1 **Title**

2 Identification of enzymes involved in the mevalonate pathway of *Flavobacterium*

3 *johnsoniae*

4

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Keywords

Mevalonate pathway, isoprenoid, mevalonate kinase, phosphomevalonate kinase, diphosphomevalonate decarboxylase

Abbreviations

BMD, bisphosphomevalonate decarboxylase; DMD, diphosphomevalonate decarboxylase; GGPP, geranylgeranyl diphosphate; IP, isopentenyl phosphate; IPK, isopentenyl phosphate kinase; IPP, isopentenyl diphosphate; M3K, mevalonate 3-kinase; MEP, methylerythritol phosphate; MVA, mevalonate; MVA-3-P, mevalonate 3-phosphate; MVA-5-P, mevalonate 5-phosphate; MVA-5-PP, mevalonate 5-diphosphate; MVK, mevalonate kinase; PMD, phosphomevalonate decarboxylase; PMK, phosphomevalonate kinase

Abstract

The mevalonate pathway is prevalent in eukaryotes, archaea, and a limited number of bacteria. This pathway yields the fundamental precursors for isoprenoid biosynthesis, i.e., isopentenyl diphosphate and dimethylally diphosphate. In the downstream part of the general eukaryote-type mevalonate pathway, mevalonate is converted into isopentenyl diphosphate by the sequential actions of mevalonate kinase, phosphomevalonate kinase, and diphosphomevalonte decarboxylase, while a partial lack of the putative genes of these enzymes is sometimes observed in archaeal and bacterial genomes. The absence of these genes has led to the recent discovery of modified mevalonate pathways. Therefore, we decided to investigate the mevalonate pathway of *Flavobacterium johnsoniae*, a bacterium of the phylum Bacteroidetes, which is reported to lack the genes of mevalonate kinase and phosphomevalonate kinase. This study provides proof of the existence of the general mevalonate pathway in *F. johnsoniae*, and it involves the kinases that are distantly related to the known enzymes.

53 **Introduction**

54 The mevalonate (MVA) pathway is one of the two biosynthetic routes that yield
55 isopentenyl diphosphate (IPP) and dimethylally diphosphate (DMAPP), which are
56 fundamental biosynthetic precursors for >70,000 diverse isoprenoids [1, 2]. The
57 pathway prevails in eukaryotes and archaea, and also in a limited number of bacteria,
58 whereas the other biosynthetic route, the methylerythritol phosphate (MEP) pathway, is
59 generally found in bacteria. In the well-known eukaryote-type (or "classical") MVA
60 pathway (Figure 1), MVA, an intermediate formed from three molecules of acetyl-CoA,
61 is converted into 5-phosphomevalonate (MVA-5-P) by the action of mevalonate kinase
62 (MVK). MVA-5-P is then phosphorylated by phosphomevalonate kinase (PMK), and
63 the product 5-diphosphomevalonate (MVA-5-PP) is converted into IPP by the action of
64 diphosphomevalonate decarboxylase (DMD). These three enzymes belong to the
65 GHMP (named from the initials of galactokinase, homoserine kinase, MVK, and PMK)
66 kinase family and show a modest degree of homology with one another [3].

67 Recently, modified MVA pathways (Figure 1) were discovered in several species
68 of archaea and bacteria. Those microorganisms are reported to lack "a part of" the
69 putative genes of the classical MVA pathway [4-6]. For example, some of the bacteria
70 of the phylum Cloroflexi, such as *Roseiflexus castenholzii*, and most of the halophilic

71 archaea of the class Halobacteria, such as *Haloferax volcanii*, lack the putative gene of
72 PMK in their genomes, while they possess those of MVK and DMD. The putative
73 DMD genes from *R. castenholzii* and *H. volcanii* were later found to encode a new
74 enzyme, phosphomevalonate decarboxylase (PMD), which converts MVA-5-P into
75 isopentenyl phosphate (IP) [7, 8]. The phosphorylation of IP by a non-GHMP kinase
76 conserved in the microorganisms, i.e., isopentenyl phosphate kinase (IPK), yields IPP.
77 The pathway involving MVK, PMD and IPK is referred to as the modified MVA
78 pathway I. In contrast, the thermoacidophilic archaea of the order Thermoplasmatales,
79 such as *Thermoplasma acidophilum*, lack the putative gene of PMK but possess
80 multiple genes of the remote homologs of DMD. The archaea utilize a relatively distant
81 homolog of DMD, i.e., mevalonate 3-kinase (M3K), which converts MVA into
82 3-phosphomevalonate (MVA-3-P) [9, 10]. MVA-3-P is phosphorylated by a
83 non-GHMP kinase to give 3,5-bisphosphomevalonate, which is then converted into IP
84 by the actions of another DMD homolog, bisphosphomevalonate decarboxylase (BMD)
85 [11]. Finally, IPK converts IP into IPP [12]. The pathway found in *T. acidophilum* is
86 referred to as the modified MVA pathway II.
87 The partial lack of the putative genes of the classical MVA pathway is reported to
88 be observed also in some bacterial species other than Chloroflexi bacteria. In their study

on the conservation of isoprenoid biosynthetic genes in the genomes of a wide range of organisms, Lombard and Moreira [4] reported the absence of the putative genes of MVK and PMK in the genomes of a portion of the bacteria of the phylum Bacteroidetes, whereas the putative genes of the other enzymes in the classical MVA pathway were present. Although these Bacteroidetes species do not have a putative gene of IPK, which is involved in the known modified pathways, this situation let us imagine that a novel modified MVA pathway might exist in them. It should be noted that the remaining Bacteroides species do possess the putative genes of the MEP pathway.

In the present study, we re-examined the genomes of the MVA pathway-utilizing Bacteroidetes species, and we found two conserved genes encoding GHMP kinase homologs that were distantly-related to the known MVKs and PMKs. An investigation into the catalytic functions of these GHMP kinase homologs and the DMD homolog from *Flavobacterium johnsoniae* demonstrated the existence of the classical MVA pathway in the bacterium, and also provided information about the evolution and prevalence of the MVA pathways.

105 **Materials and Methods**

106 *Materials* — Silica gel 60 normal-phase TLC plates and silica gel 60 RP-18 F_{254S}

107 reverse-phase TLC plates were purchased from Merck Millipore, Darmstadt, Germany.

108 The radiolabeled substrates, [2-^{14}C]MVA-5-P (55 Ci/mol) and [1-^{14}C]IPP (55 Ci/mol),

109 were purchased from American radiolabeled chemicals, USA. The Seiji Koike, ADEKA

110 Corporation, Japan, donated the (*R*)-Mevalonolactone.

111

112 *Information studies* — A database search of the sets of putative orthologous genes was

113 performed at the website of the Microbial Genome Database (MBGD;

114 http://mbgd.genome.ad.jp/). Phylogenetic analysis of the MVA pathway-related

115 enzymes of the GHMP kinase family was accomplished using the amino acid sequences

116 of the previously identified enzymes registered in Brenda (http://brenda.enzyme.org/).

117 The multiple alignments of the sequences containing those of Fjoh_1387, Fjoh_1417

118 and Fjoh_1389 were performed with the MAFFT server at the EBI website

119 (http://www.ebi.ac.uk/Tools/msa/mafft/), and the phylogenetic tree was constructed

120 based on the alignment data using CLC Sequence Viewer version 7.7 (Qiagen, USA).

121 Amino acid sequence identities shared by enzymes were calculated using LALIGN

122 (http://embnet.vital-it.ch/software/LALIGN_form.html) with a global-method mode.

123

124 *Gene cloning and recombinant expressions of enzymes* — The genes of Fjoh_1387,

125 Fjoh_1417, and Fjoh_1389 were amplified using KOD DNA polymerase (TOYOBO)

126 and the primer pairs listed below: 5'-cgcgcggcagccatatgaaaggaccactatttactc-3' and

127 5'-ggatcctcgagcatattagaattggtaaacaacttctag-3' for the Fjoh_1387 gene;

128 5'-cgcgcggcagccatatgtcaacaaccttttacagtaacg-3' and

129 5'-ggatcctcgagcatactactcctgcaaaatcatttcatc-3' for the Fjoh_1417 gene; and,

130 5'-aaaaaacatatgttaacagcagctgattttatacc-3' and 5'-aaaggatcctcaattatcaattaataatgcgccc-3'

131 for the Fjoh_1389 gene. The amplified gene of either Fjoh_1387 or Fjoh_1417 was

132 cloned into an NdeI-cut pET15b plasmid (Promega) using an InFusion Cloning kit

133 (TaKaRa) to construct pET15b-Fjoh_1387 and pET15b-Fjoh_1417, respectively. The

134 amplified gene of Fjoh_1389 was cut with NdeI and BamHI, and then ligated with an

135 NdeI/BamHI-cut pET15b plasmid to construct pET15b-Fjoh_1389. An *E. coli*

136 Rosetta(DE3) strain transformed with either pET15b-Fjoh_1387 or pET15b-Fjoh_1417

137 was grown in LB medium containing 100 μg/mL ampicillin and 30 μg/mL

138 chloramphenicol at 37°C until the OD_{600} value of the culture medium reached 0.7. After

139 induction with 1 mM IPTG, the culture was grown at 22°C for 24 h with slower

140 rotational shaking (at 90 rpm). An *E. coli* KRX strain transformed with

141 pET15b-Fjoh_1389 was grown in LB medium containing 100 μg/mL ampicillin at 37°C

142 until OD_{600} reached 0.6. After induction with 0.1% L-rhamnose, the culture was grown

143 at 37°C for 24 h.

144

145 *Purification of recombinant enzymes* — The cells of transformed *E. coli* were

146 harvested by centrifugation and then disrupted by sonication with a UP200S ultrasonic

147 homogenizer (Hielscher Ultrasonics, Germany) in a buffer containing 20 mM sodium

148 phosphate, pH7.4, 0.5 M NaCl, and 20 mM imidazole. The purification of

149 polyhistidine-tagged recombinant proteins with a HisTrap FF crude column (GE

150 Healthcare) was performed according to the manufacturer's protocol, using an elution

151 buffer containing 20 mM sodium phosphate, pH 7.4, 0.5 M NaCl, and 500 mM

152 imidazole. For the purification of Fjoh_1387, however, an elution buffer of pH8.3 was

153 used instead, and 1 M arginine and 5% glycerol were immediately added to the solution

154 of the eluted protein to avoid aggregation.

155

156 *Preparation of substrates* — [2-^{14}C]MVA and [2-^{14}C]MVA-5-PP were enzymatically

157 synthesized from [2-^{14}C]MVA-5-P, and [1-^{14}C]IP was synthesized from [1-^{14}C]IPP, as

158 described in our previous work [13]. [2-^{14}C]MVA-3-P was synthesized from

159 [2-^{14}C]MVA-5-P, as described elsewhere [9]. Non-labeled (*R*)-MVA was synthesized

160 from (*R*)-mevalonolactone. Sixty-five mg of (*R*)-mevalonolactone was hydrolyzed in 3

161 mL of 0.2 N KOH at 37°C for 1 h followed by the addition of HCl to adjust the pH to

162 7.2. After dilution with distilled water to reach a final volume of 5 mL, the solution was

163 used as 0.1 M (*R*)-MVA for the experiments described below.

164

165 *Radio-TLC analysis of the reaction products* — To analyze the products from the

166 reaction with the recombinant *F. johnsoniae* proteins, 30 μL of a reaction solution

167 containing 10.8 pmol of the ^{14}C-labeled substrate ([2-^{14}C]MVA, [2-^{14}C]MVA-5-P,

168 [2-^{14}C]MVA-3-P, [2-^{14}C]MVA-5-PP, or [1-^{14}C]IP), 10 pmol of each purified protein,

169 300 nmol of ATP, 1.5 μmol of sodium phosphate buffer at pH7.5, and 60 nmol of

170 $MgCl_2$ was prepared. After reaction for 1 h at 30 °C, 5 μL of the solution was spotted on

171 a silica gel 60 normal-phase TLC plate, and the plate was developed with

172 *n*-propanol/28% ammonium water/H_2O (6:3:1). The distribution of radioactivity on the

173 TLC plate was detected using a Typhoon FLA7000 image analyzer (GE healthcare).

174

175 *Cultivation of F. johnsoniae — F. johnsoniae* JCM8514, which was provided by the

176 RIKEN BRC through the Natural Bio-Resource Project of the MEXT, Japan, was

177 cultivated in a 1 L medium containing 5 g Bacto Peptone (Difco) and 3 g Beef Extract

178 (Difco), whose pH was adjusted to 7.0 by the addition of NaOH, at 30 °C for 2 days

179 prior to being harvested.

180

181 *Cell-free conversion assay using the radiolabeled substrates* — 0.6 g of *F. johnsoniae*

182 cells was dissolved with 1 mL of 500 mM MOPS-NaOH buffer, pH7.2, and was

183 disrupted by sonication. The homogenate was centrifuged at 15,000 rpm for 30 min, and

184 the supernatant was used as a cell-free extract. In a 100 μL volume, 100 pmol of the

185 radiolabeled substrate ([2-^{14}C]MVA, [2-^{14}C]MVA-5-P, [2-^{14}C]MVA-3-P,

186 [2-^{14}C]MVA-5-PP, [1-^{14}C]IP, or [1-^{14}C]IPP) was reacted with the cell-free extract of *F.*

187 *johnsoniae* containing 800 μg protein, 800 nmol ATP, 3 nmol DMAPP, and an

188 appropriate amount of *S. acidocaldarius* geranylgeranyl diphosphate (GGPP) synthase,

189 which was prepared as described elsewhere [14], in 10 mM MOPS-NaOH buffer, pH7.0,

190 containing 10 mM $MgCl_2$, at 30°C for 1 h. After the reaction, 200 μL of saturated saline

191 was added to the mixture, and the hydrophobic product GGPP was extracted with 600

192 μL 1-butanol that had been saturated with saline. Using a treatment with potato acid

193 phosphatase (Sigma-Aldrich) according to a method described by Fuji *et al*. [15], GGPP

194 was converted into geranylgeraniol. The alcohol was extracted with *n*-pentane and then

195 spotted on a reversed-phase silica gel 60 RP-18 F_{254S} TLC plate. The plate was

196 developed with acetone/H_2O (9:1) and visualized using a Typhoon FLA7000 (GE

197 healthcare).

198

199 *Kinetic studies of the enzymes by ADP-coupling assay* — The ATP-dependent activities

200 of MVK, PMK and DMD were assayed via rate measurement of the formation of ADP,

201 which was coupled to the oxidation of NADH via pyruvate kinase and lactate

202 dehydrogenase. To measure the MVK activity of Fjoh_1387, various amounts (1.5-100

203 nmol) of (*R*)-MVA were added to the reaction solution containing 36.5 μmol of sodium

204 phosphate buffer, pH 7.5, 80 nmol of NADH, 2.5 μmol of $MgCl_2$, 2 μmol of ATP, 2.5

205 μmol of phosphoenol pyruvate, 5 U of pyruvate kinase from rabbit muscle (Oriental

206 Yeast, Japan), and 5 U of lactate dehydrogenase from pig heart (Oriental Yeast). After

207 preincubation of the solution at 30°C for 10 min, the reaction in a total volume of 500

208 μL was started by the addition of 8.7 ng of purified Fjoh_1387. The time course for

209 decrease in the absorption of NADH at 340 nm (ε = 6,220 $M^{-1}cm^{-1}$) during the reaction

210 at 30°C was monitored using a spectrophotometer UV-2450 (Shimadzu, Japan) to

211 calculate the initial velocity. The kinetic parameters were calculated by fitting a

212 Michaels-Menten equation to the substrate concentration versus the initial velocity plot

213 using a Kaleidagraph software (SYNERGY software, USA). To measure the activities

214 of the other *F. johnsoniae* proteins, a 2/5 scale (200 μL) of the reaction mixture for the

215 MVK assay described above was used with the exception of changes in the protein and

216 substrate: To measure PMK activity of Fjoh_1417, 8-256 nmol of (*R*,*S*)-MVA-5-P

217 (Sigma-Aldrich) and 6.93 ng of the protein were used; To measure the DMD activity of

218 Fjoh_1389, 4-128 nmol of (*R*,*S*)-MVA-5-PP (Sigma-Aldrich) and 6.93 ng of the protein

219 were used. The measurement of absorption was performed using a Multiskan FC

220 microtiter plate reader (Thermo Scientific, USA).

221

222

223 **Results**

224 First, we performed the comparative genomic analysis of Bacteroidetes species
225 to obtain information about the conservation of the genes involved in the MVA pathway.
226 About half of the Bacteroidetes species whose entire genome sequences have been
227 solved possess the putative ortholog gene of the MVA pathway-related enzymes such as
228 DMD and hydroxymethylglutaryl CoA reductase, while the remaining Bacteroides
229 species possess those of the MEP pathway-related enzymes. Thus, we used the MBGD
230 database to search for putative genes that are conserved only among the MVA
231 pathway-utilizing species. The database search revealed several sets of ortholog genes
232 that are highly conserved among the MVA pathway-utilizing species, but are absent in
233 the MEP pathway-utilizing species. Among such conserved genes, our interest was
234 piqued by those encoding Fjoh_1387 and Fjoh_1417 proteins in the genome of *F.*
235 *johnsoniae*, which belong to different sets of orthologs. The gene of Fjoh_1387 is
236 located closely to that of the DMD homolog Fjoh_1389 on the chromosome, and the
237 gene of Fjoh_1417 juxtaposes that of the hydroxymethylglutaryl CoA reductase
238 homolog Fjoh_1418. Fjoh_1387 is annotated as a mevalonate kinase-like protein in
239 other databases such as KEGG, while Fjoh_1417 is annotated as a hypothetical protein.
240 Fjoh_1387 and Fjoh_1417 are, however, far removed from known MVKs and PMKs

241 from organisms other than the Bacterioidetes species. For example, the identity between

242 the amino acid sequences of Fjoh_1387 and *Staphylococcus pneumoniae* MVK is only

243 21.1%, while that between Fjoh_1417 and *S. pneumoniae* MVK is 18.1%. Sequence

244 identities between *S. pneumoniae* PMK and Fjoh_1387 or Fjoh_1417 are 19.5 and

245 19.6%, respectively. Notably, those values are lower than 23.9% identity between

246 Fjoh_1387 and Fjoh_1417. Therefore, we performed phylogenetic analysis of the

247 already-identified GHMP kinase family enzymes such as MVK, PMK, DMD, PMD,

248 M3K, and BMD that are involved in the MVA pathways of various organisms, along

249 with the hypothetical proteins found from *F. johnsoniae* such as Fjoh_1387, Fjoh_1417,

250 and the DMD homolog Fjoh_1389. As shown by the phylogenetic tree in Figure 2,

251 MVKs, PMKs, and DMDs generally form distinct clusters. Both Fjoh_1387 and

252 Fjoh_1417 are, however, distantly located from the cluster of known MVKs and also

253 from the cluster of PMKs excluding human PMK that is known to have an unusually

254 short sequence. Fjoh_1389 seems to be involved in the cluster of known DMDs, but its

255 position is relatively close to DMD homolog enzymes such as PMD, M3K, and BMD.

256 In such situations, it was difficult to judge whether the *F. johnsoniae* proteins constitute

257 the classical MVA pathway or comprise new roles in a modified MVA pathway.

258 Therefore, we cloned the genes of Fjoh_1387, Fjoh_1417, and Fjoh_1389, and

259 the proteins were recombinantly expressed in *E. coli* cells as fusion with an N-terminal

260 polyhistidine tag. After purification, each of the proteins was reacted with radiolabeled

261 substrates such as MVA, MVA-5-P, MVA-3-P, MVA-5-PP, and IP in the presence of

262 ATP and Mg^{2+}. As shown in Figure 3A, Fjoh_1387 only reacted with MVA and

263 produced MVA-5-P. In contrast, Fjoh_1417 synthesized MVA-5-PP from MVA-5-P, as

264 shown in Figure 3B. These results indicate that Fjoh_1387 and Fjoh_1417 are MVK

265 and PMK, respectively. Fjoh_1389 catalyzed the complete conversion of MVA-5-PP

266 into IPP, but showed only slight activity to convert MVA-5-P into IP (Figure 3C).

267 Although these results strongly support the existence of the classical MVA pathway in

268 *F. johnsoniae*, we performed further analysis to determine if cell-free extract from the

269 microorganism contains these enzyme activities. In the analysis, the radiolabeled

270 substrates used for the enzyme assays were reacted with the cell-free extract from *F.*

271 *johnsoniae* in the presence of ATP, Mg^{2+}, DMAPP, and GGPP synthase from *S.*

272 *acidocaldarius*. When the substrate was converted into IPP by the enzymes in the

273 cell-free extract, the condensation reaction between IPP and DMAPP catalyzed by

274 GGPP synthase gave the radiolabeled hydrophobic product GGPP, which could be

275 extracted with butanol. TLC analysis of the product following phosphatase treatment

276 demonstrated that MVA-5-P and MVA-5-PP were efficiently converted into GGPP via

277 IPP, while MVA-3-P and IP were not (Figure 3D). Although MVA was not converted

278 into GGPP, most likely due to the weak activity of MVK in the cell-free extract, an

279 explanation could be the extreme instability of the enzyme. Indeed, the recombinant *F.*

280 *johnsoniae* MVK easily aggregated and became inactivated typically in a few days after

281 purification (data not shown). These results demonstrated that the classical MVA

282 pathway actually exists in the cells of *F. johnsoniae*, even though the enzymes included

283 in the pathway, particularly MVK and PMK, are distant relatives of the ones that are

284 known. To compare the catalytic efficiencies of *F. johnsoniae* MVK (Fjoh_1387), PMK

285 (Fjoh_1417), and DMD (Fjoh_1389) with those of known enzymes from different

286 organisms, kinetic studies were performed. As shown in Table 1, the kinetic parameters

287 of the enzymes from *F. johnsoniae* are comparable to the reported parameters of their

288 orthologs, supporting the functions of the enzymes identified in this study. It is

289 noteworthy that the K_m of *F. johnsoniae* MVK for MVA is smaller than those of any

290 MVKs ever reported [16], which is particularly true for the much larger K_ms of bacterial

291 MVKs from *E. faecalis* and *S. pneumoniae*, possibly because of its unique nature with

292 respect to its amino acid sequence.

293

294 **Discussion**

295 The MEP pathway is the general route that supplies the precursors for
296 isoprenoid biosynthesis in bacteria. However, genome information [4] suggests that
297 several limited lineages of bacteria, such as parts of phyla Bacteroidetes, Chloroflexi,
298 and Firmicutes, utilize the MVA pathway, and biochemical studies have proven this in
299 some cases. This situation allows us to imagine a simple evolutionary scenario whereby
300 the MVA pathway was transferred horizontally to the ancestors of such bacterial
301 lineages and then replaced the MEP pathway that had been inherited from the a
302 common bacterial ancestor. If such a replacement has occurred, some advantage must
303 be assigned to the bacterial strains that acquired the MVA pathway. Misic et al. recently
304 proposed that escape from the immune system of animal hosts could be an advantage
305 [17]. That study found that the *Staphylococcus* species that possess the MVA pathway
306 associate with primate hosts, while those that have the MEP pathway do with
307 non-primate animal hosts. The researchers proposed that acquisition of the MVA
308 pathway allows the *Staphylococcus* species to weaken the immune response by the host
309 via Vγ2Vδ2 T cells, which reside only in primates and are activated by an intermediate
310 of the MEP pathway, (*E*)-1-hydroxy-2-methyl-2-but-2-enyl 4-diphosphate. This
311 hypothesis, however, seems not to be the case with Bacteroidetes. Most of the MVA

312 pathway-utilizing Bacteroidetes species are included in the order Flavobacteriales, and
313 the majority of them were isolated from marine environments or from marine organisms.
314 Only limited Flavobacteriales species, such as some of those belonging to genera
315 *Capnocytophaga*, *Chryseobacterium*, *Elizabethkingia*, *Myroides*, and *Weeksella*, are
316 human pathogens.

317 The positions of *F. johnsoniae* MVK and PMK in the phylogenetic tree in
318 Figure 2 are unusual because enzymes with the same function form a unified cluster, as
319 MVKs, PMKs, and DMDs do, even if they are from distantly related organisms. This
320 situation is caused by the low level of similarity that exists between the *F. johnsoniae*
321 enzymes and their homologs from organisms other than Bacteroidetes, and that can also
322 explain why the genes of both MVK and PMK were once reported to be absent in
323 Bacteroidetes [4]. The unique nature of *F. johnsoniae* enzymes could be attributed to
324 the different evolutionary history of the MVA pathway of the phylum Bacteroidetes, or,
325 practically, the order Flavobacteriales: The origin of the horizontal transfer of the MVA
326 pathway genes to their ancestor might be far removed from the origins to the ancestors
327 of other bacterial lineages.

328

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Footnotes

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338 **References**

339 [1] T. Kuzuyama, H. Hemmi, S. Takahashi, Mevalonate Pathway in Bacteria and

340 Archaea, in: L. Mander, H.-W. Liu (Eds.) Comprehensive Natural Products II Chemistry

341 and Biology, Elsevier, Oxford, UK, 2010, pp. 493-516.

342 [2] C.E. Vickers, M. Bongers, Q. Liu, T. Delatte, H. Bouwmeester, Metabolic

343 engineering of volatile isoprenoids in plants and microbes, Plant Cell Environ, 37 (2014)

344 1753-1775.

345 [3] J.L. Andreassi, II, T.S. Leyh, Molecular functions of conserved aspects of the GHMP

346 kinase family, Biochemistry, 43 (2004) 14594-14601.

347 [4] J. Lombard, D. Moreira, Origins and early evolution of the mevalonate pathway of

348 isoprenoid biosynthesis in the three domains of life, Mol Biol Evol, 28 (2011) 87-99.

349 [5] R. Matsumi, H. Atomi, A.J. Driessen, J. van der Oost, Isoprenoid biosynthesis in

350 Archaea–biochemical and evolutionary implications, Res Microbiol, 162 (2011) 39-52.

351 [6] A. Smit, A. Mushegian, Biosynthesis of isoprenoids via mevalonate in Archaea: the

352 lost pathway, Genome Res, 10 (2000) 1468-1484.

353 [7] N. Dellas, S.T. Thomas, G. Manning, J.P. Noel, Discovery of a metabolic alternative

354 to the classical mevalonate pathway, eLife, 2 (2013) e00672.

355 [8] J.C. VanNice, D.A. Skaff, A. Keightley, J. Addo, G.J. Wyckoff, H.M. Miziorko,

356 Identification in *Haloferax volcanii* of phosphomevalonate decarboxylase and
357 isopentenyl phosphate kinase as catalysts of the terminal enzymatic reactions in an
358 archaeal alternate mevalonate pathway, J Bacteriol, 196 (2014) 1055-1063.
359 [9] Y. Azami, A. Hattori, H. Nishimura, H. Kawaide, T. Yoshimura, H. Hemmi,
360 (*R*)-Mevalonate 3-phosphate is an intermediate of the mevalonate pathway in
361 *Thermoplasma acidophilum*, J Biol Chem, 289 (2014) 15957-15967.
362 [10] J.M. Vinokur, T.P. Korman, Z. Cao, J.U. Bowie, Evidence of a novel mevalonate
363 pathway in archaea, Biochemistry, 53 (2014) 4161-4168.
364 [11] J.M. Vinokur, M.C. Cummins, T.P. Korman, J.U. Bowie, An adaptation to life in
365 acid through a novel mevalonate pathway, Sci Rep, 6 (2016) 39737.
366 [12] M. Chen, C.D. Poulter, Characterization of thermophilic archaeal isopentenyl
367 phosphate kinases, Biochemistry, 49 (2010) 207-217.
368 [13] H. Nishimura, Y. Azami, M. Miyagawa, C. Hashimoto, T. Yoshimura, H. Hemmi,
369 Biochemical evidence supporting the presence of the classical mevalonate pathway in
370 the thermoacidophilic archaeon *Sulfolobus solfataricus*, J Biochem, 153 (2013) 415-420.
371 [14] H. Hemmi, K. Shibuya, Y. Takahashi, T. Nakayama, T. Nishino,
372 (*S*)-2,3-Di-*O*-geranylgeranylglyceryl phosphate synthase from the thermoacidophilic
373 archaeon *Sulfolobus solfataricus*. Molecular cloning and characterization of a

374 membrane-intrinsic prenyltransferase involved in the biosynthesis of archaeal

375 ether-linked membrane lipids, J Biol Chem, 279 (2004) 50197-50203.

376 [15] H. Fujii, T. Koyama, K. Ogura, Efficient enzymatic hydrolysis of polyprenyl

377 pyrophosphates, Biochim Biophys Acta, 712 (1982) 716-718.

378 [16] P. Nyati, C. Rivera-Perez, F.G. Noriega, Negative feedbacks by isoprenoids on a

379 mevalonate kinase expressed in the *Corpora allata* of mosquitoes, Plos One, 10 (2015)

380 e0143107.

381 [17] A.M. Misic, C.L. Cain, D.O. Morris, S.C. Rankin, D.P. Beiting, Divergent

382 isoprenoid biosynthesis pathways in *Staphylococcus* species constitute a drug target for

383 treating infections in companion animals, mSphere, 1 (2016).

384 [18] M. Hedl, V.W. Rodwell, *Enterococcus faecalis* mevalonate kinase, Protein Sci, 13

385 (2004) 687-693.

386 [19] Y.A. Primak, M. Du, M.C. Miller, D.H. Wells, A.T. Nielsen, W. Weyler, Z.Q. Beck,

387 Characterization of a feedback-resistant mevalonate kinase from the archaeon

388 *Methanosarcina mazei*, Appl Environ Microbiol, 77 (2011) 7772-7778.

389 [20] Z. Fu, N.E. Voynova, T.J. Herdendorf, H.M. Miziorko, J.J. Kim, Biochemical and

390 structural basis for feedback inhibition of mevalonate kinase and isoprenoid metabolism,

391 Biochemistry, 47 (2008) 3715-3724.

392 [21] K.X. Huang, A.I. Scott, G.N. Bennett, Overexpression, purification, and

393 characterization of the thermostable mevalonate kinase from *Methanococcus jannaschii*,

394 Protein Exp Purif, 17 (1999) 33-40.

395 [22] S.S. Doun, J.W. Burgner, 2nd, S.D. Briggs, V.W. Rodwell, *Enterococcus faecalis*

396 phosphomevalonate kinase, Protein Sci, 14 (2005) 1134-1139.

397 [23] D. Pilloff, K. Dabovic, M.J. Romanowski, J.B. Bonanno, M. Doherty, S.K. Burley,

398 T.S. Leyh, The kinetic mechanism of phosphomevalonate kinase, J Biol Chem, 278

399 (2003) 4510-4515.

400 [24] T.J. Herdendorf, H.M. Miziorko, Phosphomevalonate kinase: functional

401 investigation of the recombinant human enzyme, Biochemistry, 45 (2006) 3235-3242.

402 [25] M.L. Barta, D.A. Skaff, W.J. McWhorter, T.J. Herdendorf, H.M. Miziorko, B.V.

403 Geisbrecht, Crystal structures of *Staphylococcus epidermidis* mevalonate diphosphate

404 decarboxylase bound to inhibitory analogs reveal new insight into substrate binding and

405 catalysis, J Biol Chem, 286 (2011) 23900-23910.

406 [26] N.E. Voynova, Z. Fu, K.P. Battaile, T.J. Herdendorf, J.J. Kim, H.M. Miziorko,

407 Human mevalonate diphosphate decarboxylase: characterization, investigation of the

408 mevalonate diphosphate binding site, and crystal structure, Arch Biochem Biophys, 480

409 (2008) 58-67.

410 [27] D. Krepkiy, H.M. Miziorko, Identification of active site residues in mevalonate

411 diphosphate decarboxylase: implications for a family of phosphotransferases, Protein Sci,

412 13 (2004) 1875-1881.

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414

415 **Table 1 Kinetic parameters of *F. johnsoniae* MVK, PMK and DMD, with the**
416 **reported parameters of the enzymes from other organisms**

Enzymes Organisms/used substrates	K_m (μM^{-1})	V_{max} (μmol/min/mg)	k_{cat} (sec^{-1})
MVK			
F. johnsoniae/(*R*)-MVA	11.4	43.0	25.0
E. faecalis/(*RS*)-MVA[18]	330	11.4	-
S. pneumoniae/(*R*)-MVA[19]	236	-	11
H. sapiens/(*RS*)-MVA[20]	40.8	28.0	-
S. cerevisiae/(*R*)-MVA[19]	131	-	38
M. mazei/(*R*)-MVA[19]	68	-	4.3
M. jannaschii/(*RS*)-MVA[21]	68.5	387	-
PMK			
F. johnsoniae/(*RS*)-MVA-5-P	106	22.2	12.8
E. faecalis/(*RS*)-MVA-5-P[22]	190	3.9	-
S. pneumoniae/(*R*)-MVA-5-P[23]	4.2	-	3.4
H. sapiens/(*R*)-MVA-5-P[24]	34	46.4	-
S. solfataricus/(*RS*)-MVA-5-P[13]	77	5.1	-
DMD			
F. johnsoniae/(*RS*)-MVA-5-PP	37.7	26.6	18.5
S. epidermidis/(*RS*)-MVA-5-PP[25]	9.1	9.8	5.9
H. sapiens/(*RS*)-MVA-5-PP[26]	28.9	6.1	4.5
S. cerevisiae/(*RS*)-MVA-5-PP[27]	123	6.4	4.9

417 418 419 420 421 422 423 424 425 426 427 428 429 430 431 432 433 434 435 436 437 438 439 440 441 442 443

444 **Figure legends**

445 **Figure 1:** GHMP kinase family enzymes in the classical and modified MVA pathways.
446 The green, blue, and red arrows indicate the enzyme reactions of the classical pathway,
447 modified pathway I, and modified pathway II, respectively. The black arrows indicate
448 the upstream reactions that are held in common. Enzymes belonging to the GHMP
449 kinase family are shown in the gray boxes, while non-GHMP family kinases appear in
450 the open boxes.

451

452 **Figure 2:** Phylogenetic tree of MVA pathway-related GHMP kinase family enzymes.
453 Bootstrap values larger than 50% are shown at each node.

454

455 **Figure 3:** Radio-TLC enzyme assay of the recombinant *F. johnsoniae* proteins and
456 cell-free extract from *F. johnsoniae*.
457 (A-C) Normal-phase TLC analyses of the products from the reactions of the
458 recombinant Fjoh_1387 (A), Fjoh_1417 (B), and Fjoh_1389 (C) with the radiolabeled
459 intermediates of the classical and modified mevalonate pathways. (D) Reversed-phase
460 TLC analysis of geranylgeraniol, which was synthesized only when the radiolabeled
461 substrates were converted into IPP by *F. johnsoniae* cell-free extract.

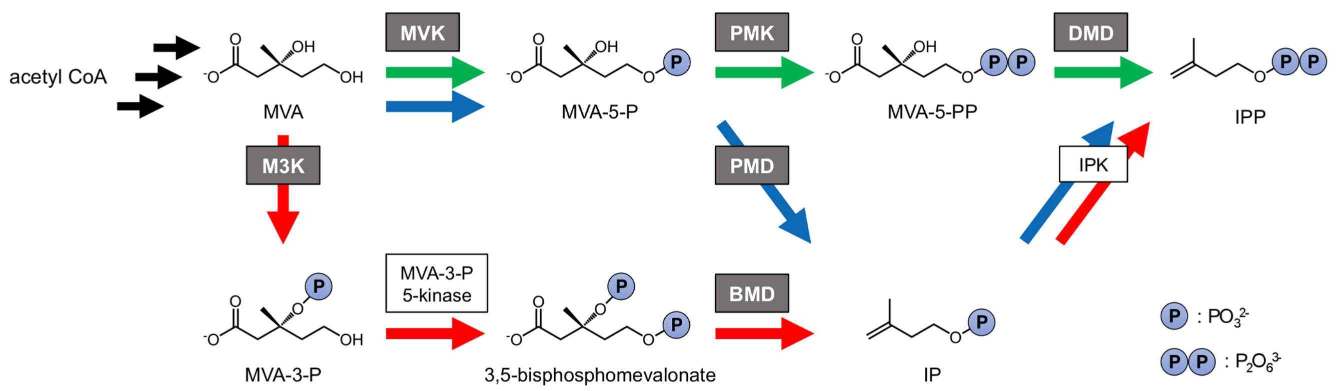

Figure 1

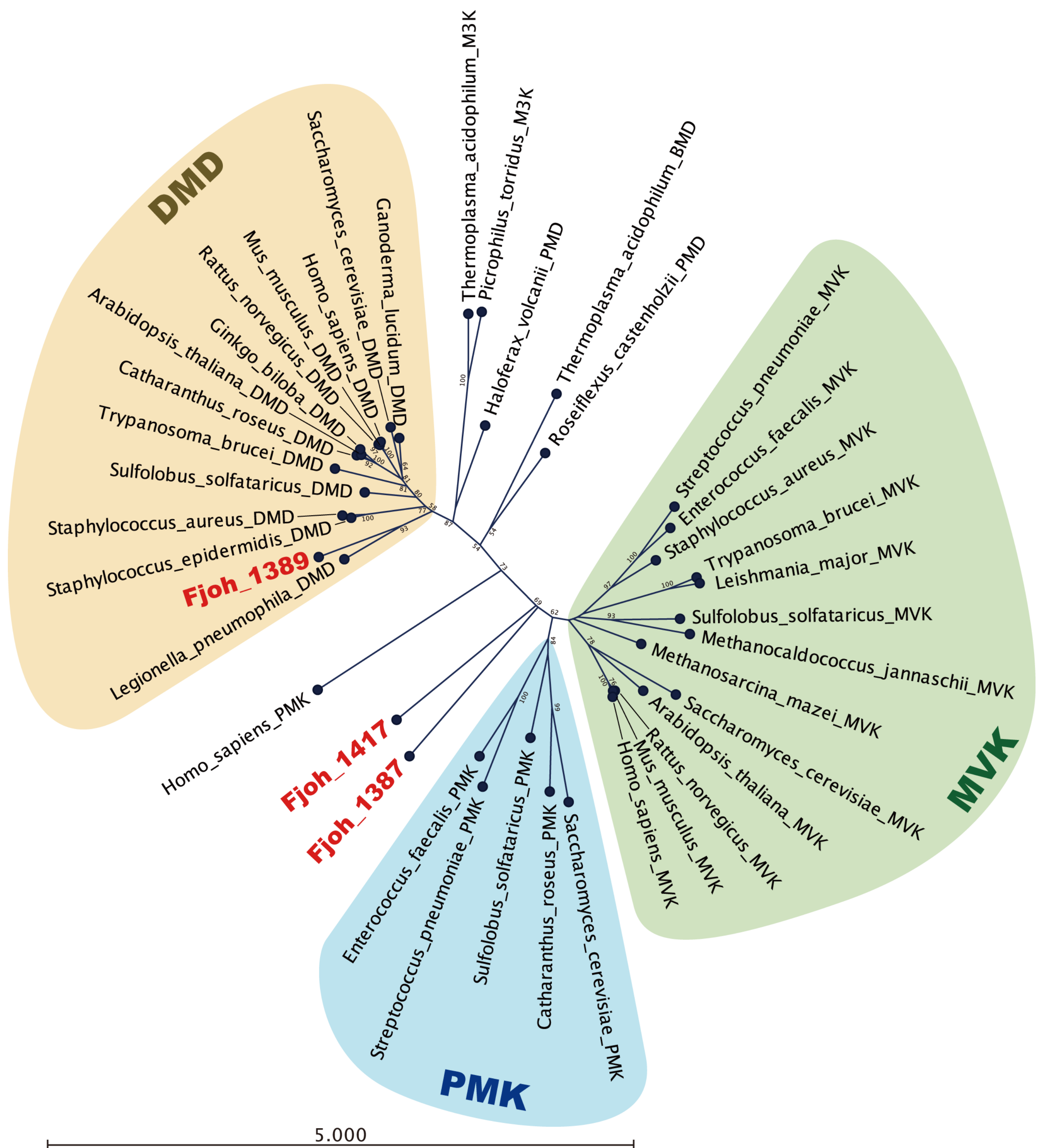

Figure 2

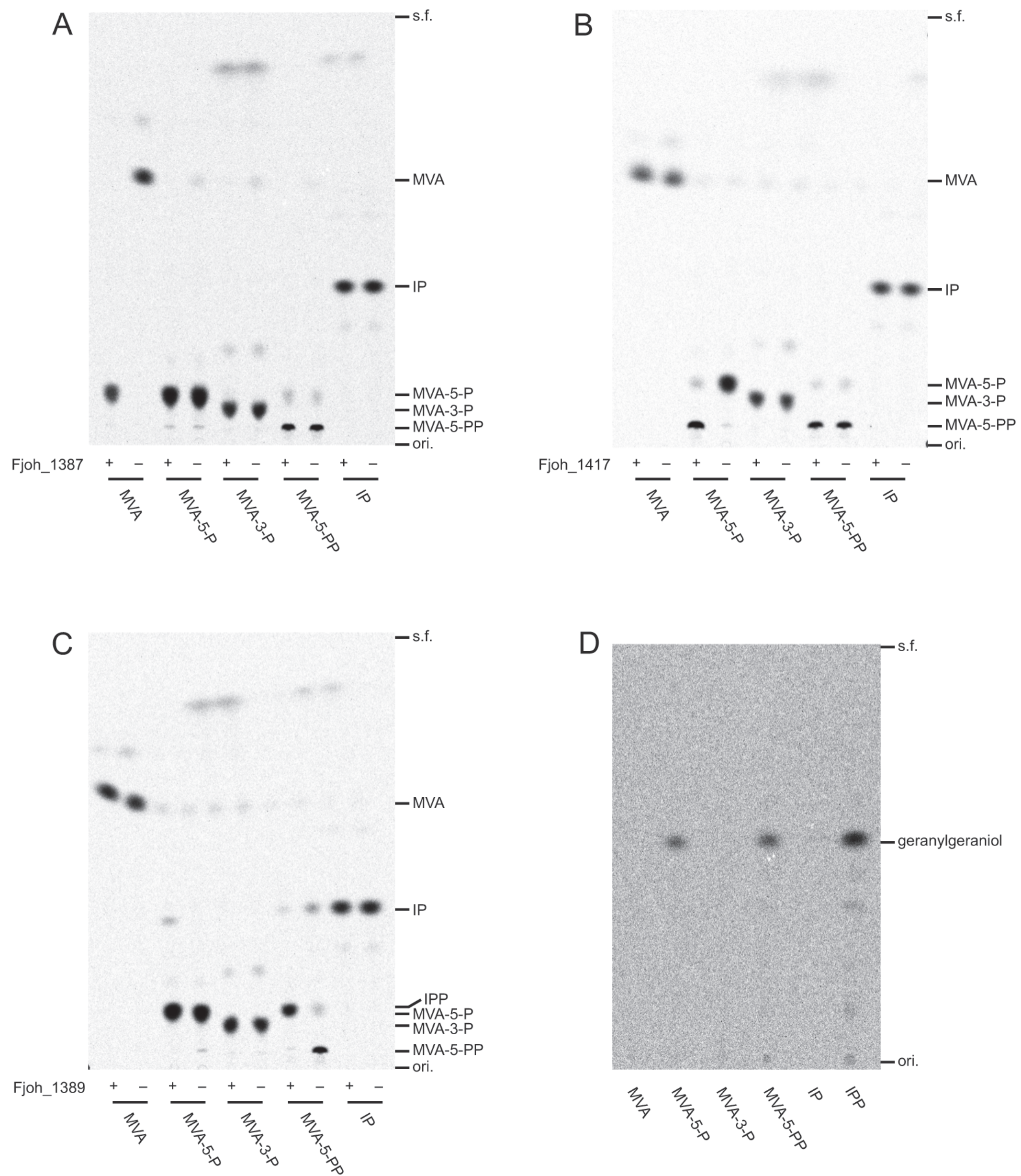

Figure 3