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PRODUCERS, BIOLOGY, SELECTION, AND GENETIC ENGINEERING

Aspartic Acid Synthesis by *Escherichia coli* Strains with Deleted Fumarase Genes as Biocatalysts

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Abstract—The work reports the construction of *Escherichia coli* strain MG1655 derivatives with deleted genes that encode fumarases (*fumAC*, *fumB*, and *fumABC*) via the phage lambda-mediated recombination system. It has been demonstrated that the deletion of *fumB* gene had almost no effect on strain growth under aerobic conditions, while the deletion of the *fumA* and *fumC* genes led to a 30% decrease in the growth rate under the same conditions. When the *E. coli* strains with deleted fumarase genes were used to catalyze L-aspartic acid synthesis from ammonium fumarate (1.5 M solution), it was observed that only the simultaneous loss of both the *fumA* and *fumC* genes led to an at least 20% increase in the aspartic acid yields and a concurrent decrease in the content of the byproduct malic acid in the reaction mixture from 40 to 1.5–2 g/L. The results obtained in the work may be used to generate more efficient novel biocatalysts of L-aspartic acid synthesis.

Keywords: aspartase, fumarase, expression, *Escherichia coli*

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INTRODUCTION

L-aspartic acid is a key cellular metabolite involved in the synthesis of amino acids and proteins. In living organisms, L-aspartic acid is synthesized via the amination of organic acids. One such reaction, the amination of fumaric acid catalyzed by the enzyme named aspartase, was taken as the basis for the industrial production of L-aspartic acid [1]. Immobilized bacterial cells with high aspartase activity were used as a catalyst in this process. However, in such cells, fumaric acid may also undergo other chemical transformations; for example, it can be hydrated to malate in the presence of the fumarase enzyme (Fig. 1).

L-aspartic acid is extensively used in the food and drug industry, as well as in agriculture [2]. When aspartic acid is produced in the course of a biocatalytic process, malic acid produced in the course of the reaction catalyzed by fumarase is a byproduct, which considerably reduces the quality of the main product. One of the means of reducing fumarase activity in the bacterial cells is the inactivation of the genes encoding the enzyme. It is well known that *E. coli* commonly used for L-aspartic acid production have three genes coding for fumarases, namely *fumA*, *fumB*, and *fumC*, and three corresponding enzymes that catalyze the transformation of fumarate to malate in the tricarboxylic acid cycle and work under aerobic, microaerophilic, and unaerobic conditions, respectively [3].

The current work reports the construction of derivatives of the *Escherichia coli* MG1655 strain with deletions of *fumAC*, *fumB*, and *fumABC* genes and the results of their use as catalysts for L-aspartic production.

MATERIALS AND METHODS

Strains, Plasmids, and Growth Conditions

The *strains and plasmids used in the work* are provided in Table 1.

Nutritive media components were purchased from BD (United States). LB broth (pH 7.2) containing the following components was used to grow *E. coli* strains (g/L): tryptone—10, yeast extract—5, and NaCl—5. The selection of sucrose-resistant clones was performed on Sula medium (pH 7.2) containing (g/L) tryptone—12, yeast extract—6, agar—12, and sucrose—60.

The strains were grown at 37°C with constant shaking (300 rpm). When necessary, antibiotics in the following concentrations were added to LB broth in which genetically marked *E. coli* strains were grown

Abbreviations: VKPM—Russian National Collection of Industrial Microorganisms (GosNIIGenetika) (RNCIM); HPLC—high-performance liquid chromatography; PCR—polymerase chain reaction; LB medium—Luria–Bertani medium; kb—thousand base pairs; OD—optic density.

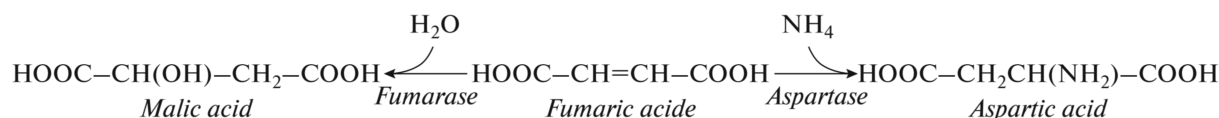

Fig. 1. Enzymatic fumaric acid transformations in *E. coli* cells.

(μg/mL): ampicillin—100 and chloramphenicol—10 (Sigma, Unites Stated).

The *oligonucleotide primers used in this study* and synthesized by ZAO Evrogen are provided in Table 2.

DNA Techniques

Plasmid DNA isolation was performed with the GeneJET Plasmid Miniprep Kit (Thermo Scientific, Vilnius, Lithuania) according to the manufacturer's recommendations. DNA fragment extraction from gels was performed with the GeneJET Gel Extraction Kit (Thermo Scientific) according to the manufacturer's instructions. Restriction endonucleases (Thermo Scientific) and T4 DNA Ligase (Thermo Scientific) were used as specified by the manufacturer or according to [6].

Construction of the accessory pICA plasmid for the subsequent generation of deletion mutants. The pICA plasmid is a derivative of the pUC19 plasmid vector. It contains the *cat* gene, which controls resistance to chloramphenicol, obtained from the pMW118-λattL-Cm^R-λattR plasmid and the *sacB* gene, which encodes the levansucrase enzyme, from the chromosomal DNA of *Bacillus subtilis* 168 (VKPM B1727). The plasmid construction outline is provided in Fig. 2.

The 5.8 kb pICA plasmid (Fig. 3) was used for the generation of deletion mutants.

Construction of the *E. coli* strain MG1655 deletion mutants. λ The red-mediated recombination [5] technique with modifications [7] was exploited to obtain the deletion mutants of the *E. coli* MG1655 strain (Fig. 4).

This technique is based on homologous recombination, which takes place between the chromosome and a linear DNA fragment introduced into the cell mediated by the bacteriophage lambda Red system. The linear DNA introduced into the cell has short regions of homology (50 bp) with the chromosomal

Table 1. Bacterial plasmids and strains used in this work

Plasmid and strain	Brief description	Source
1	2	3
pMW118	4.2 kb (Amp^R)	Nippon Gene, Tokyo Japan
pMW-attL-Cm-attR-TrrnB	5.5 kb (Amp^R Cat^R)	[4]
pUC4K	3.9 kb (Amp^R Kan^R)	GE Healthcare, Amersham, England
pUC19	2.7 kb (Amp^R)	Thermo Scientific, Vilnius, Lithuania
pKD46	6.3 kb (Amp^R Ts)	[5]
pICA	5.8 kb (Amp^R Cat^R $sacB^S$), cassette donor	This work
E. coli XL1	recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [*FproAB lacIqZΔM15 Tn10 (Tetr)*]	VKPM
E. coli TG1	supE thi-1 Δ(lac-proAB) Δ(mcrB-hsdSM)5(rk-mk-) [F' traD36 proAB $lacI^qZ$ ΔM15]	″
B. subtilis 168	VKPM B1727	″
E. coli MG1655	F^- $λ^-$ ilvG- rfb-50 rph-1	″
E. coli D30	*E. coli* MG1655 ΔiclR::cat-sacB	This work
E. coli D34 ΔiclR	*E. coli* MG1655 ΔiclR	″
E. coli D39	*E. coli* MG1655 ΔiclR ΔfumB::cat-sacB	″
E. coli D51 ΔfumB	*E. coli* MG1655 ΔiclR ΔfumB	″
E. coli D45 ΔfumAC	*E. coli* MG1655 ΔiclR ΔfumA-fumC::cat-sacB	″
E. coli D76 ΔfumABC	*E. coli* MG1655 ΔiclR ΔfumB ΔfumA-fumC::cat-sacB	″

Table 2. Oligonucleotide primers used in this work

Primer	Primer sequence 5' → 3'	Used for
1	2	3
sacBSforw	cgtctagatttttaacccatcacatatacc	pICA construction
sacBSrev	gggtcgacaaaaggttaggaatacggttag	As above
iclr-1	Caataaaaatgaaaatgatttccacgatacagaaaaaag agactgtcatgcagg gttttcccagtcacga	iclR locus elimination
iclr-2	Agaatattgcctctgcccgccagaaaaagtcagcgc attccaccgtacgatgttgtgtggaattgtgagc	As above
iclr-3	Aaaagagactgtcatggtcgcacccattcccgcgaaacgc	Marker elimination from iclR locus
iclr-4	Cctctgcccgccagaaaaagtcagcgcattccaccgtacg gcgtttcgcgggaatggg	As above
fumB-1	Ggcacgccattttcgaataacaaatacagagttacaggctg gaagctatgcagggttttcccagtcacga	fumB elimination
fumB-2	Gcatgctgccaggcgctgggccgaagaggttacttagtgc agttcgcgcaatgttgtgtggaattgtgagc	As above
fumB-3	Cgcggcacgccattttcgaataacaaatacagagttacagg ctggaagctcctcttcg	Marker elimination from fumB locus
fumB-4	Ggccaggggggatcacctggcagcatgctgccaggcgctg ggccgaagaggagcttccagc	As above
fumAC-1	Aacacccgcccagagcataaccaaaccaggcagtaagtg agagaacaatgcagggttttcccagtcacga	fumA–fumC elimination
fumAC-2	Attccacggctgcac ctgtatgttgcagattaacgcccggc tttcatactatgttgtgtggaattgtgagc	As above
autoiclr-5	cgataactctggatcatggtg	Verification of the presence of *iclR* gene
iclr-6	aaagaactacgaggaatacgag	As above
fumB-5	ctacggttattacatcctgc	Verification of the presence of *fumB* gene
fumB-6	tggtgagttcgcaaatcaggg	As above
fumAC-5	gacaatgtgcagcaccg	Verification of the presence of *fumA–fumC* genes
fumAC-6	ctggacggattttccatacc	As above
sacB-1	gctgaacaaaactggccttg	Verification of the presence of *sacB* gene
cat-1	ggtacattgagcaactgac	Verification of the presence of *cat* gene
RT-fumA-1	ggatgtagtgagcgaagtat	Detection of *fumA* gene transcription
RT-fumA-2	gcgatgtggaactggaaaa	As above
RT-fumB-1	ggtgagcgacaaacgagtgga	Detection of *fumB* gene transcription
RT-fumB-2	aggcgaagcggtgaaagttga	As above
RT-fumC-1	cgtgaggcaggctgtattcg	Detection of *fumC* gene transcription
RT-fumC-2	taacgctggggcaggagattt	As above
RT-aspA-1	agtccactaacgacgcctac	Detection of *aspA* gene transcription
RT-aspA-2	cgcagttggttaatcgcatcta	As above
RNApoly-1	tccgctctgctaactgcctta	Detection of the RNA polymerase gene transcription
RNApoly-2	gctcaacctcggtacgctgta	As above

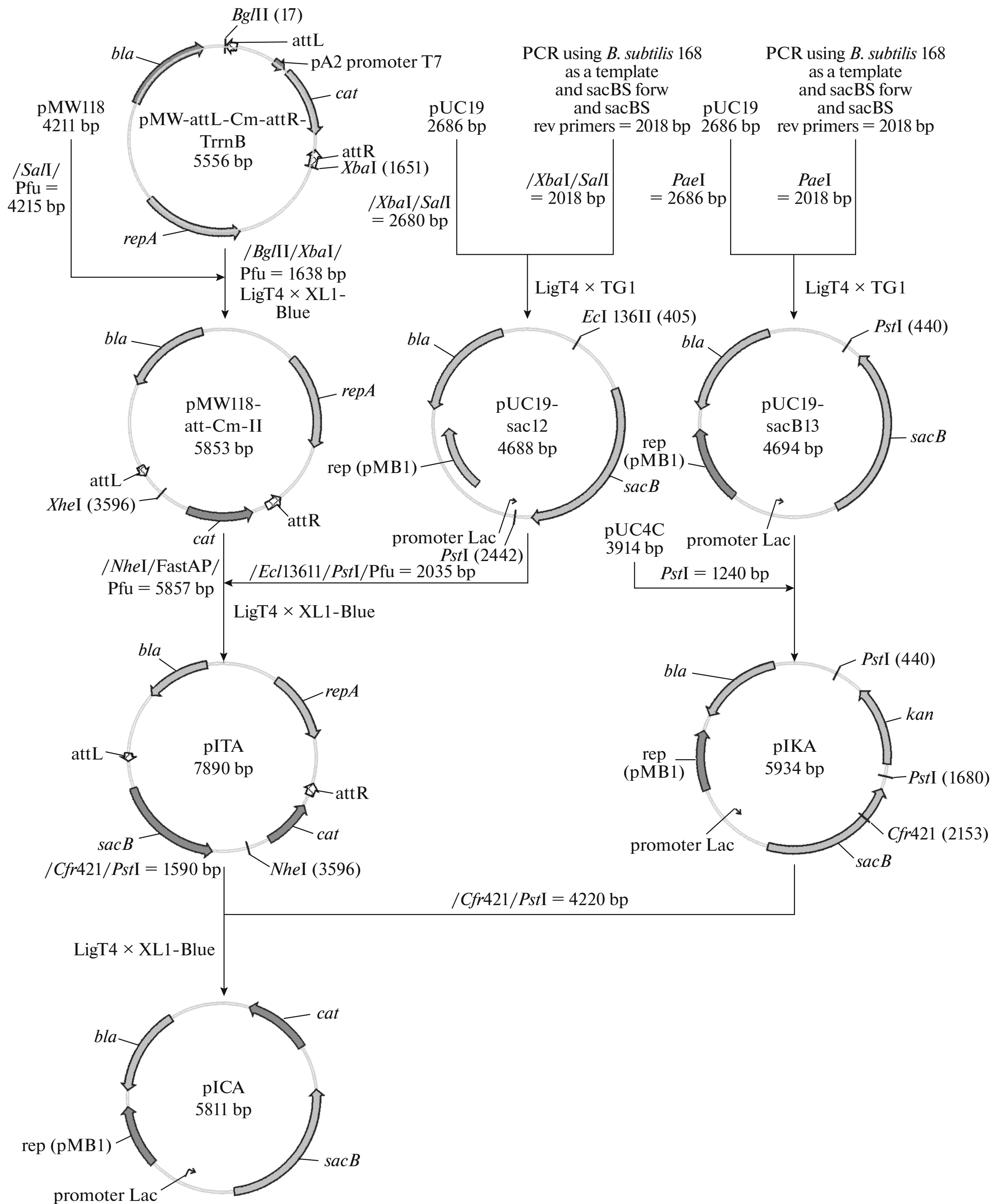

Fig. 2. pICA plasmid construction outline. *bla*—Ampicillin resistance gene; *cat*—chloramphenicol acetyltransferase gene (chloramphenicol resistance); *kan*—kanamycin resistance genes; attL and attR—phage lambda integration sites; rep(pMB1)—plasmid replication origin; *sacB*—levan sucrase gene (sucrose sensitivity).

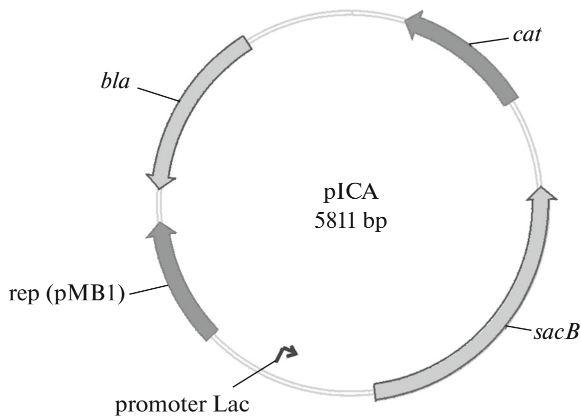

Fig. 3. pICA plasmid map. *bla*—Ampicillin resistance gene; *cat*—chloramphenicol resistance gene; rep(pMB1)—plasmid replication origin; *sacB*—levan sucrase gene (sucrose sensitivity).

DNA and in the presence of the pKD46 plasmid (provides the cell with the Redαβδ recombination proteins). It is able to integrate into the chromosome in two subsequent recombination steps, thus substituting the target gene.

The deletion variants were constructed in two steps:

(1) The gene targeted for deletion was substituted by a DNA fragment containing *cat–sacB* genes (see Fig. 4a). The fragment containing *cat* and *sacB* genes was generated by PCR with the pICA plasmid as a template and primers homologous to the flanks (see Table 2). The DNA *cat–sacB* fragment was transformed into *E. coli* MG1655 (pKD46) cells by electroporation. Clones with successful integration of the fragment into the chromosome were selected on LB agar plates by resistance to chloramphenicol. The presence of the integrated fragment was verified by PCR with primers indicated in Table 2.

(2) The second step was carried out in those cases when it was necessary to delete the *cat–sacB* fragment from the constructed chromosome (see Fig. 4b). The fragment containing the *cat–sacB* cassette was then substituted within the chromosome for a linear DNA fragment containing sequences homologous to the genome regions flanking the gene being deleted. The fragment was generated by PCR with primers that were partly homologous to each other (see Table 2). The purified DNA fragment was transformed into *E. coli* MG1655 cells containing the pKD46 plasmid by electroporation. Clones with the insertion were selected on Sula agar containing sucrose. The presence of the integrated fragment was verified by sequencing in the CEQ8000 automated sequencing device (Beckman Coulter) available in the Center for Collective Use of the GosNIIGenetika State Research Institute of Genetics and Selection of Industrial Microorganisms.

Aspartase Activity Test

Cells demonstrating aspartase activity were pelleted by centrifugation and 1.0 M ammonium fumarate solution was added to the resulting pellet. The

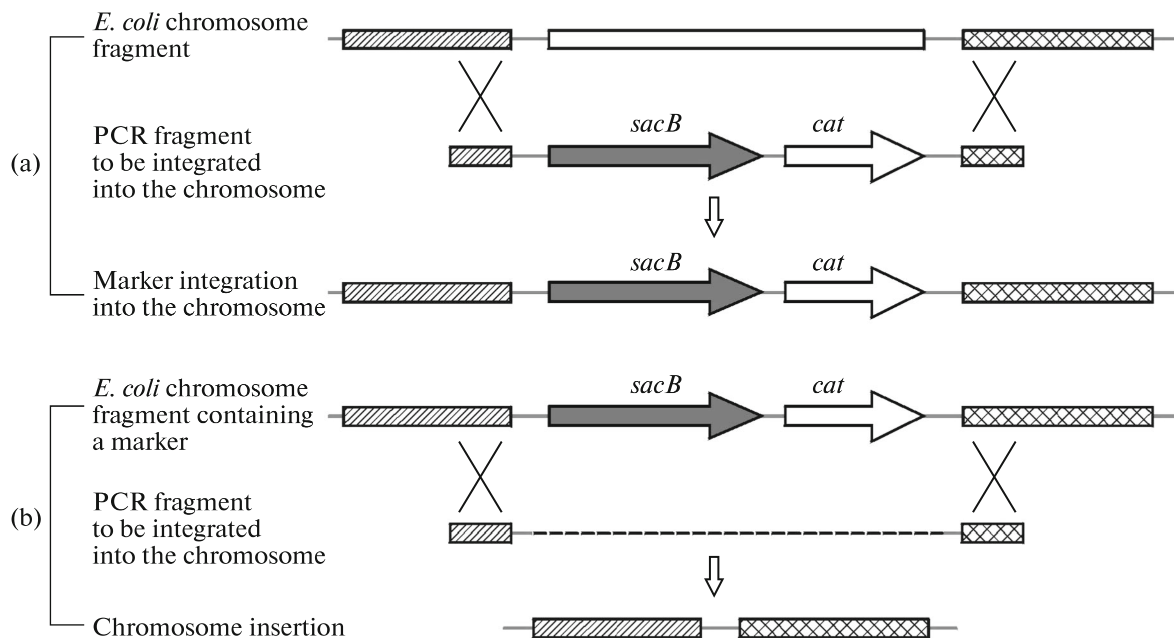

Fig. 4. Deletion mutant construction outline. a—First step, b—second step; *sacB*—levan sucrase gene (sucrose sensitivity); *cat*—chloramphenicol resistance gene.

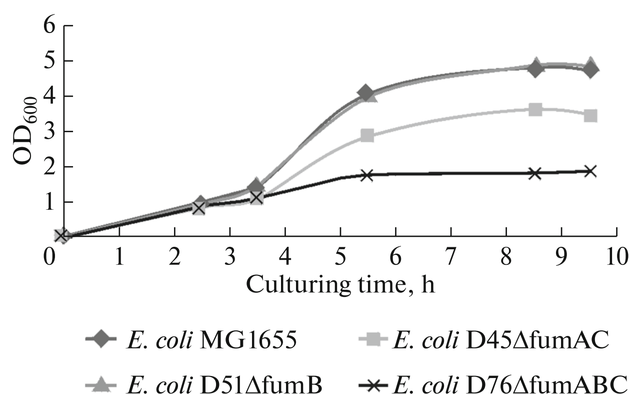

Fig. 5. Growth of strains with deleted fumarase genes.

mixture was incubated at 37°C for 45 min. Then, 9 mL of 1.5 M ammonium fumarate solution was mixed with 1 mL of the cell suspension, and the mixture was incubated at 37°C for 60 min. At the same time, the control samples were prepared by mixing 1 mL of the cell suspension with 9 mL of distilled water. The aspartic acid content in the samples was determined spectrophotometrically by absorption at 510 nm. A unit of activity was defined as the amount of enzyme that catalyzed the formation of one μmol of aspartic acid in one minute at 37°C.

After the transformation, the probe samples were centrifuged, and the resulting supernatants were 40-fold diluted and analyzed by HPLC.

HPLC Determination of the Organic Acid Content

The analysis was performed with the Alliance HPLC System (United States) (Separations Module Waters 2695 and Photodiode Array Detector Water 2996) and YMC-Triart C18 columns (5 μm, 12 nm, 250 × 4.6 mm). The mixture was 20-fold diluted with water and centrifuged at 30000 *g* for 5 min. An 0.9 mL aliquot of the resulting solution was transferred into a new tube and analyzed by HPLC under the following conditions: eluent—0.1% H_3PO_4, flow rate—1.0 mL/min, column temperature—30°C, λ—210 nm, and time of analysis—20 min. Data recording and processing was performed with the Empower Pro software.

Quantitative Analysis of Gene Expression

Quantitative real-time PCR was exploited to measure the transcription levels for the *fumA*, *fumB*, *fumC*, and *aspA* genes. RNA was isolated with the RNeasy Mini Kit (Qiagen) according to the manufacturer's recommendations. Reverse transcription was performed with the MMLV RT kit (Evrogen, Russia) according to the manufacturer's recommendations. Real-time PCR was carried out in an Applied Biosystems 7500 Fast thermal cycler with the qPCRmix-HS SYBR + LowROX PCR mix (Evrogen). The primers used for the assessment of gene transcription are listed in Table 2.

RESULTS AND DISCUSSION

Generation of E. coli Strains with Deleted Fumarase Genes

E. coli cells contain three genes controlling the synthesis of fumarases. Two of them, *fumA* and *fumC*, are located within a single chromosome locus and form a linkage group, and the third gene, *fumB*, is located in another locus [8]. To construct *E. coli* strains with the deletion of fumarase genes, we utilized an approach based on lambda Red-mediated recombination (see the Materials and Methods section). The departure strain for the generation of deletion mutants was the *E. coli* D34ΔiclR variant in which the deletion of the *iclR* repressor gene led to the activation of the glyoxylate shunt pathway (the strain was obtained in this work). It is known [9, 10] that activation of the glyoxylate pathway is essential when mutants with the complete deletion of fumarase genes have to be generated. Mutants of three different types were obtained: those with the deletion of the *fumB* gene, those with the deletion of both *fumA* and *fumC* genes (ΔfumAC with both deletions in the same locus), and a mutant in which all three fumarase genes, *fumB*, *fumA*, and *fumC*, are deleted (ΔfumABC). Among these mutants, *E.coli* strains D45ΔfumAC and *E. coli* D39 were generated via substitution of the *fumAC* and *fumB* genes for the *cat–sacB* cassette in accordance with the scheme described above (see Fig. 4). To obtain the strain in which all three fumarase genes are deleted (*E. coli* D76ΔfumABC), we used the *E. coli* D51ΔfumB strain, which lost the *fumB* gene and in which the *fumAC* genes were subsequently substituted for the *cat–sacB* cassette. The chromosome structure in each mutant was verified by PCR and sequencing of the corresponding locus.

Figure 5 shows the growth curves for the generated *E. coli* strains with deleted fumarase genes grown in flasks under aerobic conditions. The bacteria were cultured in LB broth at 37°C with constant shaking. The optical density of the bacterial culture was measured at 600 nm. It can be seen that the strain with the deletion of the *fumB* gene (*E. coli* D51ΔfumB) shows almost the same growth rate as the parental strain, which is in good accordance with the previously obtained results indicating that the fumarase encoded by *fumB* is largely inactive under aerobic conditions [11]. However, simultaneous deletion of the *fumA* and *fumC* genes (*E. coli* D45ΔfumAC) reduced the growth rate (no less than a 30% reduction) as compared to the original strain. The loss of all three fumarase genes (*E. coli* D76ΔfumABC) led to a significant (twofold or more) suppression of the mutant growth rate as compared to that of the original strain. It should be noted here that the differences in the growth rates between

the mutants became observable only after 3–4 h of incubation, which is apparently due to the accumulation of metabolites in the cells which inhibit their growth.

Transcriptional Analysis of the Strains with Deleted Fumarase Genes

The transcription levels of the genes encoding aspartase (*aspA*) and fumarases in the obtained strains were studied by real-time PCR. It was found that transcription levels of the fumarase genes in the obtained deletion mutants varied insignificantly. In the *E. coli* strain B51ΔfumB, we observed enhanced transcription of the *fumA* and *fumC* genes (by 50–70%), while we registered a slight decrease in the *fumB* gene transcription level in the strain with the deletion of the *fumA* and *fumC* genes (*E. coli* D45ΔfumAC) (Fig. 6).

At the same time, the transcription level of the aspartase gene strongly depended on the genetic structure of the strain. The minimum transcription level of the *aspA* gene was detected in the strain with the deletion of the *fumAC* genes, while the maximum transcription level (four times higher that in the original strain) was found in the strain in which all three fumarase genes were absent.

The aspartase activity (μmol/min) in the four studied strains was as follows: WT—3.0, ΔfumAC—5.7, ΔfumB—3.7, and ΔfumABC—4.5, respectively. It is evident that the level of aspartase activity in the strain doesn't correlate with the corresponding *aspA* gene transcription level, which can be apparently accounted for by the role of posttranslational modifications in the active enzyme formation [12].

Synthesis of Aspartic Acid in the Presence of the Generated Strains with Deleted Fumarase Genes

The obtained mutants with deleted fumarase genes were used as biocatalysts for aspartic acid synthesis from ammonium fumarate (1.5 M solution). To this end, the biomass of cells of each strain was obtained by growing them in LB broth at 37°C with constant shaking (300 rpm) for 16 h. Cell suspensions of each strain (OD_{600} = 0.3) were activated, treated with ethyl acetate, mixed with 1.5 M ammonium fumarate solution, and incubated at 37°C with shaking for 22 h. The contents of L-aspartic, fumaric, and malic acids in the reaction mixture were determined by HPLC. The original *E. coli* strain MG1655 was used as a control. The averaged results of the analysis are provided in Table 3.

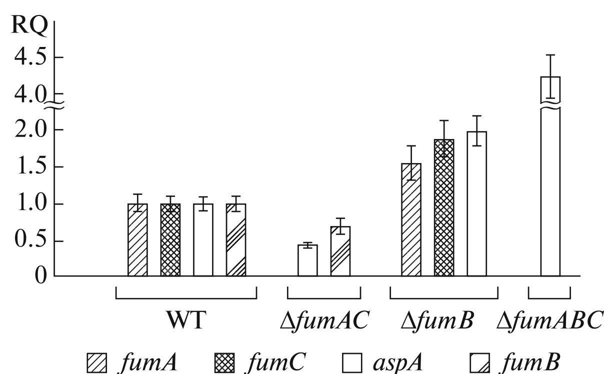

Fig. 6. Transcription levels for the *fumA*, *fumB*, *fumC*, and *aspA* genes in the *E. coli* fumarase gene deletion mutants. RQ—relative quantification.

The data in Table 3 indicates that the maximum accumulation of L-aspartic acid (193.29 mg/mL) is achieved when cells with deleted *fumA* and *fumC* genes are used as a catalyst. In this case, we also observed the lowest contents of fumarate (5 mg/mL), substrate, and the byproduct malic acid (2 mg/mL) in the reaction mixture. In the control experiment, the reaction mixture contained 150 mg/mL of L-aspartic acid, 12.97 mg/mL of fumaric acid, and 37.98 mg/mL of malic acid. The *E. coli* D76ΔfumABC biocatalysts with the deletion of all three fumarase genes showed characteristics similar to those of the ΔfumAC strain. At the same time, the *E. coli* D51ΔfumB catalyst showed almost no difference in the malic acid content in the reaction mixture as compared to the original strain.

Therefore, the use of the biomass of the *E. coli* cells as catalysts, both those with deleted *fumAC* genes and those missing all three genes encoding fumarases (*fumABC*), allows at least a 15-fold decrease in the byproduct content (malic acid) in the reaction mixture and approximately a 20% increase in the efficiency of ammonium fumarate conversion to L-aspartic acid.

The results indicate that the use of strains with deletions of fumarase genes as biocatalysts for the

Table 3. Reaction mixture composition upon the transformation of ammonium fumarate into aspartic acid in the presence of different biocatalysts

Product (acid)	Acid content in the reaction mixture in the presence of biocatalysts, mg/mL			
	E. coli MG1655	*E. coli* D45ΔfumAC	*E. coli* D51ΔfumB	*E. coli* D76ΔfumABC
L-aspartic	150.5	193.29	133.57	182.4
Malic	37.98	2.16	40.74	1.29
Fumaric	12.97	5.07	23.35	12.84

commercial production of L-aspartic acid is quite promising.

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