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Engineering *Escherichia coli* for autoinducible production of L-valine: An example of an artificial positive feedback loop in amino acid biosynthesis

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Abstract

Artificial metabolically regulated inducible expression systems are often used for the production of essential compounds. In most cases, the application of such systems enables regulating the expression of an entire group of genes in response to any internal signal such as an aerobic/anaerobic switch, a transition to stationary phase, or the exhausting of essential compounds. In this work, we demonstrate an example of another type of artificial autoinducible module, denoted a positive feedback module. This positive feedback module generates an inducer molecule that in turn enhances its own synthesis, promoting an activation signal. Due to the use of acetolactate, an intermediate of the L-valine biosynthetic pathway, as a specific inducer molecule, we realized a positive feedback loop in the biosynthetic pathway of branched chain amino acids. Such positive feedback was demonstrated to improve the production of a target compound.

Introduction

At the present time, many useful substances, e.g., L-amino acids, biofuels, and fine chemicals, are produced at a large scale by microbiological fermentation [1–5]. For this purpose, specific strain-producers are required. The basic approach for developing a producing strain is activating a biosynthetic pathway making the desired substance by enhancing key enzyme gene expression. For this goal, many specific regulatory elements, native or artificial, and metabolic toggle switches are widely used for metabolic flux redirection [6–9]. For example, the gene of interest may be placed under control of a well-characterized regulatory region, such as the *lac* promoter, *trp* promoter, P_R or P_L promoters of λ phage, and *tac* promoter [10]. These promoters have different strengths and, in the absence of corresponding repressors, provide high constitutive expression of the target genes. There are also inducer-free expression systems based on growth phase- or stress-specific promoters, such as the promoter of the *rpsF* operon or the promoter of the *pst* operon in gram-positive bacteria [11, 12].

To construct novel genetic circuits, accurate predictive design of regulatory region from versatile components can be used to "reprogram" the behavior of living microorganisms [6, 13, 14]. At the same time, exploiting dynamic sensor-regulator systems (DSRSs) to achieve the desired expression level of target genes has become increasingly popular in metabolic engineering. The DSRSs use a transcription factor that senses a key intermediate and dynamically regulates the expression of genes involved in the target compound biosynthesis in response to intracellular metabolic states [15, 16].

In the present study, an artificial autoinducible expression module with positive feedback for enhancing target gene(s) expression was constructed on the basis of one of the known DSRSs from the metabolic pathway for L-valine and L-isoleucine biosynthesis in *Escherichia coli*. L-valine, an essential nutrient for animals and humans, can be produced by microbial cultivation technology together with metabolic engineering [2, 17–20]. The development of bacterial strains with higher production of L-valine is therefore of considerable interest.

Traditionally, expression systems can be induced in response to any internal signal such as an aerobic/anaerobic switch, a transition to stationary phase, or the exhaustion of essential compounds, which were used as autoinducible signals. In this work, we demonstrate an example of another type of artificial autoinducible expression element, the positive feedback module (PFM). The PFM generates an inducer molecule that in turn enhances its own synthesis, promoting an activation signal.

Our system is based on an endogenous LysR-type protein-regulated expression module of bacteria. Despite the great interest for exploiting autoinducible gene expression systems in metabolic engineering, we found no data describing the use of LysR-type proteins in artificial expression modules working in such a mode. Originally, LTTRs (LysR-type transcriptional regulators) were described as transcriptional activators of a single divergently transcribed gene, exhibiting negative autoregulation [21–23]. Extensive research has now led to them being regarded as global transcriptional regulators, acting as either activators or repressors of single or operonic genes; they are often divergently transcribed but can be located elsewhere on the bacterial chromosome [24]. Regulation is mediated by a co-inducer of LTTR proteins. A precursor for a useful metabolite or a substrate for an enzyme under regulation may act in that capacity. A complex of a transcription regulator and its co-inducer may bind to the -35 promoter region and thus change the ability of RNA polymerase to initiate transcription of the regulated gene. Many LTTR-family members have been described in *E. coli* [22, 25]. They regulate the transcription of genes responsible for nitrogen source utilization, amino acid biosynthesis and catabolism, oxidative stress response and the detoxification of the cell. The following are several such examples: (*i*) CysB activates transcription of the genes involved in sulfur utilization and sulfonate-sulfur metabolism and acts in a complex with O-acetylserine [26]; (*ii*) MetR complexed with L-homocysteine controls transcription of several genes involved in methionine biosynthesis [27] and a gene involved in protection against nitric oxide [28]; (*iii*) ArgR in a complex with L-arginine represses transcription of its own synthesis and several genes involved in the biosynthesis and transport of arginine and the transport of histidine [29] and activates the transcription of genes responsible for arginine catabolism [30].

In the present study, as a proof of concept using PFM, we utilized an expression module regulated by LysR-type protein to improve the production of L-valine. The metabolic pathway for L-valine (and L-isoleucine) synthesis comprises several reactions catalyzed by the following enzymes: acetohydroxy acid synthase I (AHAS I) (IlvBN)/AHAS II (IlvGM)/AHAS III (IlvIH), isomeroreductase (IlvC), dihydroxyacid dehydratase (IlvD), and aminotransferase B (IlvE) (Fig 1). IlvY-mediated inducible expression of the *ilvC* gene is well characterized [31–34]. The *ilvY* and *ilvC* genes are structurally coupled in the *E. coli* chromosome and transcribed from divergently arranged promoters that partially overlap in their "upstream" regions (Fig 2).

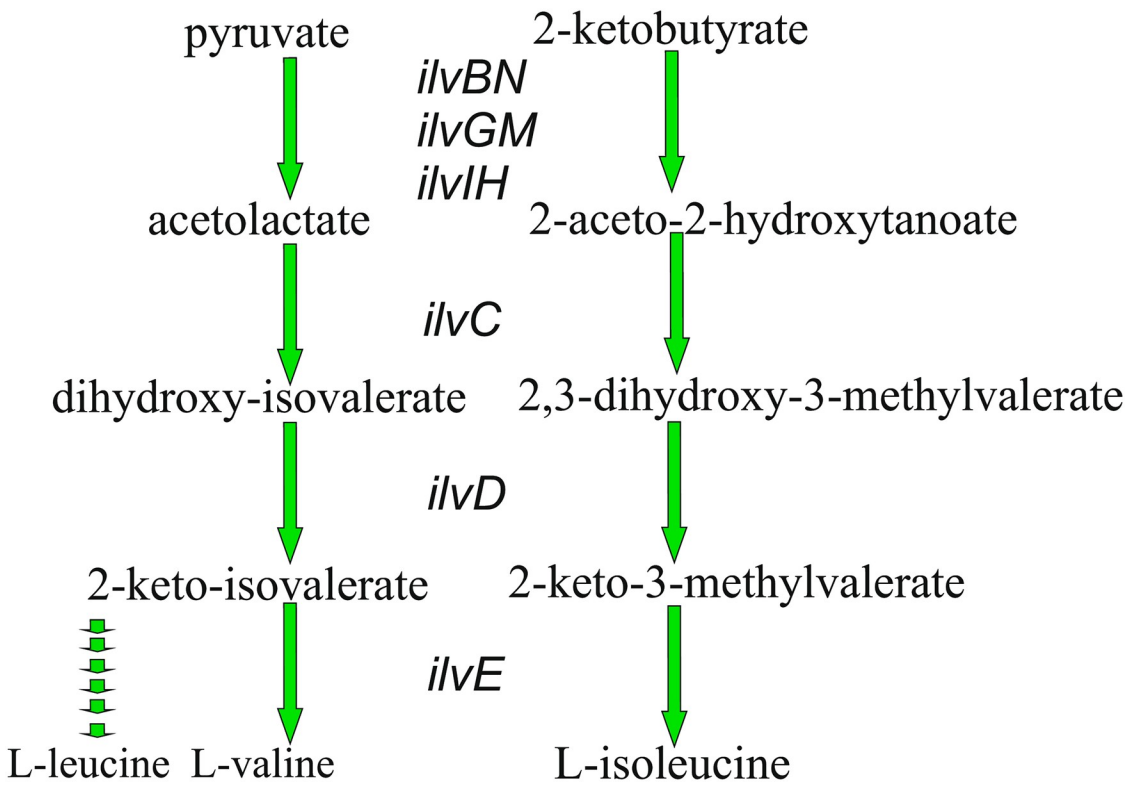

Fig 1. Schematics of BCAA biosynthesis. Metabolic pathway for BCAA (L-valine, L-leucine and L-isoleucine) synthesis consists of several reactions catalyzed by the following enzymes: acetohydroxy acid synthase I (encoded by *ilvBN* genes)/II (encoded by *ilvGM* genes)/III (encoded by *ilvIH* genes), isomeroreductase (encoded by the *ilvC* gene), dihydroxyacid dehydratase (encoded by the *ilvD* gene), and aminotransferase B (encoded by the *ilvE* gene).

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Furthermore, 2-acetolactate (AL) and 2-aceto-2-hydroxybutanoate (AHB), substrates for IlvC, are co-inducers of transcription activator IlvY, which enhances *ilvC* gene expression (Fig 2). Thus, IlvC synthesis is activated in the presence of its own substrates.

We propose to enhance and maintain stable *ilvBN* gene expression by placing it under the control of the region regulating the *ilvC* gene. The product of the AHAS I-mediated reaction is AL or AHB (co-inducers of IlvY); thus, oversynthesis of these molecules leads to the activation of transcription from P_{ilvC} and should then increase AHAS I synthesis itself. Such autoinducible positive feedback regulation can supply an appropriate level of AHAS I and, consequently, AL, the precursor of the target product L-valine. The IlvY\P_{ilvC}-determined autoinducible regulatory module shows the possibility to exploit such artificial positive feedback circuits, and their introduction into metabolic pathways for the development of industrial strains was demonstrated for the first time.

Materials and methods

Bacterial strains and growth conditions

The strains used in this study are shown in Table 1. The following media were used for bacteria cultivation: lysogeny broth (LB) [35]; minimal medium (M9), containing 11 g/L M9 minimal salts (Sigma, St. Louis, Missouri, USA), 4 g/L glucose, 10 mg/L $CaCl_2$, 0.2 mg/L $MgSO_4$; fermentation medium (FM), containing 60 g/L glucose, 15 g/L $(NH_4)_2SO_4$, 1.5 g/L KH_2PO_4, 1 g/L $MgSO_4 \times 7H_2O$, 0.1 g/L thiamine-HCl, 25 g/L $CaCO_3$, with the addition of 10% (v/v) LB

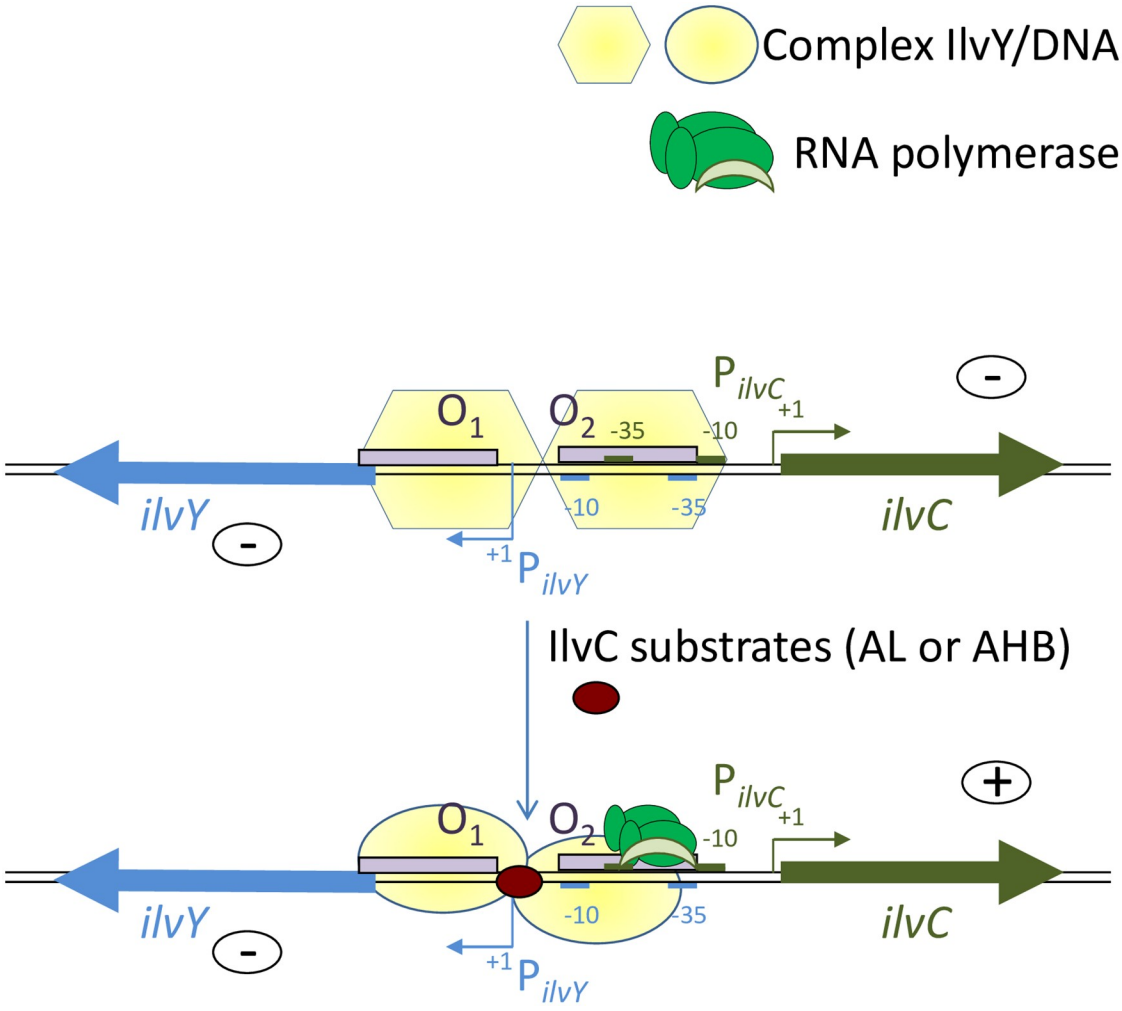

Fig 2. Schematic of the transcription regulation of the *ilvY* and *ilvC* genes. IlvY is the LysR-type transcriptional regulator designated in yellow. IlvY binds in a highly cooperative fashion to two tandem operator regions, O1 and O2, in the divergently overlapping *ilvYC* regulatory region designated (O1/$\overleftarrow{P}_{ilvY}$, $\vec{P}_{ilvC}$/O2). Upon binding to the first operator region, O1, the IlvY regulator negatively autoregulates transcription from the *ilvY* promoter, thus repressing its own further synthesis. Activation of *ilvC* transcription requires binding of the IlvY regulator to the second operator region, O2, and additional binding of a co-inducer such as 2-acetolactate (AL) or 2-aceto-2-hydroxybutanoate (AHB) to a preformed IlvY/O2 complex. When a co-inducer (red oval) is bound, a conformational change in the protein/DNA complex remodels the -35 region of the *ilvC* promoter and drastically increases the RNA polymerase binding capacity (Rhee et al., 1998). P_{ilvC} indicates the promoter of the *ilvC* gene. P_{ilvY} indicates the promoter of the *ilvY* gene. The minus sign (-) indicates a negative influence on gene transcription, and the plus sign (+) indicates a positive influence on gene transcription.

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medium. The minimal medium for the strains that possessed an *ilvYC* deletion and AHAS-deficient strains was additionally supplemented with Ile (25 mg/L) and Val (25 mg/L). Ampicillin (Ap, 100 mg/L), kanamycin (Km, 50 mg/L) and chloramphenicol (Cm, 40 mg/L) were used for selection as necessary.

Standard genetic engineering methods

Protocols for the genetic manipulation of *E. coli* and techniques for the isolation and manipulation of nucleic acids were described previously [35]. AccuTaq LA DNA polymerase (Sigma, USA) was used for PCR in accordance with the manufacturer's instructions. All primers used

Table 1. Bacterial strains used in this study.

Strain	Description	Source
MG1655	*Escherichia coli*, K-12 wild-type	VKPM[a] B6195
K12	*Escherichia coli*, K-12 wild-type	VKPM[a] B7
K12 *cat-ilvY*-P_{ilvC}-*lacZ*	K12 with expression unit *cat-ilvY*-P_{ilvC} upstream of *lacZ* gene	This work
K12 *cat*-P_{tac}-*lacZ*	K12 with constitutive promoter P_{tac} upstream of *lacZ* gene	[36]
K12 2Δ	K12 derivative with deletions of *ilvGM* and *ilvIH* genes	This work
K12 3 Δ	K12 derivative with deletions of *ilvBN*, *ilvGM* and *ilvIH* genes	This work
K12 *cat-ilvY*-P_{ilvC}-*lacZ* 3Δ	K12 3 Δ with expression unit *cat-ilvY*-P_{ilvC} upstream of *lacZ* gene	This work
K12 *cat*-P_{tac}-*lacZ* 3Δ	K12 3 Δ with constitutive promoter P_{tac} upstream of *lacZ* gene	This work
K12 2Δ P_L-*ilvBN*	K12 derivative with deletions of *ilvGM* and *ilvIH* genes, overexpression of *ilvBN* genes under control of "strong" constitutive promoter P_L	This work
K12 *cat-ilvY*-P_{ilvC}-*lacZ* 2Δ P_L-*ilvBN*	K12 2Δ P_L-*ilvBN* with expression unit *cat-ilvY*-P_{ilvC} upstream of *lacZ* gene	This work
K12 *cat*-P_{tac}-*lacZ* 2ΔP_L-*ilvBN*	K12 2Δ P_L-*ilvBN* with constitutive promoter P_{tac} upstream of *lacZ* gene	This work
K12 2Δ P_L-$ilvBN^{fbr}$	K12 derivative with deletions of *ilvGM* and *ilvIH* genes, overexpression of $ilvBN^{fbr}$ genes encoding feedback-resistant AHAS I under control of "strong" constitutive promoter P_L	This work
K12 *cat-ilvY*-P_{ilvC}-*lacZ* 2Δ P_L-$ilvBN^{fbr}$	K12 2Δ P_L-$ilvBN^{fbr}$ derivative with expression unit *cat-ilvY*-P_{ilvC} upstream of *lacZ* gene	This work
K12 *cat*-P_{tac}-*lacZ* 2ΔP_L-$ilvBN^{fbr}$	K12 2Δ P_L-$ilvBN^{fbr}$ derivative with constitutive promoter P_{tac} upstream of *lacZ* gene	This work
K12 2Δ P_L-*ilvBN* Δ*ilvYC*::*kan*	K12 derivative with deletions of *ilvGM*, *ilvIH* and *ilvYC* genes, overexpression of *ilvBN* genes under control of "strong" constitutive promoter P_L	This work
K12 *cat-ilvY*-P_{ilvC}-*lacZ* 2Δ P_L-*ilvBN* Δ*ilvYC*::*kan*	K12 2ΔP_L-*ilvBN* Δ *ilvYC*::*kan* derivative with expression unit *cat-ilvY*-P_{ilvC} upstream of *lacZ* gene	This work
K12 2Δ P_L-$ilvBN^{fbr}$ Δ*ilvYC*::*kan*	K12 derivative with deletions of *ilvGM*, *ilvIH* and *ilvYC* genes, overexpression of $ilvBN^{fbr}$ genes under control of "strong" constitutive promoter P_L	This work
K12 2Δ P_L-$ilvBN^{fbr}$ ::*cat*-P_L-$ilvBN^{fbr}$	K12 derivative with deletions of *ilvGM* and *ilvIH* genes, overexpression of $ilvBN^{fbr}$ genes under control of "strong" constitutive promoter P_L(native locus) and insertion of additional copy of P_L-$ilvBN^{fbr}$ into *ppsA* locus	This work
K12 2Δ P_L-$ilvBN^{fbr}$ ::*cat*	K12 derivative with deletions of *ilvGM* and *ilvIH* genes, overexpression of $ilvBN^{fbr}$ genes under control of "strong" constitutive promoter P_L and deletion of *ppsA* gene, marked with the *cat* gene	This work
K12 *cat-ilvY*-P_{ilvC}-*lacZ* 2Δ P_L-$ilvBN^{fbr}$ Δ*ilvYC*::*kan*	K12 2Δ P_L-$ilvBN^{fbr}$ Δ*ilvYC*::*kan* derivative with expression unit *cat-ilvY*-P_{ilvC} upstream of *lacZ* gene	This work
K12 2Δ *cat-ilvY*-P_{ilvC}-$ilvBN^{fbr}$	K12 derivative with deletions of *ilvGM* and *ilvIH* genes, overexpression of $ilvBN^{fbr}$ genes encoding feedback-resistant AHAS I under control of expression unit *cat-ilvY*-P_{ilvC}	This work
K12 2Δ $ilvY^{inactive}$-P_{ilvC}-$ilvBN^{fbr}$	K12 derivative with deletions of *ilvGM* and *ilvIH* genes, overexpression of $ilvBN^{fbr}$ genes under control of expression unit *cat*-$ilvY^{inactive}$-P_{ilvC} containing mutation in *ilvY* gene	This work
K12 Δ *ilvY* 2Δ *cat-ilvY*-P_{ilvC}-$ilvBN^{fbr}$	K12 derivative with deletions of *ilvGM*, *ilvIH* and *ilvY* (native locus) genes, overexpression of $ilvBN^{fbr}$ genes under control of expression unit *cat-ilvY*-P_{ilvC}	This work

[a] VKPM, The Russian National Collection of Industrial Microorganisms

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in this work are listed in S1 Table. λRed-mediated integration was performed according to Datsenko and Wanner [37]. The recombinant plasmid pKD46 [37] with a temperature-sensitive replicon was used as the donor of the phage λ-derived genes responsible for the λRed-mediated recombination system.

Construction of the *cat-ilvY*-P_{ilvC}-*lacZ* expression cassette

To construct the *cat-ilvY*-P_{ilvC}-*lacZ* expression unit, the *cat* gene was first introduced downstream of the *ilvY* gene on the chromosome of *E. coli* strain MG1655 using λRed-mediated integration. A DNA fragment bearing the λ*attL-cat-*λ*attR* cassette was amplified by PCR using the oligonucleotide primers P1 (for the *ilvY-attL* region) and P2 (for the *attR-ilvY* region), and the plasmid pMW118-λ*attL-cat-*λ*attR* [36] was used as the template. Second, the fragment *cat-ilvY*-P_{ilvC} including λ*attL-cat-*λ*attR*, the *ilvY* gene, and intergenic region *ilvY-ilvC* with the P_{ilvC} promoter, was PCR-amplified using the oligonucleotide primers P3 (for the *attL-lacZ* region) and P4 (for the *ilvCp-lacZ* region), and the chromosome of the *E. coli* strain MG1655 *cat-ilvY* was used as the template. The obtained PCR fragment was inserted into the *E. coli* MG1655/pKD46 chromosome region upstream of the *lacZ* gene by means of λRed-mediated integration. As a result, the strain *E. coli* MG1655 *cat-ilvY*-P_{ilvC}-*lacZ* was obtained. The *cat-ilvY*-P_{ilvC}-*lacZ* expression unit was transferred into several *E. coli* strains by P1 transduction [35].

Construction of *E. coli* strains K12 2Δ and K12 3Δ

Deletion of the *ilvBN* operon was accomplished by means of λRed-mediated integration. A DNA fragment bearing the λ*attL-cat-*λ*attR* cassette was amplified by PCR using the primers ilvBN1 and ilvBN2, and the plasmid pMW118-λ*attL-cat-*λ*attR* was used as a template. The obtained 1713 bp PCR product was used for electroporation of the *E. coli* strain MG1655/pKD46. As a result, the *E. coli* MG1655 Δ*ilvBN* strain was obtained. Deletions of the *ilvIH* operon and *ilvGM* genes were constructed by the same approach used for the deletion of the *ilvBN* operon; the primers ilvIH1 and ilvIH2 were used for the *ilvIH* deletion, and primers ilvGM1 and ilvGM2 were used for the *ilvGM* deletion. The deletion of *ilvGM* genes was specially designed to minimize polarity effects on the expression of distal genes of the isoleucine-valine *ilvGMEDA* operon. A combination of Δ*ilvBN*, *Δilv*IH and *Δilv*GM deletions was accomplished by P1 transduction [35] with intermediate elimination of the chloramphenicol resistance marker. As a result, strains K12 2Δ (= K12 Δ*ilvIH* Δilv*GM*) and K12 3Δ (= K12 Δ*ilvIH* Δilv*GM* Δ*ilvBN*) were obtained. The strain K12 2Δ was prototrophic, therefore deletion of *ilvGM* genes did not prevent expression of distal genes of the isoleucine-valine operon.

Construction of *E. coli* strains harboring different *ilvBN* and *ilvBN*^{fbr} expression units

The native regulatory region of the *ilvBN* operon was replaced with the phage lambda P_L promoter by λRed-mediated integration. For that purpose, we used the oligonucleotide ilvB-attR1, which is homologous to the region upstream of the *ilvB* gene and the region adjacent to the gene conferring antibiotic resistance, and the oligonucleotide ilvB-PLSD, which is homologous to both the *ilvB* region and the region downstream of the P_L promoter (for details of construction, see [38]). The strain K12 2Δ, with a single copy of the operon encoding AHAS I, was used for integrating the regulatory region *cat*-P_L upstream of the *ilvBN* operon. The obtained strain, K12 2Δ P_L-*ilvBN*, was L-valine sensitive.

New L-valine-resistant spontaneous mutants of AHAS I were obtained from strain K12 2Δ P_L-*ilvBN*. Spontaneous mutants that were resistant to L-valine were selected on plates with

minimal medium that had been supplemented with 1 g/L L-valine. Strains that grew better on medium with 1 g/L L-valine were characterized. Among them, the enzyme containing a mutant small regulatory subunit IlvN^{N17K} demonstrated highest AHAS specific activity (for details of construction, see [38]). Thus, K12 2Δ P_L-*ilvBN*^{fbr} was constructed.

The phage promoter P_L upstream of the *ilvBN*^{fbr} genes was substituted for the *cat*-*ilvY*-P_{ilvC} regulatory region using λRed-mediated integration, giving a strain with an autoinducible PFM for L-valine biosynthesis. To accomplish this aim, primers P7 and P8 were used.

To design an additional copy of the P_L-*ilvBN*^{fbr} construct, a PCR fragment containing *cat*-P_L-*ilvBN*^{fbr} was PCR-amplified by using primers ppsattRL and ppsilvN and integrated into *ppsA* locus of the chromosome of *E. coli* K12 3Δ by λRed-mediated integration. The resulting strain, K12 3Δ ::*cat*-P_L-*ilvBN*^{fbr}, was used as a donor for P1-transduction to combine two copies of P_L-*ilvBN*^{fbr} at one chromosome yielding K12 2Δ P_L-*ilvBN*^{fbr} ::*cat*-P_L-*ilvBN*^{fbr}. To demonstrate that deletion of the *ppsA* gene encoding phosphoenolpyruvate synthase, non-essential for L-valine biosynthesis, did not have a negative effect on the strain performance, the *ppsA* gene was inactivated in a fashion similar to the previously described method by using the primers ppsIL and ppsIR. The obtained strain, MG1655 Δ*ppsA*::*cat*, was used as donor for P1 transduction of the cassette Δ*ppsA*::*cat*.

Construction of strains harboring *cat*-P_{tac}-*lacZ*

The expression cassette *cat*-P_{tac}-*lacZ*, kindly provided by Dr. Katashkina [36], contained the exporter gene *lacZ* under control of the hybrid promoter P_{tac}, which contained the consensus sequences of the –35 and –10 regions from the natural promoters of the tryptophan and lactose (UV5) operon, respectively. This cassette was transferred into several strains by means of P1 transduction [35].

Calculation of the translation initiation rate

The translation initiation rate, TIR, for the expression cassettes containing genes encoding feedback-resistant AHAS I under the control of different regulatory elements, i.e., P_L-*ilvBN*^{fbr} and *ilvY*-P_{ilvC}-*ilvBN*^{fbr} constructs, was calculated by using the Salis Lab RBS Calculator v 2.0 https://salislab.net/software/ [39].

Construction of *ilvY*-deficient strains

An *ilvYC* deletion was constructed in two steps using λRed-mediated integration with oligonucleotide primers P5 and P6 and the plasmid pMW118-λ*attL*-*kan*-λ*attR* [36] as the template. As a result, the *E. coli* MG1655 Δ*ilvYC*::*kan* strain was obtained.

The inactivation of the *ilvY* gene in its native locus was done by introduction of microdeletion as follows. First, a PCR fragment harboring the λ*attL*-*cat*-λ*attR* cassette with the regions adjacent to an *ilvY* internal region was obtained using the oligonucleotide primers P9 and P10 and the plasmid pMIV5-JS as the template. The plasmid pMIV5-JS was constructed as described in [38]. As a result, the *E. coli* MG1655 Δ*ilvY*::*cat* strain, containing the chloramphenicol resistant marker (Cm^R) in the *ilvY* coding region, was obtained. The cassette was transferred into strain K12 2Δ *cat*-*ilvY*-P_{ilvC}-*ilvBN*^{fbr} by P1 transduction. After Cm^R marker elimination, the strain K12 2Δ Δ*ilvY cat*-*ilvY*-P_{ilvC}-*ilvBN*^{fbr} was obtained.

Inactivation of the additional copy of the *ilvY* gene, a part of the *cat*-*ilvY*-P_{ilvC} cassette, was performed as follows. The *E. coli* K12 2Δ *cat*-*ilvY*-P_{ilvC}-*ilvBN*^{fbr} strain was cured from the Cm^R marker by transient introduction of pMWts-λInt/Xis plasmid, which resulted in the markerless *E. coli* K12 2Δ *ilvY*-P_{ilvC}-*ilvBN*^{fbr} strain. The *ilvYC* genes were deleted from the *E. coli* K12 2Δ *cat*-*ilvY*-P_{ilvC}-*ilvBN*^{fbr} strain by P1 transduction as described above, using *E. coli* MG1655

Δ*ilvYC*::*kan* as a donor. Having obtained the λRed genes via the plasmid pKD46, the *E. coli* K12 2Δ *cat-ilvY*-P_{ilvC}-$ilvBN^{fbr}$ Δ*ilvYC*::*kan* strain was electrotransformed with the PCR fragment harboring the λ*attL-cat-*λ*attR* cassette with the regions adjacent to *ilvY* internal region. This PCR fragment was amplified with the oligonucleotide primers P11 and P12, and the chromosome of the *E. coli* MG1655 *ilvY*::*cat* strain was used as the template. As a result, the *E. coli* B7 K12 2Δ Δ*ilvYC*::*kan ilvY*::*cat*-P_{ilvC}-$ilvBN^{fbr}$ strain was obtained, which was then used as a donor strain to transduce the *ilvY*::*cat*-P_{ilvC}- $ilvBN^{fbr}$ cassette into the *E. coli* K12 2Δ *ilvY*-P_{ilvC}- $ilvBN^{fbr}$ strain. The P1 transduction was performed as described above. This process resulted in the strain *E. coli* K12 2Δ *ilvY*::*cat*-P_{ilvC}- $ilvBN^{fbr}$, which possesses only one active copy of the *ilvY* gene in its native locus due to inactivation of the *ilvY* gene copy in the *ilvY*::*cat*-P_{ilvC}-$ilvBN^{fbr}$ cassette as described above. The *cat* gene was eliminated using the transient introduction of the pMWts-λInt/Xis plasmid. As a result, the markerless *E. coli* B7 Δ*ilvGM* Δ*ilvIH* $ilvY^{inactive}$-P_{ilvC}-*ilvBN*4 strain was obtained.

β-Galactosidase activity assay

Cells were grown to the mid-logarithmic phase in M9:LB (9:1, v/v) medium. The medium for strains having an *ilvYC* deletion and AHAS-deficient strains was additionally supplemented with Ile (25 mg/l) and Val (25 mg/l). The activity of β-galactosidase was measured according to Miller's method [40]. The mean of triplicate experiments is presented; the standard deviation was less than 20%. MU = Miller's units.

AHAS activity assay

Cells were grown to the mid-logarithmic phase in M9: LB (9:1, v/v) medium. The activity of AHAS I in crude cell extracts was measured with or without the addition of 10 mM L-Val according to the assay described previously [41]. The mean of triplicate experiments is presented.

Test tube fermentation conditions

Strains were each cultivated at 32˚C for 18 hours in LB medium. Then, 0.2 mL of the obtained culture was inoculated into 2 mL of FM medium in 20 × 200 mm test-tubes and cultivated at 30˚C for 60 hours on a rotary shaker at 250 rpm. After cultivation, the accumulated L-valine was measured using thin-layer chromatography (TLC). TLC plates (10 x 20 cm) were coated with 0.11 mm layers of Sorbfil silica gel containing nonfluorescent indicator (Sorbpolymer, Krasnodar, Russian Federation). Samples were applied to the plates with the Camag Linomat 5 sample applicator. The Sorbfil plates were developed with a mobile phase consisting of *iso*-propanol:ethylacetate:25% aqueous ammonia:water (16:16:5:10, v/v). A solution of ninhydrin (2%, w/v) in acetone was used as the visualizing reagent. After development, plates were dried and scanned with the Camag TLC Scanner 3 in absorbance mode with detection at 520 nm using winCATS software (version 1.4.2). Average data of 4 independent test tube fermentations are shown. Optical density at wavelength 540 nm, OD_{540}, was measured by using Infinite M200 (Tecan, Austria).

Results and discussion

Properties of acetohydroxy acid-regulated expression unit based on the regulatory region of the *ilvC* gene

In the present work, the regulatory region of the *ilvC* gene was used as a metabolically regulated expression module in *E. coli*. Whereas the majority of isoleucine-valine biosynthetic

genes, e.g., *ilvGMEDA* and *ilvBN* operons, are under negative control by the end products with participation of a transcription attenuation mechanism, the *ilvC* gene in *E. coli* is positively regulated by the intermediates of BCAA biosynthesis. 2-Acetolactate (AL) and 2-aceto-2-hydroxybutanoate (AHB), the products of the AHAS-mediated reactions and also the substrates for 2-acetohydroxy acid isomeroreductase (IlvC or KARI), are co-inducers of the transcription activator IlvY, which enhances *ilvC* gene expression [34]. Thus, IlvC synthesis is activated in the presence of its own substrates. Moreover, the *ilvC* gene has overlapping promoter regions with *ilvY* gene. Both genes are divergently transcribed in a coordinated fashion, and this coordination is achieved via supercoiling in the limited space between the two promoters [31].

To elucidate the ability of the promoter P_{ilvC} to be regulated metabolically, its functioning was studied in different genetic background that might alter the pools of inducer molecules. To this end, the transcriptional fusion expression cassette *cat-ilvY*-P_{ilvC}-*lacZ* was constructed. The tested regulatory unit included the *ilvY* gene, encoding the LysR-type regulatory protein, and the intergenic region *ilvY-ilvC*, containing the P_{ilvC} promoter. The obtained expression cassette *cat-ilvY*-P_{ilvC}-*lacZ* was transferred into the following strains, which differ in their ability to synthesize and metabolize AL (in this case, we focused on AL rather than AHB):

1. K12, wild-type strain with native AL synthesis and utilization;
2. K12 3Δ, AHAS-deficient strain with deletions of *ilvBN*, *ilvGM* and *ilvIH* genes and the absence of AL synthesis;
3. K12 2Δ P_L-*ilvBN*, strain with increased AL synthesis due to the overexpression of *ilvBN* genes under the control of "strong" constitutive promoter P_L; additionally, contains disruptions of Δ*ilvGM* and Δ*ilvIH* genes;
4. K12 2Δ P_L-$ilvBN^{fbr}$, strain similar to (iii), but instead of wild-type *ilvBN* genes, the mutant operon encoding feedback-resistant AHAS I, designated as $ilvBN^{fbr}$, was used;
5. K12 2Δ P_L-*ilvBN* Δ*ilvYC*::*kan*, strain with increased AL synthesis and defect in AL utilization due to the inactivation of *ilvYC* genes;
6. K12 2Δ P_L-$ilvBN^{fbr}$ Δ*ilvYC*::*kan*, strain similar to (v) but containing mutant $ilvBN^{fbr}$ operon.

It should be noted that the deletion of *ilvGM* genes was specially constructed to minimize polarity effect on the expression of distal genes of the isoleucine-valine operon *ilvGMEDA*. A feedback-resistant AHAS I, containing the mutant small regulatory subunit $IlvN^{N17K}$, was applied. This enzyme demonstrated more than 70% residual activity in the presence of 10 mM L-Val (see [38] for details).

As expected, specific β-galactosidase (LacZ) activity measurements in strains harboring the expression cassette *cat-ilvY*-P_{ilvC}-*lacZ* indicated a correlation between the LacZ activity and the presumptive level of AL in a cell (Table 2). Thus, in the case of the AHAS-deficient strain K12 *cat-ilvY*-P_{ilvC}-*lacZ* 3Δ, i.e., in the absence of co-inductor molecule synthesis, the activity was undetectable, while strain K12 *cat-ilvY*-P_{ilvC}-*lacZ*, with native synthesis and utilization of AL, demonstrated the activity. Strain K12 *cat-ilvY*-P_{ilvC}-*lacZ* 2Δ P_L-*ilvBN*, possessing a relatively high level of AL synthesis, demonstrated LacZ activity comparable with that produced by the native LacZ regulation under isopropyl-β-D-thiogalactoside (IPTG) induction. Overexpression of the mutant operon $ilvBN^{fbr}$, encoding feedback-resistant AHAS I, led to a further increase in LacZ activity, up to 4-fold higher than that of the strain harboring the wild-type AHAS I under the same expression conditions. The maximal expression from P_{ilvC} was provided by the *E. coli* strains modified to overexpress feedback-resistant AHAS I (the product of

Table 2. Activity of β-galactosidase LacZ in strains harboring the expression cassette *ilvY*-P_{ilvC}-*lacZ* in various genetic backgrounds.

Strain	Description	LacZ activity, MU
K12 (IPTG, 1 mM)	Wild-type strain, native LacZ regulation under IPTG induction	1200
K12 *cat-ilvY*-P_{ilvC}-*lacZ*	Wild-type strain harboring the expression cassette *cat-ilvY*-P_{ilvC}-*lacZ*	150
K12 *cat-ilvY*-P_{ilvC}-*lacZ* 3Δ	3Δ = Δ*ilvBN* Δ*ilvGM* Δ*ilvIH*, AL synthesis is blocked	≤10
K12 *cat-ilvY*-P_{ilvC}-*lacZ* 2Δ P_L-*ilvBN*	2Δ = Δ*ilvGM* Δ*ilvIH*, AL is synthesized by AHAS I	940
K12 *cat-ilvY*-P_{ilvC}-*lacZ* 2Δ P_L-*ilvBN*^{fbr}	2Δ = Δ*ilvGM* Δ*ilvIH*, AL is synthesized by feedback-resistant AHAS I	3700
K12 *cat-ilvY*-P_{ilvC}-*lacZ* 2Δ P_L-*ilvBN* Δ*ilvYC*::*kan*	2Δ = Δ*ilvGM* Δ*ilvIH*, AL is synthesized by AHAS I + no utilization due to the inactivation of KARI	4400
K12 *cat-ilvY*-P_{ilvC}-*lacZ* 2Δ P_L-*ilvBN*^{fbr} Δ*ilvYC*::*kan*	2Δ = Δ*ilvGM* Δ*ilvIH*, AL is synthesized by feedback-resistant AHAS I + no utilization due to the inactivation of KARI	4400

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the *ilvBN*^{fbr} genes) and/or lacking the isomeroreductase KARI (IlvC) that normally metabolizes the inducer. In the case of KARI deficiency, there was no difference between LacZ activities in strains with wild-type and feedback-resistant AHAS I (Table 2).

The difference in LacZ activity between the tested strains can be caused, theoretically, not only by the difference in transcriptional level concerned with the AL inducer availability but also by other factors, such as significant differences in ribosomes, RNA polymerase pools, and mRNA stability, depending on the strain genotype. To confirm that the above factors had no essential impact, the same reporter, the *lacZ* gene, was put under the control of the constitutive promoter P_{tac} in the analyzed strains with different ability for AL synthesis. As the LacZ activity level was similar for all the above strains containing the same construct, *cat*-P_{tac}-*lacZ* (S2 Table), the level of AL in the cells was likely the main reason for differences in LacZ activity between strains that possessed the expression cassette *cat-ilvY*-P_{ilvC}-*lacZ* (Table 2).

Thus, the data show that P_{ilvC}-dependent expression levels may vary over a broad range, by more than a factor of 400, because of dependence on the co-inducer pool, particularly AL.

Application of positive feedback module for autoinducible production of L-valine by *E. coli*

In the present work, we developed an autoinducible gene expression module with a positive feedback loop, a so-called PFM. The idea is to place a biosynthetic gene under positive control of the product of a corresponding enzyme to incorporate a positive feedback unit into the biosynthetic pathway. The appearance of the product thus leads to activation of enzyme synthesis, which in turn results in the high accumulation of the product and a high yield of a final product. We realized this scheme by a model of an *E. coli* L-valine-producing strain (Fig 3).

As known, in *E. coli* K12 all the key enzymes of L-Val production are L-Val-sensitive: AHAS I (IlvBN) and AHAS III (IlvIH) [42]. AHAS II isozyme, L-Val-resistant, is inactive because of a frameshift mutation in *ilvG*. Thus, the wild-type strain *E. coli* K12 does not produce any detectable amounts of L-Val (Table 3); moreover, it does not grow in the presence of Val in the medium (minimal inhibiting concentration is less than 10 mg/L). As indicated above, a feedback-resistant AHAS I encoded by *ilvBN*^{fbr} operon was obtained and could be used for construction of L-Val-producing bacterium. It should be noted that in the wild-type *E. coli* cells both the operons, *ilvBN* and *ilvIH*, are subjected to transcription attenuation and their expression is depressed by BCAA [42]. So,the regulatory region of the *ilvBN*^{fbr} operon

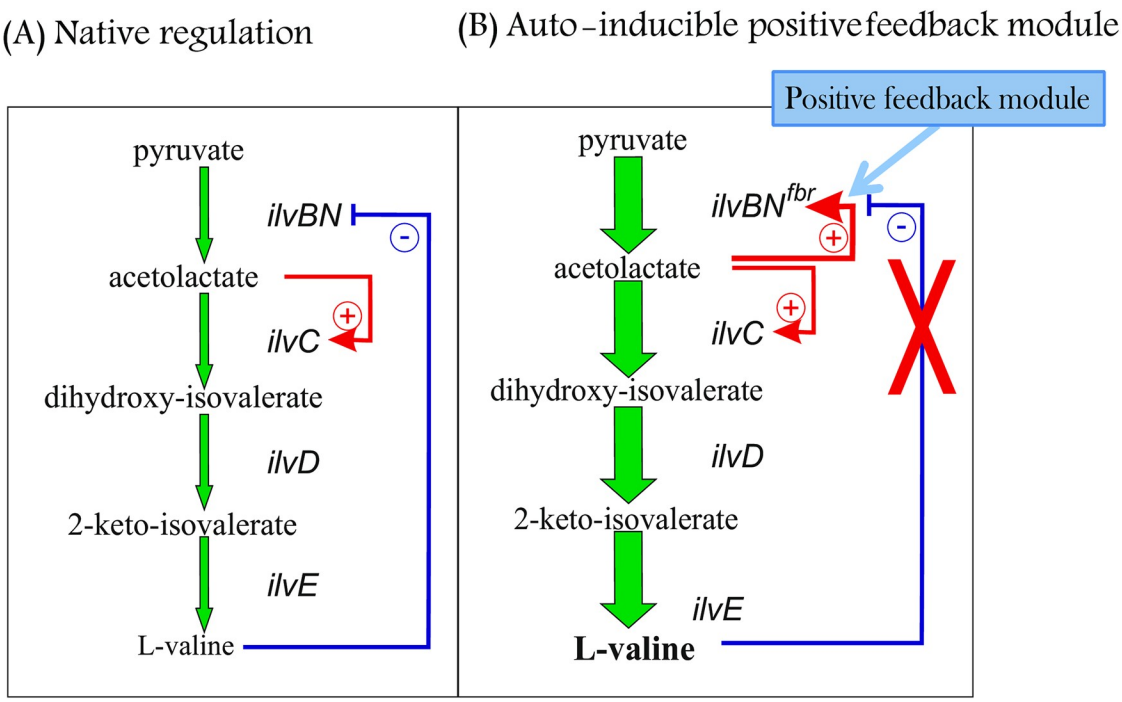

Fig 3. Schematics of L-valine biosynthesis. a) Native regulation. Negative feedback control: inhibition of AHAS I (IlvBN) activity by the pathway end product, L- valine. Positive control: AL induces its own utilization by KARI (IlvC). **b) Autoinducible positive feedback module.** Elimination of feedback control by protein modification (IlvBN^{fbr}). Positive feedback loop: autoinducibility of AL synthesis due to the replacement of *ilvBN*^{fbr} regulatory region with an *ilvY*-P$_{ilvC}$ unit. Accumulation of AL leads to the activation of transcription from P$_{ilvC}$ and increased AHAS synthesis. Thus, AL activates its own synthesis and utilization.

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was replaced with the regulatory region of the *ilvC* gene. Thus, the product of the AHAS-mediated reaction, AL, activated the transcription from the P$_{ilvC}$ promoter and increased AHAS I synthesis.

Since the native regulation of the *ilvBN* operon includes the attenuation of transcription by L-Val and L-Leu and seems to be not suitable for overproduction of L-Val, we decided to compare the performance of autoinducible expression modules based on the regulatory region of the *ilvC* gene with that of the"strong" constitutive promoter P$_L$.

Both variants of transcriptional control, constitutive by the cassette P$_L$-*ilvBN*^{fbr} or metabolic by the cassette *cat*-*ilvY*-P$_{ilvC}$-*ilvBN*^{fbr}, provided the similar level of AHAS I activity (Table 4).

Table 3. Production of L-valine by modified *E. coli* strains harboring AHAS-encoding genes as a part of different expression units.

Strain	*ilvBN*^{fbr} allele	OD$_{540}$	Production of L-Val, g/L
K12	no; only native AHAS	30.0	≤0.1
K12 2Δ P$_L$-*ilvBN*^{fbr}	1 copy; native locus; under control of 'strong' constitutive P$_L$ promoter	20.7	4.3 ± 0.7
K12 2Δ P$_L$-*ilvBN*^{fbr}:: *cat*-P$_L$-*ilvBN*^{fbr}	2 copies; native locus + Δ*ppsA* locus; both are under control of 'strong' constitutive P$_L$ promoter	21.2	4.0 ± 0.4
K12 2Δ P$_L$-*ilvBN*^{fbr} ::*cat*	1 copy; native locus; under control of 'strong' constitutive P$_L$ promoter	21.6	4.4 ± 0.4
K12 2Δ *cat*-*ilvY*-P$_{ilvC}$-*ilvBN*^{fbr}	1 copy; native locus; under control of *ilvY*-P$_{ilvC}$-based positive feedback regulatory element	21.6	6.1 ± 0.5

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Table 4. Activity of AHAS I in *E. coli* strains harboring AHAS-encoding genes as a part of different expression units.

Strain	AHAS I activity, nmol/min*mg
K12 2Δ P_L-*ilvBN*^{fbr}	93 ± 43
K12 2Δ P_L-*ilvBN*^{fbr} ::*cat*-P_L-*ilvBN*^{fbr}	83 ± 15
K12 2Δ P_L-*ilvBN*^{fbr} ::*cat*	114 ± 12
K12 2Δ *cat*-*ilvY*-P_{ilvC}-*ilvBN*^{fbr}	90 ± 37

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Nevertheless, the latter was preferable from the viewpoint of the final product accumulation, L-Val production was increased by more than 40% (Table 3). The enzyme activity level itself does not give enough information about transcription from corresponding promoters. Difference in enzyme activity can be caused by other reasons and particularly by the structure of the 5'-end of mRNA, which defines the ribosome binding. Nevertheless, for both expression cassettes, P_L-*ilvBN*^{fbr} and *ilvY*-P_{ilvC}-*ilvBN*^{fbr}, the calculated TIRs were similar (458 for the former and 482 for the latter).

Notably, a further increase in *ilvBN*^{fbr} expression by the introduction of an additional copy of P_L-*ilvBN*^{fbr} construct did not increase AHAS activity and L-valine production by K12 2Δ P_L-*ilvBN*^{fbr} (Tables 3 and 4). Most likely, an excess of AHAS I accumulated in the insoluble fraction [43]. Therefore, metabolic control of *ilvBN*^{fbr} expression seems preferable to the 'strong' promoter control.

The K12 2Δ *cat*-*ilvY*-P_{ilvC}-*ilvBN*^{fbr} strain contains two copies of *ilvY*: one in its native locus and one as a part of the *cat*-*ilvY*-P_{ilvC}- *ilvBN*^{fbr} cassette. To exclude the possibility that the positive effect on L-Val production from the introduction of the cassette *cat*-*ilvY*-P_{ilvC}-*ilvBN*^{fbr} was related to the amplification of positive regulator IlvY, we inactivated the additional copy of the *ilvY* gene that had been introduced upstream of the AHAS I genes. The copy in the upstream part of the *ilvBN*^{fbr} operon was precisely inactivated due to the introduction of a point mutation. A comparison of the resulting strain K12 2Δ *ilvY*^{inactive}-P_{ilvC}-*ilvBN*^{fbr} with the initial strain revealed that the inactivation of the additional copy of *ilvY* gene did not essentially influence L-Val accumulation (Table 5). Moreover, the precise elimination of the *ilvY* gene in its native locus also had no negative effect on L-Val production by K12 2Δ *cat*-*ilvY*-P_{ilvC}-*ilvBN*^{fbr} (Table 5). Therefore, the usage of a P_{ilvC}-based PFM for *ilvBN*^{fbr} operon expression, in addition to the amplification of the positive regulator *ilvY* gene, can be considered a reason for the strain improvement by the *cat*-*ilvY*-P_{ilvC}-*ilvBN*^{fbr} cassette.

Strategies based on the usage of PFM are not limited to the IlvY\AL\P_{ilvC} autoinducible module. PFMs as described herein can be designed based on other LysR-type regulatory elements. For example, cysteine biosynthesis could be modified via an artificial PFM by using the O-acetyl-L-serine (OAS)/CysB-inducible promoters such as P_{cysP} and P_{cysK} for the autoinducible expression of genes encoding key enzyme(s) of cysteine biosynthesis (particularly, OAS biosynthesis), e.g., feedback-resistant serine acetyltransferese (CysE^{fbr}). The regulatory factor CysB in complex with OAS activates the transcription of genes involved in high energy-

Table 5. Production of L-valine by modified *E. coli* strains harboring P_{ilvC}-based PFM for *ilvBN*^{fbr} operon expression and different copy numbers of the positive regulator IlvY.

Strain	*ilvY* allele	OD_{540}	Production of L-Val, g/L
K12 2Δ *cat*-*ilvY*-P_{ilvC}-*ilvBN*^{fbr}	2 copies; native locus + upstream *ilvBN*^{fbr} operon	21.5	5.7 ± 0.5
K12 2Δ *ilvY*^{inactive}-P_{ilvC}-*ilvBN*^{fbr}	1 copy; native locus	22.7	5.7 ± 0.5
K12 2Δ Δ*ilvY cat*-*ilvY*-P_{ilvC}-*ilvBN*^{fbr}	1 copy; upstream *ilvBN*^{fbr} operon	20.5	5.5 ± 0.5

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consuming sulfate consumption and further reduction processes, which are undesirable in the absence of the OAS acceptor molecule [44–47]. Therefore, introduction of an artificial element containing the gene encoding $CysE^{fbr}$ under the control of the OAS/CysB-activated promoter can lead to PFM formation and allow the use of CysB\OAS\P_{cysP} (or P_{cysK}, P_{cysD}, etc) as autoinducible regulatory elements. At the same time, since CysB regulon includes a wide range of genes, sometimes with unknown function, the application of OAS/CysB-based PFMs for industrial strain breeding seems a rather complicated task at this level.

Another PFM could be designed by using the regulatory region of the *metE* gene containing the *metR* gene, which encodes a LysR-type regulator, and the coupled divergent promoters P_{metR} and P_{metE}. The P_{metE} promoter is regulated by the L-homocysteine/MetR complex [48]. Thus, such a kind of hypothetical PFM, MetR\L-homocysteine\P_{metE}, can include the *metE* gene's regulatory region to positively control the synthesis of L-homocysteine, an L-methionine precursor.

Conclusions

In bacterial cells, many regulatory mechanisms are involved in negative feedback circuits that control the biosynthesis of metabolites, thereby preventing their excessive production, which is undesirable under certain conditions. Meanwhile, the activation of gene expression occurs in response to environmental or intracellular signals that indicate a necessity for adaptation to changing conditions (catabolism or transport of compounds, coordinated synthesis of separate structural elements in common biosynthetic pathways, stress defense, etc). In a native prokaryotic cell, examples of positive feedback circuits are rather rare and practically limited by signal transmission, such as "quorum sensing" [49–51]. In contrast, in artificial biological systems aimed to overproduce a target compound, such a strategy can be realized.

Here, we demonstrated an artificial way to regulate L-valine biosynthesis (Fig 3). As a first element of this artificial PFM, we used the modified AHAS I $IlvBN^{fbr}$, which is an acetohydroxy acid synthase that is resistant to feedback inhibition. This element allows negative feedback control by the end product to be avoided. Replacement of the regulatory region of the $ilvBN^{fbr}$ genes, which encode a modified AHAS I, with the regulatory region of the *ilvC* gene resulted in an artificial positive feedback loop. In this case, a portion of the AHAS I catalyzed the formation of reaction product, AL, which then simultaneously acted as a co-inducer with the regulator IlvY, induced transcription of the $ilvBN^{fbr}$ operon and, thus further enhanced its own synthesis. At the same time, AL induced its own utilization by KARI according to the native regulatory mechanism. Thus, another portion of AL or AHB can be converted into an end product of the branched chain L-amino acid (L-valine, L-leucine or L-isoleucine) pathways.

The accumulation of AL, a product of the AHAS-mediated reaction, will thus lead to the activation of transcription from P_{ilvC} and increased AHAS synthesis. Therefore, AHAS synthesis is activated by its own product. Such an autoinducible PFM can supply an appropriate level of AHAS and the consequent AL, the precursor of the final product, L-valine. Introduction of such a PFM leads to L-Val overaccumulation and may have a practical impact.

The described strategy based on the usage of PFM is not limited to BCAA biosynthesis and could be applied for the breeding of industrial strains producing other essential metabolites, which broadens the set of metabolic engineering tools.

Supporting information

S1 Table. Sequences of the PCR primers used in this study.
(DOCX)

S2 Table. Activity of β-galactosidase LacZ in strains harboring the expression cassette *cat*-P_{tac}-*lacZ* in various genetic background.
(DOCX)

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