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PRODUCERS, BIOLOGY, SELECTION, AND GENE ENGINEERING

Assessment of Effectiveness of *Corynebacterium glutamicum* Promoters and Their Application for the Enhancement of Gene Activity in Lysine-Producing Bacteria

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Abstract—A pNS-cat72 vector was constructed based on the replicative vector pNS2 and reporter gene *cat* of Tn9 transposone, which encodes chloramphenicol acetyltransferase, in order to clone various gene promoters and to assess their chloramphenicol resistance in *Corynebacterium glutamicum* strains. The strength of promoters of various genes in the *Corynebacterium glutamicum* strain GEN1-2 ($lys^{CA279T, S317A}$), which contains aspartokinase (which is resistant to lysine) and threonine feedback inhibition were studied using this vector. It was found that promoters of the genes *eftu*, *sod*, *cspB* and *leuC* provide higher level of chloramphenicol resistance than promoters of the genes *lysC*, *pyc*, *tkt*, *fbp*, which are involved in the control of lysine biosynthesis. It was shown that replacement of the natural promotor *ddh* by the promotor *sod* increases the level of transcription almost by ten times, whereas the diaminopimelate dehydrogenase activity is increases by three to four times, which results in a 9% enhancement in lysine production. The investigated set of promoters with different strengths is a necessary tool for the optimization of gene activities and the construction of metabolite-producing strains.

Keywords: enzyme activity, promoters, lysine synthesis, gene expression, *Corynebacterium glutamicum*
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INTRODUCTION

Corynebacterium glutamicum has been widely used in industrial biotechnology for over 50 years in the production of amino acids—glutamate, lysine, arginine, alanine, isoleucine, methionine, serine, and valine. In recent years, the biosynthesis of new products—α,ω-amino acids, α,ω-diamines, α-keto acids, isobutanol, carotenoids, and terpenes has been carried out with these bacteria [1].Industrial production is based on the microbial fermentation of sugars with the use of mutant *C. glutamicum* strains. Various breeding and genetic approaches, including mutagenesis and targeted design of strains via genetic and metabolic engineering, are used to obtain producer strains. One widely used approach for the design of producer strains involves alteration of the expression of some important genes via the replacement of their promoters with stronger ones. To date, more than 50 promoters of *C. glutamicum* have been described, but only the promoters of the genes *tuf* (*eftu*), *sod* and *cspB* were defined as strong and used to improve the expression level of the relevant genes [2–5]. At the same time, it should be noted that, in addition to an increase in the activity of certain genes, the provision of a coordinated activity level of different enzymes is required for the development of highly productive strains. This objective can be achieved with the use of promoters of different strengths.

The goal of this work was to test experimentally the strength of several promoters of *C. glutamicum* (by cell resistance to chloramphenicol) for their further use in effecting an increase in lysine production.

EXPERIMENTAL

Bacterial strains and conditions of culturing. The bacterial strains and plasmids are listed in Table 1. The *C. glutamicum* strains GEN1-2, GEN2-1, and GEN4-28 and derivative strains of the *C. glutamicum* strain ATCC13032 were obtained via replacement of the native allele of the genes *lysC*, *hom*, and *leuC* with mutant alleles $lysC^{A279TS317A}$, hom^{V59A}, and $leuC^{G456O}$ by homologous recombination [6].

The plasmid pNS-cat72, which contained the reporter gene *cat* for chloramphenicol resistance without the promoter region and start codon (in addition

Abbreviations: DAPD, diaminopimelate dehydrogenase; CM, culture medium; OD, optical density; PCR, polymerase chain reaction; LB medium, Luria-Bertani medium; kb, kilobase; NADPH, reduced form of nicotinamide adenine dinucleotide phosphate.

Table 1. Strains and plasmids used in the study

Strains, plasmids	Characteristics	Source
C. glutamicum ATCC13032	Wild type	VKPM* (GosNIIgenetika)
Brevibacterium flavum ATCC14067	Wild type	VKPM
B. lactofermentum ATCC13869	Wild type	DSM, Germany
B. lactofermentum B-10945	Lysin-producing	VKPM
E. coli XLI Blue	*sup*E44*hsd*R17*rec*A1endA1 *gyr*A96 *thi rel*A1 *lac*$^-$ F'[*pro*AB$^+$*lac*I^q*lac*ZΔM15Tn10]	VKPM
C. glutamicum GEN1-2	*C. glutamicum* ATCC13032 (*lysC*^{A279TS317A})	Present study
C. glutamicum GEN2-1	*C. glutamicum* ATCC13032 (*lys*^{CA279TS317A}, *hom*^{V59A})	Same
C. glutamicum GEN4-28	*C. glutamicum* ATCC13032 (*lys*^{CA279TS317A}, *hom*^{V59A}, *leu*^{CG456D})	″
Bacillus subtilis 168	Wild type	VKPM
pNS2	8.05 kb ApR KmR *E.coli-C. glutamicum* vector	Rostova et al., 1993 [7]
pACYC184	4.2 kb Cm^R Tc^R	GenBank/EMBL Accession Number: X06403
pNS-cat72	8.87 kb ApR KmR, obtained from pNS2, *cat* of pACYC184	Present study
pIKA-sac13	pUC19 + KmR of pUC4K+ *sacB* of *B. subtilis* 168	Present study
pIKA-sod-ddh	pIKA-sac13 + (psod-*ddh*)	″
pIKA-leu-ddh	pIKA-sac13 + (pleu-*ddh*)	″
pIKA-2xddh	pIKA-sac13 +2x*ddh*	″

to standard structural elements), was constructed to assess the strength of promoters (Fig. 1).

The pNS-cat72 plasmid is a derivative of the replicative vector pNS2 [7], which comprises the *cat* gene of Tn9 transposon. It is cloned in the form of *Bam*HI–*Sac*I fragment as a reporter gene. The resulting vector pNS-cat72 (8.87 kb) contains unique sites *Sal*I and *Bam*HI before the reporter gene *cat*, which can be used for cloning different promoter sequences from the genome of *C. glutamicum*.

Derivatives of the pNS-cat72 plasmid with different promoters were obtained by cloning the PCR fragments of *Bam*HI–*Sac*I, which wre obtained with the use of the primers and template given in Table 1 and 2.

E.coli strains were cultured on LB medium (Difco) at 37°C. If necessary, antibiotics were added to the medium: kanamycin (Synthesis, Russia) to a concentration of 50 μg/mL, ampicillin (Synthesis)—μg/mL, and chloramphenicol (Synthesis)—μg/mL. The *C. glutamicum* strains were cultured on 2×LB medium with 1% maltose at 30°C. For the transformation of *C. glutamicum*, BHIS medium of the following composition was used: 37 g/L brain heart infusion (BHI, Difco), 95 g/L sorbitol supplemented with 15 g/L Bacto agar to obtain a solid medium. Selection of the transformants was carried out by the addition of kanamycin to a concentration of 17 μg/mL.

Evaluation of the promoter strength in the *C. glutamicum* cells GEN1-2 was performed by the level of resistance to increasing chloramphenicol concentrations. For this purpose, overnight cultures of strains containing the plasmid with chloramphenicol resistance gene under the control of different promoters in different dilutions were applied to plates with different concentrations of chloramphenicol (2 μg/mL, 4, 6, 8, 10, 12, 14, 20 and 25 μg/mL) by a replicator. The culture of the *C. glutamicum* strain GEN1-2 (pNS-cat72), which contained a plasmid with the reporter gene *cat* without a promoter, was used as a control. The culture growth was evaluated after incubation of the plates for 2 days at 30°C.

Genetic engineering techniques. Isolation of the plasmids and *E. coli* transformation were carried out according to standard procedures [8]. DNA restriction and ligation was carried out under the conditions recommended by the manufacturer (Thermo Scientific). Amplification was performed with a Master-cycler Gradient (Eppendorf) under the following conditions: 94°C for 30 s; 53°C for 30 s; 68°C for 1–2 min; 25 cycles. Amplification was performed with a mixture of polymerases *Pfu* (no. EP0502) and *Taq* (no. EP0404) (Fermentas, Lithuania) in the ratio of 1 : 9. Chromosomal DNA from *C. glutamicum* strains was isolated according to the method described in [9].

The preparation of competent *C. glutamicum* culture and electroporation was performed according to the method of Van der Rest et al. [10]. The electroporation conditions were the following: 2500 V, 25 μF, 200 Ohm. The duration of the electric impulse was 4.5–5.5 ms depending on the cell properties and DNA purity. The transformation frequency of *C. glutamicum*

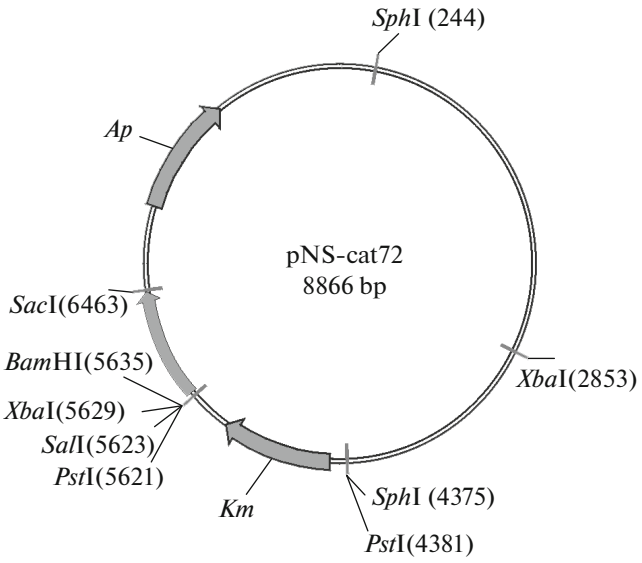

Fig. 1. pNS-cat72 plasmid map for promoter strength assessment. Start codon and promoter region are absent before the structural part of the *cat* gene. *Ap* and *Km*—the genes of ampicillin and kanamycin resistance, *cat*—the chloramphenicol resistance gene.

cells ATSS13032 was 1×10^4 transformants/μg of DNA of the pNS2 plasmid and 1×10^{-2} transformants/μg of DNA of the plasmid based on the vector pIKA-sac13.

Measurement of the *ddh* gene expression level by real-time PCR. To isolate bacterial total RNA, the cells from 50 mL of grown culture of *C. glutamicum* with an optical density OD600 = 15–25 were pelleted at 4000 rpm in an Eppendorf 5810R centrifuge for 10 min at 4°C. The supernatant was removed, and 100 μL of precipitate was resuspended in 0.8 mL of TRIzol reagent (Invitrogen, United States) supplemented with 0.1 g of glass beads of 0.1 mm. The mixture was homogenized with a high speed vortex (Heidolph, Germany) for 5 min. Further isolation was performed according to the TRIzol reagent manufacturerXs protocol. After RNA extraction the samples were treated with DNase I (ThermoScientific) for 30 min to remove DNA.

Reverse transcription was performed with a MMLV RT kit (Evrogen, Russia) according to the attached instructions using a random decanucleotide primer. The resulting cDNA may be stored at −20°C for 1 month.

PCR was performed on a ABI 7500 PCR System Fast device (Applied Biosystems, United States) with a HS Taq DNA polymerase reagent kit (Evrogen). The conditions of cDNA fragment amplification were the following: 95°C, 2 min for the first cycle; 94°C, 10 s and 60°C, 60 s for 40 cycles. PCR was performed with the following primers and probes:

the endogenous control gene 16SC16S-*FJ*, CAGCAGTGGGGAATATTG, 16S-*RJ*, GTGCTTCTTCTCCAGGTA, 16S-FAM, FAM- CGTCACCATAAGGCTTCGTCC-BHQ-1;

the *ddh* geneCDdh-*FJ*, CGTGGAATACATCCTCAA, Ddh-*RJ*, CAGGTATGGAGCAACTTC, Ddh-FAM, FAM-TCACCGCTTCCTCACAGATCGBHQ-1.

Analysis of the expression level was carried out with SDS 1.4 software according to the ddCt method.

Determination of the diaminopimelate dehydrogenase (DAPD) activity was carried out with cell-free extracts according to the protocol [11]. The specific activity of DAPD was expressed as nmol of NADPH generated for 1 min under the treatment with 1 mg of the enzyme preparation.

RESULTS AND DISCUSSION

Construction of pNS-cat72 Plasmid Derivatives Containing Different C. glutamicum Promoters

For a comparative evaluation of promoter strength, derivatives of the pNS-cat72 plasmid containing the reporter gene *cat* under the control of different promoters were constructed. In total, ten promoters were used for this purpose, including promoters of the genes *eftu* (elongation factor thermo unstable), *sod* (superoxide dismutase), *lysC* (aspartokinase), *pyc* (pyruvate carboxylase), *tkt* (transketolase), *fbp* (fructose-1,6-bisphosphatase), *leuC Cgl1315* (3-isopropylmalate dehydratase large subunit), *Cgl0284* (Acyl-CoA synthetases (AMP-forming)/AMP-acid ligases II), *Cgl2628* (periplasmic component, ABC-type transporter), and *cspB* (PS2, S-layer protein) (Table 3). Natural strains of *C. glutamicum* ATSS13032 (promoters of the genes *tkt*, *Cgl0284, fbp, Cgl2628, pyc, lysC, sod, Cgl1315-leuC, eftu*), *B. flavum* ATSS14067 (*cspB* gene promoter), and lysine-producing *B. lactofermentum* VKPM B-10945 (promoters of the genes *cspB* and *lysC*) were used as sources of promoters.

The structure of fragments containing different promoters was identical in all cases (Fig. 2). To obtain them the primers complementary to 5X and 3X ends of promoter regions were used, while *Sal*GI and *Eco*RV sites were included in the primer composition at the 5X end and *Age*I and *Bam*HI sites were included at the 3X end. The promoter regions contained a start codon typical for each promoter, which was before the site *Age*I and coincided with the reading frame of the gene *cat f*rom the vector pNS-cat72. The exception was the promoter of the *eftu* gene, the start codon of which, GTG, was replaced with ATG. As a result of this construction, the Cat protein additionally contained four amino acids at the N-terminus, ThrGlyGlySer, encoded by the sequences of *Bam*HI and *Age*I sites (see Fig. 2).

A total of 13 plasmids containing different promoters from three strains were constructed (see Table 3). Additional sequencing with primers Km20seqforw and catseqrev (see Table 2) confirmed the structure of

Table 2. Site-specific primers used in the study

Primer	Gene	Primer sequence
1	2	3
catforw	*cat*	GG*GGATCC*GAGAAAAAAATCACTGGATATACCACCG
catrev	*cat*	GC*GAGCTC*CGGTAAACCAGCAATAGACATAAGCGGC
pcspBforw	*cspB*	CG*GTCGACGATATC*CTGTGAATTAGCTGATTTAGT
pcspBrev	*cspB*	CC*GGATCCACCGGT*CATAGAGGCGAAGGCTCCTTG
Km20seqforw	*Km*	CTACCTTCTTCACGAGGCAGACCTC
catseqrev	*cat*	GCATGTTCTTTACGATGCCATTGGG
sodforw	*sod*	GG*GTCGACGATATC*TAGCTGCCAATTATTCCGGG
sodrev	*sod*	GC*GGATCCACCGGT*CATGGGTAAAAAATCCTTTCGTAGG
eftuforw	*eftu*	GG*GTCGACGATATC*TGGCCGTTACCCTGCGAATG
efturev	*eftu*	CG*GGATCCACCGGT*CATTGTATGTCCTCCTGGACTTC
plysCforw	*lysC*	GG*GTCGACGATATC*ATTGTTAATGCCGATGCTAGG
plysCrev	*lysC*	CG*GGATCCACCGGT*CACCTTTGTGCACCTTTCGATC
prpycforw	*pyc*	GT*GTCGAC*CATATCGTTAAACTTGGCCAAATGTG
prpycrev	*pyc*	TC*GGATCCACCGGT*CACTAGAGTAATTATTCCTTTCAAC
prtktforw	*tkt*	GG*GTCGACGATATC*GACGCTTGATTGGCGGACGG
prtktrev	*tkt*	TC*GGATCCACCGGT*CAATCCTTCCTGGGTTAAACCGGG
prfbpforw	*fbp*	GG*GTCGACGATATC*TCAGCCACCCGCCTAGTATG
prfbprev	*fbp*	GC*GGATCCACCGGT*CATCTGAAGGGCCTCCTGGG
pr1315forw	*leuC*	CC*GTCGAC*CATATCGTCCCATACCTTGAATTTAGC
pr1315rev	*leuC*	CC*GGATCCACCGGT*CATGGAACTCACCGTCCTTTA
pr0284forw	*Cgl0284*	GC*GTCGAC*CATATCGATCCACAAAGGTGCAGCTAAG
pr0284rev	*Cgl0284*	CG*GGATCCACCGGT*CATTCTCCTTTGTGCTTTAGATC
pr2628forw	*Cgl2628*	GG*GTCGACGATATC*TGTAGGCGTTGGACACTGTG
pr2628rev	*Cgl2628*	TC*GGATCCACCGGT*CATACCTGCCATTATATGCTTATTG
P5	*ddh*	*CGATTAGAGCTC*TGAACGGCAACGGATCAAAA
P6	*ddh*	TT*TCTAGA*ATTATTTTCTAGATTCCTCTTGGTCCAGCGAAG
P7	*ddh*	TT*TCTAGA*ATTATTTTCTAGATGAACGGCAACGGATCAAAAA
P8	*ddh*	*GTCACTGTCGAC*TTCCTCTTGGTCCAGCGAAG
F-up-ddh	*ddh*	*GTCGAC*TGTTCTTGTAATCCTCCAAAATTGTGGTGG
R-up-ddh	*ddh*	*TCTAGA*TCGCTCAAGGCTGCTGCTGACCTGGTCA
Fddh-2787716	*ddh*	AGGACGGTGAGTTCCATGACCAACATCCGCGTAGCTATC
Fddh	*ddh*	AATCCAGATTTTTGTCACGCCTGTCT
Rddh	*ddh*	ACCGCGCATCATTCCAATGCTGATCC
R-sod-ddh	*sod-ddh*	CGCGGATGTTGGTCATGGGTAAAAAATCCTTTCGTAGG
R-leuC-pr	*leuC-ddh*	CGCGGATGTTGGTCATGGAACTCACCGTCCTTTA
RleuC-456	*leuC*	CCAGGTGGGTGCGGTCTCCTGGTCCTTGGCGTCCT
FleuC-456	*leuC*	CAACCGAAACTTCGAAGGACGCCAAGGACCAGGAGACCGCACCCACC
FleuC-1381281	*leuC*	TTT*GGATCC*TGCCAACCGACGAAGGCGCAACCTTT
FleuC-1381301	*leuC*	TTT*GGATCC*ACCTTTGACAAGGTCGTAGAAATCGAT
RleuC-1382350	*leuC*	TTT*GGATCC*ACGTCGCCTGCGGTGACGATCTGCTT
RleuC-1382324	*leuC*	TTT*GGATCC*TTCCAGGTTCACGGTCAGTTCGAG
F leu-mut	*leuC*	CCGAAACTTCGAAGGACGCCAAGGACCAGGAGA

Restriction sites are marked in italics in the sequences.

all constructed plasmids except the plasmid containing the promoter region of the *cspB* gene from *Brevibacterium* B-10945. Along with the plasmid pcsp44cat, which had the expected structure, a variant of this plasmid that contained a substitution of the triplet GGT to AGT, which resulted in the inactivation of the *Age*I site, was found. This replacement was localized out of the promoter region immediately after the start codon. The plasmid with this replacement was specified as pcsp*40cat.

Table 3. Assessment of *C. glutamicum* promoter strength in cells of the GEN1-2 strain (*lys*C^{A279TS317A})

Plasmid	Promoter	Strength of promoter	Source of promoter
pNS-cat72	–	–	Promoter is absent
ptktcat1	tkt	±	From strain ATCC13032
p0284cat6	Cgl0284	±	From strain ATCC13032
pfbpcat1	fbp	±	From strain ATCC13032
p2628cat1	Cgl2628	+	From strain ATCC13032
plysCcat57	lysC	+	From strain B-10945
pcsp1cat	cspB	+	From strain ATCC14067
ppyccat3	pyc	++	From strain ATCC13032
pcsp44cat	cspB	++	From strain B-10945
plysCcat3	lysC	+++	From strain ATCC13032
psodcat5	sod	++++	From strain ATCC13032
p1315cat72	Cgl1315(leuC)	+++++	From strain ATCC13032
pcsp*40cat	cspB	++++++	From strain B-10945
peftucat5	eftu	++++++	From strain ATCC13032

The strength of promoters has the following correspondence with chloramphenicol resistance of the cells: ±—antibiotic resistance at a concentration up to 2 μg/mL; +—up to 4 μg/mL; ++—8 μg/mL; +++—10 μg/mL; ++++—12 μg/mL; +++++—14 μg/mL; ++++++—20 μg/mL.

Assessment of Promoter Strength in the C. glutamicum Strain GEN1-2 Cells by the Level of Resistance to Increasing Chloramphenicol Concentrations

The *C. glutamicum* strain GEN1-2, the aspartokinase of which is insensitive to feedback inhibition by lysine and threonine, served as a comparison strain for the assessment of promoter strength. Plasmids containing the *cat* gene under the control of different promoters were introduced into this strain (GEN1-2 (*lys*C^{A279TS317A})). The clones that received the plasmids were maintained on LB medium supplemented with kanamycin (17 μg/mL). The evaluation assessment of promoter strength in the *C. glutamicum* GEN1-2 strains containing different plasmids was carried out by the level of resistance to increasing chloramphenicol concentrations as described in Experimental. The experimental results are represented in Fig. 3.

The tested promoters can be arranged in the following order by level of chloramphenicol resistance (from weak to strong): tkt—Cgl0284—fbp—Cgl2628—lysC(B10945)—cspB(ATCC14067)—pyc – cspB(B-10945)—lysC(ATCC13032)—sod—leuC(Cgl1315)—cspB*(B-10945)—eftu(tuf) (see Table 3). The highest level of resistance (up to 20 μg/mL) was provided by the *eftu* gene promoter.The promoters of the genes *sod* and *leuC* were attributed to the group of strong promoters (a resistance level of 12–14 μg/mL). Our results on the strength of the promoters tuf (eftu) and sod coincide with the data obtained previously [3]; this indicates the adequacy of the used system.

A high resistance level (20 μg/mL) was also provided by the plasmid pcsp*40cat, which contained a point replacement in the fragment cloned from the promoter region, at the beginning of the *cat* gene. It is known that the level of gene expression is determined not only by the strength of the promoter but also by the structure of surrounding sequences [11].

Comparative analysis of the promoter strength also showed that the promoters of the genes *lysC, fbp and pyc*, which are involved in the control of lysine biosynthesis, are weak (the level of chloramphenicol resistance 2–4 μg/mL) and can serve as targets for intensification of gene functioning during the design of lysine-producing strains.

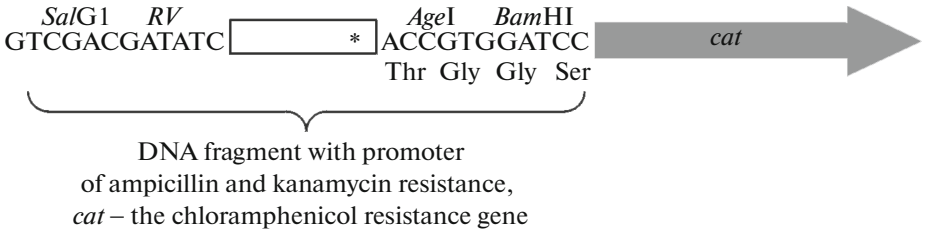

Fig. 2. Structure of DNA fragment containing promoter region in the plasmid pNS-cat72. *Sal*GI, RV, *Age*I and *Bam*HI—restriction sites of corresponding endonucleases; start codon is indicated by *.

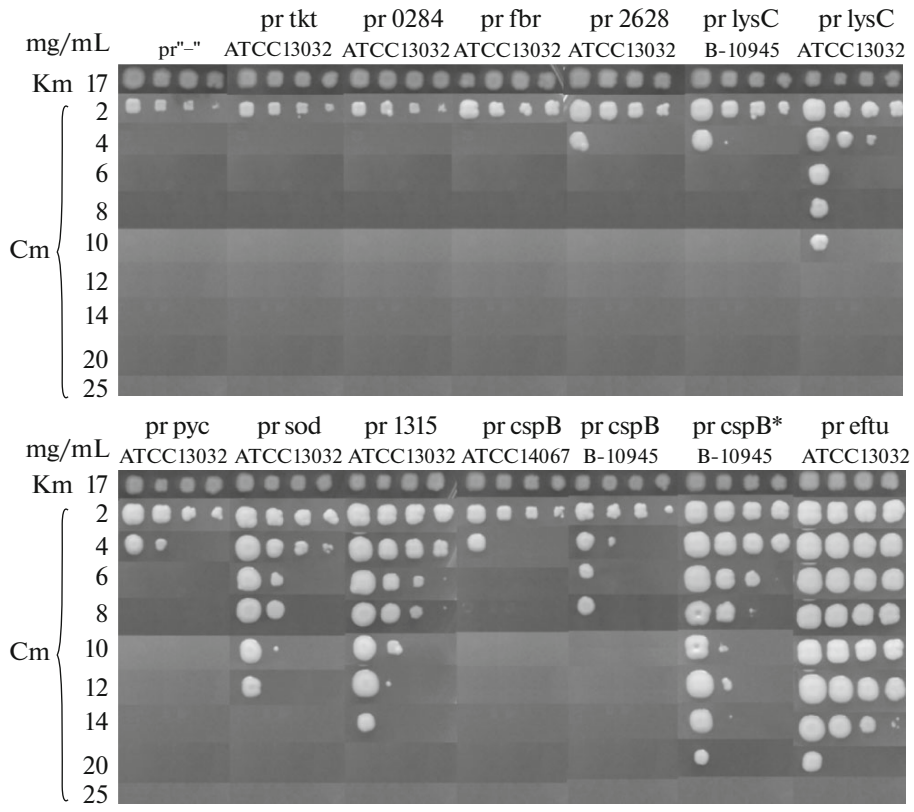

Fig. 3. Assessment of promoter strength in *C. glutamicum* strain GEN1-2 (*lys*C^{A279TS317A}), in which different plasmids (captions in the top of the figure) were introduced, by resistance to different chloramphenicol concentrations (captions in the left). Promoter from the strain pcsp40cat is indicated as prcspB*. The experiment was carried out in triplicate.

Enhanced Lysine Production via Increased ddh Expression

It is known that a change in the expression level of the genes controlling the limiting stages of lysine synthesis increases the production of amino acid by *C. glutamicum cells* [12]. The *ddh* gene, which controls the formation of diaminopimelate dehydrogenase (which catalyzes the synthesis of D,L-2,6-diaminopimelate, a key intermediate metabolite in the synthesis of lysine), was chosen as a target for lysine production enhancement. The derivatives of the strain *C. glutamicum* GEN2-1 (*lys*C^{A279TS317A}, *hom*^{V59A}), which contained either two copies of the *ddh* gene under the control of its own promoter or the *ddh* gene under the control of a strong promoter of the *sod* gene, were constructed with genetic engineering techniques. Such strains were obtained with the use of the plasmids pIKA-sod-ddh and pIKA-2-ddh, which led to substitution of the native gene by *sod-ddh* or 2×*ddh* by homologous recombination (see Experimental). The *ddh* transcription level in the obtained strains was investigated by real-time PCR (Fig. 4).

It was found that the level of *ddh* gene transcription in the strain with gene duplication, GEN2-1 (*2*×*ddh*), was two times higher than the gene transcription level of the starting strain, GEN2-1. The substitution of its own promoter with the strong promoter of the *sod* gene resulted in a significant (almost tenfold) increase in the level of the *ddh* gene transcription.

The level of the diaminopimelate dehydrogenase activity in the constructed strains was also significantly increased (Table 4). The enzyme activity in the

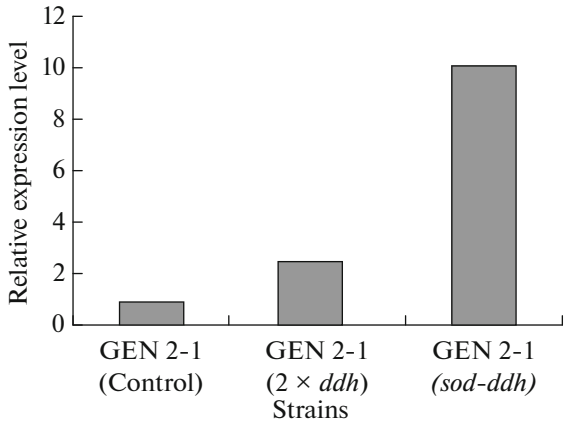

Fig. 4. Level of *ddh* gene transcription determined by real-time PCR.

Table 4. Activity of diaminopimelate dehydrogenase in *C. glutamicum* strains

Strain	DAPD activity, nmol NADPH/min/mg of protein
GEN2-1 (starting)	310
GEN2-1(2×*ddh*)	580
GEN2-1(*sod-dhh*)	1200

Table 5. Lysine production by *C. glutamicum* strains with different levels of *ddh* gene expression

C. glutamicum strain	Lysine accumulation in CM, g/L
GEN2-1 (starting)	16.0
GEN2-1(2×*ddh)*	17.3
GEN2-1(*sod-ddh*)	17.4

GEN2-1 strain (2×*ddh*) with *ddh* gene duplication was increased by 1.9 times, while it increased 3.9 times in the GEN2-1 strain (sod-dhh) as compared with the parent strain.

Lysine production in the obtained strains with an altered level of diaminopimelate dehydrogenase activity was studied in tests in vitro (Table 5). As can be seen from Table 5, an increase in diaminopimelate dehydrogenase activity due to *ddh* gene duplication leads to an 9% increase in lysine production as compared with the starting strain. However, further enhancement of the enzyme activity by the strong promoter of the *sod* gene did not lead to a further increase in amino acid production. Therefore, the highest level of lysine accumulation in CM is achieved at an optimal level of the activity of biosynthesis enzymes.

Thus, the set of promoters with different strengths obtained in this study is an important tool for the optimization of gene functioning when designing lysine-producing strains. In addition to the gene promoters (*sod* and *eftu*) previously attributed to the group of strong promoters, the promoter of the *leuC* gene can be also attributed to this group; less strong is the *cspB* gene promoter. The studied gene promoters, which affect the level of lysine synthesis (*lysC*, *pyc*, *tkt*, *fbp*), are rather weak, so it is reasonable to study the effect of promoters of different strengths to modulate of the activity of the genes of interest in further construction of the producer strain.

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REFERENCES

1. Heider, S.A.E. and Wendisch, V.F., Engineering microbial cell factories: metabolic engineering of *Corynebacterium glutamicum* with a focus on non-natural products, *J. Biotechnol.*, 2015, vol. 10, pp. 1170–1184.
2. Patek, M., Nesvera, J., Guyonvarch, A., Reyes, O., and Leblon, G., Promoters of *Corynebacterium glutamicum*, *J. Biotechnol.*, 2003, vol. 104, pp. 311–323.
3. Becker, J., Klopprogge, C., Zelder, O., Heinzle, E., and Wittmann, C., Amplified expression of fructose 1,6-bisphosphatase in *Corynebacterium glutamicum* increases in vitro flux through the pentose phosphate pathway and lysine production on different carbon sources, *Appl. Environ. Microbiol.*, 2005, vol. 71, no. 12, pp. 8587–8596.
4. Tateno, T., Fukuda, H., and Kondo, A., Direct production of L-lysine from raw corn starch by *Corynebacterium glutamicum* secreting Streptococcus bovis 0-amylase using cspB promoter and signal sequence, *Appl. Microbiol. Biotechnol.*, 2007, vol. 77, pp. 533–541.
5. Becker, J., Zelder, O., Hafner, S., Schroderand, H., and Wittman, C., From zero to hero—design-based systems metabolic engineering of *Corynebacterium glutamicum* for L-lysine production, *Metabolic Eng.*, 2011, vol. 13, pp. 159–168.
6. Schrumpf, B., Eggeling, L., and Sahm, H., Isolation and prominent characteristics of an L-lysine hyperproducing strain of *Corynebacterium glutamicum*, *Appl. Microbiol. Biotechnol.*, 1992, vol. 37, pp. 566–571.
7. Construction of shuttle vector for coryneform bacteria and E. coli. Cloning and expression study of lysC gene Rostova, Yu.G., Peredel'chuk, M.Yu., Okorokov, A.L., Gusftiner, M.M., and Debabov, V.G., *Biotekhnologiya*, 1993, no. 4, pp. 14–17.
8. Maniatis, T., Fritsch, E. F., and Sambrook, J., *Molecular Cloning*, Cold Spring Harbor, New York: Cold Spring Harbor Lab. Press, 1982.
9. Sidoruk, K.V., Levitin, E.I., and Piksasova, O.V., Universal method for isolating high-molecular-weight DNA from microorganisms based on biomass pretreatment with ammonium acetate solution, in *Aktual'nye voprosy genetiki, radiobiologii i radioekologii: Vtorye chteniya, posvyashchennye pamyati V.I. Korogodina i V.A. Shevchenko: Tez. dokl.* (Abstr. Second Readings in Memory of V.I. Korogodin and V.A. Shevchenko "Topical Issues of Genetics, Radiobiology, and Radioecology"), Dubna: OIYaI, 2008, p. 100.
10. Van der Rest, M.E., Lange, C., and Molenaar, D., A heat shock following electroporation induces highly efficient transformation of *Corynebacterium glutamicum* with xenogeneic plasmid DNA, *Appl. Microbiol. Biotechnol.*, vol. 52, no. 4, pp. 541–545.
11. Cremer, J., Treptow, C., Eggeling, L., and Sahm, H., Regulation of enzymes of lysine biosynthesis in *Corynebacterium glutamicum*, *J. Gen. Microbiol.*, 1988, vol. 134, pp. 3221–3229.
12. Merkamm, M. and Guyonvarch, J., Superoxide dismutase in cellular viability of *Corynebacterium melassecola* and role of cellular viability, *J. Bacteriol.*, 2001, vol. 183, no. 4, p. 1284.

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