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APPLIED GENETICS AND MOLECULAR BIOTECHNOLOGY

Development of a high-copy-number plasmid via adaptive laboratory evolution of *Corynebacterium glutamicum*

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Abstract

Beyond its traditional role as an L-amino acid producer, *Corynebacterium glutamicum* has recently received significant attention regarding its use in the production of various biochemicals and recombinant proteins. However, despite these attributes, limitations in genetic tools are still hampering the engineering of *C. glutamicum* for use in more potential hosts. Here, we engineered a *C. glutamicum* via adaptive laboratory evolution to enhance the production of recombinant proteins. During the continuous cultivation, *C. glutamicum* producing enhanced green fluorescent proteins was screened using high-speed flow cytometer, and in the end, we successfully isolated an evolved strain with a fluorescence intensity 4.5-fold higher than that of the original strain. Extensive analysis of the evolved strain confirmed that the plasmid prepared from the evolved strain contains the nonsense mutation in the *parB* locus, which mutation contributed to increasing the copy number of plasmid by approximately 10-fold compared to that of the wild type. To validate the usefulness of the high-copy-number plasmid, we examined the secretory production of endoxylanase and the bioconversion of xylose to xylonate using xylonate dehydrogenase. In the fed-batch cultivation, the use of the high-copy-number plasmid led to 1.4-fold increase in the production of endoxylanase (~ 1.54 g/L in culture medium) without cell growth retardation comparing cultivation with cells harboring original plasmid. The expression of xylonate dehydrogenase in the high-copy-number plasmid also improved the bioconversion into xylonic acid by approximately 1.5-fold compared to the original plasmid.

Keywords *Corynebacterium glutamicum* · Adaptive evolution · Plasmid copy number · *ParB*

Introduction

Corynebacterium glutamicum is an important industrial host that is capable of producing various biochemicals, including amino acids, nucleic acids, and vitamins (Becker and Wittmann 2012; Choi et al. 2015; Wieschalka et al. 2013). In addition, *C. glutamicum* has many other attractive properties to become a major bacterial host for the production of recombinant proteins. Specifically, it has notable potential for secretory production of recombinant proteins into an extracellular medium because there is no contamination of endogenous proteins including protease, many recombinant proteins have been successfully produced with high yields and purity (Liu et al. 2016; Yim et al. 2014). Because *C. glutamicum* is generally recognized as safe (GRAS), much effort has been made to develop it as a host for the production of pharmaceutical proteins (e.g., antibody fragments), industrial enzymes (for use in food additives), and value-added biochemicals (Freudl 2017; Wendisch et al. 2016).

The primary goal in bio-industry is the economic production of target biomolecules (i.e., higher production in shorter operation time), and to achieve this goal, considerable effort has been put into the engineering of host–vector systems. For higher level gene expression, various strategies such as engineering of key genetic components including promoter, UTR (untranslated region)/TIR (translation initiation region), codon

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optimization, co-expression of protein for folding such as foldase and molecular chaperon, supplementation of amino acids or tRNA etc. have been employed (Angov 2011; Correa and Oppezzo 2011; Terpe 2006). In addition to above traditional approaches, recent development of systems and synthetic biology tools enable us to engineer *C. glutamicum* as more potential cell factory (Heider and Wendisch 2015). By combining various genetic parts, multiple gene-expression systems and metabolic pathways can be optimized, and high-level producers can be developed (Zahoor et al. 2012). Many successful results have been achieved through the above efforts, however, those works also require extensive trial-and-error to find the optimal host-vector system, which are laborious and time consuming (Nešvera and Pátek 2011). In addition, compared with *Escherichia coli*, much less accumulation of knowledge and experience in *C. glutamicum* and limited toolbox for genetic engineering make it difficult to engineer *C. glutamicum* as more superior cell factory. To fulfill the potential of *C. glutamicum* in full, there is a strong demand for developing more efficient approaches.

An alternative strategy for cell engineering, an adaptive laboratory evolution has been used as a powerful tool in various bacterial hosts, including *E. coli*, *Bacillus* sp., *Lactobacillus* sp., yeast etc. (Dragosits and Mattanovich 2013; Ju et al. 2016; Pal et al. 2014). During continuous cultivation under the relevant selection pressure, a host cell can adapt the genetic mutations (e.g., insertion, deletion, point mutation, duplication, and recombination) that are the major driving forces of evolution. For the success of adaptive evolution, it is also necessary to develop an efficient screening tool by which the best mutants having the desired function can be isolated from the huge undesired population. For example, through the use of biosensor systems, intracellular product formation can be translated into screenable phenotypes such as color, fluorescence, or antibiotics resistance (De Paepe et al. 2016; Williams et al. 2016). During adaptive evolution, the desired change acquired by host can induce the biosensor systems which can be represented as a phenotypic change such as high fluorescent intensity or higher resistance to antibiotics etc., and the desired mutants can be isolated efficiently from the undesired populations. Recently, Mahr et al. (Mahr et al. 2015) reported the adaptive evolution of *C. glutamicum* to improve metabolite (L-valine) production. A L-valine-responsive sensor based on the transcriptional regulator Lrp of *C. glutamicum* was used, and the high L-valine producer (2-fold higher production yield than wild type strain) could be isolated. However, the adaptive evolution of *C. glutamicum* for the high-level gene expression has not been reported yet.

Here, we evolved *C. glutamicum* to engineer it toward high-level production of recombinant proteins. Enhanced green fluorescent protein (eGFP) expression was employed as a biosensor system, and a fluorescence-activated cell sorting (FACS)-based high-throughput screening was carried out to isolate the evolved strain in which the eGFP expression level was greatly increased. In the end, we isolated an evolved strain and confirmed that the isolated clone exhibited a significant increase in the copy number of plasmid (to almost 45 copies per cell). We also demonstrated the usefulness of a high-copy-number plasmid in *C. glutamicum* via two examples: (i) production of endoxylanase in both shake flasks and lab-scale bioreactor cultivations, and (ii) bioconversion of xylose into value-added chemical xylonic acid via the expression of xylonate dehydrogenase in a high-copy-number plasmid.

Materials and methods

Bacterial hosts and cultivation

The bacterial hosts and plasmids used in this study are listed in Table 1. *E. coli* XL1-blue was used for gene cloning and plasmid maintenance. *C. glutamicum* ATCC 13032 was used for FACS screening and the production of recombinant proteins. *E. coli* was cultivated in Luria-Bertani medium (BD, Franklin Lakes, NJ, USA) at 37 °C and 200 rpm and *C. glutamicum* was cultivated in brain heart infusion (BHI) medium (BD) at 30 °C and 200 rpm. Fed-batch fermentation was performed in a semi-defined medium containing, per liter, 3 g K_2HPO_4, 1 g KH_2PO_4, 2 g urea, 10 g $(NH_4)_2SO_4$, 2 g $MgSO_4$, 200 μg biotin, 5 mg thiamine, 10 mg calcium pantothenate, 10 mg $FeSO_4$, 1 mg $MnSO_4$, 1 mg $ZnSO_4$, 200 μg $CuSO_4$, 10 mg $CaCl_2$, 15 g yeast extract, and 7 g casamino acid. To produce xylonic acid from xylose, we added 20 g of xylose per liter to the semi-defined medium. In all cultivations, we added kanamycin (25 μg/mL) to the culture medium as the sole antibiotic.

Plasmid construction

All the restriction enzymes used for the recombinant DNA was purchased from Enzynomics Co. (Daejeon, Republic of Korea). Polymerase chain reaction (PCR) was performed with the C1000™ Thermal Cycler (Bio-Rad, Hercules, CA, USA). In PCR, a Prime STAR HS polymerase (Takara Bio Inc., Shiga, Japan) and a PfuX polymerase (SolGent, Daejeon, Korea) were used for general PCR and for amplification of the whole plasmid, respectively. All oligonucleotides used for PCR are listed in Table 2. To introduce the *parB* nonsense mutation (C→A at the 21st position) into the plasmids, pCES-H36-GFP, and pCES-PLPV were amplified by PCR with two primers, *parB*-non-F and *parB*-non-R. After PCR, T4 polynucleotide kinase (Enzynomics Co.) was used to add phosphates to the 5`-end of PCR products and, they were ligated with T4 DNA ligase (Enzynomics Co.), yielding pHCP-H36-GFP and pHCMS, respectively. To construct a

Table 1 Bacterial strains and plasmids used in this study

Strain	Relevant characteristics	Reference or source
E. coli		
XL1-blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proABlacIq ZΔM15 Tn10 (Tet^r)]*	Stratagene[a]
C. glutamicum		
ATCC 13032	Biotin-auxotrophic wild type	ATCC
Plasmids	Relevant Characteristics	Reference or source
pCES208	shuttle vector between *E. coli* and *C. glutamicum*, Km^r	(Park et al. 2008)
pCES-H36-GFP	pCES208 derivative; P_{H36}, eGFP, Km^r	(Yim et al. 2013)
pHCP-H36-GFP	pCES-H36-GFP derivative; *parB* nonsense mutation, eGFP, Km^r	This study
pCES-PLPV	pCES208 derivative; multiple cloning site, Km^r	(Yim et al. 2016)
pHCMS	pCES-PLPV derivative; *parB* nonsense mutation, Km^r	This study
pCG-S	pCES208 derivative; P_{cg1514}, signal peptide of *cg1514*, Km^r	(Yim et al. 2016)
pCG-S-XynA	pCG-S derivative; *xynA* from *Streptomyces coelicolor* A3(2), Km^r	(Yim et al. 2016)
pHCP-S-XynA	pHCMS derivative; *xynA* from *Streptomyces coelicolor* A3(2), Km^r	This study
pX	pCES208 derivative; P_{H36}, *xdh* from *Caulobacter crescentus*	(Yim et al. 2017)
pHCP-X	pHCMS derivative; P_{H36}, *xdh* from *Caulobacter crescentus*	This study

[a] New England Biolabs, Beverly, MA, USA

XynA expression system in a high-copy-number plasmid, we amplified the system from a pCG-S-XynA template by PCR with pCES-F and pCES-R primers. The PCR product was digested with *Kpn*I and *Not*I before being ligated into pHCMS, yielding pHCP-S-XynA. To construct a Xdh expression system in a high-copy-number plasmid, the system was amplified from pX by PCR with pCES-F and pCES-R primers. The PCR product was digested with *Kpn*I and *Not*I before being ligated into pHCMS, yielding pHCP-X.

Table 2 List of oligonucleotides in PCR reaction

Primer name	Sequences (5′ to 3′)
parB-non-F[a]	CAGTAGGCtCAACTGATTCG
parB-non-R	CGGTGGCCTGATTCCT
p15A-ori-F	CTCATTCCACGCCTGACACT
p15A-ori-R	GCGCAGTCACCAAAACTTGT
sigB-F	TGTCCCTTGCTAAGCGCTAC
sigB-R	GGTTGCGTAGGTGGAGAACT
pCES-F	AGTACTGATCCTCCGGCG
pCES-R	TCAGTACCGACGGTGATATGG

[a] Small letter indicate point mutation on *parB*

FACS analysis and cell sorting

After overnight cultivation of *C. glutamicum* harboring pCES-H36-GFP, the cells were transferred to 50 mL of BHI medium in 250-mL flasks. After cultivation for 24 h, the cells were harvested by centrifugation for 10 min at 4 °C and 6000 rpm. After washing with 1X phosphate-buffered saline (PBS; 135 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 14 mM KH_2PO_4; pH 7.2), the cells were resuspended in the same buffer and fluorescence intensities were analyzed using a flow cytometer (MoFlo XDP, Beckman Coulter Inc., FL, USA) and a plate reader (Infinite M200 Pro ELISA, Tecan Group Ltd., Männedorf, Switzerland). In the FACS screening, the cells were excited with a laser at 488 nm and detected with a 530/40 band-pass filter for the eGFP emission spectrum. The high-fluorescence cells (the top 1% of the population) were sorted and dropped into fresh BHI medium. The sorted cells were grown overnight and transferred to 50 mL of BHI medium in a 250-mL flask for the next round of screening.

Protein fractionation and analysis

After cultivation, cells were harvested by centrifugation (10 min at 4 °C and 6000 rpm), and resuspended in 300 μL of PBS. Then, cells were disrupted by sonication for 9 min at

50% pulse and 20% amplitude (Sonics, Newtown, CT, USA), and then the total protein fraction were collected. After centrifuging of cell lysates for 10 min at 4 °C and 10,000 rpm, the soluble proteins were collected from the supernatant. The fractionated protein samples were analyzed using SDS-PAGE and western blot. For the SDS-PAGE analysis, the protein samples were loaded on 12% polyacrylamide gels. After gel electrophoresis, the gels were stained with Coomassie brilliant blue [50% (*v*/*v*) methanol, 10% (*v*/*v*) acetic acid, and 1 g/L Coomassie brilliant blue R-250] for 1 h and then destained with a destaining solution [10% (*v*/*v*) methanol, 10% (*v*/*v*) acetic acid]. For the western blotting, the proteins bands on the SDS-PAGE gel were transferred onto a polyvinyl difluoride (PVDF) membrane (Roche, Rotkreuz, Switzerland) using a transblot apparatus (Bio-Rad). Then, the membrane was incubated with a blocking solution (Tris-buffered saline, 24.7 mM Tris, 137 mM NaCl, 2.7 mM KCl, and 0.5% Tween-20) and 5% (*w*/*v*) skim milk for 1 h at room temperature. Then, the membrane was incubated with blocking solution containing a horseradish peroxidase-conjugated monoclonal anti-FLAG M2 antibody (Sigma-Aldrich, St. Louis, MO, USA) for immune-detection of the FLAG-tagged protein. After incubation for 1 h, each membrane was washed four times with Tris-buffered saline for 5 min each and protein bands were detected using an ECL kit (GE Healthcare Bio-Science AB, Buckinghamshire, UK).

Plasmid segregational stability test

After cultivating *C. glutamicum* harboring either pCES-H36-GFP or pHCP-H36-GFP overnight in BHI medium, the cells were transferred to 50 mL of BHI medium in 250-mL flasks without kanamycin and sub-cultured repeatedly every 12 h (approximately six generations). In every culture, diluted cultures were spread on plates with and without kanamycin. The colony forming unit (CFU) was determined by counting the number of cells grown on each plate, and the plasmid stability was the percentage of cells that retained antibiotic resistance (i.e., the ratio of CFU on the kanamycin plate to CFU on the kanamycin-free plate).

Quantitative PCR (qPCR)

The relative quantification of the plasmid copy number (PCN) was measured using a quantitative PCR method (Lee et al. 2006; Škulj et al. 2008). First, the genomic DNA, including the plasmids, was extracted from the cells using the MasterPure™ DNA Purification Kit (Epicenter®, Madison, WI, USA). Then, a real-time quantitative PCR (RT-qPCR) was performed with two primer sets (p15A-ori-F and p15A-ori-R; SigB-F and SigB-R). The p15A origin gene (p15A) in the plasmid and the *sig*B gene in the chromosome were used as the RT-qPCR specific sites for the plasmid (P) and for chromosome (C), respectively. The value of the quantification cycle (C_q) for the target sequence was determined using a LightCycler instrument (Roche) with a PrimeScript RT-PCR kit (Takara Bio Inc.). The amplification efficiency (E) of each gene was based on a 10-fold dilution of pCES-H36-GFP from 0.1 to 100 ng/μL. PCN was determined with Cq value and the amplification efficiency (E) of plasmid and chromosomal gene. PCN was calculated using this standard curve ($E = 10^{(-1/slope)}$) and the C_q mean value of each gene by following equation; $PCN = (E_c)^{Cqc}/(E_p)^{Cqp}$ (Lee et al. 2006).

Endoxylanase activity assay

The activity of endoxylanase (*xynA*) in extracellular medium was determined by the 3,5-dinitrosalicylic acid (DNS) method (Yim et al. 2016). After cell cultivation, 1 mL of cultured cells was harvested by centrifugation for 10 min at 4 °C and 13,000 rpm. Then, the supernatant were diluted 10 times with PBS and 500 μL of diluted sample was mixed with 2 mL of 2.5% (*w*/*v*) birchwood xylan (Sigma-Aldrich) in PBS. After incubating the reaction samples for 10 min at 30 °C, they were centrifuged for 10 min at 4 °C and 13,000 rpm, and 0.5 mL of supernatant was mixed with 0.5 mL of DNS solution. The absorbance of reactant was measured at 550 nm after 5 min boiling.

Analysis of xylonic acid concentration

After cultivation, cells were removed by centrifugation and supernatant samples were collected. After removing insoluble residues in supernatants by passing them a 0.45-μm syringe filter (Sartorius Stedim Biotech, Goettingen, Germany), the prepared samples were diluted 30-fold in distilled water and were analyzed by high performance liquid chromatography (HPLC, LC-20AD, CTO-20A, SPD-20A; Shimadzu, Japan) equipped with Aminex HPX-87H column (300 × 7.8 mm, Bio-Rad). For a mobile phase, 5 mM H_2SO_4 was used. Operation condition was 60 °C with 0.5 mL/min flow rate. Xylonic acid was detected with UV at 210 nm.

Fed-batch fermentation

C. glutamicum harboring pHCP-G-XynA was inoculated in BHI medium. After cultivation for 24 h, cells were transferred into 200 mL of semi-defined medium in four 250 mL baffled flasks. The seed culture was poured into 2 L of fresh semi-defined medium in a 5 L jar bioreactor (BioCNS, Daejeon, Republic of Korea). The cultivation temperature was set at 30 °C. The pH was controlled at pH 7 by 5 N ammonia salutation and sulfuric acid with online monitoring. The dissolved oxygen (DO) concentration was maintained at 30% (*v*/*v*) by increasing the agitation to 1200 rpm and then mixing

pure oxygen. The glucose concentration was measured by YSI 2700 SELECTTM Biochemistry Analyzer (YSI Life Science, Yellow Springs, OH, USA). When glucose concentration was lower than 20 g/L, a glucose solution (90 g in 150 mL) was added to the culture. The cell growth (optical density at 600 nm) was monitored by spectrophotometer.

Results

Adaptive laboratory evolution of *C. glutamicum*

With *C. glutamicum* producing eGFP, we performed an adaptive laboratory evolution based on the fluorescence intensity of each cell. *C. glutamicum* harboring pCES-H36-GFP in which the eGFP gene was expressed under the strong constitutive promoter (P_{H36}) were cultivated and the cells exhibiting higher fluorescence intensities (the top 1% of the population) were selectively sorted by FACS. In the first five rounds of sorting, we could not find any increase in fluorescence intensity in the sorted cells (Fig. 1a). However, in the sixth round, a significant increase in fluorescence intensity (mean fluorescence, $M = 6288$) was observed, and in the seventh round, a slightly higher intensity ($M = 7805$) that was about 4.5-fold higher than that of the original library ($M = 1748$) was also observed (Fig. 1a). In addition to FACS analysis, we analyzed the level of eGFP expression in each sorting round using SDS-PAGE. As with the FACS results, the expression levels of eGFP significantly increased in the sixth and seventh rounds (Fig. 1b).

Characterization of mutation in isolated clones

After the seventh round screening, the sorted cells were spread on an agar plate and 10 individual clones were randomly picked for an analysis of eGFP expression. In the FACS analysis, all 10 clones exhibited much higher fluorescence intensities than that of cell harboring the original pCES-H36-GFP (data not shown). The 10 isolated clones were further analyzed to identify the critical mutations for the enhanced expression of eGFP. After cultivating each isolated clone, the plasmids were purified from the same amount of cells and were analyzed by agarose gel electrophoresis. Interestingly, we found that the intensities of plasmids from all isolated clones were much higher than that of the original clone (Fig. 2a), which indicates that the plasmid copy number significantly increased in the isolated clones. The plasmid copy number can change via mutations in the plasmid itself or via mutations in the host's chromosomes. To confirm this point, we purified plasmids from each clone and transformed them into fresh *C. glutamicum*. When we checked the plasmid intensity via agarose gel electrophoresis, every clone showed similar intensity to that of each isolated clone and thus a higher intensity than that of the original clone (Fig. S1), which

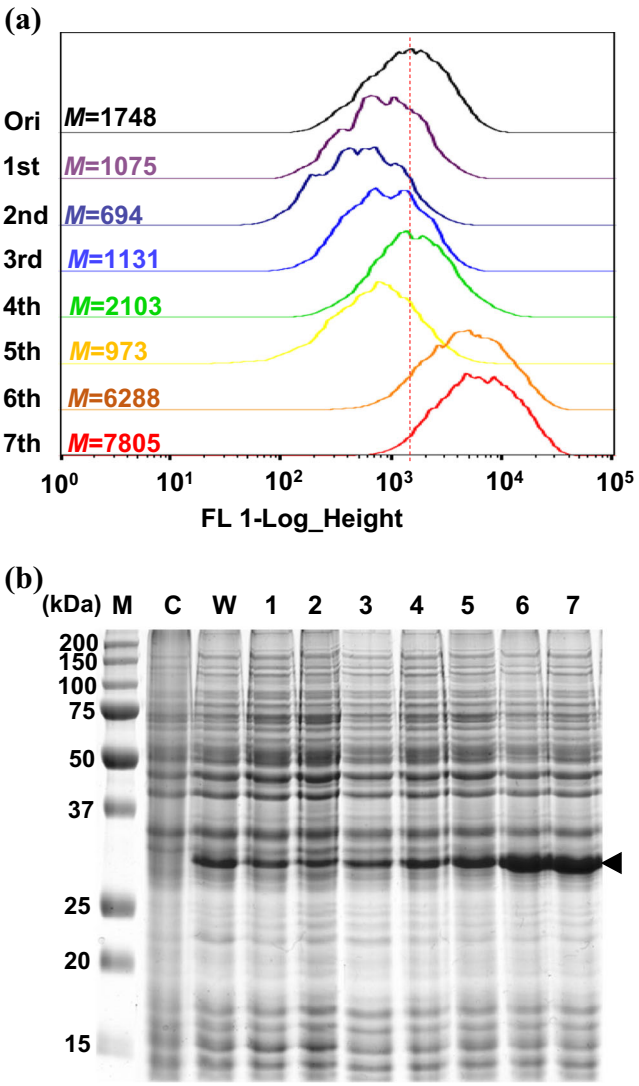

Fig. 1 Adaptive evolution by FACS-based screening. **a** Histogram of FACS-based screening. Wildtype *C. glutamicum* harboring pCES-H36-GFP was cultivated in flask, and in each round, high fluorescent cells (top 1% of total population) were sorted by FACS. Black, purple, indigo, blue, green, yellow, orange, and red color represent the wildtype cell and first to seventh sorted cell population, respectively. *M* is the mean fluorescence intensity. Red-color dashed line indicates the mean fluorescent value of original clone. **b** SDS-PAGE analysis of proteins in the cells sorted in each round. Lane M, molecular weight markers (kDa). Lanes C and W represent negative control (no plasmid) and *C. glutamicum* harboring pCES-H36-GFP. Lanes 1 to 7 represent the protein samples of first to seventh round sorted cells, respectively. Arrowhead indicates the band of eGFP (~ 27 kDa)

implies that mutations in the plasmid itself caused the change in the plasmid copy number. The backbone of pCES-H36-GFP is the *E. coli–C. glutamicum* shuttle vector (pCES208), and the pCG1 region in this plasmid was originated from a *C. glutamicum* cryptic plasmid (Park et al. 2008). The pCG1 region has three genes including (i) *repA* which encodes plasmid replication initiator and (ii) *parA* and *parB* which plays a role in partitioning system for autonomous plasmid replication

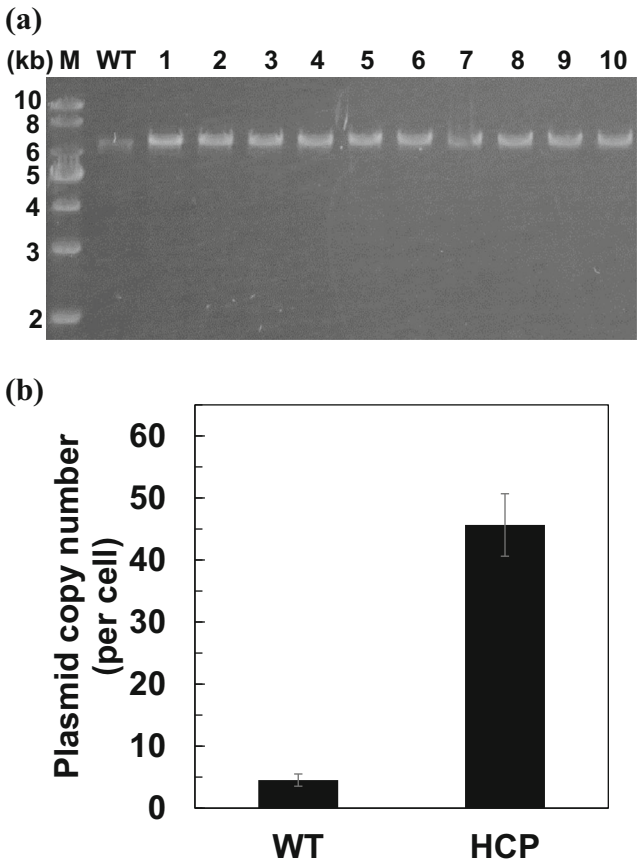

Fig. 2 Determination of plasmid copy number. **a** Agarose gel analysis of plasmid prepared from each evolved strain. Lanes M and WT represent DNA size markers (kb) and plasmids from *C. glutamicum* harboring pCES-H36-GFP, respectively. Lanes 1 to 10 represent plasmids prepared from 10 evolved clones. **b** Determination of plasmid copy number. WT and HCP represent cells harboring pCES-H36-GFP and pHCP-H36-GFP, respectively. All results were obtained from biological triplicate experiment

in *C. glutamicum* (Fig. S2) (Ozaki et al. 1984). Therefore, to identify the mutations in the plasmid, we decided to analyze the sequences of the pCG1 region in each purified plasmid. From the sequencing experiment, it was clear that all plasmids contain mutations in the *parB* locus only and that none contain mutations in replicase or the *parA* locus. Among the 10 clones, 2 clones (#8 and #10) were same clones in which 17-bp DNA fragment was inserted in the *parB* region, and the other clones contain single-nucleotide substitutions that result in nonsense mutations (TGA or TAA) on the *parB* locus (Table S1). Therefore, the *parB* gene could not be expressed in any of the isolated clones.

Verification of nonsense mutation in the *parB* locus

To verify that the nonsense mutation in the *parB* gene contributed to the increase in the plasmid copy number, we introduced a point mutation (C→A at the 21st position) in the *parB* locus of the original plasmid (pCES-H36-GFP), yielding pHCP-H36-GFP, by which the TGC codon was changed to the stop codon (TGA), causing the *parB* gene to not be expressed. After transforming the wild-type *C. glutamicum* with pHCP-H36-GFP, the plasmid intensity was analyzed using agarose gel electrophoresis, and as expected, we observed a higher intensity in pHCP-H36-GFP than in the original plasmid (data not shown). Next, the relative plasmid copy number was determined using qPCR. The standard curve of each plasmid gene (p15A) and chromosomal gene (sigma factor (*sigB*)) had high linearity ($R^2 = 0.97$ and 0.98, respectively), and the calculated amplification efficiencies of the p15A and *sigB*, as references, were 2.03 and 2.02, respectively. As a result, the copy number of pHCP-H36-GFP was 45.6 ± 5.0 per cell, which is 10-fold higher than that of the original, pCES-H36-GFP (4.52 ± 0.98) (Fig. 2b). Taken all together, we concluded that the nonsense mutation in *parB* locus of the plasmid was responsible for the increase of copy number in the plasmid.

Characterization of engineered high-copy-number plasmids

In general, the maintenance of high-copy-number plasmids in bacteria can give high metabolic load on the hosts, which consequently causes poor cell growth and reduces production of recombinant proteins (Bower and Prather 2009; Jones et al. 2000). To determine the effect of an increased copy number on cell growth and protein production, cells were cultivated in two media: defined media (without any complex sources) and semi-defined media (with complex sources: yeast extract and casamino acid). In the defined medium, cells harboring pHCP-H36-GFP showed slightly slower growth rates (specific growth rate, $\mu = 0.306 \pm 0.012\ h^{-1}$) than did cells harboring the wild-type plasmid ($\mu = 0.330 \pm 0.032\ h^{-1}$), but similar maximum cell densities (Optical density, $OD_{600} = \sim 40$) were achieved (Fig. 3a). In the semi-defined medium, both cells harboring pCES-H36-GFP or pHCP-H36-GFP exhibited shorter lag phases and higher specific growth rates compared to those in the defined medium (Fig. 3a). Both cells also showed very similar growth profiles: the specific growth rates (μ) of pCES-H36-GFP and pHCP-H36-GFP were 0.120 ± 0.035 h^{-1} and 0.127 ± 0.047 h^{-1}, respectively. After cultivation, we analyzed the fluorescence intensity of cells in each cultivation using a flow cytometer to compare the protein production. In both defined and semi-defined media, cells harboring pHCP-H36-GFP exhibited higher fluorescence intensities than that of the original plasmid (Fig. 3b). Particularly in the semi-defined medium, cells harboring pHCP-H36-GFP exhibited 3.8-fold higher fluorescence intensity than that of cells harboring pCES-H36-GFP (Fig. 3b).

Due to a high metabolic load, high-copy-number plasmids may have lower segregational stability than that of low-copy-number plasmids, which problem can become quite serious in

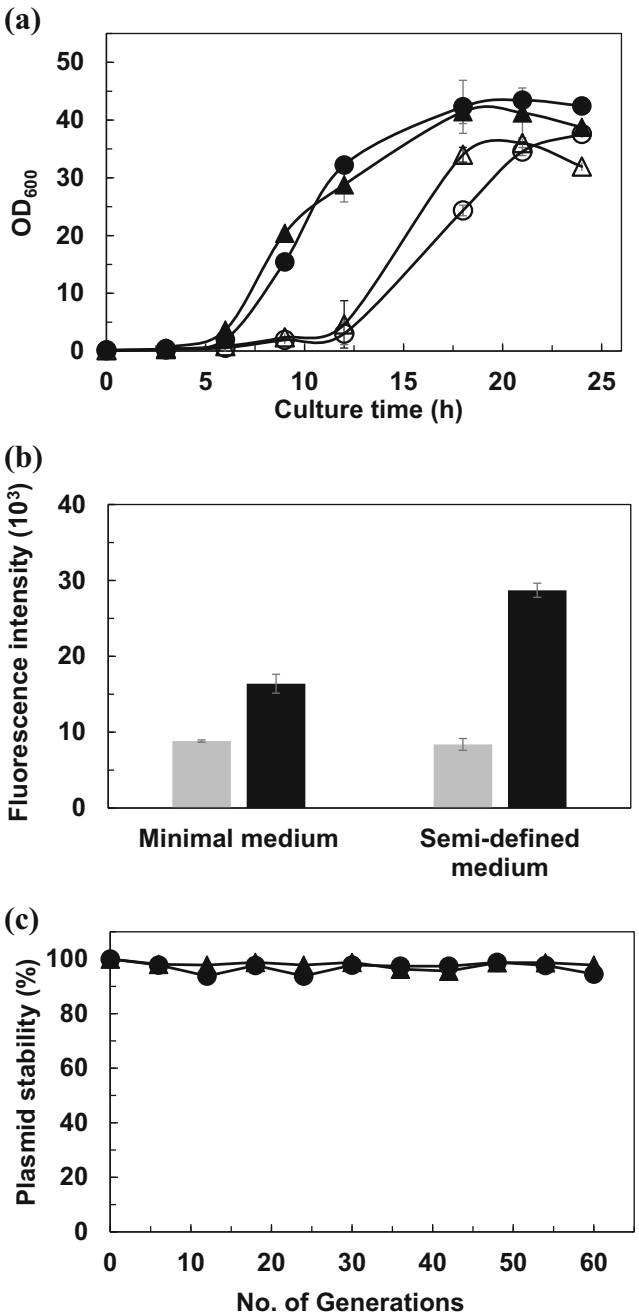

Fig. 3 Characterization of high copy plasmid. **a** Time profiles of cell growth in defined and semi-defined medium. Symbols: triangles (Δ, ▲), *C. glutamicum* harboring pCES-H36-GFP; circles (○, ●), *C. glutamicum* harboring pHCP-H36-GFP. Open and closed symbols represent cultivation in minimal and semi-defined medium, respectively. **b** Analysis of fluorescence intensity. *C. glutamicum* harboring pCES-H36-GFP (gray bar) or pHCP-H36-GFP (black bar) were cultivated in defined or semi-defined medium without antibiotic. **c** Analysis of segregational stability of plasmid in *C. glutamicum*. Symbols triangles (▲), cell harboring pCES-H36-GFP; circles (●), cell harboring pHCP-H36-GFP. All results were obtained from biological triplicate experiment

long-term cultivation (Jones et al. 2000; Summers and Sherratt 1984). To check the long-term stability of high-copy-number plasmid in *C. glutamicum*, cells harboring pHCP-H36-GFP were cultivated in antibiotic-free media. Until the 60th generation (almost 5 days), pHCP-H36-GFP maintained well (> 95%), and we did not find any difference in stability between the original plasmid (pCES-H36-GFP) and the high-copy-number plasmid (pHCP-H36-GFP) (Fig. 3c). Moreover, in the 60th generation, cells harboring pHCP-H36-GFP exhibited about 5-fold higher fluorescence intensity than did the cells harboring pCES-H36-GFP in both cultivations—with and without antibiotics (Fig. S3). These results clearly indicated that high-copy-number plasmids have high stability in *C. glutamicum* and that these plasmids can be used for overproduction in long-term cultivation.

Production of endoxylanase using high-copy-number plasmids in flask cultivation

To demonstrate the versatility of the high-copy-number plasmids, we examined the secretory production of endoxylanase from *Streptomyces coelicolor* which can degrade hemicellulose (xylan). The endoxylanase gene (*xynA*) linked to *Cg1514* signal peptide was cloned into high-copy-number plasmid, yielding pHCP-G-XynA. After flask cultivation, the proteins in the extracellular fraction were analyzed by SDS-PAGE and we found that cells harboring pHCP-G-XynA could produce more endoxylanase in the culture medium than that of cells harboring original pCG-G-XynA (Fig. 4a). We also determined the activities of endoxylanases in the culture medium using the DNS method, and similar to the SDS-PAGE data, the cells harboring pHCP-G-XynA exhibited higher (approx. 4.5-fold) activity of endoxylanase (58.7 ± 6.3 U/ml) than that of pCG-G-XynA (13.2 ± 2.2 U/ml) (Fig. 4b).

Production of endoxylanase in fed-batch cultivation

Next, we examined the production of endoxylanase in fed-batch cultivation (working volume 2 L) with *C. glutamicum* harboring pHCP-G-XynA. With glucose supplementation, the cells continued to grow at a specific growth rate (0.116 h^{-1}) and reached an OD_{600} of 308.4 in 33 h (Fig. 5a). In pHCP-G-XynA, the endoxylanase gene was expressed under the constitutive promoter (P_{H36}), so the endoxylanase concentration in the culture medium increased gradually after the initial time point. The maximum concentration of endoxylanase (1.54 g/L) could be obtained at 33 h, and the maximum productivity was 47 mg/L/h. Comparing to our previous results for low-copy-number plasmids (endoxylanase concentration 1.07 g/L, productivity 33 mg/L/h) (Yim et al. 2016), we found that high-copy-number plasmids exhibited an approximately 1.3-fold increase in the productivity of endoxylanase in fed-batch cultivation. The proteins in the culture medium were analyzed using SDS-PAGE, and we found that endoxylanase was successfully produced in a high-purity (> 70% in a densitometric analysis) culture medium (Fig. 5b), which means that the use

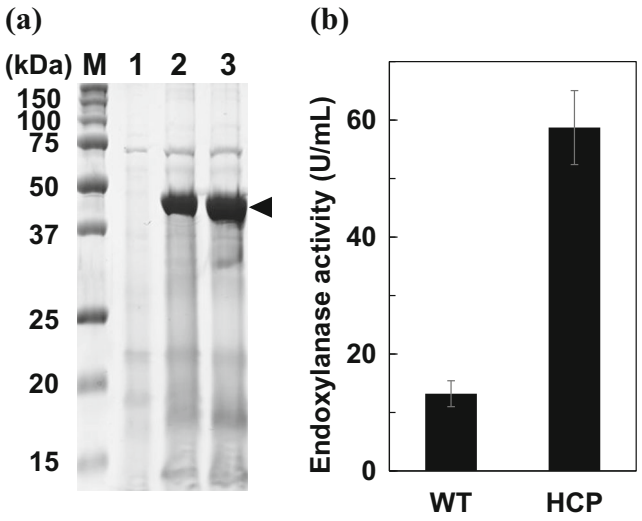

Fig. 4 Shake flask cultivation with *C. glutamicum* harboring pHCP-G-XynA. **a** SDS-PAGE analysis of proteins in culture medium Lanes 1 to 3, cells harboring pCES208 (negative control), pCG-G-XynA, or pHCP-G-XynA, respectively. The arrowhead indicates endoxylanse. **b** Determination of endoxylanse activity by DNS method. WT and HCP represent the endoxylanase activities from cultivation of cells harboring pCG-G-XynA and pHCP-G-XynA, respectively. All results were obtained from biological triplicate experiment

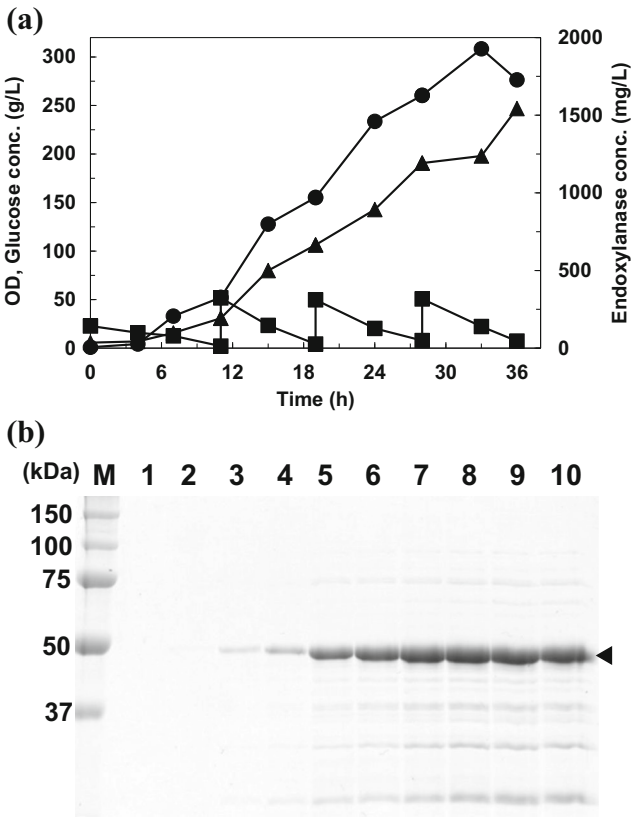

Fig. 5 Fed-batch cultivation with *C. glutamicum* harboring pHCP-G-XynA. **a** Time profiles of cell growth, glucose concentration, and endoxylanase concentration in culture medium. Symbols: circles (●), cell density (OD_{600}); squares (■), glucose concentration (g/L); triangles (▲), endoxylanase concentration in culture medium (mg/L). **b** SDS-PAGE analysis of proteins in culture medium. In each time point, total 8 μL of culture supernatant was loaded in each lane. Lane M, molecular weight markers (kDa); Lanes 1 to 10, protein samples at 0, 4, 7, 11, 15, 19, 24, 28, 33, and 36 h, respectively. The arrowhead indicates endoxylanase

of a high-copy-number plasmid did not cause a severe cell lysis problem during the high-level secretory production of endoxylanase in fed-batch cultivation.

Enhanced xylonic acid bioconversion rate

In addition to the recombinant proteins, various biochemicals are also important products of *C. glutamicum*, and the production of such biochemicals can also be increased by increasing the expression level of the gene that encodes the key enzyme for the synthesis of the target biochemicals. To demonstrate the usability of high-copy-number plasmids for the enhanced production of biochemicals, we examined the expression of the xylose dehydrogenase (Xdh) gene from *Caulobacter crescentus*; A Xdh can convert xylose to xylonic acid, which is one of the 30 highest-value biochemicals according to the US Department of Energy (DOE) and has been applied in the fields of food, pharmaceuticals, and agriculture (Williamson 2010). A *xdh* gene was cloned into a high-copy-number plasmid (pHCP-X), and cells were cultivated in defined media containing xylose (20 g/L) as a substrate. The SDS-PAGE analysis confirmed that the expression level of the *xdh* gene in pHCP-X was higher than that in the original plasmid (pX) (Fig. 6a). At 24 h, the cells harboring pHCP-X exhibited 1.5-fold higher conversion (> 90%) and bioconversion rates (0.75 g/L/h) than did the cells harboring pX (approximately 61% and 0.51 g/L/h, respectively) (Fig. 6b).

Discussion

In this work, we performed an adaptive laboratory evolution for the engineering of *C. glutamicum* toward high-level production of recombinant proteins. We isolated high-copy-number (approximately 45 copies per cell) plasmids for which the copy number was 10-fold higher than that of the original plasmid. As described in the introduction, it is important to develop proper screening tools for the success of adaptive evolution. As one general method for this purpose, growth-dependent screening strategy has been employed, in which the mutants with desired property can exhibit better cell growth on the selective condition (Olson-Manning et al. 2012). But, in many cases, those method need longer times (several weeks or months) to ensure the isolation of candidates (Dragosits and Mattanovich 2013; Ju et al. 2016) which is one of the obstacles in adaptive evolution. In the present study, we used an eGFP-expression system as a biosensor; by combining this with FACS-based high-throughput screening, we completed

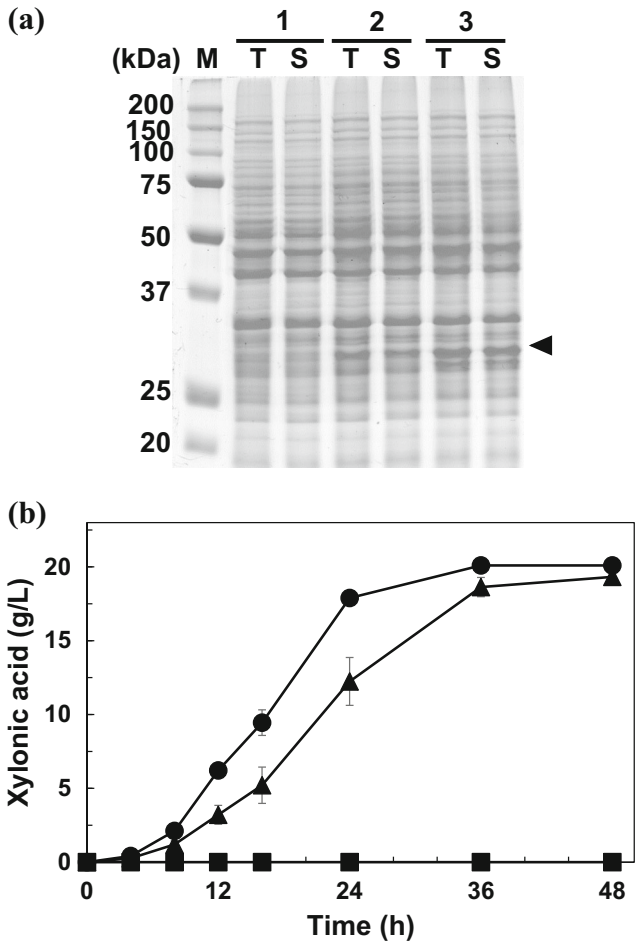

Fig. 6 Production of xylonic acid in *C. glutamicum* harboring pHCP-X. **a** SDS-PAGE analysis of proteins in cytoplasm. Lanes 1, 2, and 3 represent protein sample from cells harboring either pCES208 (negative control), pX, or pHCP-X, respectively. Lanes M, T, and S represent molecular weight markers (kDa), total, and soluble protein fraction, respectively. The arrowhead indicates xylose dehydrogenase (Xdh). **b** Time profile of xylonic acid in the culture medium during cell cultivation. Symbols: squares (■), cell harboring pCES208 (negative control); triangles (▲), cell harboring pX; circles (●), cell harboring pHCP-X

all seven rounds of FACS screening in 2 weeks and the mutant with desired function (high-level production of recombinant proteins) was successfully isolated. Compared to random mutagenesis-driven engineering of bacterial hosts, another beneficial point in adaptive evolution is the easy identification of critical mutations for the evolved function. In random mutagenesis, the artificial mutation can be accomplished using a chemical method (ethylmethanesulfonate treatment, etc.) or a physical method (ultraviolet light illumination, etc.); these methods induce at least hundreds of mutations in the entire genome of host, and after isolating the candidates, it is a major challenge to identify the mutations that are linked to a phenotype change (desired property) in the genome of an isolated clone (Harper and Lee 2012; Sarin et al. 2010). In contrast, clone isolated using adaptive evolution without artificial mutagenesis have only a few mutations which can be easily identified. As shown here, it was easily identified that all mutants contained single mutation in *parB* locus (nonsense mutation) although mutation point in each cell was different, and the effect of this nonsense mutation on the plasmid copy number was also clearly confirmed. In the FACS screening, the sudden enrichment from sixth round (Fig. 1a), may not be resulted from the sudden change in the growth of the isolated mutants. During the iterative cultivation, the spontaneous mutations may arise very rarely, so the portion of positive clones (high-fluorescence) could not be high and FACS signals were very low in the early rounds. But, if the positive clones appear once, those cells can be selected and enriched by FACS screening, so the portion of positive clones can be increased suddenly. As shown in Fig. 3, cells harboring high copy plasmid showed similar or slower cell growth compared with cells harboring the original plasmid. We also performed a growth competition experiments: after mixing cells harboring either the original plasmid or high-copy-number plasmid with same ratio (1:1), the change of each population in the next six rounds was monitored by FACS. As expected, it was observed that the population of cell harboring the original plasmid increase gradually up to 86.8% (Fig. S4). Taken all results, we concluded that the cells harboring high copy plasmid were isolated not by high-growth rate but by higher fluorescence of cells because of high copy plasmid.

In the pCG1 family, which are well-known plasmids in *C. glutamicum*, it is known that the *par* locus (*parA*, *parB*, *parC*, and *parS*) plays a key role in plasmid replication and copy-number control (Tauch et al. 2003). Of these, *parB* is a centromere binding protein; together with *parC* and *parS*, it can bind the specific sequence around the *parAB* locus in the plasmid and serves as an autorepressor (Donovan et al. 2010). The genetic modification in the *par* locus enables manipulation of plasmid copy number to varying degrees. Okibe et al. examined the effects of *parB* and *crrI*, which is an antisense RNA for a replication initiator protein, had on the plasmid copy number in pCGR2 (Okibe et al. 2010). However, they found that the deletion of *parB* resulted in only a small increase in the copy number (2.5→3.1 copies per cell) but that the simultaneous deletion of *parB* and *crrI* resulted in an extreme increase in the copy number (up to 218 copies per cell). In our study, we used a pCES208-based plasmid, which also belongs to pCG1 family, for adaptive evolution, and all the mutations were found only in the *parB* locus; we confirmed that the nonsense mutation in *parB* without the *crr*I mutation can still result in a significant increase (~ 10-fold) in a plasmid copy number. Although both pCES208 and pCGR2 belong to the pCG1 family, there is no sequence homology of *parA* and *parB*. Thus, we supposed that the pCES208-based plasmid has a distinct mechanism of plasmid replication, which may explain why only *parB* nonsense mutations were isolated in our adaptive evolution.

Although an increase in copy number allows for an increased expression level due to the high dosage of a target gene in a cell, maintaining a high-copy-number and the high-level synthesis of proteins can give heavy metabolic loads on a cell and have a deleterious effect on both cell growth and plasmid stability; this is critical for practical applications (Jones et al. 2000; Silva et al. 2012). The engineered plasmids in this study exhibited great stability for long-term (~5-day) cultivation, and the cells showed no growth retardation problems during the high-level production of any of the examined proteins in either flask or fed-batch cultivation. Of course, cell growth can differ according to the characteristics of the target protein; in some cases (for example, with toxic or highly insoluble proteins), the overexpression of gene in a high-copy-number plasmid can cause serious cell-growth problems (Swartz 2001). Particularly in secretory production, excessive production of proteins can cause protein aggregations in the cytoplasm or cell lysis by jamming of proteins in the secretion channel. In those cases, it may be necessary to reduce the expression level by choosing low-copy-number plasmids (An et al. 2013; Humphreys et al. 2002). In this context, the development of plasmids with various copy numbers can help overcome the limitation of plasmids in the engineering of *C. glutamicum*. Considering the characteristics of the target proteins (toxicity, solubility, secretion, etc.), we can construct the optimal expression system by choosing the proper copy-number plasmid.

In conclusion, we developed a high-copy-number plasmid (~45 copies per cell) through the adaptive laboratory evolution of *C. glutamicum*, and the increase of gene expression in high-copy-number plasmid was successfully demonstrated with several models including eGFP, endoxylanase, and xylose dehydrogenase. To the best of our knowledge, this is the first report on the adaptive laboratory evolution of *C. glutamicum* toward the enhanced production of recombinant proteins. The observation of high stability of plasmid and no retardation of cell growth during flask and fed-batch cultivation also indicates its suitability for the practical and industrial-scale application.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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