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Bioconversion of methanol to value-added mevalonate by engineered *Methylobacterium extorquens* AM1 containing an optimized mevalonate pathway

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Abstract Methylotrophic biosynthesis using methanol as a feedstock is a promising and attractive method to solve the over-dependence of the bioindustry on sugar feedstocks derived from grains that are used for food. In this study, we introduced and engineered the mevalonate pathway into *Methylobacterium extorquens* AM1 to achieve high mevalonate production from methanol, which could be a platform for terpenoid synthesis. We first constructed a natural operon (MVE) harboring the *mvaS* and *mvaE* genes from *Enterococcus faecalis* as well as an artificial operon (MVH) harboring the *hmgcs1* gene from *Blattella germanica* and the *tchmgr* gene from *Trypanosoma cruzi* that encoded enzymes with the highest reported activities. We achieved mevalonate titers of 56 and 66 mg/L, respectively, in flask cultivation. Introduction of the *phaA* gene from *Ralstonia eutropha* into the operon MVH increased the mevalonate titer to 180 mg/L, 3.2-fold higher than that of the natural operon MVE. Further modification of the expression level of the *phaA* gene by regulating the strength of the ribosomal binding site resulted in an additional 20 % increase in mevalonate production to 215 mg/L. A fed-batch fermentation of the best-engineered strain yielded a mevalonate titer of 2.22 g/L, which was equivalent to an overall yield and productivity of 28.4 mg mevalonate/g methanol and 7.16 mg/L/h, respectively. The production of mevalonate from methanol, which is the initial, but critical step linking methanol with valuable terpenoids via methylotrophic biosynthesis, represents a proof of concept for pathway engineering in *M. extorquens* AM1.

Keywords Methanol · Mevalonate pathway · *Methylobacterium extorquens* AM1 · Pathway engineering · Terpenoid synthesis

Introduction

Industrial biotechnology has great potential to develop a green and sustainable economy and to solve environmental issues by replacing traditional, petroleum-based industries. However, large-scale applications of industrial biotechnology need to find suitable feedstocks, as the current fermentation industry mainly relies on sugars derived from grains that are used as foods. The *OECD-FAO Agricultural Outlook 2014* stated that 12 % of coarse grains was used for the production of biofuels worldwide in 2014 (OECD 2014). In China, the fermentation industry accounted for approximately 20 % of the total grain production in 2013 (National Bureau of Statistics 2014; Shi 2014). Therefore, non-food, or even non-sugar, alternative feedstocks for industrial biotechnology are being exploited, such as lignocellulose-derived sugars (Nieves et al. 2015), the cultivation of microalgae for biofuels (Behera et al. 2015), the conversion of carbon dioxide to higher chemicals by integrating electricity and microbes (Li et al. 2012; Bar-Even et al. 2013), and methylotrophic biosynthesis (Schrader et al. 2009). Abundant and sustainable methane and methanol supplies from diverse chemical or biochemical pathways make methylotrophic cell factories (MeCFs) one of the most promising methods for future industrial

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biotechnology applications, especially after the shale gas revolution (Olah et al. 2009; Schrader et al. 2009). In 2013, the Reducing Emissions using Methanotrophic Organisms for Transportation Energy (REMOTE) project sponsored by the US Department of Energy, which focuses on the conversion of methane to liquid fuels, was initiated (ARPA-E 2013).

Methylotrophs, which have been used to produce amino acids, single-cell proteins, and biodegradable plastics since the 1960s, are of unique practical significance to biotechnology because of their ability to utilize C1 compounds (Schrader et al. 2009). *Methylobacterium extorquens* AM1, a facultative methylotrophic α-proteobacterium, is a model strain in methylotrophic research that is capable of utilizing methanol as the sole carbon and energy source (Anthony 1982). In recent years, the genome of *M. extorquens* AM1 has been sequenced and its central carbon metabolism has been elucidated (Chistoserdova et al. 2003; Kiefer et al. 2008; Xiaofeng 2008; Peyraud et al. 2009). These achievements provided essential knowledge for the genetic engineering of *M. extorquens* AM1 at a systematic level via generally available genetic tools (Marx and Lidstrom 2001, 2002, 2004; Choi et al. 2006; Marx 2008; Chubiz et al. 2013; Kaczmarczyk et al. 2013; Metzger et al. 2013; Borzyskowski et al. 2014). Using such tools, *M. extorquens* AM1 was successfully engineered to produce value-added compounds, such as 1-butanol (Hu and Lidstrom 2014), mesaconic, and methylsuccinic acid (dicarboxylic acids) (Sonntag et al. 2014), and high-quality polyhydroxyalkanoate (PHA) copolymers (Orita et al. 2014) after complex or systematic genetic modifications. Based on these exciting studies, much broader applications can be expected if a variety of synthetic pathways for the production of value-added compounds can be introduced into *M. extorquens* AM1 via appropriate engineering.

Terpenoids, which are used as flavors, fragrances, pharmaceuticals, bulk chemicals, and biofuels, represent the largest and most structurally diverse family of chemicals in nature, consisting of at least 40,000 compounds (Bohlmann and Keeling 2008; Zhang et al. 2011). To synthesize considerable amounts of terpenoids, a commonly used strategy in bacteria is to introduce an exogenous mevalonate (MEV) pathway to bypass the native methylerythritol phosphate (MEP) pathway, thereby avoiding endogenous regulation and enhancing terpenoid production (Martin et al. 2003; Zahiri et al. 2006; Yoon et al. 2009; Wang et al. 2010; Bentley et al. 2014). In the MEV pathway, mevalonate is synthesized from acetyl-CoA (Ac-CoA) and then it is converted to the building blocks of isopentenyl pyrophosphate/dimethylallyl pyrophosphate (IPP/DMAPP) for terpenoid synthesis. Thus, pathway engineering for mevalonate production is a feasible first step as a platform for terpenoid synthesis. To the best of our knowledge, no studies have attempted to synthesize mevalonate from methanol via pathway engineering in *M. extorquens* AM1.

Three enzymes [acetoacetyl-CoA thiolase (AACoAT), hydroxymethylglutaryl-CoA synthase (HMGS), and hydroxymethylglutaryl-CoA reductase (HMGR)] are essential for mevalonate production from Ac-CoA via the MEV pathway. In *M. extorquens* AM1, AACoAT can be expressed natively; thus, heterologous introduction of HMGS and HMGR in *M. extorquens* AM1 could be used to synthesize mevalonate from Ac-CoA using methanol as a feedstock (Fig. 1c). In this study, to introduce the MEV pathway into *M. extorquens* AM1, a commonly used natural operon encoding the top portion of the MEV pathway, as well as an artificially constructed operon, was introduced into the host strain. Mevalonate production by the artificial operon was superior to that of the natural operon, and AACoAT played a key role in enhancing the performance of the heterologous MEV pathway (Fig. 1a). Furthermore, we balanced the heterologous MEV pathway by regulating the AACoAT expression level, which significantly increased mevalonate production (Fig. 1b). A fed-batch fermentation of the best-engineered strain achieved a considerable titer and yield of mevalonate from methanol.

Materials and methods

Materials

Metabolite standards (acetyl-CoA, hydroxymethylglutaryl-CoA (HMG-CoA), and mevalonate) and antibiotics were purchased from Sigma-Aldrich (Shanghai, China). DNA polymerase, restriction enzymes, ligases, and Gibson Assembly Master Mix kits were purchased from New England Biolabs (Beijing, China). Media components were purchased from Sinopharm Chemical Reagent Corporation (Shanghai, China).

Bacterial strains and culture medium

The strains and plasmids used in this study are listed in Table 1. *Escherichia coli* DH5α (Biomed, Beijing, China) was used for plasmid construction, and it was grown at 37 °C in Luria-Bertani (LB) medium; tetracycline (12.5 mg/L) was added to the medium when necessary. *M. extorquens* AM1 was purchased from NCIMB (NCIMB 9133; Aberdeen, Scotland). MC minimal medium, which was developed by our laboratory for fast and consistent growth of *M. extorquens* AM1, was used in this study. Compared with MP minimal medium developed by Delaney et al. (2013), trace metals and chelator were the same in composition and concentration whereas the rest components in MC medium were the same in concentration as Choi medium (Choi et al. 1989). Components of MC medium were stored as solutions A, B,

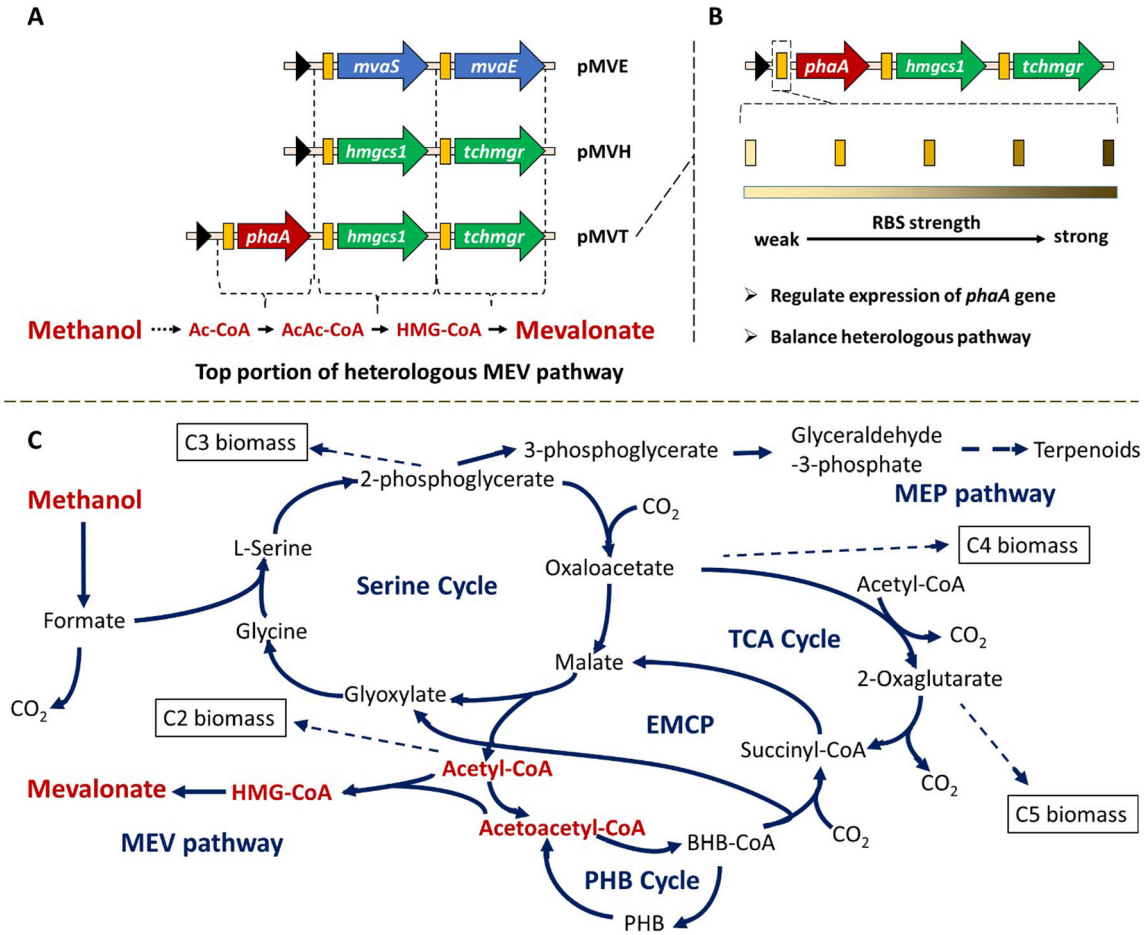

Fig. 1 Construction and balance of the heterologous MEV pathway for high mevalonate production in *M. extorquens* AM1. **a** *Black triangles* represent the P_{mxaF} promoter. *phaA* encodes acetoacetyl-CoA thiolase (AACoAT) from *Ralstonia eutropha*. *mvaS* encodes HMG-CoA synthase (HMGS), and *mvaE* encodes a dual-function protein, with both HMG-CoA reductase (HMGR) and AACoAT activities, from *Enterococcus faecalis*. *hmgcs1* encodes HMGS from *Blattella germanica*, and *tchmgr* encodes HMGR from *Trypanosoma cruzi*, both of which possess the highest reported HMGS and HMGR activities, respectively. **b** Replacement of the RBS in front of the *phaA* gene in the MVT operon with a series of RBSs that differ significantly in strength. **c** The central metabolism and heterologous MEV pathway in the engineered *M. extorquens* AM1 strain for mevalonate production. *Ac-CoA* acetyl-CoA, *AcAc-CoA* acetoacetyl-CoA, *HMG-CoA* hydroxymethylglutaryl-CoA, *MEV* mevalonate, *MEP* methylerythritol phosphate, *TCA* tricarboxylic acid, *EMCP* ethylmalonyl-CoA pathway, *PHB* poly-3-hydroxybutyrate, *BHB-CoA* 3-hydroxybutyryl-CoA

C, and D. The composition of solution A was 75.68 mM $(NH_4)_2SO_4$, 18.26 mM $MgSO_4{\cdot}7H_2O$, and 0.22 mM $CaCl_2{\cdot}2H_2O$ in Milli-Q (EMD Millipore, Billerica, MA, USA) water. The composition of solution B was 45.53 mM $Na_3C_6H_3O_7{\cdot}2H_2O$, 1.2 mM $ZnSO_4{\cdot}7H_2O$, 1.0 mM $MnCl_2{\cdot}4H_2O$, 18 mM $FeSO_4{\cdot}7H_2O$, 2 mM $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 1 mM $CuSO_4{\cdot}5H_2O$, 2 mM $CoCl_2{\cdot}6H_2O$, and 0.33 mM $Na_2WO_4{\cdot}2H_2O$ in Milli-Q water. The composition of solution C was 0.485 mM H_3BO_3 in Milli-Q water. The composition of solution D was 0.959 M KH_2PO_4 and 1.50 M $Na_2HPO_4{\cdot}7H_2O$ in Milli-Q water, adjusted to pH 6.8 with NaOH. To prepare 1 L of MC medium, 100 mL of solution A, 1 mL of solution B, and 1 mL of solution C were first mixed with 878 mL of Milli-Q water. The mixed solution and solution D were sterilized separately. Then, 10 mL of sterilized solution D was added to the mixed solution to obtain the final MC medium. Tetracycline (10 mg/L) was added when necessary. A total of 100 mL of MC medium in a 500-mL baffle flask was used for *M. extorquens* AM1 strain cultivation and mevalonate production. The growth condition of cells was monitored by the optical density (OD) at 600 nm. The standard procedure of *M. extorquens* AM1 strain cultivation was as follows: *M. extorquens* AM1 strains were pre-cultivated in 100 mL of medium containing 1 % (*v*/*v*) methanol at 30 °C on rotary shakers at 170 rpm for about 2.5 days until they reached an OD_{600} of 3.5±0.1. Then, a proper volume of the cells to yield an initial OD_{600} of 0.25 was subcultured in 100 mL of fresh medium containing 1 % (*v*/*v*) methanol. The cultivation was conducted at 30 °C on rotary shakers at 170 rpm for 5 days. During the cultivation process, measurements of the cell growth, methanol concentration, and mevalonate titer were conducted in triplicate every 12 h.

Table 1 Strains and plasmids used in this study

Strains or plasmids	Characteristics	Source
Strains		
AM1-wt	Wild-type *M. extorquens* AM1	NCIMB (NCIMB 9133)
AM1-mve	*M. extorquens* AM1/pMVE	This study
AM1-mvh	*M. extorquens* AM1/pMVH	This study
AM1-mvt	*M. extorquens* AM1/pMVT	This study
AM1-ARBSD	*M. extorquens* AM1/pARBSD	This study
AM1-ARBSU1	*M. extorquens* AM1/pARBSU1	This study
AM1-ARBSU2	*M. extorquens* AM1/pARBSU2	This study
AM1-ARBSU3	*M. extorquens* AM1/pARBSU3	This study
Plasmids		
pCM110	*M. extorquens* expression vector (*PmxaF*, Tc^R)	Marx and Lidstrom (2001)
pMVE	pCM110 (*PmxaF*:: RBS of *PalkB*:*mvaS*:: RBS of *PalkB*::*mvaE*)	This study
pMVH	pCM110 (*PmxaF*:: RBS of *PalkB*::*hmgcs1*:: RBS of *PalkB*::*tchmgr*)	This study
pMVT	pCM110 (*PmxaF*:: RBS of *PalkB*::*phaA*:: RBS of *PalkB*::*hgmcs1*:: RBS of *PalkB*::*tchmgr*)	This study
pARBSD	pMVT with RBS in front of *phaA* gene replaced by RBS of pCM110	This study
pARBSU1	pMVT with RBS in front of *phaA* gene replaced by RBS of segB	This study
pARBSU2	pMVT with RBS in front of *phaA* gene replaced by RBS of gp46	This study
pARBSU3	pMVT with RBS in front of *phaA* gene replaced by RBS of gp4	This study

DNA manipulations

The protein sequences used in this study were retrieved from GenBank, and the accession numbers were follows: AACoAT form *Ralstonia eutropha* (GenBank: CAJ92573.1); HMGCS1 from *Blattella germanica* (GenBank: P54961); MvaS from *Enterococcus faecalis* (GenBank: AAG02438.1); soluble tHMGR, HMGR from *Trypanosoma cruzi* (GenBank: EAN94850.1) without the N-terminal membrane anchor (Peña-Díaz et al. 1997); and MvaE from *E. faecalis* (GenBank: AAG02439.1). Genes encoding these enzymes were optimized in the Gene Designer software according to the codon usage frequency of *M. extorquens* AM1 using a critical frequency of 0.12. Accession numbers of the codon-optimized genes were as follows: *phaA* (GenBank: KR911779), *mvaS* (GenBank: KR911780), *mvaE* (GenBank: KR911781), *hmgcs1* (GenBank: KR911782), and *tchmgr* (GenBank: KR911783). The *mxaF* promoter (Marx and Lidstrom 2001) was used to induce operon expression. DNA sequences of the MVE and MVH operons, with the ribosomal binding site (RBS) of promoter P_{alkB} from plasmid pCom10 (5′-TTGGAGAATTCCAT-3′) placed in front of each gene, were directly synthesized by DNA2.0 (Menlo Park, CA, USA). *Hin*dIII and *Kpn*I restriction sites were placed at the 5′ and 3′ ends of the sequences, respectively. After digestion, fragments of the MVE and MVH operons were ligated to *Hin*dIII/*Kpn*I-digested pCM110 to construct the plasmids pMVE and pMVH, respectively. To construct pMVT, the optimized *phaA* gene (encoding AACoAT from *R. eutropha*) was synthesized and used as template for PCR amplification with the primers Pr-alkB-phaA-F/phaA-alkB-hmgs-R (Table S1). Then, pMVH was used as template for the amplification of the promoter and MVH operon using primers pCM110-*Hin*dIII-F/Pr-alkB-phaA-R (Table S1) and phaA-alkB-hmgs-F/*Kpn*I-pCM110-R (Table S1). The above three fragments were assembled with *Hin*dIII/*Kpn*I-digested pCM110 via Gibson assembly. The constructions of pARBSD, pARBSU1, pARBSU2, and pARBSU3 were similar to those of pMVT, although different primers with corresponding RBSs in the flanking regions were used to amplifying the promoter and *phaA* gene by PCR (Table S1). The sequences of the RBSs used were as follows: the RBS of pCM110 in pARBSD (5′-GATCCTAAGGGCGAATTC-3′), RBS of *segB* in pARBSU1 (5′-ATACTCTTAGAAATAGGAGATATTT-3′), RBS of *gp46* in pARBSU2 (5′-AGATACATTAAAAGGAATCTTTT-3′), and RBS of *gp4* in pARBSU3 (5′-ATTATCTATTTA TAAGGAGAATCCA-3′).

Measurement of mevalonate and methanol

Methanol and mevalonate concentrations were measured using a Shimadzu GC-2010 Plus instrument (Shimadzu Corp., Shanghai, China). The column was a DB-Wax (30 m×0.320 mm×0.25 μm film; Agilent Corp., Shanghai, China). Ultra high-purity helium was used as the carrier gas at a constant flow mode of 50 cm/s, and 1 μL of sample was injected in split-less mode. The culture filtrate was directly analyzed for methanol by gas chromatography. The

temperatures of the column box, inlet, and flame ionization detector (FID) detector were set to 90, 250, and 250 °C, respectively. For the analysis of mevalonate, the culture filtrate was adjusted to pH 2 with 3 M H_2SO_4 and incubated in a water bath at 45 °C for 1 h to convert mevalonate to mevalonolactone. Then, ethylacetate was added to extract the mevalonolactone. The organic layer was analyzed for mevalonate. The temperatures of the column box, inlet, and FID detector were set to 200, 230, and 260 °C, respectively.

Extraction and measurement of intracellular metabolites

Cells were cultivated in 100 mL of medium in a 500-mL baffle flask at 30 °C on rotary shakers at 170 rpm. In the mid-exponential phase (OD_{600}=3.5±0.2), 10 mL of culture was placed on a membrane filter (0.22 μm, 47 mm; Sartorius) using a pipette and rapidly extracted. The filter was immediately transferred to a 50-mL tube in liquid nitrogen for quenching. The sample was stored at −80 °C for further extraction.

The extraction method for in vivo metabolites was modified based on a previous study (Yang et al. 2013). Briefly, 10 mL of boiled water was added to the tube containing the sample and incubated in a water bath at 100 °C for 12 min. The sample was centrifuged at 5000×*g* at 4 °C for 10 min to remove cell debris. The supernatant was dried in a freeze dryer (FDU-1100, EYELA). The dried sample was dissolved in 1.5 mL of water and centrifuged at 13,000×*g* at 4 °C for 10 min to remove debris. The supernatant was dried again in the freeze dryer.

For liquid chromatography-mass spectrometry (LC-MS) analysis of acetyl-CoA, the dried sample was dissolved in 100 μL of water and analyzed on an Agilent 6460 system. The positive electrospray ionization (ESI) mode was used for detection, and the multiple reaction monitoring (MRM) mode was used for scanning. The MRM pair of the standard metabolite was acetyl-CoA (ESI+, 809.9→303). The dwell time for the MRM transition was 100 s. The sample was separated on an Acquity UPLC BEH Amide (Waters, USA) column. Mobile phase A was 0.1 % ammonium hydroxide, 0.2 % formic acid, and acetonitrile/water (2:98 *v*/*v*). Mobile phase B was 0.075 % ammonium hydroxide, 0.1 % formic acid, and acetonitrile/water (95:5 *v*/*v*). The linear gradient was as follows: 0–0.5 min, 100 % B; 0.5–5.5 min, 100–81 % B; 5.5–11 min, 81–72 % B; 11–16 min, 72–40 % B; 16–17 min, 40–100 % B; and 17–29 and 29–39 min, 100 % B. The total run time was 39 min at 0.2 mL/min. The injection volume was 10 μL. All of the peaks were integrated using the Qualitative Analysis B.05.00 software.

For gas chromatography-mass spectrometry (GC-MS) analysis of HMG-CoA, the dried sample was derivatized in advance. First, keto groups were methoximated by adding 30 mL of methoxyamine solution (25 mg/mL methoxyamine hydrochloride in pyridine) and incubating at 60 °C for 30 min. Then, trimethylsilylation was performed by adding 30 mL of TMS reagent (BSTFA/TMCS, 99:1) and incubating at 30 °C for 90 min (Fiehn et al. 2000). Derivatized samples were detected using an Agilent 5975B/6890N instrument (Agilent Corp., Santa Clara, CA, USA). The column was a DB-5MS (50 m×0.32 mm×0.25 μm film; Agilent Corp., Santa Clara, CA, USA). Ultra high-purity helium was used as the carrier gas at a constant flow mode of 1 mL/min, and 1 μL of the given sample was injected in split-less mode via an Agilent 7683 auto sampler. The inlet and transfer line temperatures were set to 280 °C. The temperature program began at 60 °C with a hold time of 0.25 min and then increased at 5 °C/min to 280 °C, followed by a hold time of 10 min at 280 °C. The ion source and quadrupole temperatures were set to 250 and 150 °C, respectively. Mass spectra were collected from *m*/*z* 40 to 500 at a rate of 3 spectra/s after a 4.5-min solvent delay. The peaks were evaluated using the Agilent data analysis software.

Fed-batch fermentation

A 5-L stirred tank bioreactor (Applikon, Delft, The Netherlands) was used for the fermentation. The working volume was 3 L. An optimized medium based on MC medium was used for fermentation because of its better performance in mevalonate production. The new medium, named MO, contained 45 % less solution B and 60 % more solution D compared with MC medium. Pre-cultivation and inoculation of the engineered cells for fermentation were the same as for the flask cultivation. The bioreactor was operated at 30 °C with shaking at 450 rpm. The dissolved oxygen concentration was maintained at 25± 5 % (saturated oxygen was 100 %) using pure oxygen and compressed air. The pH of the bioreactor was maintained at 7 ±0.2 using 3 M H_2SO_4 and 1 M ammonia in water.

Results

Construction of the top portion of the MEV pathway

To introduce the top portion of MEV pathway in *M. extorquens* AM1, the enzymes HMGCS1 from *B. germanica* and tHMGR from *T. cruzi* were chosen, as they have the highest activities reported thus far (Cabañó et al. 1997; Hrtado-Guerrrero et al. 2002). The enzymes MvaS and MvaE from *E. faecalis*, which were widely used in previous studies, were also introduced into *M. extorquens* AM1 (Tabata and Hashimoto 2004; Yoon et al. 2009; Wang et al. 2010; Yang et al. 2012). The engineered strains were named AM1-mvh and AM1-mve, respectively.

As shown in Fig. 2a, both of the engineered strains showed significant mevalonate titers in flask cultivation using methanol as the sole carbon and energy source. After 5 days of

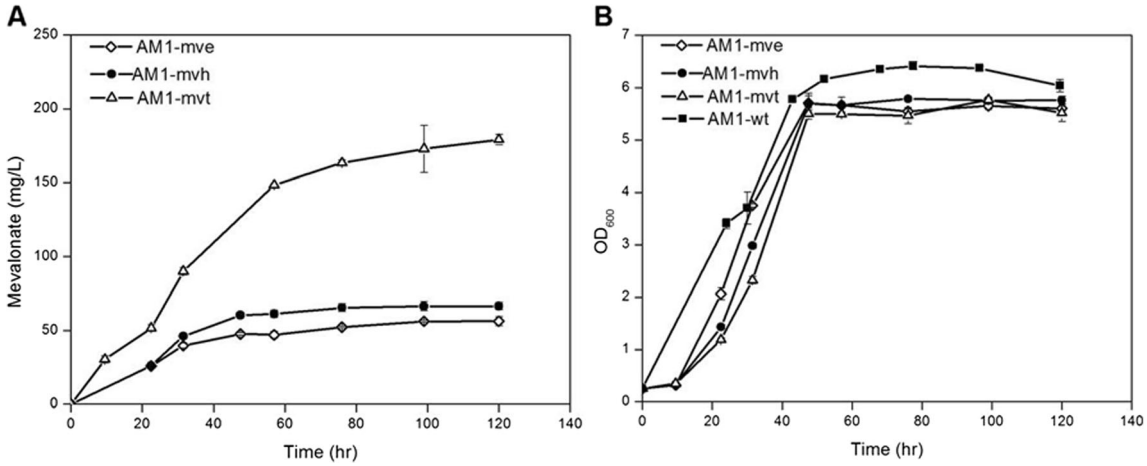

Fig. 2 Characterization of engineered strains with different operons. **a** Mevalonate production. **b** Growth conditions. *Error bars* indicate standard deviations from three replicates

cultivation, the mevalonate titer of AM1-mvh harboring the artificial operon reached 66 mg/L, which was about 18 % higher than that of AM1-mve (56 mg/L) that harbors the natural operon. Further engineering of the artificial operon was conducted on the basis of AM1-mvh. Heterologous AACoAT (encoded by the *phaA* gene from *R. eutropha*) was introduced into AM1-mvh, resulting in the engineered strain AM1-mvt. AM1-mvt achieved a mevalonate titer of 180 mg/L after 5 days of flask cultivation, which was 3.2-fold higher than that of AM1-mve (Fig. 2a).

Regarding growth (Fig. 2b), the engineered strains showed relatively slower growth rates and lower final cell densities than the wild-type strain. Comparing all the engineered strains (AM1-mve, AM1-mvh, and AM1-mvt), a correlation between a high mevalonate titer and low growth rate in exponential phase was observed.

Balancing the MEV pathway by regulating the expression of heterologous AACoAT

To balance the MEV pathway further, we placed a series of RBSs of different strengths in front of the *phaA* gene in the artificial MVT operon. The strengths of the RBSs were calculated using "Reverse Engineering RBSs" in an online RBS calculator (https://salis.psu.edu/software/reverse) (Salis et al. 2009; Espah Borujeni et al. 2014). Five RBS scores for the expression of AACoAT calculated using the RBS calculator are listed in Table 2.

As shown in Fig. 3a, with a moderate upregulation in AACoAT expression, AM1-ARBSU1 achieved the highest mevalonate titer of 215±9 mg/L after 5 days of flask cultivation, which was a 20 % increase over the starting strain AM1-mvt (179±4 mg/L). Further upregulation of AACoAT expression caused a smaller increase in mevalonate production, as indicated by the MEV titers during flask cultivation: 201±5 mg/L for AM1-ARBSU2 and 205±4 mg/L for AM1-ARBSU3. Reducing the expression of AACoAT resulted in a concomitant reduction in the mevalonate titer of AM1-ARBSD (170±3 mg/L).

As shown in Fig. 3b, c, mevalonate production showed semi-coupling with growth, as indicated by the fact that mevalonate was continuously produced even after the strains entered stationary phase. For all the strains with various *phaA* expression levels, there were no obvious differences in their growth during the course of cultivation.

Analysis of intracellular metabolites in the MEV pathway

To better understand the changes in the heterologous MEV pathway that occurred at the different AACoAT expression levels in the engineered strains, intracellular metabolite concentrations in the MEV pathway were measured. As shown in Fig. 4, the intracellular Ac-CoA concentration did not differ between the wild-type and engineered strains. In contrast, with the introduction of the MEV pathway and an increase in AACoAT expression, HMG-CoA gradually accumulated in the four tested strains. Interestingly, we found that mevalonate production increased accordingly with the accumulation of intracellular HMG-CoA (Fig. 4b), indicating that an increased supply of HMG-CoA could enable greater mevalonate synthesis.

Table 2 RBS strengths calculated using a RBS calculator

No.	Engineered strains	RBS in front of the *phaA* gene	Score	Length (bp)
1	AM1-ARBSD	pCM110	22.87	18
2	AM1-mvt	*PalkB*	93.02	14
3	AM1-ARBSU1	segB	259.87	25
4	AM1-ARBSU2	gp46	768.76	23
5	AM1-ARBSU3	gp4	10,410.21	25

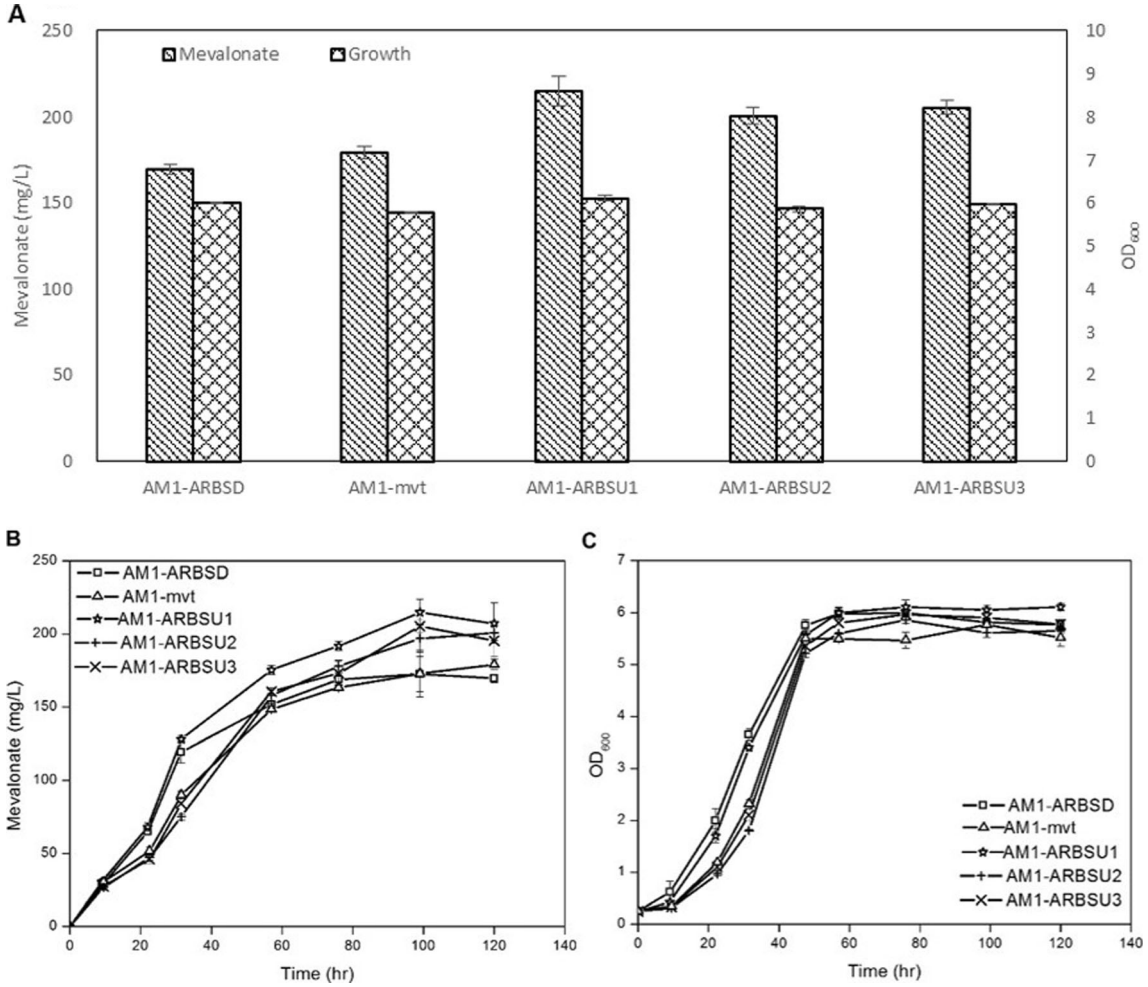

Fig. 3 Regulation of heterologous AACoAT expression by engineering the RBS in front of the *phaA* gene in the MVT operon. **a** Comparison of the engineered strains in terms of the maximum mevalonate titer and terminal growth conditions. **b** Mevalonate production at different AACoAT expression levels via the MVT operon. **c** Growth conditions for the engineered strains. *Error bars* indicate standard deviations from three replicates

Fed-batch fermentation

The best strain for mevalonate production, which carried the ARBSU1 operon, was chosen for fed-batch fermentation. The initial concentration of methanol was set to 0.177 % (*v*/*v*) in the bioreactor, which reportedly resulted in a fast growth rate for *M. extorquens* AM1 (Bourque et al. 1992). The methanol concentration was measured every 12 h during the exponential phase, and methanol was added immediately after measurement to maintain a concentration of 0.177 % (*v*/*v*). When the strains entered stationary phase, the methanol concentration was adjusted to 1 % (*v*/*v*) to ensure an abundant supply of feedstock without a significant inhibition of cell growth (Peel and Quayle 1961).

As shown in Fig. 5, cell growth increased exponentially at 77 h to an OD_{600} of 11 and mevalonate production was associated with cell growth during this period, reaching a titer of 0.746 g/L. Subsequently, the cell growth slowed and the bacteria entered stationary phase. However, mevalonate was still produced continuously in this period, albeit with a lower production rate. After 310 h of fermentation, the cell concentration reached an OD_{600} of 14.8 and the mevalonate titer reached 2.22 g/L, which was equivalent to an overall yield of 28.4 mg mevalonate/g methanol and a volumetric productivity of 7.16 mg/L/h. Comparing mevalonate production in the exponential and stationary phases, the former had a higher productivity and yield along with rapid cell growth, while the latter contributed to mevalonate production to a large extent.

Discussion

Because the exploration of alternative, non-sugar feedstocks for industrial biotechnology is important and urgent, MeCFs on the basis of *M. extorquens* AM1 show promising potential because of the advantages of using methanol as the sole carbon and energy source, their non-pathogenic feature, the availability of complete genome sequences, and the prior

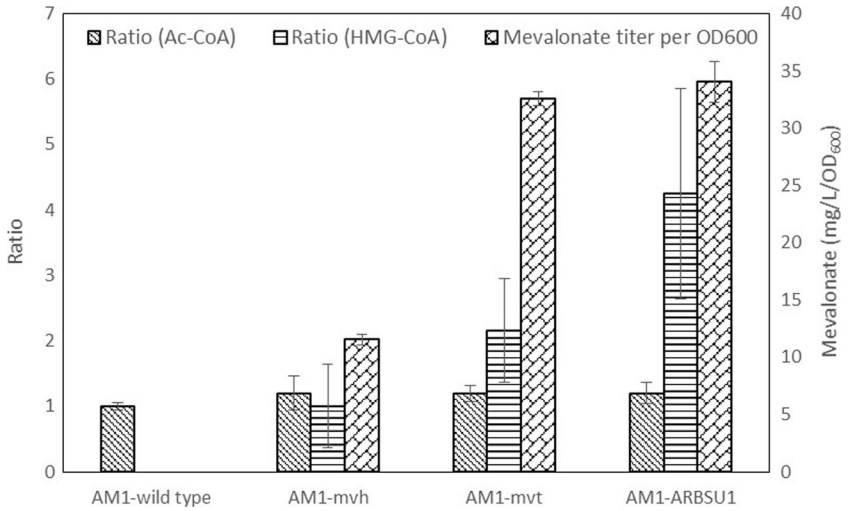

Fig. 4 Effect of heterologous AACoAT on the concentrations of intracellular intermediates in the MEV pathway and terminal mevalonate production during exponential phase. The results are ratios of the intracellular Ac-CoA concentration, normalized to the OD_{600}, compared to that of the wild-type strain, and the HMG-CoA concentration, normalized to the OD_{600}, compared to that of AM1-mvh. Mevalonate production was also normalized to the OD_{600}. Acetoacetyl-CoA could not be detected due either to its small pool size or its instability during sample pretreatment. *Error bars* indicate standard deviations from three replicates

elucidation of the metabolism and feasibility of bioprocess engineering (Vuilleumier et al. 2009; Peyraud et al. 2011; Ochsner et al. 2014). In this study, we succeeded in constructing the heterologous MEV pathway in *M. extorquens* AM1 for the biosynthesis of mevalonate from methanol. By systematically introducing genes encoding key enzymes with high activities of the MEV pathway, elucidating and overcoming the rate-limiting step for mevalonate synthesis, and conducting a fed-batch fermentation, mevalonate production reached 2.22 g/L, with a yield of 28.4 mg mevalonate/g methanol. According to the comparison in titers and yields between various compounds produced by the engineered *M. extorquens* AM1 strain (Table 3), the successful introduction of the MEV pathway, as well as the considerable yield of mevalonate from methanol, made this work a promising application for methylotrophic synthesis by providing a platform for the future synthesis of terpenoids.

Thus far, the introduction and modification of the MEV pathway for terpenoid production have been commonly studied in *E. coli* and *Saccharomyces cerevisiae* (Li and Pfeifer 2014). Related enzymes from various species have been used in these studies. Considering evolution by natural selection, HMGS and HMGR from the same species, such as *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *E. faecalis*, were naturally assumed to be a good match for pathway balancing (Wilding et al. 2000). Yoon et al. (2009) engineered and compared recombinant *E. coli* expressing the enzymes HMGS and HMGR from *S. pneumoniae*, *S. aureus*,

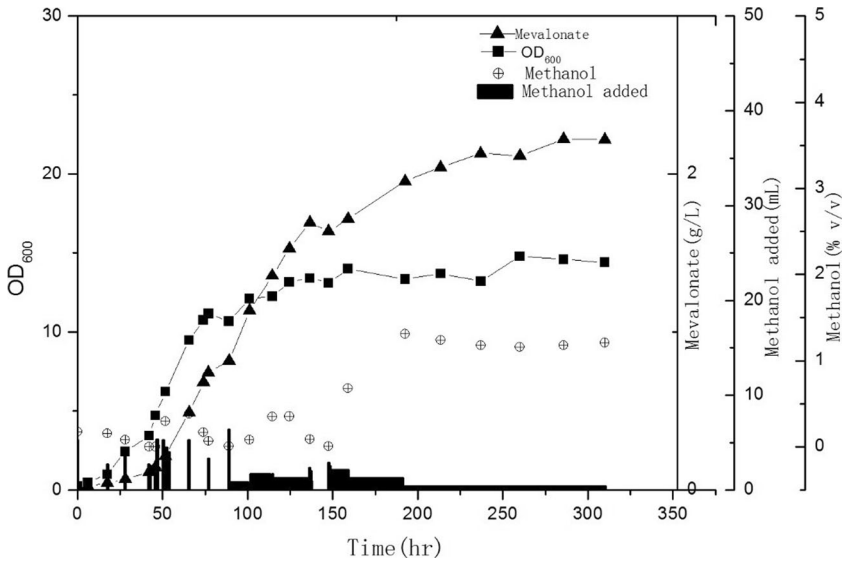

Fig. 5 Fed-batch fermentation of the engineered *M. extorquens* AM1 strain with the ARBSU1 operon in a 3 L working volume of MO medium

Table 3 The titers and yields of various compounds produced by the engineered *M. extorquens* AM1

Substance class	Product	Titer	Yield	Substrate	Fermentation strategy	Reference
Amino acids	L-Serine	54.9 g/L	0.272 g/g methanol 0.550 g/g glycine	Methanol Glycine	Fermentation	Sirirote et al. (1986)
PHAs	PHB (900–1800 kDa)	52.9 g/L	0.09–0.12 g/g MeOH	Methanol	Fed-batch fermentation	Bourque et al. (1995)
	PHB-co-3HV	0.33 g/L	0.968 g/g MeOH	0.5 % (*v*/*v*) methanol and 0.05 % (*v*/*v*) *n*-amyl alcohol	Shake flask cultivation	Ueda et al. (1992)
	PHB-co-3HV and PHB-co-3HV-co-3HHx		43 % of cdw	Methanol	Shake flask cultivation	Orita et al. (2014)
Dicarboxylic acids	Mesaconate	70 mg/L	0.0175 g/g MeOH	Methanol	Shake flask cultivation	Sonntag et al. (2014)
	2-Methylsuccinate	60 mg/L	0.015 g/g MeOH			
Alcohols	1-Butanol	15.2 mg/L		Ethylamine	Shake flask cultivation	Hu and Lidstrom (2014)
Proteins	Green fluorescent protein (GFP)	1.4 g/L	80 mg/g cdw; 16 % of total protein	Methanol	Fed-batch fermentation	Bélanger et al. (2004)
	Haloalkane dehalogenase (DhlA)		10 % of total protein; 50 mg/g biomass	Methanol	Shake flask cultivation	FitzGerald and Lidstrom (2003)
Acids	Mevalonate	2.22 g/L	28.4 mg/g MeOH	Methanol	Fed-batch fermentation	This study

and *E. faecalis*, and they found that the enzymes MvaS (a type of HMGS) and MvaE (a dual-function protein with HMGR and AACoAT activities) from *E. faecalis* showed the best mevalonate production. These two enzymes were used in this work as a control. However, with an expectation for better performance due to their high enzymatic activities, we constructed an artificial MVH operon encoding HMGS and HMGR with the highest activities included in the BRENDA enzyme database. As these enzymes expressed by the artificial operon were superior to the natural operon in both substrate affinity and maximum catalytic rate (Table S2), mevalonate production of the artificial operon could possibly have better performance than the natural one. As indicated by our results, the mevalonate titer of the strain with the artificial operon was 18 % higher than that of the strain with the commonly used natural operon from *E. faecalis*. Our study supplied a good genetic source for constructing the top portion of the MEV pathway in heterologous strains.

An insufficient supply of substrates might hamper the potential of the artificial operon to produce mevalonate. The properties of HMGS reveal that it is more sensitive to acetoacetyl-CoA (AcAc-CoA) than Ac-CoA (Table S2). Additionally, the supply of Ac-CoA tends to be ample and stable due to its position at a key node in the metabolic flux (Peyraud et al. 2011), which was also demonstrated by the measurement of intracellular Ac-CoA in the engineered strains in this study (Fig. 4). Therefore, more AcAc-CoA should be synthesized to satisfy the need of high flux to the MEV pathway. We succeeded in achieving a 1.7-fold increase in mevalonate production via the heterologous expression of AACoAT from *R. eutropha* and then a further 20 % increase by upregulating the expression of AACoAT via RBS engineering. As intracellular AcAc-CoA is very difficult to analyze with LC-MS (Hu and Lidstrom 2014), HMG-CoA was detected instead. With the introduction and upregulation of AACoAT, the concentration of intracellular HMG-CoA was significantly increased, which confirmed the effect of AACoAT overexpression on the potential of the artificial operon. However, a comparison of the concentrations of intracellular intermediates between the AM1-ARBSU1 and AM1-mvt strains (Fig. 4) showed that the significant increase of HMG-CoA resulted in only a small increase in mevalonate production, suggesting that HMGR became the rate-limiting step after the overexpression of AACoAT in AM1-ARBSU1. HMGR is found to be the rate-limiting enzyme for the MEV pathway in many studies (Ohto et al. 2009), and attempts to search for HMGR with high activity in *E. coli* have been made (Ma et al. 2011). The discovery, or engineering, of HMGR with higher activity is necessary to further enhance mevalonate production in *M. extorquens* AM1.

In *M. extorquens* AM1, the native MEP pathway, using pyruvate and glyceraldehyde-3-phosphate (GAP) as primary precursors, satisfies the necessary terpenoid demand for

growth and metabolism. The heterologous MEV pathway in the engineered *M. extorquens* AM1 strains operated in parallel with the native MEP pathway and bypassed the regulatory elements, which contributes to a further enhancement in terpenoid synthesis. A genome-scale metabolic network of *M. extorquens* AM1 for methanol utilization revealed that the synthesis of C2 compounds, with Ac-CoA as the entry metabolite, consumes considerable carbon flux, while little flux flows to pyruvate and GAP (Peyraud et al. 2011). This means that significant metabolic flux to Ac-CoA guarantees a stable and sufficient carbon supply to the MEV pathway, which provides adequate space for further improvement in mevalonate production. With a titer of 2.22 g mevalonate/L (15.09 mM) in 310 h, this study was just a beginning compared with the well-studied microbial systems. For example, a 2-L jar fermentor of recombinant *E. coli* achieved 47 g mevalonate/L (319.40 mM) in fed-batch culture in 50 h (Tabata and Hashimoto 2004). Aiming at a economically competitive process for terpenoid synthesis, an industrial production of semi-synthetic artemisinin via MEV pathway developed by Keasling et al. provided some referenced value for further study; the production of artemisinic acid by engineered strain of *S. cerevisiae* (baker's yeast) achieved a titer of 25 g/L (106.68 mM) in 140 h in fermentation (Paddon et al. 2013). To reach this goal, the titer of mevalonate should be at least 31 g/L (213.36 mM) accordingly. To enhance mevalonate production to such a level in *M. extorquens* AM1, overcoming the rate-limiting step of HMGR, balancing the heterologous pathway, and increasing flux to Ac-CoA by engineering global metabolism would be helpful. This work provides an essential basis for the further biosynthesis of value-added terpenoids from methanol.

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Conflict of interest The authors declare that they have no competing interests.

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