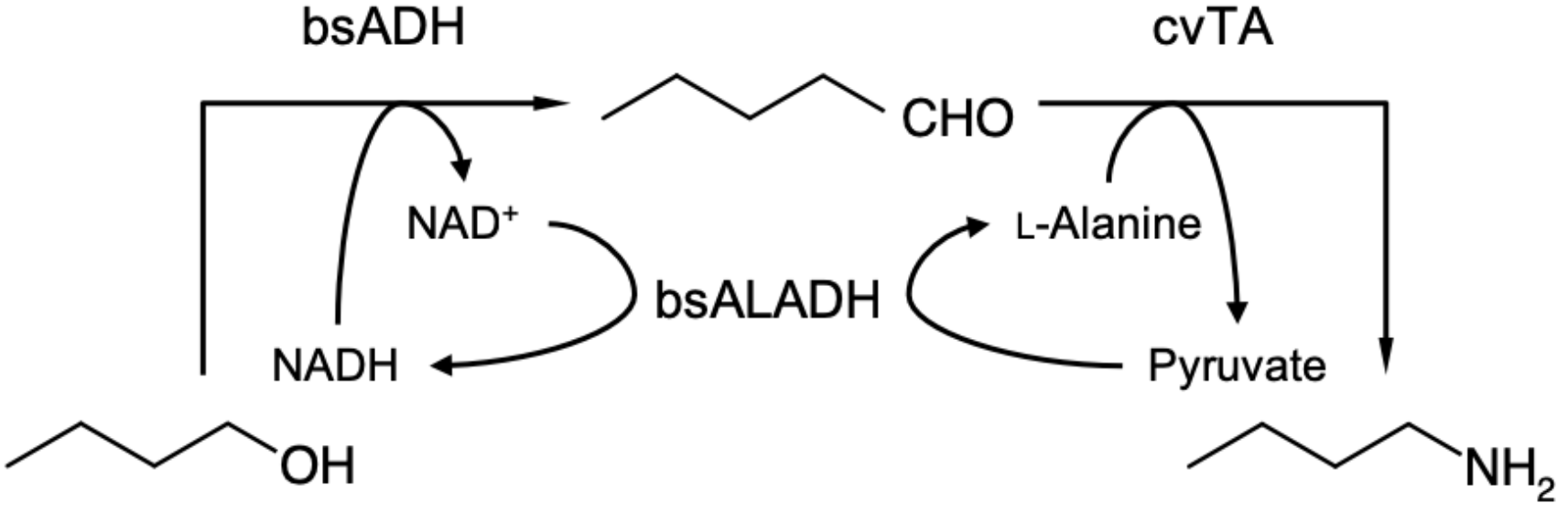

Figure 1

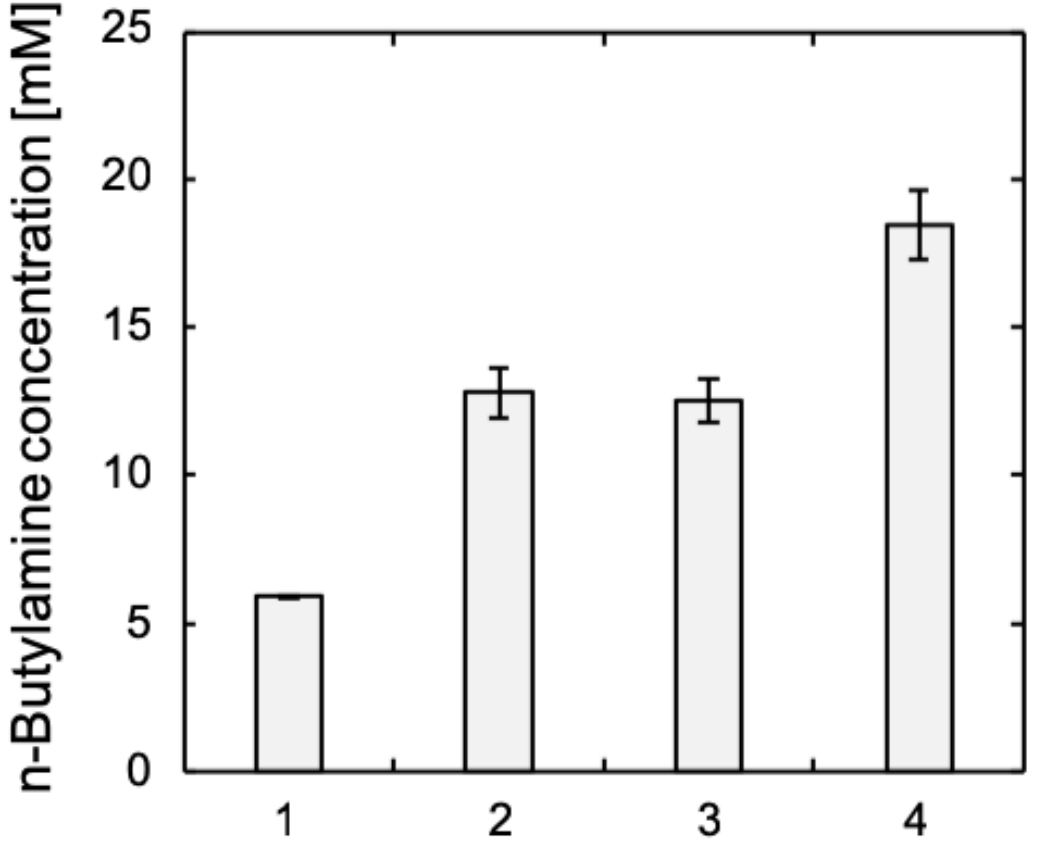

Figure 2

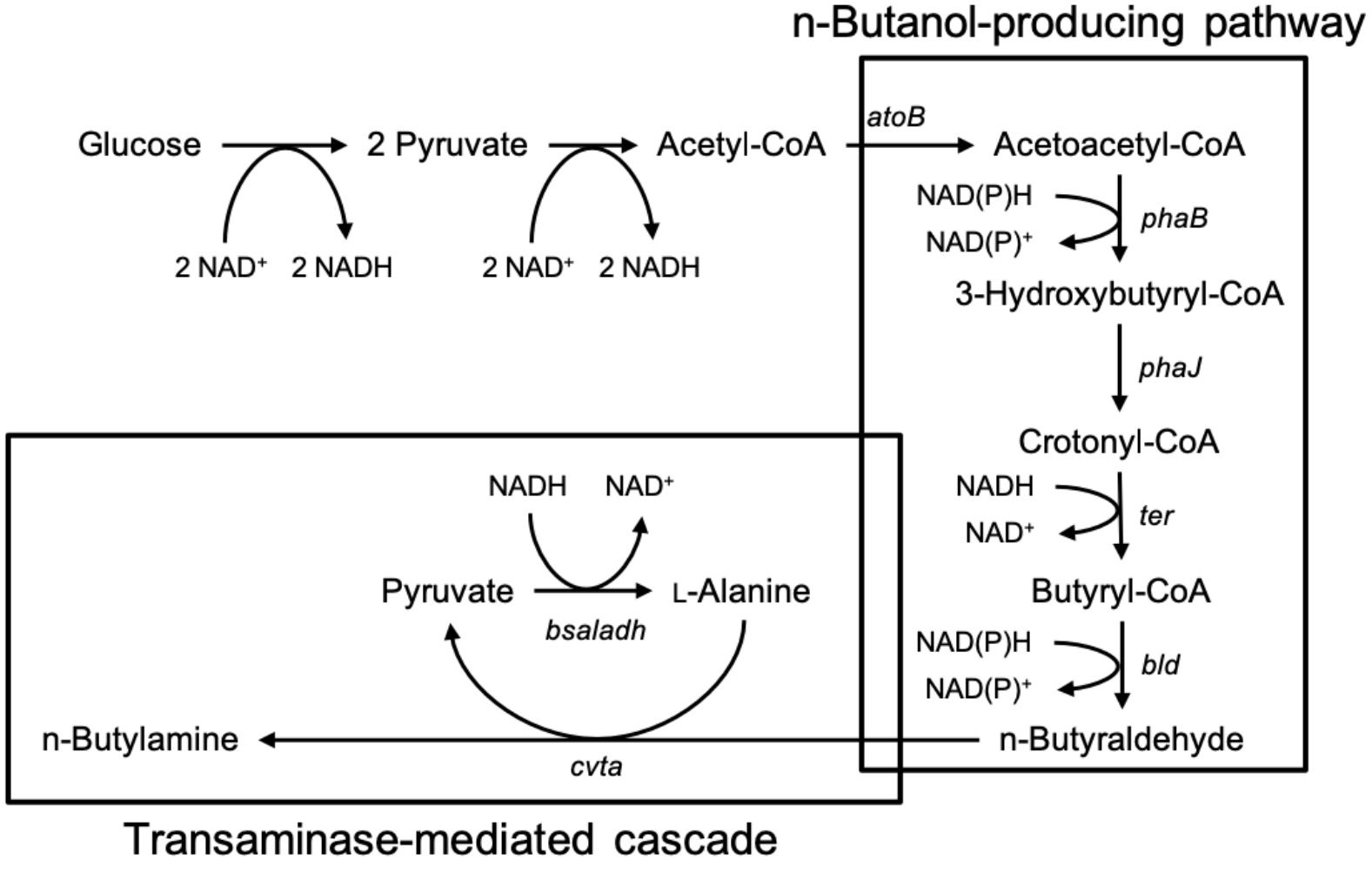
n-Butanol-producing pathway
Glucose
2 Pyruvate
Acetyl-CoA
2 NAD+
2 NADH
2 NAD+
2 NADH
atoB
Acetoacetyl-CoA
NAD(P)H
NAD(P)+
phaB
3-Hydroxybutyryl-CoA
phaJ
Crotonyl-CoA
NADH
NAD+
ter
Butyryl-CoA
NAD(P)H
NAD(P)+
bld
n-Butyraldehyde
NADH
NAD+
Pyruvate
L-Alanine
bsaladh
n-Butylamine
cvta
Transaminase-mediated cascade

Figure 3

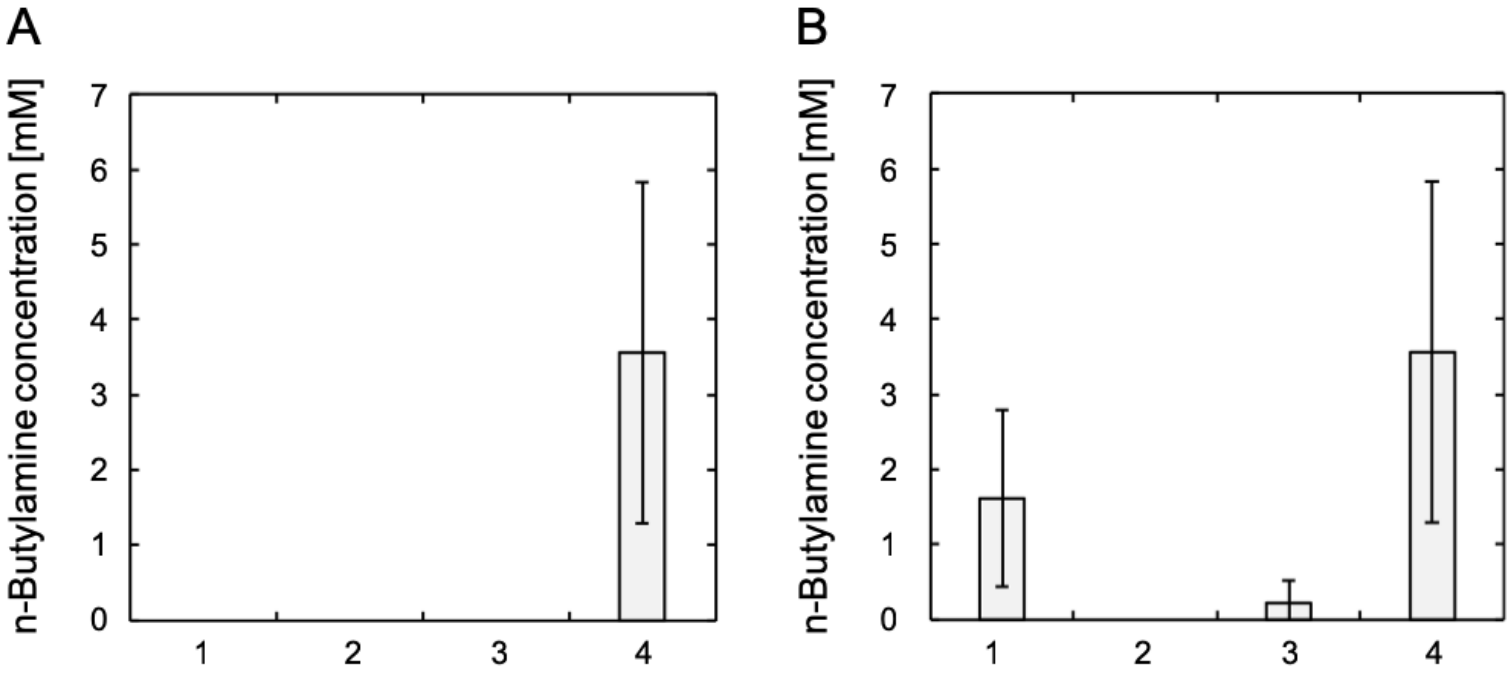
A
n-Butylamine concentration [mM]
0
1
2
3
4
5
6
7
1
2
3
4
B
n-Butylamine concentration [mM]
0
1
2
3
4
5
6
7
1
2
3
4

Figure 4

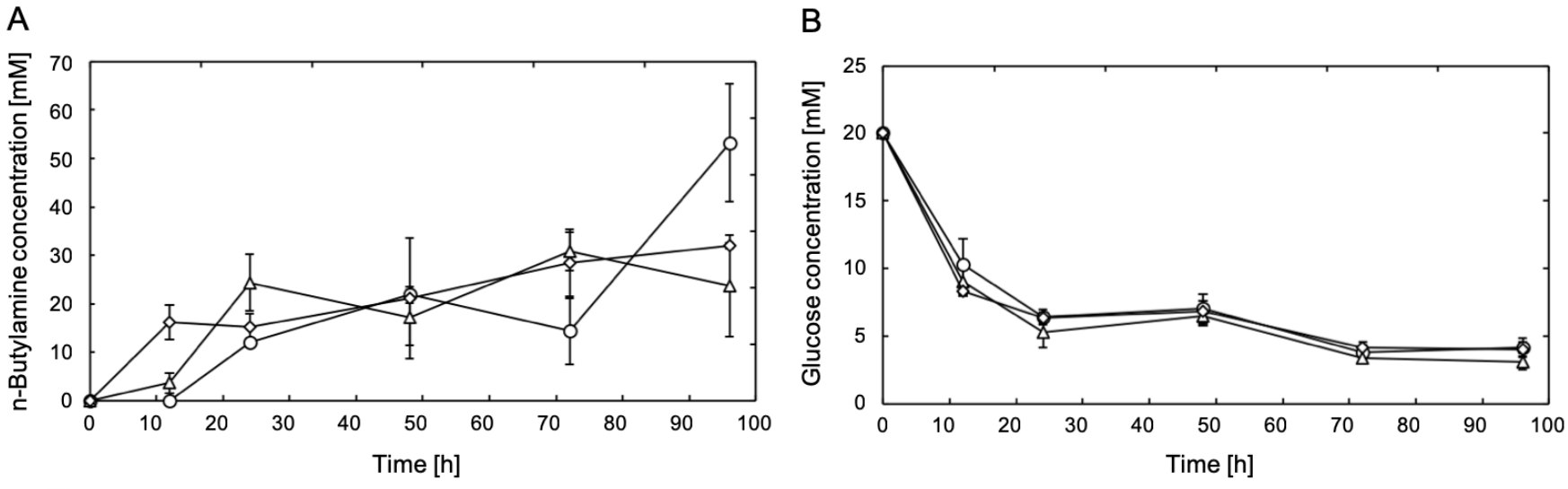
A
n-Butylamine concentration [mM]
0
10
20
30
40
50
60
70
0
10
20
30
40
50
60
70
80
90
100
Time [h]
B
Glucose concentration [mM]
0
5
10
15
20
25
0
10
20
30
40
50
60
70
80
90
100
Time [h]

Figure 5

Table

Table 1. Strains and plasmids used in this study

Strains or plasmids	Characteristics	Source of reference
Strain		
E. coli NovaBlue	*endA1 hsdR17* ($rk^- mk^+$) *supE44 thi-1 gyrA96 relA1 lac recA1*/F' [*proAB lacI*^{q} *Z* ΔM15 Tn*10*(*tet*^{r})]	Novagen
E. coli BL21(DE3)	*F*$^-$ *ompT hsdS*$_B$(*r*$_B^-$, *m*$_B^-$) *gal dcm λ(DE3)*	Novagen
E. coli MG1655	K-12; $F^- \lambda^-$ rph-1	NBRP
MG1655Δ*ppc*	as MG1655, but Δ*ppc*	This study
MG1655Δ*glcB*	as MG1655, but Δglc*B*	This study
MG1655Δ*ppc*Δ*glcB*	as MG1655, but Δ*ppc*Δglc*B*	This study
Plasmid		
pET-22b(+)	pBR322 ori; Amp^R; P_{T7}::MCS	Novagen
pCOLA Duet-1	ColA ori; Km^R; P_{T7-1}::MCS; P_{T7-2}::MCS	Novagen
pET22-*bsadh*	pBR322 ori; Amp^R; P_{T7}::*bsadh*	This study
pCOLA-*bsaladh-cvta*	ColA ori; Km^R; P_{T7-1}::*bsaladh*; P_{T7-2}::*cvta*	This study
pZE12MCS	ColE1 ori; Amp^R; $P_{LlacO-1}$::MCS	Expressys
pZA23MCS	p15A ori; Km^R; $P_{A1lacO-1}$::MCS	Expressys
ATPP-pZE12	ColE1 ori; Amp^R; $P_{LlacO-1}$::*atoB ter phaB phaJ*	This study
BCA-pZA23	p15A ori; Km^R; $P_{A1lacO-1}$::*bld cvta bsaladh*	This study
pCas	repA101 (Ts) ori; Km^R; P_{cas}::*cas9*; P_{araB}::*Red*; $lacI^q$; P_{trc}::sgRNA-pMB1	Addgene (21)
pTargetF	pMB1 ori; Spe^R; P_{J23199}::sgRNA	Addgene (21)
pTargetT- *ppc*	pMB1 ori; Spe^R; P_{J23199}::sgRNA-*ppc*; Donor-*ppc*	This study
pTargetT-*glcB*	pMB1 ori; Spe^R; P_{J23199}::sgRNA-*glcB*; Donor-*glcB*	This study

ATGATCAAAGATACCCTGGTGAGCATTACCAAAGACCTGAAACTGAAAACCAATGTGGAAAACGCCAACCTGAAAAACT
ATAAAGATGACAGCAGCTGTTTTGGCGTGTTTGAAAACGTTGAAAATGCCATTAGCAATGCAGTTCATGCCCAGAAAATTCT
GAGCCTGCATTATACCAAAGAACAGCGCGAAAAAATCATTACCGAAATTCGTAAAGCAGCCCTGGAAAACAAAGAAATTCT
GGCAACCATGATTCTGGAAGAAACCCACATGGGTCGTTATGAAGATAAAATCCTGAAACATGAACTGGTGGCCAAATATAC
ACCGGGTACAGAGGATCTGACCACCACCGCATGGTCAGGTGATAATGGTCTGACCGTTGTTGAAATGAGCCCGTATGGTGTT
ATTGGTGCAATTACCCCGAGCACCAATCCGACCGAAACCGTTATTTGTAATAGCATTGGTATGATTGCAGCCGGTAATACCG
TTGTGTTTAATGGTCATCCGGGTGCCAAAAAATGTGTTGCATTTGCAGTGGAAATGATCAACAAAGCCATTATTAGCTGTGG
TGGTCCGGAAAATCTGGTTACCACCATTAAAAACCCGACAATGGATAGCCTGGATGCCATTATCAAACATCCGAGCATTAA
ACTGCTGTGTGGCACAGGCGGTCCGGGTATGGTTAAAACCCTGCTGAATAGCGGTAAAAAAGCAATTGGTGCCGGTGCAGG
TAATCCGCCTGTTATTGTTGATGATACCGCAGATATTGAGAAAGCCGGTAAAAGCATTATTGAAGGCTGCAGCTTTGATAAT
AATCTGCCGTGTATTGCCGAAAAAGAGGTGTTTGTTTTTGAGAATGTTGCCGATGATCTGATCAGCAACATGCTGAAAAATA
ACGCCGTGATCATTAACGAAGATCAGGTGAGCAAACTGATTGATCTGGTGCTGCAGAAAAACAACGAAACCCAAGAATACA
GCATCAACAAAAAATGGGTGGGCAAAGATGCAAAACTGTTTCTGGATGAAATTGATGTTGAAAGCCCGAGCAGCGTGAAAT
GTATTATTTGTGAAGTTAGCGCAAGCCACCCGTTTGTTATGACCGAACTGATGATGCCGATTCTGCCGATTGTTCGTGTTAAA
GATATTGATGAAGCCATCGAGTATGCCAAAATTGCAGAACAGAATCGTAAACACAGCGCCTATATCTACAGCAAAAACATC
GATAATCTGAACCGCTTTGAACGCGAAATTGATACCACCATCTTTGTGAAAAACGCGAAAAGCTTTGCCGGTGTTGGTTATG
AAGCAGAAGGTTTTACCACCTTTACCATTGCAGGTAGCACCGGTGAAGGTATTACCAGCGCACGTAATTTTACCCGTCAGCG
TCGTTGTGTTCTGGCAGGT

Fig. S1. Sequence of synthetic gene: BLD from *Clostridium saccharoperbutylacetonicum*.

ATGATCGTTAAACCGATGGTGCGCAATAACATTTGTCTGAATGCACATCCGCAGGGTTGTAAAAAAGGTGTTGAAGATCA
GATCGAATATACCAAAAAACGCATTACCGCTGAAGTTAAAGCCGGTGCAAAAGCACCGAAAAATGTTCTGGTTCTGGGTTG
TAGCAATGGTTATGGTCTGGCAAGCCGTATTACCGCAGCATTTGGTTATGGCGCAGCAACCATTGGTGTTAGCTTTGAAAAA
GCAGGTAGCGAAACCAAATATGGTACACCGGGTTGGTATAATAACCTGGCATTTGATGAAGCAGCAAAACGTGAAGGTCTG
TATAGCGTTACCATTGATGGTGATGCATTTAGCGACGAAATTAAAGCGCAGGTTATTGAAGAAGCCAAAAAAAAAAGGCATC
AAATTCGACCTGATTGTGTATAGCCTGGCAAGTCCGGTTCGTACCGATCCGGATACCGGCATCATGCATAAAAGCGTTCTGA
AACCGTTTGGCAAAACCTTTACCGGCAAAACCGTTGATCCGTTTACCGGTGAACTGAAAGAAATTAGCGCAGAACCGGCAA
ATGATGAAGAAGCAGCAGCAACCGTTAAAGTTATGGGTGGTGAAGATTGGGAACGTTGGATTAAACAGCTGAGCAAAGAA
GGTCTGCTGGAAGAAGGTTGTATTACCCTGGCATATAGTTATATTGGTCCGGAAGCAACCCAGGCACTGTATCGTAAAGGCA
CCATTGGTAAAGCAAAAGAACATCTGGAAGCCACCGCACATCGTCTGAATAAAGAAAATCCGAGCATTCGTGCATTTGTGA
GCGTTAATAAAGGTCTGGTTACCCGTGCAAGCGCAGTGATTCCGGTTATTCCGCTGTATCTGGCCAGCCTGTTTAAAGTGAT
GAAAGAAAAAGGTAACCACGAAGGTTGCATTGAGCAGATTACCCGTCTGTATGCAGAACGTCTGTATCGCAAAGATGGCAC
CATTCCGGTGGATGAAGAAAATCGTATTCGTATCGATGATTGGGAGCTGGAAGAGGATGTTCAGAAAGCAGTTAGCGCACT
GATGGAAAAAGTGACCGGTGAAAATGCAGAAAGCCTGACCGATCTGGCAGGTTATCGTCATGATTTTCTGGCAAGTAATGG
CTTTGATGTGGAAGGCATTAACTATGAAGCAGAAGTGGAACGTTTTGACCGCATCTAA

Fig. S2. Sequence of synthetic gene: TER from *Treponema denticola*.

atgCAGAAACAGCGTACCACCTCTCAGTGGCGTGAACTGGATGCAGCACATCATCTGCATCCGTTTACCGATACCGCAAGCC
TGAATCAGGCAGGTGCACGTGTTATGACCCGTGGTGAAGGTGTTTATCTGTGGGATAGCGAAGGCAACAAAATTATTGATG
GTATGGCAGGTCTGTGGTGTGTTAATGTTGGTTATGGTCGCAAAGATTTTGCAGAAGCAGCACGTCGTCAGATGGAAGAACT
GCCGTTTTATAATACCTTTTTTAAAACCACCCATCCGGCAGTTGTTGAACTGAGCAGCCTGCTGGCCGAAGTTACACCGGCA
GGTTTTGATCGTGTGTTTTATACCAATAGCGGTAGCGAAAGCGTTGATACCATGATTCGCATGGTTCGTCGTTATTGGGATGT
TCAGGGCAAACCGGAAAAAAAAAACCCTGATCGGTCGTTGGAATGGTTATCATGGTAGCACCATTGGTGGTGCCAGCCTGGG
TGGTATGAAATATATGCATGAACAGGGTGATCTGCCGATTCCGGGTATGGCACATATTGAACAGCCGTGGTGGTATAAACAT
GGCAAAGATATGACACCGGATGAATTTGGTGTTGTTGCAGCACGTTGGCTGGAAGAAAAAATTCTGGAAATTGGTGCCGAT
AAAGTTGCAGCATTTGTGGGTGAACCGATTCAGGGTGCAGGTGGTGTTATTGTTCCGCCTGCAACCTATTGGCCTGAAATTG
AACGTATCTGCCGCAAATATGATGTTCTGCTGGTTGCCGATGAAGTTATTTGTGGTTTTGGTCGTACCGGTGAATGGTTTGGT
CATCAGCATTTTGGTTTTCAGCCGGACCTGTTTACCGCAGCCAAAGGCTTATCTTCTGGCTATCTGCCGATTGGTGCAGTTTT
TGTTGGTAAACGTGTTGCAGAAGGTCTGATTGCAGGCGGTGATTTTAATCATGGCTTTACCTATAGCGGTCATCCGGTTTGTG
CAGCAGTTGCACATGCAAATGTTGCAGCACTGCGTGATGAAGGTATTGTTCAGCGCGTGAAAGATGATATTGGTCCGTATAT
GCAGAAACGTTGGCGTGAAACCTTTAGCCGTTTTGAACATGTTGATGATGTTCGTGGTGTTGGTATGGTTCAGGCATTTACC
CTGGTGAAAAACAAAGCAAAACGCGAACTGTTTCCGGATTTTGGTGAAATTGGCACCCTGTGCCGTGATATTTTTTTTCGCA
ATAATCTGATTATGCGTGCCTGTGGTGATCACATTGTTAGCGCACCGCCTCTGGTGATGACCCGTGCCGAAGTTGATGAAAT
GCTGGCCGTTGCAGAACGCTGTCTGGAAGAATTTGAACAGACCCTGAAAGCACGTGGTCTGGCCtaa

Fig. S3. Sequence of synthetic gene: TA from *Chromobacterium violaceum*.

atgAAAGCAGCAGTTGTGGAACAGTTTAAAGAACCGCTGAAAATTAAAGAAGTGGAAAAACCGACCATTAGCTATGGTGA
AGTTCTGGTTCGTATTAAAGCCTGTGGTGTTTGTCATACCGATCTGCATGCAGCACATGGTGATTGGCCTGTTAAACCGAAA
CTGCCGCTGATTCCGGGTCATGAAGGTGTTGGTATTGTTGAAGAAGTTGGTCCTGGCGTTACCCATCTGAAAGTTGGTGATC
GTGTTGGTATTCCGTGGCTGTATAGCGCATGTGGTCATTGTGATTATTGTCTGAGCGGTCAAGAAACCCTGTGCGAACATCA
GAAAAATGCAGGTTATAGCGTGGATGGTGGTTATGCAGAATATTGTCGTGCAGCAGCAGATTATGTTGTGAAAATTCCGGAT
AATCTGAGCTTTGAAGAAGCAGCACCGATTTTTTGTGCCGGTGTTACCACCTATAAAGCACTGAAAGTTACCGGTGCAAAAC
CGGGTGAATGGGTTGCAATTTATGGTATTGGTGGTCTGGGCCATGTTGCAGTTCAGTATGCAAAAGCAATGGGTCTGAATGT
TGTTGCAGTGGATATTGGTGATGAAAAACTGGAACTGGCAAAAGAACTGGGTGCAGATCTGGTTGTTAATCCGCTGAAAGA
AGATGCAGCCAAATTTATGAAAGAAAAAGTGGGTGGTGTTCATGCAGCAGTTGTTACCGCAGTTAGCAAACCGGCATTTCA
GAGCGCATATAATAGCATTCGTCGTGGTGGTGCATGTGTTCTGGTTGGTCTGCCTCCGGAAGAAATGCCGATTCCGATTTTT
GATACCGTGCTGAATGGCATTAAAATTATTGGTAGCATTGTGGGCACCCGTAAAGATCTGCAAGAAGCACTGCAGTTTGCAG
CAGAAGGTAAAGTTAAAACCATTATTGAAGTGCAGCCGCTGGAAAAAATTAATGAAGTGTTTGATCGCATGCTGAAAGGTC
AGATTAATGGTCGTGTTGTTCTGACCCTGGAAGATAAAtaa

Fig. S4. Sequence of synthetic gene: ADH from *Bacillus stearothermophilus*.

ATGATCATAGGGGTTCCTAAAGAGATAAAAAACAATGAAAACCGTGTCGCATTAACACCCGGGGGCGTTTCTCAGCTCAT

TTCAAACGGCCACCGGGTGCTGGTTGAAACAGGCGCGGGCCTTGGAAGCGGATTTGAAAATGAAGCCTATGAGTCAGCAGG

AGCGGAAATCATTGCTGATCCGAAGCAGGTCTGGGACGCCGAAATGGTCATGAAAGTAAAAGAACCGCTGCCGGAAGAAT

ATGTTTATTTTCGCAAAGGACTTGTGCTGTTTACGTACCTTCATTTAGCAGCTGAGCCTGAGCTTGCACAGGCCTTGAAGGAT

AAAGGAGTAACTGCCATCGCATATGAAACGGTCAGTGAAGGCCGGACATTGCCTCTTCTGACGCCAATGTCAGAGGTTGCG

GGCAGAATGGCAGCGCAAATCGGCGCTCAATTCTTAGAAAAGCCTAAAGGCGGAAAAGGCATTCTGCTTGCCGGGGTGCCT

GGCGTTTCCCGCGGAAAAGTAACAATTATCGGAGGAGGCGTTGTCGGGACAAACGCGGCGAAAATGGCTGTCGGCCTCGGT

GCAGATGTGACGATCATTGACTTAAACGCAGACCGCTTGCGCCAGCTTGATGACATCTTCGGCCATCAGATTAAAACGTTAA

TTTCTAATCCGGTCAATATTGCTGATGCTGTGGCGGAAGCGGATCTCCTCATTTGCGCGGTATTAATTCCGGGTGCTAAAGCT

CCGACTCTTGTCACTGAGGAAATGGTAAAACAAATGAAACCCGGTTCAGTTATTGTTGATGTAGCGATCGACCAAGGCGGC

ATCGTCGAAACTGTCGACCATATCACAACACATGATCAGCCAACATATGAAAAACACGGGGTTGTGCATTATGCTGTAGCG

AACATGCCAGGCGCAGTCCCTCGTACATCAACAATCGCCCTGACTAACGTTACTGTTCCATACGCGCTGCAAATCGCGAACA

AAGGGGCAGTAAAAGCGCTCGCAGACAATACGGCACTGAGAGCGGGTTTAAACACCGCAAACGGACACGTGACCTATGAA

GCTGTAGCAAGAGATCTAGGCTATGAGTATGTTCCTGCCGAGAAAGCTTTACAGGATGAATCATCTGTGGCGGGTGCTTAA

Fig. S5. Sequence of synthetic gene: ALADH from *Bacillus subtilis* subsp*. subtilis str.168*.

ATGAGCGCACAGAGCCTGGAAGTTGGTCAGAAAGCACGTCTGAGCAAACGTTTTGGTGCAGCAGAAGTTGCAGCATTTGC
AGCACTGAGCGAAGATTTTAATCCGCTGCATCTGGACCCTGCATTTGCCGCAACCACCGCATTTGAACGTCCGATTGTTCAT
GGTATGCTGCTGGCAAGCCTGTTTAGCGGTCTGCTGGGTCAGCAGCTGCCTGGTAAAGGTAGCATTTATCTGGGTCAGAGCC
TGTCATTTAAACTGCCGGTTTTTGTTGGTGATGAAGTTACCGCAGAAGTGGAAGTTACAGCACTGCGTGAAGATAAACCGAT
TGCAACCCTGACCACCCGTATTTTTACCCAGGGTGGTGCACTGGCAGTTACCGGTGAAGCAGTTGTGAAACTGCCGTAA

Fig. S6. Sequence of synthetic gene: PHAJ from *Aeromonas caviae*.

Table S1 Primers used in this study

No.	Primer name	
1	NcoI pETd atoB for	ggagatataccatggcgATGAAAAATTGTGTCATCGTCAGTGCGGTA
2	atoB RBS re	ggtaccCTAGTTTGTCCCCTCTTTCgaattcttattaATTCAACCGTTCAATCACCATCGCAATTCC
3	RBS ter for	taataagaattcGAAAGAGGGGACAAACTAGggtaccATGATCGTTAAACCGATGGTGCGCAATAAC
4	NotI pETd ter re	gcattatgcggccgcTTAGATGCGGTCAAAACGTTCCACTTCTGC
5	NdeI pETd phaB for	ggagatatacatatgatgactcagcgcattgcgtatgtgaccggc
6	phaB RBS re	ggtaccCTAGTTTGTCCCCTCTTTCgaattcttattagcccatatgcaggccgccgttgagcga
7	RBS phaJ for	taataagaattcGAAAGAGGGGACAAACTAGggtaccATGAGCGCACAGAGCCTGGAAGTTGGTCAG
8	AvrII pETd phaJ re	tggcagcagcctaggTTACGGCAGTTTCACAACTGCTTCACCGGT
9	NcoI pETd R	ccatggtatatctccttattaaagttaaacaaaat
10	NotI pETd F	gcggccgcataatgcttaagtc
11	NdeI pETd R	catatgtatatctccttcttatacttaactaatatactaag
12	AvrII pETd F	cctaggctgctgccaccgctga
13	XhoI pZE12 M1-93 F	CGTCTTCACCTCGAGTTATCTCTGGCGGTGTTGACAAGAGATAACAACGTTGATATAATTGAGCCCGTATTGTTAGC ATGTACG
14	M1-93 rbs RFP F	AGCCCGTATTGTTAGCATGTACGTTTAAACGAATTCATTAAAGAGGAGAAAGGTACCATGGACAACACCGAGGACG TC
15	XbaI pZE12 RFP F	TTTGATGCCTCTAGAttaACTGGGAGCCGGAGTGGCGGG
16	XbaI pZE12 F	TCTAGAGGCATCAAATAAAAC
17	XhoI pZE12 R	CTCGAGGTGAAGACGAAAGGG
18	KpnI pMR1 phaA F	GAGGAGAAAGGTACCATGACTGACGTTGTCATCGTATC
19	XbaI pMR1 ter R	TTTGATGCCTCTAGAttaGATGCGGTCAAAACGTTCCACTTC
20	KpnI pMR1 phaB F	GAGGAGAAAGGTACCATGactcagcgcattgcgtatgtg
21	XbaI pMR1 phaJ R	TTTGATGCCTCTAGAttaCGGCAGTTTCACAACTGCTTCA
22	KpnI pMR1 R	GGTACCTTTCTCCTCTTTAATGAATTCGTTTAAAC
23	XbaI pMR1 F	TCTAGAGGCATCAAATAAAACGAAAGGCTCAG
24	AvrII pMR op2 F	CTAGGCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAA
25	AvrII pMR op2 R	CTAGGTCTAGGGCGGCGGATTTGTCCTACTCAGGAGAGCG
26	XbaI ter IF M1-93 F	CCGCATCtaaTCTAGTTATCTCTGGCGGTGTTGACAAGA
27	XbaI IF Term R	ATTTGATGCCTCTAGTCTAGGGCGGCGGATTTGT
28	AvrII IF M1-93 F	CCGCCCTAGACCTAGTTATCTCTGGCGGTGTTGACAA
29	AvrII IF Term R	CAGCCGAACGCCTAGgTCTAGGGCGGCGGATTTG
30	inv pMR1 ABJt 1cassette F	aagcttAGGAGGGTACCatgactcagcgcattgcgtatgtgaccggc
31	inv pMR1 ABJt 1cassette R	GGTACCCTCCTaagcttttaGATGCGGTCAAAACGTTCCACTTCTGC
32	Xho1 atoB fo	CGGGCCCCCCCTCGAGatgaaaaattgtgtcatcgtcagtgcggta
33	Xma1 phaJ re	CATGGGATCCCCCGGGttaCGGCAGTTTCACAACTGCTTCACCGGT
34	KpnI pMR2 csbld F	GAGGAGAAAGGTACCATGATCAAAGATACCCTGGTGA

35	cxbld rbs R	ggtaccCTAGTTTGTCCCCTCTTTCgaattcttattaACCTGCCAGAACACAACG
36	rbs cvta F	taataagaattcGAAAGAGGGGACAAACTAGgtaccatgCAGAAACAGCGTACCAC
37	XbaI pMR2 cvta R	TTTGATGCCTCTAGAttaGGCCAGACCACGTG
38	kpn1 bld for	ATTAAAGAGGAGAAAGGTACCATGATCAAAGATACCCTGGTGAGCATTAC
39	cvta sodo-rbs2 re	GGTACCTTTCTCCTCTTTAATGAATTCGTTttaGGCCAGACCACGTGCTTTCAGGGTCTG
40	sodo-rbs2 aladh for	AACGAATTCATTAAAGAGGAGAAAGGTACCatgATCATAGGGGTTCCTAAAGAGATAAAA
41	aladh hindlll re	ATTCGATATCAAGCTTttaAGCACCCGCCACAGATGATTCATCCTG
42	NcoI_aladh_Bs_for	aggagatataccatgggcATCATAGGGGTTCCTAAAGAGATAAAAAAC
43	NotI_aladh_Bs_re	aagcattatgcggccgcTTAAGCACCCGCCACAGATGATTCATCCTG
44	NdeI_tacv_for	aaggagatatacatatgatgCAGAAACAGCGTACCACCTCTCAGTGG
45	AvrII_tacv_re	aagcattatgcggccgcTTAAGCACCCGCCACAGATGATTCATCCTG
46	p22 IF ADH-Bs for 2	AAGGAGATATACATATGAAAGCAGCAGTTGTGGAACAGTTT
47	p22 IF ADH-Bs re 2	TGCTCGAGTGCGGCCGCTTTATCTTCCAGGGTCAGAACAAC
49	sgRNA ppc Rv	caggtatcgagcacttcgattatacctaggactgagct
50	sgRNA ppc Fw	agtgctcgatacctgccgttttagagctagaaatagc
51	HomSeqUp ppc Fw	gctttttttgaattcGTGCCGCAATAATGTCGGATGCGAT
52	HomSeq Up ppc Rv	TGCAGAAGAGGAAGAATTACCCCAGACACCCCATCTTATC
53	HomSeq Dw ppc Fw	TCTTCCTCTTCTGCAAACCCTCGTG
54	HomSeq Dw ppc Rv	cagggtaatagatctGCCCATAGCACCACGCCGATTACTG
55	sgRNA glcB Rv	TTTCAGAGCCATCGGCGGGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGC
56	sgRNA glcB Fw	GCCGATGGCTCTGAAATTGCTAGCATTATACCTAGGACTGAGCTAGCTGTCAAGG
57	HomSeqUp g;cB Fw	TGCTTTTTTTGAATTCGGTGGAAACCACGGGCATTGACAG
58	HomSeq Up glcB Rv	CACAATATAGACGCTGCAGCTTCGACGATAACATCGTTGATGTGC
59	HomSeq Dw glcB Fw	CGATGTTATCGTCGAAGCTGCAGCGTCTATATTGTGAAACCGAAAATGCAC
60	HomSeq Dw glcB Rv	GCTTCTGCAGGTCGACGTGACGGAACCCAGGCTGTGTTTGC

3