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Joint antisense RNA strategies for regulating isoprene production in *Escherichia coli*

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Abstract: Isoprene (C_5H_8) is a key chemical ingredient for the production of synthetic rubber and plastic. Isoprene is commonly petro-chemically produced. Enabling a sustainable microbial fermentation for isoprene production from the potential biofuel is an attractive alternative to the original derivation. To this end, antisense RNA strategies, for redirection of weakening genes and control of metabolic pathways, were introduced to regulate the isoprene production. The isoprene titer, the intermediates of the methylerythritol 4-phosphate (MEP) pathway at the metabolic level and mRNA at the transcriptional level were all successfully affected as a consequence of simultaneous weakening of the farnesyl diphosphate synthase (ispA), octaprenyl diphosphate synthase (ispB) and undecaprenyl pyrophosphate synthase (ispU) of the MEP pathway in *Escherichia coli BL21 (DE3)*. The finally obtained strain IAUB accumulated isoprene up to 16 mg/L in a flask culture, which was about eight times of what was achieved by the control strain Idi. Detailed knowledge about the mechanisms of the novel strategies may benefit the development of many other bio-derived products.

Keywords: antisense RNA strategies, isoprene, *Escherichia coli*, MEP pathway

1. Introduction

Shortage of energy and increasing global environmental concerns are problems that require immediate attention. Over the past few decades, many chemicals are still synthesized from petroleum, causing environmental problems. In nature, non-petroleum derived isoprene is synthesized by the enzyme isoprene synthase (IspS), which converts dimethylallyl diphosphate (DMAPP) to isoprene and pyrophosphate [1]. A large amount of isoprene is released by woody plants and deciduous broad-leaved trees into the atmosphere [2, 3]. Due to an increasing global need for isoprene and a simultaneous environmental concern, many researchers and companies have used genetic engineering techniques to develop microorganisms possessing a plant-like isoprene metabolic pathway to enable conversion of sugar into isoprene.

DMAPP and its isomer isopentenyl diphosphate (pyrophosphate; IPP), the precursors of isoprene, are basic components of all isoprenoids, and are synthesized using two different pathways: the methylerythritol 4-phosphate (MEP) pathway and the mevalonate (MVA) pathway (Fig.1) [4]. For several decades, the mevalonate pathway was believed to be the unique source of isoprenoid building blocks. Later, the inconformity of certain biosynthetic data with the mevalonate paradigm was independently recognized by Rohmer, Arigoni and their respective co-workers [5-7]. Isoprenoids are highly variable natural compounds, all synthesized using either of these two pathways. The structural diversity of terpenes is also immense, including acyclic, monocyclic and polycyclic compounds [8], and the numerous terpenoids have

important medical aspects. Isoprene is the simplest terpenoid and its production has been assessed after introducing the IspS gene as characterized from poplar species into *Escherichia coli BL21 (DE3)* [9]. The IspS gene derived from *Populus alba* was chemically synthesized after codon optimization and modification of the native MEP pathway. Some reports show an enhancement of the isoprene production by over-expression of the key genes of the MEP pathway [10] and by construction of the MVA pathway [11] in *E. coli*. These strategies have proven to be successful redirections [12, 13]. However, there are no reports on the regulation of the native MEP pathway for enhancement of the isoprene production using joint antisense RNA strategies to down-regulate the lower pathway.

Farnesyl diphosphate synthase (ispA) [9], octaprenyl diphosphate synthase (ispB) [14] and undecaprenyl pyrophosphate synthase (ispU) [15], relating to the isoprenoids synthesis, are essential genes that cannot be knocked out from the lower MEP pathway in *E. coli*. The joint antisense RNA strategies can down-regulate multi genes through antisense RNA molecules hybridizing with complementary mRNA transcripts. Therefore, more precursor DMAPP was transformed to isoprene. Studies have focused on either determining the effectiveness of different antisense RNA strategies or on elucidating the role of a structural gene targeted for down regulation [16-19].

In this paper, based on our previous experiments, the efficiency of joint antisense RNA strategies on isoprene production has been improved. Each 620 bps single DNA with the putative ribosome binding sites of the three genes was inversely inserted into the plasmid pRSF-Idi supplied with the T7 promoter, T7 terminator and the native

ribosome binding site removed. The three weakening cassettes were put into the same plasmid pRSF-Idi in order to obtain the joint weakening plasmid. The constructed plasmids were co-transformed into *E. coli BL21 (DE3)* with the pETD-DXS-IspS plasmid. The final genetic strain IABU accumulated isoprene up to16 mg/L after flask shaking, which was approximately an 8-fold increase in isoprene production compared with the control strain Idi. The strain IABU also displayed favourable effects both on the transcriptional level and the metabolic level.

Figure 1

2. Materials and methods

2.1 Reagents, strains, plasmids and media

Restriction enzymes and ligase were purchased from New England Bio-labs (Ipswich, MA). Primer STAR HS and Ex Taq DNA Polymerase and other enzymes were obtained from TAKARA (Dalian, China). The genomic DNA isolation kits, plasmid extraction kits and gene retrieval kits were offered by OMEGA (USA). The DNA recovery kit was purchased from Biomed (Beijing, China). Isoprene standard (Sigma-Aldrich) and DMAPP standard (Sigma-Aldrich) were used for qualitative and quantitative analysis. All other chemicals used in this study were of analytical or chromatographic grade and obtained from the Beijing Chemical Company (Beijing, China).

LB medium (5 g /L yeast extract powder, 10 g /L tryptone, 10 g /L NaCl) was used

for cloning and shake-flask fermentation. Optimized TB medium (1.2% (W/V) tryptone, 2.4% (W/V) yeast extract, 0.4% (V/V) glycerol, 17 mM KH_2PO_4, 72 mM K_2HPO_4) and M9 media (Na_2HPO_4 33.7 mM, KH_2PO_4 22.0 mM, NaCl 8.55 mM, NH_4Cl 9.35 mM, $MgSO_4$ 1 mM, $CaCl_2$ 0.3 mM, biotin 1 μg, thiamin 1 μg, EDTA 0.134 mM, $FeCl_3$-$6H_2O$ 31 μM, $ZnCl_2$ 6.2 μM, $CuCl_2$-$2H_2O$ 0.76 μM, $CoCl_2$-$2H_2O$ 0.42 μM, H_3BO_3 1.62 μM, $MnCl_2$-$4H_2O$ 0.081 μM) were prepared as described [20]. Ampicillin (100 mg /ml) /kanamycin (50 mg /ml) were added into the media as needed.

All plasmids listed in Table 1 were sequenced to verify the cloning accuracy.

Table 1

2.2 Cloning of genes and construction of the plasmids

The plasmids of the weakening genes were constructed on the previous plasmid pRSF-Idi [9]. Including the 20 bps upstream of the ispA, ispB and ispU genes ATG start codon and the putative ribosome binding site, 620 bps or 635 bps of target sequence were inversely inserted into the pRSF-Idi plasmid after the native ribosome binding site was removed. The PCR was performed in a Mastercycler Personal (Eppendorf) with Primer STAR HS DNA Polymerase. All primers and restriction enzyme cutting sides are listed in Table 2. Every fragmentary antisense DNA of ispA, ispB and ispU was inserted into an expression cassette with T7 promoter and T7 terminator and without ribosome binding site. The constructed plasmids and genotypes of the

recombinant strains are shown in Fig 2.

Table 2

Figure 2

2.3 Flask culture of recombinant strains and analysis of isoprene production with GC-MS

Single colonies of *E. coli BL21 (DE3)* harboring different recombinant plasmids were picked from the solid LB culture media into 4 ml liquid LB media. The cultures were inoculated into 20 ml M9 media in flasks after 12-16 h cultivating at 37℃. Then, 3 ml cultures were transformed and induced by 0.8 mM isopropyl β-D-thiogalactoside (IPTG) until reaching an OD600 of 0.6 in 20 ml airtight bottles at 30℃. After 20 hours, the produced isoprene was examined by GC-MS.

Vapor samples from the sealed headspace of the transformed strains were injected with an automatic headspace injector and examined by GC-MS (Thermo Fisher, Trace ISQ). A TG-WAXMS column (30 m × 0.25 mm; 0.25 μm film thickness) was used, with 1 ml /min helium as the carrier gas. The temperature of the EI detector was 250℃. The samples were incubated at 60℃, shaken for 10 seconds and stirred 10 seconds, 10 minutes before injection with the headspace needle at 70℃. The sample drawn was 0.5 ml and split after the column in a ratio of 1/50. The following oven temperature program was carried out: the initial temperature was 40℃, then increased to 150℃ at 20℃/min, and finally remained at that temperature for 1 min. The

157 injector was maintained at 200℃.

158

159 **2.4 Extraction of cellular metabolites and detection of DMAPP and GPP with**

160 **HPLC–MS**

161 LC-MS analysis was used to determine intracellular levels of IPP /DMAPP and

162 GPP in the strains Idi and IAUB. After 0.8 mM IPTG induction, 4 ml cell culture was

163 harvested by centrifugation, and the cell pellets were lysed with 1 ml 50% methanol

164 /water solution at the temperature of -40℃. The combined solutions were kept at

165 -80℃, lyophilized and resuspended in 360 μl 50% methanol /water and subjected to

166 LC-MS analysis [21].

167 The extracted cellular metabolites were determined using HPLC–MS. The HPLC

168 system consisted of a Surveyor quaternary gradient pump, a vacuum degasser, a

169 column temperature controller, and an autosampler (Agilent Technologies 1200

170 Series). A 4.6 × 150 mm Agilent Extend-C18 column with the temperature

171 maintaining at 20 ℃ was used. The intermediates DMAPP and GPP of the pathway

172 were separated by a linear gradient between solution A (H_2O: $NH_3.H_2O$, 20: 1) and

173 solution B (acetonitrile: H_2O, 4:1). The gradient was as follows 0–3 min, 65% A to

174 65% A; 3–10 min, 65% A to 20% A; 10–15 min, 20%A to 65%; equilibration with

175 65% A. The flow rate was set at 0.8 ml /min and 10 μl of sample was injected onto

176 the column, producing an inlet flow into the tandem mass spectrometer of 100 μl/min.

177 The total analysis time, including the equilibration, was 20 min for each analysis.

For the detection of intermediate, a TSQ Quantum AM (Agilent.) was applied, and operated in the negative electrospray ionization mode and with the mass spectrometer in multiple reaction monitoring (MRM) mode. Nitrogen and argon were used as the nebulizing gas and the collision gas, respectively at a pressure of 1.8 mTorr. The ion spray voltage was set at -3500 V, and the capillary temperature was 300℃. The collision cell energy was optimized for each particular intermediate of the isoprenoid biosynthesis pathway [22].

2.5 **Quantification of mRNA and ssRNA levels**

An RNA sample Total RNA Kit (TianGen) was used to isolate RNA from the IPTG-induced bacterial cells of Idi and IAUB. After the total RNA concentration was measured using the Biophotometer (Eppendorf), cDNA was synthesized with random primers using reverse transcriptase (TianGen) and the above mentioned RNA as template. To quantify the ispA, ispB and ispU relative to the mRNA and ssRNA levels, each gene was designed to two primers listed in Table 2, one for the inversely inserted DNA and the other for the left genome gene. The synthesized cDNA was amplified to quantify the copies using an UV spectrophotometer with primers. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an internal reference to eliminate differences between samples, and the primers for the GAPDH reference are also listed in Table 2. The QPCR reactions were performed with 2 μl of cDNA product in a 20-μl reaction system with Sybr Green I (TianGen) in the real-time Analytik Jena fluorescence PCR instrument. Each sample was measured in

triplicate, and the results reported herein are the averages of the replicates.

3. Results and discussion

3.1 Analysis of isoprene production and functions of weakening genes

From the titer of isoprene as shown in the Fig. 3, we can see that it enhanced a little when the separate gene was weakened while the three genes joint weakening strain IAUB achieved a significant increase in comparison with the strain Idi. Isoprene titer of the recombinant strain IAUB was up to 16 mg/ L, about 8 times higher than that of Idi.

Figure 3

3.2 The effect of joint weakening genes on transcription level

The relative mRNA levels in both the control *E. coli BL21 (DE3)* Idi and the *E. coli BL21 (DE3)* ABU recombinant are listed in Fig. 4. The mRNA levels of ispA, ispB and ispU in the engineered strains were normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and the normalized mRNA levels of ispA, ispB and ispU in the *E. coli BL21 (DE3)* Idi were assumed to be 1.0. As shown in Fig. 4, the total relative mRNA and ssRNA were much higher than those in the control strain Idi. Obviously, the mRNA relative levels of ispA, ispB and ispU in *E. coli BL21 (DE3)* ABU were almost 35%, 42% and 49% lower than those in the control strain *E. coli BL21 (DE3)* Idi, respectively. Every weakening gene's transcription was quantified by QPCR with two pairs of primers: one is used to

quantify mRNA transcribed by the native gene, and the other is used to quantify the total of the mRNA and ssRNA transcribed by the inverse target fragments of the plasmid pRSF-ispA-ispU-ispB-Idi. In fact, the mRNA+ssRNA levels of Idi were the mRNA levels because there were no inverse target fragments in the strain Idi. Also, ssRNA levels of AUBI increase significantly relating with the copies of the plasmid pRSF-ispA-ispU-ispB-Idi and the strong T7 promoter.

From quantification of the mRNA level, there was a distinct effect of weakening the genes at the transcriptional level. The decrease in mRNA content resulted in an effective decrease in the expression of the target genes.

Figure 4

3.3. The effect of joint weakening genes on intermediates IPP/DMAPP and GPP

To judge from quantitative analysis of the intermediates IPP/DMAPP and GPP, there were an obvious accumulation of IPP/DMAPP and a significant decrease of GPP after the genes ispA, ispB and ispU had been weakened. There should hence be more DMAPP to be transformed into isoprene by the gene IspS: the improvements observed were due in part to carbon flux redirection as designed. We can conclude that the antisense RNA strategies successfully induced the disturbance of the intermediates.

Figure 5

Both the two genes ispB and ispU need to be down regulated on top of ispA

down regulation for a better yield of IPP. When there was only one of the three genes weaken, the isoprene accumulation was enhanced a little or almost no enhancement, while it was significantly enhanced when they were weakened at the same time. Genes weakening just makes the genes expression and metabolism slow down and down-regulated. If the down pathway of consumption IPP is down-regulated four times, the IPP accumulates more than that of only ispA down regulation. If the gene ispA was knockout, ispA down regulation itself would be enough to block IPP consumption. Therefore, only the three genes were simultaneously weakened, the consequence was significantly achieved.

Metabolic engineering of various pathways is a common strategy to improve the production of high value-added metabolites. It has been suggested that over-expression and deletion of some genes are the traditional strategies. The antisense RNA strategies applying the repression strategy have rarely been used in metabolic engineering. This study can provide a basis for further fine tuning of the native MEP pathway in *E. coli* aiming at more efficient production of isoprenoids. Comparing the differences between the MVA and MEP pathways, the MEP pathway shows a significant superiority regarding stoichiometry, energy consumption, and the equilibrium between oxidation and reduction of glucose to IPP/DMAPP conversion [23]. Therefore, we continued to choose manipulating the native MEP pathway to enhance the isoprene production. In this work, as a follow-up to our previous systematic study of over-expression the three key genes, we have focused on decreasing the consumption of the precusor DMAPP to other isoprenoids in order to enhance the isoprene production. It showed the push trends to isoprene from the two directions, with the key genes' over-expression of up pathway and the reductases' repression of

271 the low pathway. Therefore, we applied two strategies to enhance the isoprene
272 production. The three genes ispA, ispB and ispU were weakened by inverting a
273 contiguous sequence inversely, separately and jointly.

274 Recently, the antisense RNA strategies were used in metabolically engineered
275 strains of *Clostridium acetobutylicum* [12] and *Klebsiella pneumonia* [24]. *Wang*
276 *Miaomiao* et.al [24] successfully applied the strategies to produce 1-butanol and
277 down-regulated by-products in *K. pneumonia* fermentation, with the resulting titer of
278 1, 3-propanediol and 2, 3-butanediol reduced by 81% and 15%, respectively. Desal
279 Ruchir P. et.al [12] obtained the strain ATCC 824(pRD4) exhibiting 85 to 90% lower
280 butyrate kinase (BK) and acetate kinase specific activities, strain ATCC 824(pRD1)
281 exhibiting 70 and 80% lower phosphotransbutyrylase (PTB) and BK activities than
282 the control strain. In this report, it is shown that they can also be used to regulate the
283 isoprenoids pathway in *E. coli*.

284 The antisense RNA strategies are very useful and effective for weakening and
285 silencing of the normal expression of some specific genes, thus down regulating the
286 concentration of related metabolites, particularly the products of the essential genes
287 that cannot be knocked out. Kevin et.al [13] exogenously manipulated glucokinase (Glk)
288 through engineered antisense RNA by inverting a contiguous sequence stretch of the
289 glk operon, and they also detected the effect of different lengths of complementarity
290 of objective genes. It was shown that the longer complementarity has a better effect
291 than the shorter ones. Several possible mechanisms have been suggested, for example
292 inhibition of translation because the duplex RNA structure prevents accessing to the

293 ribosome binding site, rapid degradation of the mRNA, possibly by duplex
294 RNA-specific RNases, and inhibition of transcription of mRNA due to premature
295 termination [25- 27]. Although the mechanism of an antisense RNA action is not
296 completely understood, the antisense RNA strategies have been used to down-regulate
297 levels of targeted gene products in prokaryotes [16-19]. These joint antisense RNA
298 strategies are however generally useful and effective in the isoprene production.
299

300 **4. Conclusion**

301 The isoprene production can be regulated using the engineered antisense RNA
302 strategies in *E. coli* based on our previous experiments. Quantification of the
303 produced isoprene was performed by GC-MS with an automatic headspace injector
304 from the previously established SPME-GC determination. From the data obtained, we
305 can conclude that the isoprene production is greatly enhanced when three genes are
306 weakened jointly. The isoprene production of the recombinant strain IAUB was 16
307 mg/ L, about 8 times higher than that of the recombinant strain Idi. The joint antisense
308 RNA strategy is a useful and effective method to accumulate metabolites of particular
309 interest and to decrease the production of certain by-products by control and
310 regulation of the metabolism.
311

312 **Conflict of interest** All the authors declare that they have no conflict of interest.
313

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

Acknowledgments

This work was supported by the State key laboratory of organic-inorganic composites， the National Basic Research Program of China (973 program), (2013CB733600，2012CB725200), the National Nature Science Foundation of China (21390202, 21476017).

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Fig. 1

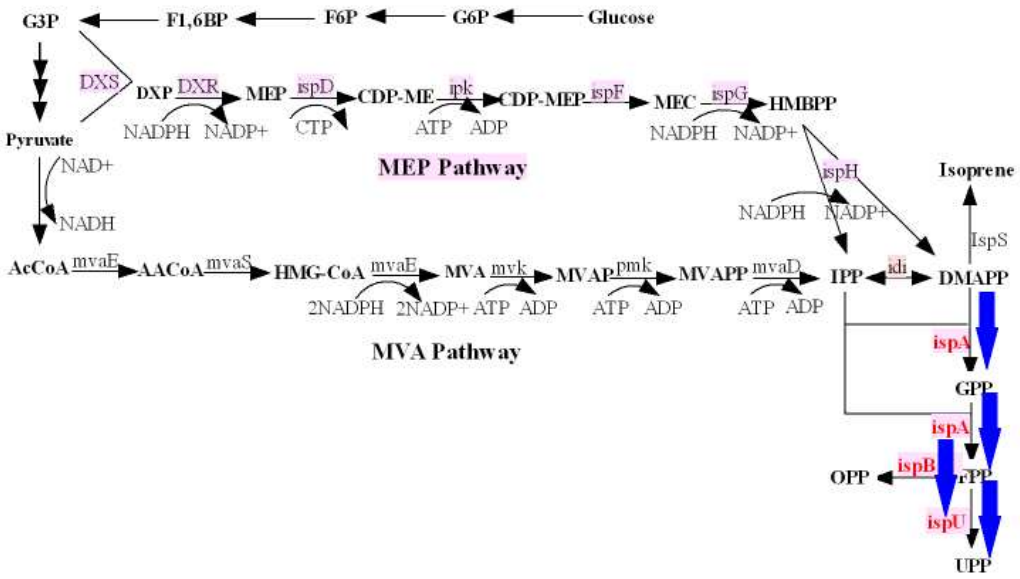

Fig.1 The two isoprenoid pathways to central metabolism, MEP pathway and MVA pathway.

Abreviation		Abreviation	
G6P	glucose 6-phosphate	DXS	1-deoxy-D-xylulose-5-phosphate synthase
F6P	fructose-6-phosphate	DXR	1-deoxy-D-xylulose-5-phosphate reductoisomerase
F16BP	fructose 1,6 diphosphate	IspD	4-diphosphocytidyl-2-C-methyl-D-erythritol synthase
G3P	glyceraldehyde-3-phosphate	ipk	4-diphosphocytidyl-2-C-methyl-D-erythritol kinase
DXP	1-deoxy-D-xylulose 5-phosphate	IspF	2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase
MEP	2-C-methyl-D-erythritol4-phosphate	IspG	1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase
CDP-ME	4-(Cytidine 5'-diphospho)-2-C-methyl-D-erythritol	IspH	1-hydroxy-2-methyl-butenyl 4-diphosphate reductase
CDP-MEP	2-Phospho-4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol	Idi	isopentenyl pyrophosphate isomerase
MEC	2-C-methyl-D-erythritol-2,4-cyclo-diphosphate	IspA	farnesyl diphosphate synthase
HMBPP	(E)-4-hydroxy-3-methylbut-2-enyl-diphosphate	IspS	isoprene synthase
DMAPP	dimethylallyl pyrophosphate	MvaE	3-hydroxy-3-methylglutaryl-coenzyme A reductase
GPP	geranyl diphosphate	MvaS	mevalonate synthase
AcCoA	acetyl coenzyme A (acetyl-CoA)	MVK	mevalonate kinase
AACoA	acetoacetyl-CoA	PMK	phosphomevalonate kinase
HMG-CoA	hydroxymethylglutaryl-CoA	mvaD	diphosphomevalonate decarboxylase
MVA	mevalonate	ispA	geranyltranstransferase
MVAP	mevalonate-5-phosphate	ispB	octaprenyl diphosphate synthase
MVAPP	mevalonate-5-diphosphate	ispU	undecaprenyl pyrophosphate synthase

Fig. 2

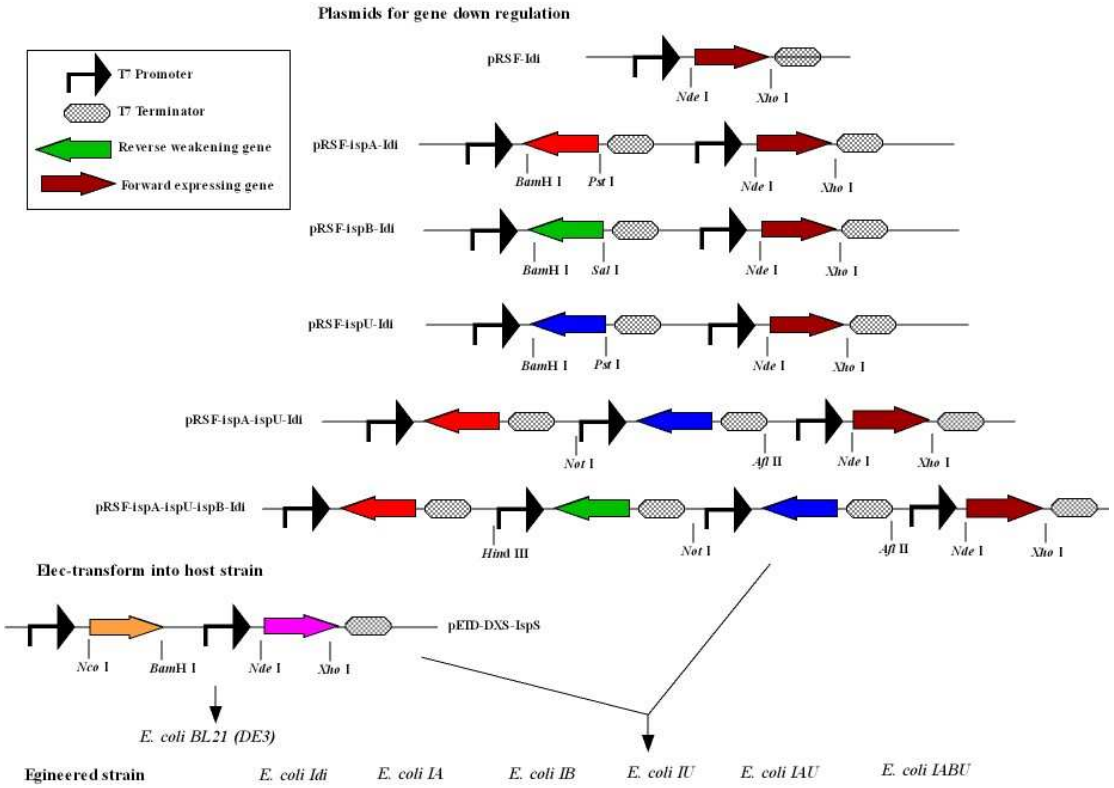

Fig. 2 Strategy for plasmids and strain construction.

Fig. 3

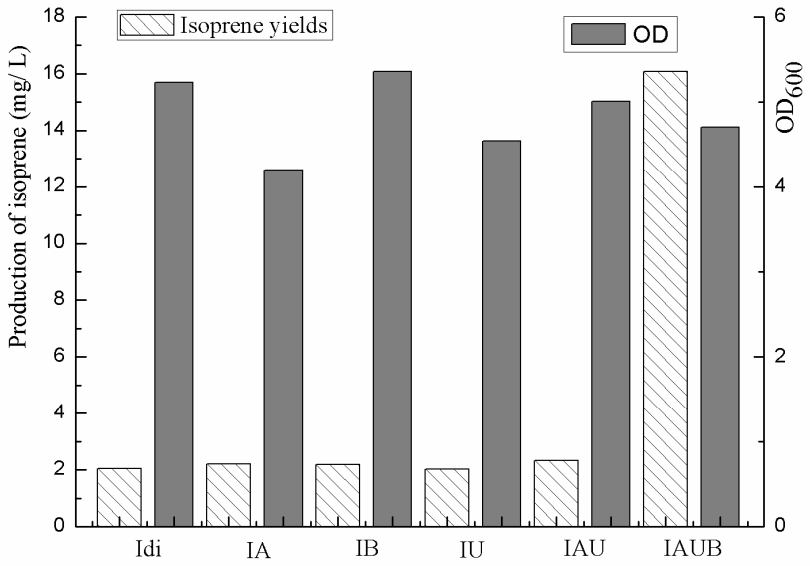

Fig. 3 *Production of isoprene of different strains harboring different plasmids.*

Fig. 4

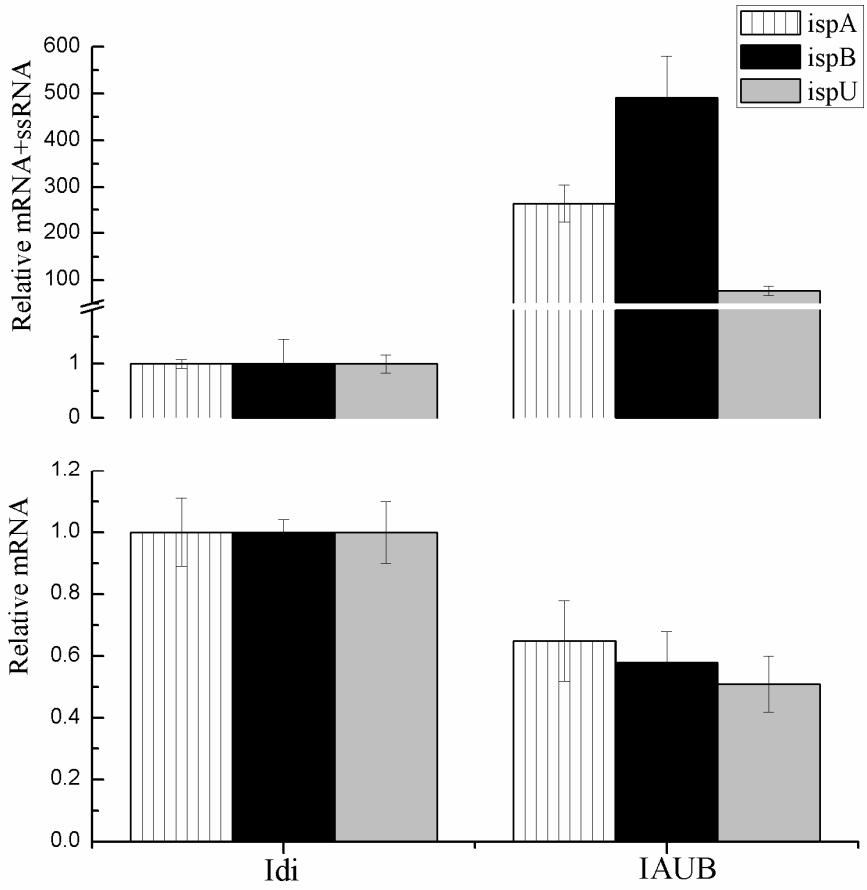

Fig. 4 Characterization at the relative mRNA level and ssRNA of the control strain Idi and joint weakening strain IAUB.

Fig. 5

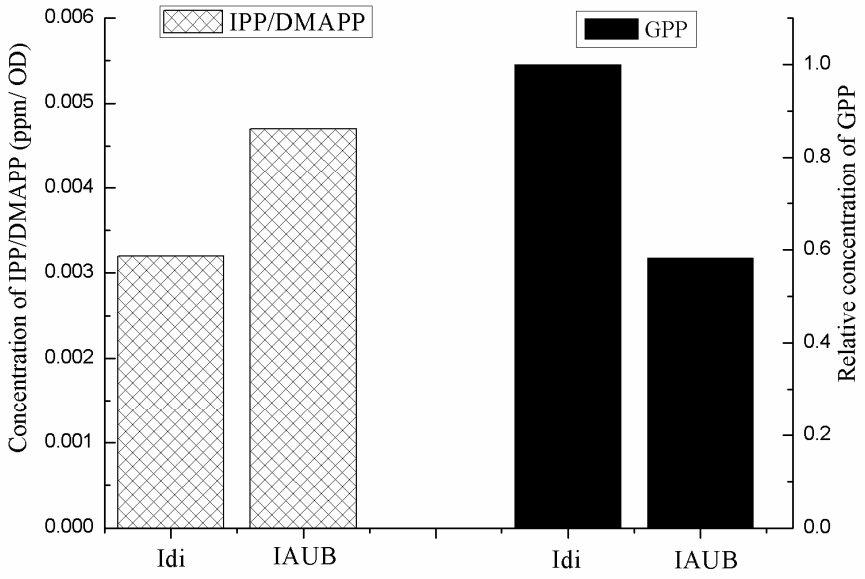

Fig. 5 Relative quantification of extracted cellular metabolites DMAPP and GPP in the control strain Idi and joint weakening strain IAUB.

Table 1 Strains and plasmids used in this study.

Strains or plasmids	Relevant characteristics	Reference or source
Strains		
Escherichia coli TOP10	Applied for harvesting plasmid	Purchased from Biomed
Escherichia coli BL21 (DE3)	Applied for expression genes	Purchased from Biomed
E. coli Idi	pETD-DXS-IspS, pRSF-Idi	DXS-Idi-IspS[(Liu et al., 2014)]
E. coli I-A	pETD-DXS-IspS, pRSF-ispA-Idi	DXS-Idi-IspS-ispA[(Liu et al., 2014)]
E. coli I-B	pETD-DXS-IspS, pRSF-ispB-Idi	This study
E. coli I-U	pETD-DXS-IspS, pRSF-ispU-Idi	This study
E. coli I-A-U	pETD-DXS-IspS, pRSF-ispA-ispU-Idi	This study
E. coli I-A-B-U	pETD-DXS-IspS, pRSF-ispA-ispB-ispU-Idi	This study
plasmids		
pETDUET-1	pBR322 Ori Amp^r	This study
pRSFDUET-1	RSF Ori $Kana^r$	This study
pETD-DXS-IspP	pBR322 Ori Amp^r DXS, IspS	pETDuet-1-Dxs-IspS[(Liu et al., 2014)]
pRSF-Idi	RSF Ori $Kana^r$ Idi	pRSFDuet-1-Idi[(Liu et al., 2014)]
pRSF-ispA-Idi	RSF Ori $Kana^r$ Idi, $ispA^a$	pRSFDuet-1-IDI-ispA[(Liu et al., 2014)]
pRSF-ispB-Idi	RSF Ori $Kana^r$ Idi, $ispB^a$	This study
pRSF-ispU-Idi	RSF Ori $Kana^r$ Idi, $ispU^a$	This study
pRSF-ispA-ispU-Idi	RSF Ori $Kana^r$ Idi, ispA a,$ispU^a$	This study
pRSF-ispA-ispB-ispU-Idi	RSF Ori $Kana^r$ Idi, ispA a,ispB a,$ispU^a$	This study

A^a, the reverse complement of the truncated gene ORF and 20 bps upstream of the start codon. $Kana^r$, Kanamycin resistance; Amp^r, Ampicillin resistance.

Table 2. Primers used in this study.

Genes	Primers	Sequences of oligonucleotides
ispB[a]	ispBa-*Bam*HⅠ-F	cgc**ggatcc**g CCCATAATCCTGCAGGCCTT
	ispBa-*Sal*Ⅰ-R	acgc**gtcgac**CGAAAAGCCCGGCTTTTGCGATG
ispU[a]	ispU-*Bam*HⅠ-F	cgc**ggatcc**g TCAGGCTGTTTCATCACCG
	ispU-*Pst*Ⅰ-R	aa**ctgcag** TGTCAGGGAATAAAAAACGC
T7 Ter	T7R-*Sal*Ⅰ-F	acgc**gtcgac**TCTACTAGCGCAGCTTAAT
	T7R -*Hin*d III-R	ccc**aagctt**ATTGACTACCGGAAGCAGTGTGA
T7-IspB-T7R	T7IspBT7R-*Hin*d III-F	ccc**aagctt**CGGGATCTCGACGCTCTC
	T7IspBT7R-*Not*I -R	ataagaat**gcggccgc**ATTGACTACCGGAAGCAGTGTGA
T7-ispU-T7R	T7ispUT7R-*Not*I –F	ataagaat**gcggccgc**CGGGATCTCGACGCTCTC
	T7ispUT7R-*Afl* II -R	aatg**cttaag**ATTGACTACCGGAAGCAGTGTGA
GAPDH	Qpcr-GAPDH-F	TATGACTCCACTCACGGC
	Qpcr-GAPDH-R	AACCACTTTCTTCGCACC
ispA[a]	Qpcr-ispA-F	GCCTGACCACCGCACATT
	Qpcr-ispA-R	GCTGGCGACGCTTTACAA
ispA	mRNA-ispA-F	GATGTGGTGGGAGATAC
	mRNA-ispA-R	CTGTTCAGCCAGTTGTT
ispB[a]	Qpcr-ispB-F	CAGCACTTTGAGCGAACC
	Qpcr-ispB-R	TTTATCCACACGGCGACT
ispB	mRNA-ispB-F	CGAACAGGGTAACGGTC
	mRNA-ispB-R	CTTCTCGCCAAGGGGTG
ispU[a]	Qpcr-ispU-F	CCTTTTCAGCCAGTTGCCT
	Qpcr-ispU-R	CGTGCGTCTGCGTATTATT
ispU	mRNA-UPPS-F	GTAACTTTTTGCTTTGGC
	mRNA-UPPS-R	TCAGGCTGTTTCATCA