Mevalonate diphosphate decarboxylase (MVD) is an ATP-dependent enzyme that catalyzes the phosphorylation/decarboxylation of \((R)-\text{mevalonate-5-diphosphate}\) to isopentenyl pyrophosphate in the mevalonate (MVA) pathway. MVD is a key enzyme in engineered metabolic pathways for bioproduction of isobutene, since it catalyzes the conversion of 3-hydroxyisovalerate (3-HIV) to isobutene. The enzyme’s potential in isobutene formation is due to the conversion of 3-HIV to an unstable 3-phosphate intermediate that undergoes consequent spontaneous decarboxylation to form isobutene. Isobutene production rates were as high as 507 pmol min\(^{-1}\) g cells\(^{-1}\) using *Escherichia coli* cells expressing the enzyme and 2,880 pmol min\(^{-1}\) mg protein\(^{-1}\) with the purified histidine-tagged enzyme, significantly higher than reported previously. M3K is a key enzyme of the novel MVA pathway discovered very recently in *Thermoplasma acidophilum*. We suggest that *P. torridus* metabolizes MVA by the same pathway.

The mevalonate (MVA) pathway (1) is the main route for the production of isopentenyl pyrophosphate (IPP) from acetyl coenzyme A (acetyl-CoA) in a diverse cohort of organisms, including higher eukaryotes, archaea, a few eubacteria, fungi, and plants (2). IPP is a key building block for a large family of metabolites such as isoprenoids, dolichols, and sterols that are crucial for a wide variety of cellular functions (3). Many of these molecules have found applications in medicine and agriculture or are used as nutraceuticals, flavors, and fragrances (4, 5). Inhibition of the MVA pathway may also be a target for the design of antibacterial drugs (6).

More recently, the emergence of industrial synthetic biology has led to more and more examples of metabolic engineering using enzymes from the MVA pathway for sustainable production of bioactive and industrial chemicals (7). Products include amorpha-4,11-diene (the precursor to artemisinin) (8, 9), limonene and β-carotene (10–12), a range of sesquiterpenes with potential applications in cosmetics, pharmaceuticals, and jet fuels (13–15), and other biofuels, such as prenol, isoprenol, or other C\(_5\) compounds (16, 17).

A noteworthy example is the use of mevalonate diphosphate decarboxylase (EC 4.1.1.33) (MVD) to produce isobutene. In the classical MVA pathway, MVD catalyzes the final step, where it produces IPP from \((R)-\text{mevalonate-5-diphosphate}\) (MVAPP) in an irreversible reaction dependent upon ATP (Fig. 1A). MVAPP is phosphorylated first, and consequent decarboxylation occurs with the concomitant release of inorganic phosphate. With the same mechanism, classical MVDs also catalyze the conversion of the nonphosphorylated 3-hydroxyisovalerate (3-HIV) to isobutene (Fig. 1B) (18). Isobutene is a small, highly reactive molecule, used extensively as a platform chemical to manufacture a wide variety of products (19, 20) including fuel additives, rubbers, and specialty chemicals. The global demand for isobutene produced from petrochemical sources was estimated to be around 10 million metric tons per year with a market value higher than 15 billion euros (21, 22). Sustainable conversion of renewable feedstocks to isobutene using engineered pathways containing MVD is, therefore, an important target, although current microbial processes are still unsatisfactory (20) compared with current petrochemical production. Nevertheless, Global Bioenergies (Evry, France) has recently announced a second pilot plant study (23).

The first demonstration of isobutene production via MVD employed *Escherichia coli* engineered with the *Saccharomyces cerevisiae* mevalonate diphosphate decarboxylase homologue (SmMVD) to catalyze the conversion of 3-HIV (18). A patent regarding the production of 3-HIV from acetone and acetyl-CoA was filed subsequently (24), followed by further patents on the utilization of MVDs from different sources to produce isobutene more efficiently (25, 26). The concept was also extended to production of other molecules such as isoprenol (27, 28).

Among the enzymes tested in the patents, one of the most...
efficient decarboxylases for isobutene production (25, 27) is the enzyme from *Picrophilus torridus* (GenBank [29] accession no. AAT43941; locus tag PTO1356), which has been annotated as an MVD (29, 30). The utilization of the putative *P. torridus* MVD (*PutMVD*) alone resulted in greater production titers, but even higher yields were obtained when *PutMVD* was mixed with MVDs from other organisms (26, 28). To explain this, it has been proposed that *PutMVD* catalyzes phosphorylation of the substrate faster than the subsequent decarboxylation. It has further been proposed that the phosphorylated intermediate is released and the additional mixture of other MVDs catalyzes decarboxylation of this intermediate to isobutene, providing an overall increase in efficiency (26, 28). However, this hypothesis remains to be tested, since *PutMVD* has not yet been purified and characterized, and the reaction mechanism has not been confirmed.

In the interest of unveiling more information on this poorly characterized enzyme and its potential for isobutene formation, we investigated *PutMVD* in more detail. After sequence analysis, *PutMVD* was tested on the putative natural substrate MVAPP and on other potential substrates. We found that the enzyme is not an MVD. Instead, it belongs to a new class of enzyme, mevalonate-5-diphosphate (MVAPP), which is converted to isopentenyl pyrophosphate (IPP). (B) MVD is also active on 3-hydroxisovalerate (3-HIV) to produce isobutene in *S. cerevisiae* (18).

**FIG 1** MVD reaction scheme. (A) The natural substrate of MVD is (R)-mevalonate-5-diphosphate (MVAPP), which is converted to isopentenyl pyrophosphate (IPP). (B) MVD is also active on 3-hydroxisovalerate (3-HIV) to produce isobutene in *S. cerevisiae* (18).

**MATERIALS AND METHODS**

**Materials.** Bacterial strains and plasmids were purchased from Novagen. Enzymes for gene cloning and strain transformation were purchased from Fermentas, while purification kits for genes and plasmids were purchased from Qiagen. Materials for protein purification and SDS-PAGE were purchased from Fermentas, while purification kits for genes and plasmids were purchased from Qiagen. All other chemicals were purchased from Sigma-Aldrich unless stated otherwise.

**Expression and purification.** Overnight precultures (50 ml) of *E. coli* strains were grown in LB medium (500 ml) with antibiotics and incubated at 37°C with shaking at 160 rpm. At an optical density at 600 nm (OD₆₀₀) of approximately 0.6, protein expression was induced by adding isopropyl-β-D-1-thiogalactopyranoside (IPTG) (0.4 mM). After 14 h, cells were removed by centrifugation and resuspended in buffer A (20 mM sodium phosphate, 30 mM imidazole, 500 mM NaCl [pH 7.4]) for subsequent purification or in buffer B (50 mM Tris-HCl, 10 mM MgCl₂, 20 mM KCl [pH 7.5]) when the supernatants were to be used directly as crude extracts. The cells were lysed with a Constant Systems cell disruption system, cell debris was removed by centrifugation at 15,000 × g for 15 min, and protein expression was checked by SDS-PAGE (see below).

**Enzyme assays.** The activity of purified *PutMVD* and ScMVD was determined by coupling the release of ADP with NADH oxidation by pyruvate kinase/lactate dehydrogenase (PK/LDH) (37-41), measured at 340 nm at 10-s intervals using an Agilent 8453 UV-visible spectrophotometer (Thermo Scientific) using a computed extinction coefficient of 22.015 and 53,650 M⁻¹ cm⁻¹, respectively.

**Sequence and structure analysis.** Sequences were searched with BLASTp (33) using the *PutMVD* amino acid sequence (GenBank [29] accession no. AAT43941) within nonredundant sequences database (November 2014). The E value cutoff was set at 0.1, and the 47 most significant hits were aligned with MUSCLE (34). Phylogenetic reconstruction was performed with MEGA6 (35) by the maximum likelihood test.

For multiple-sequence alignment of classical MVDs and *Thermoplasmatales* putative MVDs, amino acid sequences used were from the following organisms (with the respective GenBank [29] accession no. in parentheses): *Staphylococcus epidermidis* (AAO03959), *Saccharomyces cerevisiae* (AAC49252), *Legionella pneumophila* (AAU28109), *Trypanosoma brucei* (EAN7828), *Candida albicans* (AAT19399), *Homo sapiens* (AAC50440), *Mus musculus* (CAG53731), *Rattus norvegicus* (AA001912), *Arabidopsis thaliana* (AEE79204), *Ginkgo biloba* (AAY23433), *Staphylococcus aureus* (BAB56733), *Streptococcus pyogenes* (AASK3797), *Streptococcus pneumoniae* (CCP34068), *Thermoplasma voltae* (BAB59465), *Thermoplasma acidophilum* (CACC12426), *Ferroplasma acidarmanus* (AGO61795), and *Picrophilus torridus* (AAT43941). Alignment was generated using MUSCLE (34) and rendered with ESPript (36).

**Cloning.** Genes encoding *PutMVD* and ScMVD, optimized for expression in *E. coli* (see Fig. S2 and Fig. S3 in the supplemental material), were synthesized by Biomatik with the inclusion of Ndel and NotI restriction sites to facilitate insertion into a modified version of plasmid pET-20b(+) (EAN7828), adapted for N-terminal histidine tagging. *Escherichia coli* BL21(DE3)LysS was transformed by heat shock, and the transformants were selected on LB agar plates supplemented with carbenicillin (50 μg/ml), chloramphenicol (34 μg/ml), and glucose (10 g/liter). *E. coli* was also transformed with the empty plasmid and used as a control. Glycerol stocks (8%) of transformants were stored at −80°C, and experimental cultures were derived from single colonies grown overnight on LB agar at 37°C. For expression or whole-cell biotransformation assays, single colonies from those plates were used to inoculate precultures.
pure enzyme. Assays were equilibrated for 2 min and initiated by the addition of substrate (see below). NADH concentrations were calculated using an extinction coefficient of 6,220 M⁻¹·cm⁻¹. One unit of enzyme activity corresponds to production of 1 μmol NADH per 1 min.

**Substrate range and kinetic characterization.** **PrM** and **SvM** were tested for activity with (R,S)-mevalonate-5-diphosphate (MVAPP), (R,S)-mevalonate-5-phosphate (MVA-5P), and (R)-mevalonate (MVA), all at 500 μM. **PrM** was also tested for activity with 3-hydroxyisovalerate (3-HIV), 3-hydroxybutyrate (3-HB), and 3-hydroxypropionate (3-HP), at the same concentration.

For **PrM** kinetic characterization, the MVAPP concentration range was 75 to 900 μM, and the 3-HIV and 3-HB concentrations range from 1 to 25 mM. For **SvM** kinetic characterization, the MVAPP concentration range was 50 to 300 μM. When performing kinetic analyses for the cosubstrate ATP, MVA, and MVAPP were kept in excess (2 mM) and ATP was kept in a range between 18.75 and 300 μM. Maximum velocities ($V_{max}$) and Michaelis constants ($K_m$) were estimated using the Michaelis-Menten model of GraphPad Prism version 6.04.

**Product identification.** For isoprenol analysis by gas chromatography-mass spectrometry (GC-MS) and phosphorylated MVA identification by electrospray ionization-mass spectrometry (ESI-MS), products were generated in reaction mixtures (1 ml) containing 5 mM MVA, 6 mM ATP, and 50 μg pure **PrM** in buffer B at 30°C with shaking at 200 rpm. Incubation times are stated in the text. Control samples were prepared in the same way but without adding the enzyme.

Enzymatic reaction mixtures for nuclear magnetic resonance (NMR) were incubated for 24 h at 30°C with shaking at 200 rpm in reaction mixtures described previously (31) but using a larger volume (300 μl) and a higher concentration of MVA and PEP (40 mM). Analytical standards of MVA and MVA-5P were prepared at a concentration of 40 mM.

**Formation of isobutene and other gaseous alkenes.** Isobutene formation was investigated for both **PrM** and **SvM** using whole cells, crude extracts, and pure enzymes. Isobutene formation was linear until 48 h, so production rates were calculated during this period. **PrM** was also tested for ethene and propene formation from 3-HP and 3-HB, respectively, in crude extracts and with purified enzyme. Quantification of all gases was performed by GC-MS.

(i) **Whole-cell assays.** *E. coli* strains were grown and induced as described above. After 5 h, cells were collected by centrifugation at 15,000 × g for 15 min and resuspended in fresh medium containing IPTG. Reaction mixtures (10 ml) containing harvested cells (20 g/liter) and 3-HIV (50 mM) were incubated at 37°C with shaking at 200 rpm in 30 ml glass vials closed with gas-tight caps with polytetrafluoroethylene (PTFE)-silicone septa.

(ii) **Crude extract and pure enzyme assays.** Reaction mixtures (1 ml) containing crude extracts (1 mg/ml total protein) or pure **PrM** and **SvM** (50 μg), ATP (40 mM), and either 3-HIV, 3-HB, or 3-HP (40 mM) in buffer B were incubated at 30°C with shaking at 200 rpm in sealed GC vials (nominal volume of 2 ml) with PTFE screw caps. For controls, samples without the addition of enzymes were also analyzed. The reaction mixtures were incubated at the temperatures stated in the text.

**Analytical methods.** (i) **GC-MS.** Samples were analyzed using an Agilent 7890A GC-MS system equipped with a Restek Rxi-5ms capillary column (0.25 mm by 30 m by 0.25 μm) and a 5975C inert selective detector (MSD) with a quadrupole mass analyzer. Helium was used as a carrier gas at 1.2 ml/min. Data analysis was done using the Agilent MSD ChemStation software and by comparison with the mass spectra and retention times of authentic standards.

For detection and quantification of isoprenol, samples of reaction mixtures (250 μl) were extracted with 750 μl ethyl acetate, and the solvent phase (1 μl) was injected at an inlet temperature of 250°C and at a split ratio of 100:1. The oven temperature was kept at 40°C for 5 min, increased to 200°C at 20°C/min, and then held for 5 min. The retention times of authentic isoprenol were 3.32 min, and a linear calibration curve was obtained over a concentration range from 0.1 to 10 mM.
AGED TO GENERATE THE DATA USED IN THE FIGURES. THE MASSES OF THE NEGATIVELY CHARGED IONS DISCUSSED ARE MEVALONATE-3-PHOSPHATE (227 m/z) AND MEVALONATE (147 m/z), AND THE PHOSPHATE SPECIES ARE H2PO4 (97 m/z) AND PO3 (79 m/z). FOR TANDEM MS (MS-MS) ACQUISITION, THE ISOLATION WIDTH WAS SET AT 1 m/z, AND THE COLLISION ENERGY WAS SET AT 10 eV.

(iii) NMR. The samples were diluted to 600 μl with 99.9% D2O. Spectra were acquired at ambient temperature using a 500-MHz Bruker AVIII500 spectrometer equipped with a cryoprobe. Data were processed and analyzed using ACD/NMR Processor version 12.01.

![FIG 3](https://example.com/figure3.png)

Multiple-sequence alignment. Conventional MVDs are indicated in black, and “MVD-like” proteins from the Thermoplasmatales order are shown in red. Only highly conserved regions (outlined in blue) are depicted. Conservation of residues is denoted by colors: white amino acids on a red background are strictly conserved; red amino acids on a white background are high in similarity. The numbering annotation is based on S. epidermidis MVD. Stars denote residues involved in MVAPP interaction (cyan for C-1 carboxylate group and blue for diphosphate moiety and hydroxyl group). Triangles denote residues involved in ATP interaction (red for phosphate groups, orange for purine ring and ribose).

### TABLE 1

| Enzyme          | Reference(s) | Activity (U · mg⁻¹) | Kₘ (μM)ᵃ | Kₘ(AVP) (μM) | kcat (s⁻¹) | kcat/Kₘ (s⁻¹ · μM⁻¹)ᵇ |
|-----------------|---------------|---------------------|----------|-------------|------------|----------------------|
| P. torridus     | 3  ±  0.1     | 131 ± 18 (MVA)      | 23 ±  0.1| 1.9         | 1.5 × 10⁻² (MVA) |
| S. cerevisiae   | This study    | 7 ± 0.7             | 133 ± 23 (MVAPP) | 61 ±  6 | 5.4 | 4 × 10⁻² (MVAPP) |
| S. cerevisiae   | 39, 40        | 6.4 ± 0.2           | 123 ± 22 (MVAPP) | 61 ±  6 | 4.9 | 4 × 10⁻² (MVAPP) |
| H. volcanii MVD | 44            | 5.6 ± 0.1           | 159 ± 15 (MVA-5P) | 289 ± 15 | 3.5 | 2.2 × 10⁻² (MVA-5P) |
| R. castenholzii | 45            | NA                  | 152 ± 38 (MVA-5P) | NA        | 1.7 | 1.1 × 10⁻² (MVA-5P) |
| T. acidophilum  | 31            | NA                  | 97 ± 6 (MVA)     | NA        | 5.0 | 5.1 × 10⁻² (MVA)  |

ᵃ Activity values refer to the rate of ADP formation coupled to NADH oxidation. Values for P. torridus MVD and ScMVD are calculated from enzyme assays at 30°C in 50 mM Tris-HCl buffer, pH 7.5, with the addition of 10 mM MgCl₂, 20 mM KCl, and 10 μg of pure purMVD or ScMVD. Assays were done in triplicate, and the means ± standard errors of the means for the kinetic constants are shown. Previous data for ScMVD, H. volcanii MVD, R. castenholzii MVD, and T. acidophilum M3K are taken from the literature. NA, not available.

ᵇ Values are given for the respective substrate (in parentheses) of each enzyme (MVAPP, MVA-5P, or MVA).
RESULTS

Sequence analysis. Initially, a bioinformatics approach was used to gain insights into the possible functionality of putPtMVD. A BLASTp search for putPtMVD homologues and a phylogenetic analysis revealed that proteins with the highest sequence identity and similarity with putPtMVD are found mostly in members of the Archaea domain, with higher values with enzymes from Ferroplasma acidarmanus, Thermoplasma volcanium, and T. acidophilum, all members of the Thermoplasmatales order (Fig. 2; see Table S1 in the supplemental material). Interestingly, putPtMVD has sequence identity and similarity of only 21% and 41%, respectively, with Staphylococcus epidermidis MVD (SeMVD) (38), and only 18% and 36% with ScMVD (42), two enzymes that have been verified to act catalytically as MVDs. This significant difference opens the possibility that putPtMVD, and the other Thermoplasmatales homologues, may be unrelated to classical MVDs.

To further investigate this hypothesis, a multiple-sequence alignment was made between Thermoplasmatales homologues and classical MVDs to compare important conserved regions and residues known to be involved in substrate binding and enzyme activity (Fig. 3). The classical MVDs were chosen on the basis of their experimentally verified ability to catalyze decarboxylation of MVAPP and the availability of structural, functional, and mutation data. The regions for ATP binding and interaction with the

FIG 4 ESI-MS analysis. All spectra were collected in negative ion mode. The mass spectra are shown. (A) Control sample containing MVA but without added enzyme; (B) sample containing MVA and putPtMVD; (C) MS-MS spectrum of 227 m/z ion yielding phosphate ions (97 and 79 m/z). The reaction was performed in 50 mM Tris-HCl buffer, pH 7.5, with the addition of 10 mM MgCl₂ and 20 mM KCl. Samples were analyzed after 24 h.
The crystal structure of TaM3K (31,32) (Fig. 3), which was also sequence identity (38%) with the newly discovered gene encoding putM3K was very ready in use for the analysis of substrate phosphorylation by classical MVDs (37–41). There was no activity when MVAPP was tested as the substrate. (R,S)-Mevalonate-5-phosphate (MVA-5P) was also tested, since this is the substrate for another enzyme, MVA-5P decarboxylases (phosphomevalonate decarboxylase [PMD]), similar to MVD, and recently discovered in Archaea and Bacteria (44, 45). However, there was no detectable activity on MVA-5P. Finally, MVA was tested as well, since it is the substrate for TaM3K (31, 32). With MVA, activity was detected at a rate of 3.01 U mg⁻¹ (Table 1). Overall, the substrate range is consistent with the hypothesis that putPtMVD is actually an M3K, similar to TaM3K, rather than a classical MVD, since it did not act on MVAPP. As a comparison, the classical ScMVD (sequence available in Fig. S3 in the supplemental material) was expressed and purified in the same way as putPtMVD and tested on the same substrates. As expected, ScMVD activity was observed only with MVAPP and not with MVA-5P or MVA (Table 1).

In order to understand whether MVA is putPtMVD’s natural substrate, we measured and compared the kinetic parameters for MVA phosphorylation by putPtMVD with MVAPP phosphorylation by ScMVD (Table 1). The kinetic parameters for ScMVD were very similar to those reported previously (Table 1) (39, 40). The magnitude of the kinetic constants and activities of putPtMVD with MVA were similar to those of ScMVD with MVAPP, consistent with the hypothesis that MVA is the natural substrate of putPtMVD, as MVAPP is for ScMVD. In addition, the kinetic parameters reported in the literature for the newly discovered PMDs from Haloferax volcanii (44) and Roseiflexus castenholzii (45), and of M3K from T. acidophilum are also similar in magnitude (Table 1).

New enzyme classification of putPtMVD. To discriminate between putPtMVD being an MVD or an M3K, further experiments were needed, since the coupled enzyme assay monitors only ADP release and not the actual decarboxylation of the intermediate. The expected reaction product would be 3-methylbut-3-en-1-ol (isoprenol) if MVA is phosphorylated and then decarboxylated. Therefore, attempts were made to detect isoprenol by gas chromatography-mass spectrometry (GC-MS). Pure putPtMVD was mixed with a high concentration of MVA (5 mM), and the presence of isoprenol was checked at intervals (24, 48, and 96 h). Even after a long incubation time (96 h), isoprenol was not observed,
suggesting that P423P424 PMV does not catalyze MVA decarboxylation per se.

Therefore, we tested purP425 PMV for formation of a phosphorylated product from MVA, analyzing the reaction product by electrospray ionization-mass spectrometry (ESI-MS). An ion with m/z 227 was detected only in the samples where purP426 PMV was added to the reaction mixture, which corresponded to the mass of the expected phosphorylated MVA (Fig. 4B). In the control without added enzyme, this ion was not present. Instead, an ion with 147 m/z was among those with the highest intensity, corresponding to unreacted MVA (Fig. 4A). A higher background noise was observed in the MS trace for the control compared to the test. This was probably due to a lower response of MVA in the ESI-MS analysis compared to the phosphorylated MVA. Unreacted MVA was also observed in samples where the enzyme was added, meaning that full conversion was not reached (Fig. 4B). As a confirmation that the 227 m/z ion corresponded to phosphorylated MVA, MS-MS analysis of the isolated product generated peaks corresponding to phosphate ions (H2PO4− [97 m/z] and PO43− [79 m/z] [Fig. 4C]).

Although the phosphorylated MVA product is likely to be MVA-3P, it must be mentioned that the observed 227 m/z ion could also correspond to MVA-5P, the alternative phosphorylation product. Therefore, we used 13C NMR analysis to distinguish between the two isomers by analyzing the coupling between 13C and 31P. Any coupling will result in fine structure (doublets) corresponding to phosphate ions (H2PO4− [97 m/z] and PO43− [79 m/z] [Fig. S4 and Fig. S5 in the supplemental material]). In contrast, the 227 m/z ion corresponded to phosphorylated MVA, MS-MS analysis of the isolated product generated peaks corresponding to phosphate ions (H2PO4− [97 m/z] and PO43− [79 m/z] [Fig. 4C]).

TABLE 2 Kinetic constants of PtM3K on four different substrates

| Substrate                  | Activity (U · mg−1) | Km (μM) | kcat (s−1) | kcat/Km (s−1 · μM−1) |
|----------------------------|---------------------|---------|------------|----------------------|
| Mevalonate (MVA)           | 3 ± 0.1             | 131 ± 18| 1.9        | 1.5 × 10−3           |
| 3-Hydroxyisovalerate (3-HIV)| 0.17 ± 0.01         | 2,348 ± 574| 0.1        | 4.25 × 10−5         |
| 3-Hydroxybutyrate (3-HB)   | 0.14 ± 0.01         | 3,163 ± 637| 0.1        | 2.9 × 10−5         |
| 3-Hydroxypropionate (3-HP) | ND                  | ND      | ND         | ND                   |

a Activity values refer to the rate of ADP formation coupled to NADH oxidation. Values are calculated at 30°C in 50 mM Tris-HCl buffer, pH 7.5, with the addition of 10 mM MgCl2, 20 mM KCl, and 50 μg of pure PtM3K (for MVA, the amount of pure PtM3K added was 10 μg). Assays were done in triplicate, and the means ± standard errors of the means for the kinetic constants are shown. ND, not detected.

showed splitting at carbons 2 to 4 and 6, consistent with phosphorylation at position 3 (Fig. 5; see Fig. S6 in the supplemental material).

Thus, analysis of the sequence, substrate range, and reaction products demonstrated that purP426 PMV has been annotated incorrectly and that it is actually a mevalonate-3-kinase that acts on MVA to produce MVA-3P (Fig. 6A). Therefore, we propose that “P427 PMV” should be renamed PtM3K. From now on, it is referred to as PtM3K throughout the text.

Formation of isobutene. PtM3K was identified as a preferred enzyme for the production of isobutene from 3-HIV (25). Therefore, activity of PtM3K on 3-HIV was first investigated. Compared to the natural substrate MVA, the catalytic efficiency was very low, with a 20-fold-higher Km and a 1,000-fold-lower kcat/Km (Table 2). The Km and kcat/Km were lower (2.35 mM) and higher (4.25 × 10−5 s−1 μM−1), respectively, than those disclosed previously (9.17 mM and 2 × 10−5 s−1 μM−1 [26]).

Next, isobutene formation was compared using whole cells expressing PtM3K, crude extract containing PtM3K, and pure PtM3K (Table 3). Production rates were compared with ScMVD under the same conditions, since ScMVD also catalyzes isobutene formation from 3-HIV (18). The experiments were performed in sealed vials so that isobutene could be measured by GC-MS analysis of the headspace. Since isobutene can form spontaneously from 3-HIV (46), controls with no enzyme were always compared, and the rate of isobutene formation in controls was subtracted from the enzymatic reactions. As expected (18, 25, 27), both PtM3K and ScMVD catalyzed formation of isobutene (Table 3). In controls without ATP, isobutene formation was similar to those observed for controls without enzyme (data not shown). This confirms that the reactions catalyzed by PtM3K and ScMVD are ATP dependent. PtM3K catalyzed isobutene formation at rates consistently higher than ScMVD under all assay conditions. With the pure enzyme, an 11-fold improvement was observed (Table 3). Very interestingly, a further 110-fold enhancement (from 26 to 2,880 pmol min−1 mg protein−1) was observed for purified PtM3K when the temperature of the assay was increased from 30 to 50°C (Table 3). However, it must be noted that PtM3K is not a true decarboxylase but a kinase, and therefore, isobutene production depends on formation of an unstable intermediate, 3-phosphoisovalerate (3-PIV) (Fig. 6B) that decarboxylates nonenzymatically. For this reason, it is possible that the higher production rate observed at higher temperature is due to an increased rate of spontaneous decarboxylation of 3-PIV. We wish to note that the rates of isobutene formation obtained using PtM3K were even higher than reported previously using enhanced variants of ScMVD obtained with protein engineering tools (18).

Formation of other gaseous alkenes. In order to test the use of PtM3K for production of other small, industrially useful alkenes,
we investigated its activity on two other substrates, 3-hydroxypropionate (3-HP) and 3-hydroxybutyrate (3-HB). These substrates have the potential to be converted to ethene and propene (19) if they can be phosphorylated at position 3 and if the intermediates are sufficiently unstable to decarboxylate spontaneously.

Although the enzyme was able to phosphorylate 3-HB (Fig. 6B) with kinetics similar to those for 3-HIV (Table 2), no propene was detected by GC-MS using either crude extract or purified enzyme, even at a temperature of 50°C. This suggests that, as for MVA, the phosphorylated intermediate is too stable to decarboxylate. As for 3-HP, the enzyme was not even able to catalyze its phosphorylation. This suggests that one or two methyl substituents are required at position C-3 to enable binding of the substrate to the enzyme and/or for catalytic activity.

**DISCUSSION**

We demonstrated that the *P. torridus* enzyme (GenBank [29] accession no. AAT43941; locus tag PTO1356), so far classified as mevalonate diphosphate decarboxylase, is actually a mevalonate-3-kinase, since it acted on MVA to produce MVA-3P (Fig. 6A). This intermediate appeared to be stable, since no decarboxylation of MVA was observed and MVA-3P could be detected in the reaction mixture. Classical MVDs also act as kinases, but MVAPP is the substrate instead. The phosphorylation results in formation of a transient tertiary phosphorylated intermediate that quickly releases inorganic phosphate with concomitant decarboxylation (38, 47, 48) or even in a concerted manner (49). Thus, the difference in substrate specificity (MVA versus MVAPP) may account for the differences in the ability of PtM3K and MVDs to act as decarboxylases. Differences in amino acid composition in regions suggested to be involved in the catalytic mechanism (Fig. 3, positions 281 to 284) may also affect the enzymatic mechanism, as recently confirmed for *TaM3K* (43).

The results of our sequence analysis suggest that M3Ks may also be found in other members of the *Thermoplasmatales* order in addition to *T. acidophilum* (31, 32) and *P. torridus*. This raises questions about the pathway for MVA metabolism in these organisms, since there is little evidence for the operation of the classical MVA pathway. In the classical MVA pathway, MVA is doubly phosphorylated to MVAPP and consequently converted to IPP by MVD (Fig. 7). In the absence of MVD, MVA must undergo a different route to be converted to IPP. Indeed, the discovery of *TaM3K* led to the description of a completely new type of MVA pathway in *T. acidophilum*, where M3K catalyzes the initial step in the metabolism of MVA (31, 32) (Fig. 7) and MVA-3P-5-kinase (M3P5K) (GenBank [29] accession no. CAC11895) catalyzes the conversion of MVA-3P to isopentenyl phosphate (IP) (44, 47, 48). The enzymes catalyzing conversion of MVA-3PP to isopentenyl phosphate (IP) have not yet been identified, and this provides an excellent target for future research. IP is finally converted to IPP via IP kinase (IPK) (50).

We have now shown that an M3K is also present in *P. torridus*. Since a strongly similar counterpart of M3P5K is also found in *P. torridus* (see Table S7 in the supplemental material), *P. torridus* is also likely to contain this new type of MVA pathway (Fig. 7). It should be noted that this pathway is distinct from the mevalonate-5-kinase (M5K) pathway found in *Haloflexus volcanii* (44) and *Roseiflexus castenholzii* (45) (Fig. 7). Neither of the key enzymes, (M5K and PMD) are present in *T. acidophilum* (50), and there is no experimental or sequence homology evidence to support their presence in *P. torridus*. Overall, this suggests that *P. torridus* produces IPP via the newly discovered M3K pathway.

**FIG 7** Classical MVA pathway and modified routes. The classical MVA pathway and the modified routes (via M5K and PMD and the newly suggested route in *T. acidophilum*) are specifically indicated. Enzyme abbreviations are as follows: M5K, mevalonate-5-kinase; PMK, phosphomevalonate (mevalonate-5-phosphate) kinase; MVD, mevalonate-3-diphosphate decarboxylase; PMD, phosphomevalonate (mevalonate-5-phosphate) decarboxylase; IPK, isopentenyl phosphate kinase; M3K, mevalonate-3-kinase.
Regarding the use of PtM3K and MVDs for isobutene production, we found that PtM3K exhibits much higher catalytic efficiency than ScMVD. Unlike ScMVD, the potential of PtM3K in forming the gas is not due to direct decarboxylation but to the generation of the unstable intermediate, 3-PIV (Fig. 6B). The isobutene production rates for PtM3K were not disclosed until now and were found here to be significantly higher than reported previously for ScMVD (18). Having an enzyme with a higher rate of isobutene formation from 3-HIV is an important step for the production of renewable isobutene, since this further reduces the gap toward commercial application. With ScMVD, it was estimated that the level of cellular activity obtained after enhancement of the enzyme with protein engineering (98 pmol min \(^{-1}\) g cells \(^{-1}\) with whole cells [18]) was about 10\(^{-2}\)-fold below what is needed for a commercial process (18, 20). With PtM3K, the rate is increased 5-fold (307 pmol min \(^{-1}\) g cells \(^{-1}\)). Although this is still far from industrial application, the discovery of a new M3K class of enzymes opens the possibility to screen other, related enzymes for isobutene formation or to undertake protein engineering to improve the production rates still further.

As for the potential of PtM3K to be used for the production of other gaseous alkenes such as ethene and propene, unfortunately, we were not able to observe any product formation. The enzyme was able to phosphorylate 3-HB (Fig. 6B and Table 2), but under the tested conditions, the 3-P intermediate did not spontaneously decarboxylate to propene. This could be due to the 3-phosphobutyrate (3-PB) intermediate being more stable than 3-PIV. The absence of an electron-donating methyl group on 3-PB could reduce the electron density relative to the tertiary carbon of the intermediate and slow down the rate of the decarboxylation, thought to occur via a carbocation intermediate (42).

To conclude, from a microbiological/evolutionary point of view, our study raises important questions on the presence of alternative MVA pathways in Archaea. It is even possible that variant MVA pathways are present among members of all kingdoms in nature (45). Many plant, bacterial, and fungal enzymes are believed to participate in the classical MVA pathway, but like PtM3K, they are still only computationally annotated and need to be investigated experimentally. Discovery of new enzymes is an important goal, since this may yield biocatalysts with improved properties. This can lead to better process efficiencies where production yields with current enzymes are not yet sufficient for commercial viability and where enzyme stability can be a problem. Therefore, discovery of new enzymes of the MVA pathways could aid in improved metabolic engineering for efficient, sustainable bioproduction of chemicals and fuels.

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