Metabolic engineering of *Methylobacterium extorquens* AM1 for the production of butadiene precursor

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**Abstract**

**Background:** Butadiene is a platform chemical used as an industrial feedstock for the manufacture of automobile tires, synthetic resins, latex and engineering plastics. Currently, butadiene is predominantly synthesized as a byproduct of ethylene production from non-renewable petroleum resources. Although the idea of biological synthesis of butadiene from sugars has been discussed in the literature, success for that goal has so far not been reported. As a model system for methanol assimilation, *Methylobacterium extorquens* AM1 can produce several unique metabolic intermediates for the production of value-added chemicals, including crotonyl-CoA as a potential precursor for butadiene synthesis.

**Results:** In this work, we focused on constructing a metabolic pathway to convert crotonyl-CoA into crotyl diphosphate, a direct precursor of butadiene. The engineered pathway consists of three identified enzymes, a hydroxyethylthiazole kinase (THK) from *Escherichia coli*, an isopentenyl phosphate kinase (IPK) from *Methanothermobacterthermautotrophicus* and an aldehyde/alcohol dehydrogenase (ADHE2) from *Clostridium acetobutylicum*. The $K_m$ and $k_{cat}$ of THK, IPK and ADHE2 were determined as 8.35 mM and 1.24 s$^{-1}$, 1.28 mM and 153.14 s$^{-1}$, and 2.34 mM and 1.15 s$^{-1}$ towards crotonol, crotyl monophosphate and crotonyl-CoA, respectively. Then, the activity of one of rate-limiting enzymes, THK, was optimized by random mutagenesis coupled with a developed high-throughput screening colorimetric assay. The resulting variant (THK$^{MB2V}$) isolated from over 3000 colonies showed 8.6-fold higher activity than wild-type, which helped increase the titer of crotyl diphosphate to 0.76 mM, corresponding to a 7.6% conversion from crotonol in the one-pot in vitro reaction. Overexpression of native ADHE2, IPK with THK$^{MB2V}$ under a strong promoter mxaF in *M. extorquens* AM1 did not produce crotyl diphosphate from crotonyl-CoA, but the engineered strain did generate 0.60 μg/mL of intracellular crotyl diphosphate from exogenously supplied crotonol at mid-exponential phase.

**Conclusions:** These results represent the first step in producing a butadiene precursor in recombinant *M. extorquens* AM1. It not only demonstrates the feasibility of converting crotonol to key intermediates for butadiene biosynthesis, it also suggests future directions for improving catalytic efficiency of aldehyde/alcohol dehydrogenase to produce butadiene precursor from methanol.

**Keywords:** *Methylobacterium extorquens*, Butadiene, Crotyl diphosphate, High throughput screening, In vitro reaction, Pathway engineering

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Background

Butadiene is the simplest conjugated diene and a major commodity of the petrochemical industry, used for the manufacture of automobile tires, synthetic resins, latex and plastics [1]. It is one of the most widely-used chemicals in the world with over ten million tons produced per year [2]. Currently, butadiene is predominantly synthesized from non-renewable petroleum as a byproduct of ethylene production [3]. Butadiene can also be produced by the dehydrogenation of butane or butene, but this process has some drawbacks including large consumption of steam and harsh operation conditions [4]. In contrast with traditional approaches, microbial synthesis of butadiene from renewable sources such as lignocellulose, biogas and carbon dioxide represents an environmentally friendly approach requiring lower investment [5, 6]. So far, there is no successful example of butadiene synthesis using biological platforms, despite the fact that three different pathways for butadiene production have been proposed, starting with crotonyl-CoA, erithrose-4-phosphate and malonyl-CoA respectively [2].

*Methylobacterium extorquens* AM1, a facultative methylotrophic α-proteobacterium, is capable of utilizing methanol as the sole carbon and energy source [7]. Methanol is known as an important C1 feedstock, which can be generated from synthesis gas (a mixture of CO and H₂) or from biogas with relatively cheap cost [8]. The methylotrophic metabolism in *M. extorquens* AM1 involves three interlocked cycles: the serine cycle, the ethylmalonyl-CoA pathway (EMC pathway) and the poly-3-hydroxybutyrate (PHB) cycle (Fig. 1) [9]. Notably, the ethylmalonyl-CoA pathway (EMC pathway) and the methylotrophic metabolism in *M. extorquens* AM1 are well established.

Methods

Culture medium and condition

Cultures of *E. coli* strain Top10 and *E. coli* strain BL21-Z were grown at 37 °C in Luria–Bertani (LB) medium. *M. extorquens* AM1 and its recombinant strains were routinely cultured in a minimal medium as described previously [21]. Briefly, the strains were first inoculated in tubes and pre-cultivated to mid-exponential phase at 30 °C, and then 0.5 mL of a sub-culture was transferred into 50 mL of minimal medium in 250 mL flasks and grown on rotary shaker at 200 rpm. Substrates and antibiotics were supplied at the following concentrations: succinate (15 mM), methanol (125 mM), 20 μg/mL tetracycline (Tet) and 50 μg/mL ampicillin. MC minimal medium was adapted from the previous description to cultivate *M. extorquens* AM1 in 96-well plates [22]. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. Crotyl monophosphate and crotyl diphosphate were synthesized by Kare-Bay Biochem, Inc (Ningbo, Jiangsu Province, China). Milli-Q (Billerica, MA, USA) was used for preparing all the media, buffers, standards, and sample solutions.

Plasmids and strain construction

The *erg12* gene encoding mevalonate kinase from *Saccharomyces cerevisiae* (GenBank accession: BK006946.2), *thik* gene encoding thiamine kinase from *Salmonella enterica* (GenBank accession: AMG28242.1), *MTH_47* encoding isopentenyl phosphate kinase (IPK) from
**Methanothermobacter thermautotrophicus** (GenBank accession: AAB84554.1), **far** gene encoding fatty acyl-CoA reductase (FAR) from *Hahella chejuensis* and *Marinobacter manganoxydans* (GenBank accession: ABC31684.1, WP_008171430.1), and **adhe2** gene encoding aldehyde/alcohol dehydrogenase (ADHE2) from *Clostridium acetobutylicum* (GenBank: AF321779.1) were synthesized into the vector pUC57 (GenScript, Nanjing, China) with codon usage optimized for expression in *M. extorquens* AM1. The **thiM** gene encoding hydroxyethylthiazole kinase (THK) from *E. coli* K-12 (GenBank accession: AVI56602.1), and **gck** gene encoding glycerate kinase (GCK) from *M. extorquens* AM1 were amplified from the genomic DNA [23]. The genes were amplified using the corresponding primers listed in Additional file 1: Table S1. Resulting PCR products of **erg12**, **thik**, **MTH_47**, **thiM**, and **gck** genes were digested with *Bam*HI and *Sac*I. The PCR products of **far** and **adhe2** were digested with *Hind*III and *Bam*HI. The fragments of genes were assembled into the same restriction sites.
of pCM80 [24] and pET.32M.3C. All the plasmids were transformed into M. extorquens AM1 by electroporation as described before [25] or into E. coli BL21-Z, respectively (Table 1).

PCR was performed using PrimeSTAR HS, and error-prone PCR was performed using Taq™ (Tankala, Dalian, China). The restriction enzymes were purchased from Fisher Scientific (Pittsburgh, PA, USA). T4 ligase was purchased from Takala (Dalian, China). Amplified DNA was purified by PCR purification Kit and plasmid DNA was purified from E. coli by SanPrep Column Plasmid Mini-Preps Kit (Sangon Biotech, Shanghai, China). Primers and nucleotide sequences were confirmed by Sangon Biotech (Shanghai, China).

Recombinant protein expression and purification
Escherichia coli BL21-Z cultures harboring recombinant plasmids were grown overnight in tubes at 37 °C and at 200 rpm in LB medium containing ampicillin (100 μg/mL). 0.5 mL of overnight cultures were transferred into 50 mL of fresh LB medium in 250 mL flasks, and grown at 37 °C and at 200 rpm until OD600 reached 0.8. Then, isopropyl-β-D-thiogalactopyranoside (IPTG) was added at a final concentration of 1 mM for THK and GCK, 200 μM for IPK and 50 μM for both FAR and ADHE2. The cell cultures were incubated for an additional 20 h at 18 °C and at 160 rpm. The cells were harvested by centrifugation, and resuspended and washed with a buffer (50 mM Tris–HCl, 5 mM imidazole, pH 8.0). The cells were lysed by One Shot cell disruptor (Constant Systems Ltd, United Kingdom) at 3.5 × 107 psi and cell debris was removed by centrifugation at 13,000 rpm for 30 min. The soluble fraction was used for His-tagged purification by Ni-nitrilotriacetic acid (NTA) resin (Pointbio, Shanghai, China). Non-specifically bound proteins were washed out with a buffer (50 mM Tris–HCl, 10 mM MgCl2, 20 mM KCl, 30 mM imidazole, pH 8.0), while bound His-tagged proteins were eluted with elution buffer (50 mM Tris–HCl, 10 mM MgCl2, 20 mM KCl, 200 mM imidazole, 200 mM NaCl).

| Table 1 Strains and plasmids used in this study |
|-----------------------------------------------|
| **Strains or plasmids** | **Description** | **Source or references** |
| **Strains** | | |
| E. coli BL21-Z | F−ompT hsd52(rB_MgI)gal dcm | A gift from Dr. Yu-Long Zhao at the Tianjin Medical University |
| M. extorquens AM1 | Wild-type, pink color, rifamycin-resistant strain | [21] |
| YCB0 | M. extorquens AM1/pCM80 | This study |
| YJM | M. extorquens AM1/pCM80-thiM | This study |
| YJK | M. extorquens AM1/pCM80-thiK | This study |
| YJG | M. extorquens AM1/pCM80-erg12 | This study |
| YCB1 | M. extorquens AM1/pCB1 | This study |
| YCB3 | M. extorquens AM1/pCB3 | This study |
| YCB4 | M. extorquens AM1/pCB4 | This study |
| **Plasmids** | | |
| pCM80 | M. extorquens expression vector, mxaF promoter; TcR | [24] |
| pET.32M.3C | Expression vector, T7 promoter, Amr | Lab storage |
| pCM80-thiM | thiM from E. coli K-12 inserted into pCM80 | This study |
| pCM80-thiK | thiK from S. enterica inserted into pCM80 | This study |
| pCM80-erg12 | erg12 from S. cerevisiae inserted into pCM80 | This study |
| pCM80-far | far from Hahella chejuensis inserted into pCM80 | This study |
| pET.32M.3C-thiM | thiM from E. coli K-12 inserted into pET.32M.3C | This study |
| pET.32M.3C-thiK | thiK from S. enterica inserted into pET.32M.3C | This study |
| pET.32M.3C-erg12 | erg12 from S. cerevisiae inserted into pET.32M.3C | This study |
| pET.32M.3C-ipk | ipk from M. thermautotrophicus inserted into pET.32M.3C | This study |
| pET.32M.3C-gck | gck from M. extorquens AM1 inserted into pET.32M.3C | This study |
| pET.32M.3C-far | far from Hahella chejuensis inserted into pET.32M.3C | This study |
| pCB1 | pCM80 (PmxaF::thiM:THM_47) | This study |
| pCB3 | pCM80 (PmxaF::adhE2:thiM:THM_47) | This study |
| pCB4 | pCM80 (PmxaF::adhE2) | This study |
pH 8.0). To remove imidazole and concentrate protein, the eluted solution was centrifuged through a centrifugal filter with a molecular cutoff of 10 kDa (Millipore, Billerica, MA) and purified protein was verified by 12% SDS-PAGE followed by Coomassie Blue staining. The concentration of protein was determined according to Modified BCA Protein Assay Kit (Sangon Biotech, Shanghai, China).

Characterization of kinases
To characterize the optimal temperature of THK and IPK towards crotonol and crotyl monophosphate, a total reaction volume of 100 μL contained 500 μM crotonol or 500 μM crotyl monophosphate, 5 mM ATP and 0.5 mg/mL THK or 0.02 mg/mL IPK, 10 mM MgCl₂ and 20 mM KCl in 50 mM Tris–HCl buffer with pH 7.5. For THK, the reaction was performed at temperatures of 29.5 °C, 32 °C, 34.5 °C, 37 °C, 39.5 °C, 42 °C and 44.5 °C for 20 min. All enzymatic reactions were performed at temperatures of 29.5 °C, 32 °C, 34.5 °C, 37 °C, 39.5 °C, 42 °C and 44.5 °C for 1 h, and for IPK the reaction was carried out at temperatures of 29.5 °C, 32 °C, 34.5 °C, 37 °C, 39.5 °C, 42 °C and 44.5 °C for 20 min.

For determining the optimal pH of THK on crotonol, a total reaction volume of 100 μL contained 500 μM crotonol, 5 mM ATP, 0.5 mg/mL THK, 10 mM MgCl₂ and 20 mM KCl in either 50 mM sodium phosphate buffer with pH 6.0 and 6.5 or 50 mM Tris–HCl buffer with pH 7.0, 7.5, 8.0, 8.5 and 9.0. The reaction was performed at 39.5 °C for 1 h. To evaluate the optimal pH of IPK on crotyl monophosphate, the 100 μL reaction contained 500 μM crotyl monophosphate, 5 mM ATP, 0.02 mg/mL IPK, 10 mM MgCl₂ and 20 mM KCl in either 50 mM sodium phosphate buffer with pH 6.0 and 6.5 or 50 mM Tris–HCl buffer with pH 7.0, 7.5, 8.0, 8.5 or 9.0. The reaction was performed at 39.5 °C for 20 min. All enzymatic reactions were terminated by adding 50 μL cold methanol.

The activity of IPK was determined through coupling the release of ADP with NADH oxidation by pyruvate kinase/lactate dehydrogenase (PK/LDH) [26]. Briefly, enzymatic assays were performed at the optimized condition with the addition of 0.2 mM NADH, 0.5 mM phosphoenolpyruvate, 5 mM ATP and 20 μL aqueous glycerol solution of PK/LDH (Sigma-Aldrich, MO, USA). NADH consumption was measured at 340 nm at 10 s intervals using a UV–visible spectrophotometer [27, 28]. One unit of IPK activity corresponds to the consumption of 0.5 to 13 mM and 0.2 to 8 mM, respectively. To determine the activity of FAR and ADHE2, NADPH and NADH consumptions were measured at 340 nm at 10 s intervals using a UV–visible spectrophotometer. One unit of FAR and ADHE2 activity corresponds to the consumption of 10 nmol NADPH and 10 nmol NADH per minute, respectively.

Kinetics of THK, IPK, FAR and ADHE2
The kinetics of each enzyme was processed via a proportional weighted fit using a nonlinear regression analysis program based on Michaelis–Menten enzyme kinetics. For THK, a total reaction volume of 100 μL contained 5 mM ATP, 0.5 mg/mL THK, 10 mM MgCl₂ and 20 mM KCl in 50 mM Tris–HCl buffer (pH 8.0) at 39.5 °C. For IPK, the 100 μL reaction contained 5 mM ATP, 0.02 mg/mL IPK, 10 mM MgCl₂ and 20 mM KCl in 50 mM Tris–HCl buffer (pH 7.5) at 39.5 °C. For FAR, the 100 μL reaction contained 5 mM NADPH, 0.5 mg/mL FAR, 50 mM NaCl₂ in 50 mM Tris–HCl buffer (pH 7.5) at 37 °C [27]. For ADHE2, the 200 μL reaction contained 0.4 mM NADH, 0.4 mg/mL ADHE2, 1 mM dithiothreitol in 100 mM Tris–HCl buffer (pH 7.5) at 37 °C [28]. The kinetic parameters of THK and IPK were determined when crotonol and crotyl monophosphate were added in a concentration range of 0.1 to 30 mM and 0.062 to 3.0 mM, respectively. The kinetic parameters of FAR and ADHE2 were determined when crotonyl-CoA were added in a concentration range of 0.5 to 13 mM and 0.2 to 8 mM, respectively. To determine the activity of FAR and ADHE2, NADPH and NADH consumptions were measured at 340 nm at 10 s intervals using a UV–visible spectrophotometer. One unit of FAR and ADHE2 activity corresponds to the consumption of 10 nmol NADPH and 10 nmol NADH per minute, respectively.

Extraction and measurement of crotyl monophosphate and crotyl diphosphate
20 mL of samples at the late of exponential phase (OD₆₀₀ = 1.2 ± 0.1) were rapidly harvested by vacuum filtration using MILLEX-GP PES membrane filters (0.22 μm, 33 mm) and quickly washed with culture medium [29]. Extraction of crotyl monophosphate and crotyl diphosphate was performed as described previously for measuring sugar phosphates in M. extorquens AM1 [30]. Briefly, 10 mL of boiling water was added to a given sample and incubated at 100 °C for 10 min. The extracted cell suspension was cooled on ice for 5 min, and then cell debris was removed by centrifugation at 5000 rpm for 5 min. The cell-free extract was centrifuged at 14,000 rpm for 8 min. The supernatant was dried in a rotational vacuum concentrator (Christ, Osterode, Germany) and stored at −80 °C for further use. For LC–MS analysis, each dried sample was dissolved in 100 μL of purified water. The sample analysis was carried out on an Agilent LC-QQQ-MS system (Agilent 1290 Infinity-6460, Agilent Technologies, Santa Clara, CA, USA). Multiple reaction monitoring (MRM) precursor/product ion pairs were crotyl monophosphate (ESI—m/z 151.0 to m/z 79.0) and crotyl diphosphate (ESI—m/z 231.0 to m/z
the following conditions: 500 μL of reaction mixture contained 50 mM Tris–HCl (pH 8.0), 10 mM MgCl₂, 20 mM KCl, 5 mM ATP, 5 mM (360 μg/mL) crotonol, 1 mM dithiothreitol, 5% glycerol (v/v) and cell extract. The amount of cell extract was added according to semi-quantification of the band intensity of THK (0.5 mg/mL) by Adobe Photoshop CS 6.0. Crotyl monophosphate was analyzed by LC–MS as described above.

Homology modeling of hydroxyethylthiazole kinase
To explore the structure of the complex between THK and the substrate crotonol, three-dimensional structure models were constructed using the program Swiss-Model (http://swissmodel.expasy.org/), followed by enzyme-ligand docking using the AutoDockVina program [31]. The structures of THK were modeled using a complex structure template including ATP (pdb id: 1esq). The structure model was subjected to energy minimization using the Swiss-PdbViewer. Afterwards, the docking studies were run with crotonol as ligand and the built THK structure models as receptor. The crotonol structure file (ligand) was retrieved from ZINC site [32]. Docking cluster analysis was performed in the AutoDockVina program environment, and characterized by binding energy. Establishment of eventual complex structural model was based on the energy minimization. The built complex structural analysis was done using Pymol software [33]. The mutation at the specific amino acid site was also introduced using this software, which allowed exploration of the spatial and molecular interactions among amino acids.

Crude enzymatic assay of M. extorquens AM1
For constructing the sequential reactions in M. extorquens AM1, DNA fragments of thiM and MTH_47 and the fragments of adhE2, thiM and MTH_47 were assembled into the BamHI–SacI restriction sites of pCM80 plasmid under the mxaF promoter to obtain pCB1 plasmid and pCB3 plasmid. Cell extracts of recombinants of M. extorquens AM1 were generated as described previously with slight modifications [15, 34]. Briefly, 50 mL of cells at the late exponential phase (OD₆₀₀ = 1.2 ± 0.1) were harvested and resuspended in 7 mL of buffer (50 mM Tris–HCl, 10 mM MgCl₂, 20 mM KCl, pH 8.0). Crude cell extracts were obtained by passing the cells through One Shot cell disruptor at 3.8 × 10⁷ psi. Crude cell extracts were concentrated to 1 mL via centrifugal filter as described above. For detecting the production of crotonol, crotyl monophosphate and crotyl diphosphate from crotonol-CoA, 500 μL of the reaction mixture contained 50 mM Tris–HCl (pH 8.0), 10 mM MgCl₂, 20 mM KCl, 4 mM NADH, 5 mM ATP, 1 mM dithiothreitol, 5% glycerol (v/v), and 0.8 mg cell extracts. The enzymatic reaction was started by adding
4 mM crotonyl-CoA into the reaction mixture. The reaction was stopped by adding 2 mM HCl at 4 h. The samples were extracted by 500 μL dichloromethane, and the mixtures were vortexed for 5 min and then centrifuged at 5000 rpm for 10 min to separate the aqueous phase and dichloromethane. Crotonol was analyzed by GCMS-QP2020 system (Shimadzu, Kyoto, Japan) equipped with a Rtx-5MS column (30 m × 0.25 mm × 0.25 μm) via the auto-sampler. 1 μL samples were analyzed with the following program: set initial temperature at 40 °C for 4 min, ramped to 250 °C at 40 °C/min, maintained at 250 °C for 5 min. The ion source temperature was set to 250 °C. GC–MS data were processed using GCMS solution software. For measuring the production of crotyl diphosphate from crotonol, 500 μL of the reaction mixture contained 50 mM Tris–HCl (pH 8.0), 10 mM MgCl2, 20 mM KCl, 5 mM ATP, 1 mM dithiothreitol, 5% glycerol (v/v) and 0.3 mg cell extracts. The enzymatic reaction was started by adding 0.2 mM crotonol into the reaction mixture. At time points (2, 4, 6 and 8 h), the reaction was stopped by adding 2.5 mL cold methanol. The samples were centrifuged at 13,000 rpm for 30 min to remove precipitated protein. Crotyl monophosphate and crotyl diphosphate were analyzed by LC–MS as the described above.

Results and discussion
Phosphorylation of crotonol into crotyl monophosphate
The direct phosphorylation of crotonol into crotyl monophosphate has no precedent in the published biochemical literature, but mevalonate kinase (MK) has been well characterized to phosphorylate mevalonate to mevalonate 5-phosphate in the isoprene biosynthesis pathway (Fig. 1) [35]. We first expressed the erg12 gene encoding MK from Saccharomyces cerevisiae in M. extorquens AM1 and detected crotyl monophosphate in the culture after the addition of 1 mM crotonol. Intracellular crotyl monophosphate was quantified by LC–MS (Fig. 2a). As shown in Fig. 2b, crotyl monophosphate was detected at a low level slightly above the control. Then, two other types of kinases, i.e. hydroxyethylthiazole kinase (THK) and thiamine kinase (TK), which catalyzed 4-methyl-5-(2-hydroxyethyl)thiazole to 4-methyl-5-(2-phosphonoxyethyl)thiazole and thiamine to thiamine phosphate, respectively, were evaluated for phosphorylation of crotonol. A thiM gene encoding THK from Escherichia coli and a thiK gene encoding TK from Salmonella enterica were respectively introduced into M. extorquens AM1, and the resultant strains produced 2.4-fold and 1.3-fold higher crotyl monophosphate, respectively, than the control (Fig. 2b). This result indicated that THK was the most suitable kinase among three candidates for phosphorylating crotonol to crotyl monophosphate in M. extorquens AM1. Interestingly, crotyl monophosphate was also detected in the control, suggesting wild-type M. extorquens AM1 may contain one or more native enzymes capable of phosphorylating crotonol. One of the potential enzymes is the glycerate kinase encoded by the
The kinetic behavior of THK towards crotonol was 44.5 °C respectively (Additional file 2: Fig. S3a). The optimization was evaluated in the range between 29.5 and 44.5 °C at which THK generated 1.8-fold and 1.2-fold higher crotonol monophosphate than at 29.5 °C and 44.5 °C respectively (Additional file 2: Fig. S3a). The optimal activities of THK were also determined to be from pH 8.0 to 9.0 in 50 mM Tris–HCl, showing a broad optimum occurred in that range (Additional file 2: Fig. S3b). The kinetic behavior of THK towards crotonol was further characterized at this optimized condition. In vitro enzymatic activity was then assayed by measuring the production of crotonol monophosphate along a time course. The values of \( K_m \) and \( k_{cat} \) were determined to be 8.35 mM and 1.24 s\(^{-1} \) (Additional file 2: Fig. S4 and Table 2). The specific activity of THK for 0.5 mM crotonol was 0.1 U/mg, which was 300-fold lower than that for the native substrate 4-methyl-5-(2-hydroxyethyl)thiazole at the same concentration [38]. This result suggested that the activity of THK needed to be further improved through protein engineering in order to supply more crotonol monophosphate for the subsequent reaction.

Phosphorylation of crotonol monophosphate into crotyl diphosphate

Similar to bioconversion of crotonol into crotyl monophosphate, no enzyme in nature has been reported to be able to catalyze crotonol monophosphate to crotyl diphosphate. Initially, a phosphomevalonate kinase (PMK) described to convert mevalonate-5-phosphate into mevalonate-5-pyrophosphate was selected for evaluation (Fig. 1). We also identified another kinase (isopentenyl phosphate kinase, IPK) from *Methanothermobacter thermautotrophicus* that was able to catalyze a phosphorylation from 3-butenyl phosphate (BEP) to 3-butenyl diphosphate [39]. The structures of BEP and crotyl diphosphate are quite similar and the only difference is the location of the carbon–carbon double bond. The double bond of crotyl monophosphate is between the position of \( C_2 \) and \( C_3 \) whereas the double bond of BEP is between \( C_3 \) and \( C_4 \). Additionally, IPK from *M. thermautotrophicus* or *Thermoplasma acidophilum* was demonstrated to be promiscuous over a broad range of substrates, such as dimethylallyl phosphate, isopentenyl thiolophosphate, 1-butyl phosphate, 3-buten-1-yl phosphate, and geranyl phosphate [39]. Therefore, we expressed the IPK gene in *M. extorquens* AM1 and set up a crude enzymatic assay to evaluate the conversion of crotonol monophosphate. As shown in extracted ion chromatograms, crotyl diphosphate was found to be accumulated after the addition of crotonol monophosphate (Fig. 3a). Accordingly, 3.8 \( \mu \)M of crotyl diphosphate was detected at 0.5 h and increased to 16.6 \( \mu \)M at 4 h after incubation (Fig. 3b). Purified IPK was analyzed by SDS-PAGE, and shown to have a molecular mass of 44 kDa consistent with calculated molecular weight based on amino acid sequence (Additional file 2: Fig. S2). Initial reaction rates of IPK were determined by an assay in which ADP production was coupled to consumption of NADH within 10 min [26]. The optimal temperature and pH of IPK was identified to be 39.5 °C and 7.5 (Additional file 2: Fig. S3). Under the optimal reaction conditions, the kinetic values of \( K_m \) and \( k_{cat} \) for crotyl monophosphate were measured to be 1.28 mM and 153.14 s\(^{-1} \), respectively (Additional file 2: Fig. S4 and Table 2).

High-throughput screening approach for discovering high activity of THK variants

In order to improve the catalytic efficiency of THK, error-prone PCR libraries were screened with a high-throughput screening (HTS) method to identify a mutated variant with increased enzyme activity. First, a HTS colorimetric assay was developed based on a pink–purple

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**Table 2** Kinetic parameters of THK, THK\(^{MB2V}\), IPK, FAR and ADHE2

| Enzymes   | Sources          | Substrates        | \( K_m \) (mM) | \( k_{cat} \) (s\(^{-1} \)) | \( k_{cat}/K_m \) (mM\(^{-1}\) s\(^{-1} \)) | \( V_{max} \) (µmol/min/mg) |
|-----------|-----------------|-------------------|----------------|------------------|--------------------------------|--------------------------|
| THK       | *E. coli*       | Crotonol          | 8.35 ± 2.24    | 1.24 ± 0.26      | 0.15 ± 0.01                    | 0.86 ± 0.18              |
| THK\(^{MB2V}\) | *E. coli*    | Crotonol          | 4.79 ± 0.51    | 8.58 ± 0.31      | 1.80 ± 0.13                    | 5.97 ± 0.21              |
| IPK       | *M. thermautotrophicus* | Crotyl-monophosphate | 1.28 ± 0.50    | 5.03 ± 0.18      | 1.00 ± 0.01                    | 77.83 ± 9.5              |
| FAR       | *H. chejuensis* | Crotonyl-CoA      | 3.22 ± 0.07    | 0.015 ± 0.001    | 0.005 ± 0.001                  | 0.030 ± 0.002            |
| ADHE2     | *C. acetobutylicum* | Crotonyl-CoA      | 2.34 ± 0.28    | 1.15 ± 0.27      | 0.49 ± 0.04                    | 0.25 ± 0.06              |
colored agent (potassium permanganate), which loses its color when reduced by crotonol. Thus, the level of residual crotonol after introduction of THK variants can be evaluated by the reduction of potassium permanganate calculated from the absorbance change at 490 nm.

The assay development work was carried out with *M. extorquens* AM1 grown on methanol or succinate as the sole carbon source. When *M. extorquens* AM1 was grown on succinate in the presence of crotonol, the colorimetric assay showed a linear correlation ($R^2$ of 0.9677) of crotonol decrease and absorbance increase in the range between 0.16 and 0.2 mM of crotonol (Additional file 2: Fig. S5). For operational convenience, we also tried to grow single colonies of mutant strains in 96-well plates. However, the strains stopped growth at an OD$_{600}$ of about 0.15. This phenomenon was in line with the previous report that the growth curves of *M. extorquens* AM1 in 96-well plates had large deviations in the exponential phase [40]. Therefore, the mutant strains were pre-grown in tubes and then the supernatants were transferred to 96-well plates for OD readout in a high-throughput way (Fig. 4). In addition, we measured the level of extracellular crotyl monophosphate in *M. extorquens* AM1 culture to determine whether the produced crotyl monophosphate could be excreted to interfere with the colorimetric assay. No crotyl monophosphate was detected, thereby eliminating the interference of crotyl monophosphate on the screening.

**Improving THK activity by directed evolution**

A random mutagenesis library of *thiM* was made with an average of 1 to 2 point mutations per gene. The generated library was transformed into *M. extorquens* AM1 and a library of around 3100 colonies was created and screened by the HTS for improved enzyme activity. A total of 18 mutants displayed higher enzyme activity than wild-type *thiM* (Fig. 5a), among which two THK variants, i.e. THKM82V and THKM82V/G180R, showed the highest activity. *In vitro* assay indicated that purified THK$^{M82V}$ and THK$^{M82V/G180R}$ had 8.6-fold and 1.2-fold higher activities than wild-type THK (Fig. 5b). Subsequently, a
saturation mutagenesis on position 82 was carried out to discover whether there was a more favorable mutation that could further increase the activity of THK. We found that three (M82V, M82I, and M82F) of the 18 variants were highly soluble in the recombinant *E. coli* and M82A, M82P, and M82C were relatively insoluble (Additional file 2: Fig. S6). Enzyme assays with crude extracts from *E. coli* were carried out to detect the production of crotyl monophosphate. None of these variants showed higher crotyl monophosphate compared to THK M82V (Additional file 2: Fig. S6). Enzyme assays with crude extracts from *E. coli* were carried out to detect the production of crotyl monophosphate. None of these variants showed higher crotyl monophosphate compared to THK M82V (Additional file 2: Fig. S6). It has been reported that the activity of a heterologous protein was affected by its solubility [41]. The significant decrease in production of crotyl monophosphate for the variants THK M82D, THKM82N, THKM82E, THKM82G, THKM82W and THKM82K was likely due to insolubility resulting in inactivity as well.

In order to surmise the molecular mechanisms conferring higher enzymatic activity, we conducted a homology modeling analysis. As shown in the modeled structure of THK (Fig. 5c), M82 is adjacent to the significant loop 28–33 (distance < 5 Å) which is part of the substrate-binding pocket. Probably, this impedes binding of smaller substrate molecules and, thus, contributes to the specificity of the enzyme. When M82 was mutated to V82, the above-mentioned hindrance around M82 was relieved, likely improving the catalytic activity to the smaller molecule substrate such as crotonol. As shown in Additional file 2: Fig. S4 and Table 2, the *K*<sub>m</sub> value of THK<sup>M82V</sup> was determined to be 4.79 mM, 42% lower than wild-type THK, indicating a higher affinity to crotonol. And the *k*<sub>cat</sub> value was 8.58 s<sup>−1</sup>, representing a 6.9-fold improvement in turnover rate. As a consequence, the *k*<sub>cat</sub>/*K*<sub>m</sub> value of THK<sup>M82V</sup> was 12-fold higher than that of wild-type THK.

**Optimization of THK<sup>M82V</sup> and IPK concentrations for in vitro reaction**

The optimal amount of IPK and THK<sup>M82V</sup> required for efficient conversion of crotonol further into crotyl diphosphate in a one-pot reaction was also evaluated. As shown in Additional file 2: Fig. S7a, the production of crotyl monophosphate was increased by 1.4-fold at 8 h when the addition of THK<sup>M82V</sup> was increased from 0.5 to 1.5 mg/mL, then reached a plateau at 2.0 mg/mL. Thus, 1.5 mg/mL of THK<sup>M82V</sup> was used for the one-pot reaction system. In Additional file 2: Fig. S7b, the titers of crotyl diphosphate reached 69.7, 87.5, 99.1 and 102.7 μg/mL at 4 h corresponding to 0.25, 0.5, 1.0 and 1.5 mg/mL of IPK, respectively. As the catalytic efficiency of IPK (*k*<sub>cat</sub>/*K*<sub>m</sub> = 127.94 mM<sup>−1</sup> s<sup>−1</sup>) was 71-fold higher than that of THK<sup>M82V</sup> (*k*<sub>cat</sub>/*K*<sub>m</sub> = 1.80 mM<sup>−1</sup> s<sup>−1</sup>), the lower concentration of IPK (0.5 mg/mL) was chosen for one-pot reactions. Moreover, the in vitro reaction was conducted under the optimal temperature and pH for THK<sup>M82V</sup> in order
to enhance the supply of crotyl monophosphate. Notably, the production of crotyl monophosphate and crotyl diphosphate showed a decreasing rate after 2 h for all the tested conditions (Additional file 2: Fig. S7), implying that those kinases were possibly deactivated gradually along with the reaction. We did not find any literature studying the stability of these two kinases [42], but did notice the conversion was significantly decreased in our preliminary experiments when the enzymes were stored in the reaction buffer overnight before usage.

**In vitro reaction to convert crotonol into crotyl diphosphate**

The two kinases and crotonol were co-incubated under the aforementioned conditions (i.e. 10 mM of crotonol, 1.5 mg/mL THKM82V, 0.5 mg/mL of IPK, 200 μL reaction system, at 39.5 °C and pH 8.0) for production of crotyl diphosphate. As expected, the crotyl diphosphate was produced in a linear manner to 0.22 mM within 2 h, equal to a 2.2% conversion rate from crotonol (Fig. 6a). Beer et al. demonstrated that NADH oxidase was not stable at O2 saturated conditions in the one-pot reaction for converting glucuronate to α-ketoglutarate, and this oxidase was added three times resulting in the increase of conversion rate by 4.76-fold [43]. In our case, a similar phenomenon was observed by replenishing THKM82V and IPK at 2 h and 4 h, which increased the conversion rate by 3.45-fold. Eventually, the titer of crotyl diphosphate was increased to 0.76 mM at 6 h, corresponding to a 7.6% conversion (Fig. 6b). Notably, crotyl monophosphate was barely detected in the whole time course (Fig. 6), suggesting THK was still a rate-limiting step. Future work is possible to significantly increase the activity of THK by a combination of homology modeling established in this work and iterative evolution based on the THKM82V.

**Conversion of crotonyl-CoA to crotonol by an aldehyde/alcohol dehydrogenase**

To identify a candidate enzyme for producing crotonol from crotonyl-CoA involved in the EMC pathway, we tested aldehyde/alcohol dehydrogenase (ADHE2) from *C. acetobutylicum* and fatty acyl-CoA reductase (FAR) from *H. chejuensis* and *M. manganoxydans*. ADHE2 has been used to convert short chain acyl-CoA to alcohols in either engineered *M. extorquens* AM1 or Clostridium species [16, 44]. FAR is another promiscuous enzyme which has good rates of reduction for longer (C20) and shorter (C8) fatty acyl-CoA groups or longer (C8) and shorter (C2) aldehyde groups to produce various alcohols [27]. We found that purified ADHE2 from *C. acetobutylicum* could reduce crotonyl-CoA to crotonol more efficiently compared with FAR from *H. chejuensis* and *M. manganoxydans* by measuring the production of crotonol on GC–MS (Fig. 7). The purified ADHE2 was confirmed by SDS-PAGE gel (Additional file 2: Fig. S2), showing the expected molecular weight of 109 kDa. The kinetic values of ADHE2 were then determined as \( K_m \) and \( k_{cat} \) values of 2.34 mM and 1.15 s\(^{-1}\) (Additional file 2: Fig. S4,
Table 2). This catalytic efficiency towards crotonyl-CoA ($k_{cat}/K_m = 0.49$ mM$^{-1}$ s$^{-1}$) was lower than that of ADHE2 towards butanyl-CoA ($k_{cat}/K_m = 152$ mM$^{-1}$ s$^{-1}$) [45].

Constructing a heterologous pathway to produce butadiene precursor in *M. extorquens* AM1

Following the in vitro reaction, we further expressed thiM and the MTH_47 gene (encoding IPK) in *M. extorquens* AM1 and the engineered strain was named YCB1. Cell lysates of YCB1 were incubated with appropriate amounts of ATP and crotonol. As shown in Fig. 8a, 0.34 μg/mL of crotyl diphosphate was detected at 2 h in the engineered strains while the controls lacking THK and IPK did not yield detectable crotyl diphosphate in the presence of crotonol. We also detected the production of crotyl diphosphate in vivo by adding
2 mM of crotonol during inoculation. About 0.60 μg/mL (2.59 μM) of intracellular crotyl diphosphate was accumulated at mid-exponential phase (Fig. 8b), which was comparable to the individual intermediates in assimilation pathways, which ranged from 0.13 to 55.6 μM in M. extorquens AM1 grown on methanol [46, 47].

In order to explore the possibility of producing crotyl diphosphate from crotonyl-CoA, the engineered M. extorquens AM1 (YCB3 strain) containing the genes for ADHE2, THK^M82V and IPK was further constructed and tested for crotyl diphosphate production. However, no crotyl diphosphate was produced by YCB3 during growth on methanol. The engineered M. extorquens AM1 (YCB4 strain) containing adhE2 was also analyzed for crotonol production when OD reached 0.6, 1.2 and 1.4. No crotonol was detected either. One bottleneck was probably insufficient supply of crotonyl-CoA [14, 17]. Thus, we performed a crude enzymatic assay where a high concentration of crotonyl-CoA was added to drive the reaction from crotonyl-CoA to crotyl diphosphate. Trace concentration of crotyl diphosphate was detected, and 0.51 μg/mL of crotonol (7.0 μM) was produced at 4 h. This result indicated that low activity of ADHE2 was one major bottleneck resulting in the lack of reduction of crotonyl-CoA into crotyl alcohol. Just recently, Becher et al. characterized a novel CoA-acylating aldehyde dehydrogenase responsible for prenal (3-methyl-2-butenal) to 3-methylcrotonyl-CoA oxidation [48], which could possibly improve the activity for the first step of reduction of crotonyl-CoA to crotonaldehyde. Future direction will be focused on improving catalytic efficiency of crotyl-CoA into crotyl alcohol to realize the production of butadiene precursor from methanol.

**Conclusions**

In this work we engineered a metabolic pathway in M. extorquens AM1 for converting crotonol into crotyl diphosphate, a direct precursor of butadiene. The pathway contains a hydroxyethylthiazole kinase (THK) from E. coli and isopentenyl phosphate kinase (IPK) from M. thermautotrophicus. Directed evolution of the rate-limiting THK resulted in a variant (M82V) with the $k_{cat}/K_m$ value 12-fold higher than that of wild-type THK. As a consequence, 7.6% of crotonol was converted into crotyl diphosphate at an optimized in vitro condition. Moreover, the pathway of crotonyl-CoA into crotyl diphosphate was constructed in M. extorquens AM1. 0.60 μg/mL of intracellular crotyl diphosphate was accumulated at the middle of exponential phase with crotonol feeding, and 0.51 μg/mL of crotonol was produced from crotonyl-CoA in vitro crude enzymatic assay. The engineered M. extorquens AM1, however, cannot produce crotyl diphosphate from methanol yet. This was likely because of low activity of ADHE2 towards crotonyl-CoA reduction. In the future, it should be possible to address this issue including protein design and manipulating the flux through the EMC pathway [14, 48, 49]. Although further enzymes and strain optimization are required to make this system industrially relevant, this novel work is the first example for biosynthesis of butadiene precursors. Future work will focus on increasing the activity of ADHE2 in M. extorquens AM1 to realize the bioconversion of methanol into economically important product of butadiene.
Additional files

Additional file 1: Table S1. All the primers are used in this work.

Additional file 2: Fig. S1. Specific activity of purified glycerate kinase (GCK) toward crotonol. The crotonol is added at 500 μM and GCK is 0.5 mg/mL. The control has no added crotonol.

Fig. S2. SDS-PAGE analysis of purified THK, THK<sub>82V</sub>, IPK and FAR. a: M1, M2: Protein markers; 1: Purified THK, b: M1, M2: Protein markers; 1: Purified THK<sub>82V</sub>, 2: Purified IPK, c: M1: Protein marker, 1: Purified ADHE2. d: M1: Protein marker, 1: Purified FAR, originated from Halothallus cheyensis.

Fig. S3. Determining the optimal temperature and pH for THK and IPK. a: The optimal temperature of THK and IPK. b: The optimal pH of THK and IPK. Data represent mean and standard deviations calculated from three biological replicates.

Fig. S4. Enzymatic kinetics of THK, THK<sub>82V</sub>, IPK, FAR and ADHE2. a: Wild-type THK toward crotonol. b: IPK toward crotonyl monophosphate. c: THK<sub>82V</sub> toward crotonol. d: FAR toward crotonyl-CoA. e: ADHE2 toward crotonyl-CoA. Data represent mean and standard deviations calculated from three biological replicates.

Fig. S5. Development of a high throughput screening method. Wild-type M. extorquens AM1 was grown on succinate to mid-exponential phase (OD<sub>600</sub> = 0.60), then crotonol from 160 to 200 mM was added into the culture medium. The supernatants were then transferred into 96-well plate and potassium permanganate was added at a final concentration of 200 μM. a: Color reaction between crotonol and potassium permanganate for 3 min. b: The linear correlation between crotonol concentration and OD<sub>550</sub> value. Data show the mean with error bars indicating standard deviations calculated from three independent biological replicates.

Fig. S6. The effect of targeted mutation on THK activity. a: Activity for mutants of the 82th amino acid of THK. A crude enzymatic assay detects the production of crotonyl monophosphate by LC-MS at 1 h. b: Crude proteins extracted from E. coli are analyzed by SDS-PAGE gel. Data show the mean with error bars indicating standard deviation calculated from three biological replicates.

Abbreviations
THK: Hydroxyethylhydrazine kinase; IPK: isopentenyl phosphate kinase; EVC: pathway ethylmalonyl-CoA pathway; PHB: poly-3-hydroxybutyrate; LB: Luria-Bertani; Tett: tetracycline; FAR: fatty acyl-CoA reductase; ADHE2: aldehyde/alcohol dehydrogenase; GCK: glycerate kinase; IPTG: isopropyl-β-D-thiogalactopyranoside; NTa: Nα-nitrotriacetic acid; PK: pyruvate kinase; LDH: lactate dehydrogenase; MRM: multiple reaction monitoring; MK: mevalonate kinase; EIC: the extracted ion chromatogram; TK: thiamine kinase; 2-PGA: 2-phosphoglycerate; PMK: phosphomevalonate kinase; BEP: 3-butyryl phosphate; HTS: high-throughput screening.

Authors’ contributions
JY, XHM, LPZ and SY conceived and designed the project. JY, CTZ, XJY, MZ and SY performed the experiments. JY, CTZ, XHM, LPZ, LLT, MDY, BH and SY interpreted the data. All authors contributed to the preparation of the manuscript. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

Availability of data and materials
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Not applicable.

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