Biochemical and Biophysical Research Communications xxx (2017) 1–7

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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

Hajime Hayakawa [1], Fumiaki Sobue [1], Kento Motoyama, Tohru Yoshimura, Hisashi Hemmi*

Department of Applied Molecular Bioscience, Graduate School of Bioagricultural Sciences, Nagoya University, Furo-cho, Chikusa-ku, Nagoya, Aichi 464-8601, Japan

ARTICLE INFO

Article history:
Received 18 April 2017
Accepted 22 April 2017
Available online xxx

Keywords:
Mevalonate pathway
Isoprenoid
Mevalonate kinase
Phosphomevalonate kinase
Diphosphomevalonate decarboxylase

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*, although the pathway involves the kinases that are distantly related to the known enzymes.

1. Introduction

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

Recently, modified MVA pathways (Fig. 1) were discovered in several species of archaea and bacteria. Those microorganisms are reported to lack "a part of" the putative genes of the classical MVA pathway [4–6]. For example, some of the bacteria of the phylum Cloroflexi, such as *Roseiflexus castenholzii*, and most of the halophilic archaea of the class Halobacteria, such as *Haloferax volcanii*, lack the putative gene of PMK in their genomes, while they possess those of MVK and DMD. The putative DMD genes from *R. castenholzii* and *H. volcanii* were later found to encode a new enzyme, phosphomevalonate decarboxylase (PMD), which converts MVA-5-P into isopentenyl phosphate (IP) [7,8]. The phosphorylation of IP by a non-GHMP kinase conserved in the microorganisms, i.e., isopentenyl phosphate kinase (IPK), yields IPP. The pathway involving MVK, PMD and IPK is referred to as the modified MVA pathway I. In contrast, the thermoacidophilic archaea of the order Thermoplasmatales, such as *Thermoplasma acidophilum*, lack the putative gene of PMK but possess multiple

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.

* Corresponding author.
E-mail address: hhemmi@agr.nagoya-u.ac.jp (H. Hemmi).

[1] These authors contributed equally.

http://dx.doi.org/10.1016/j.bbrc.2017.04.120
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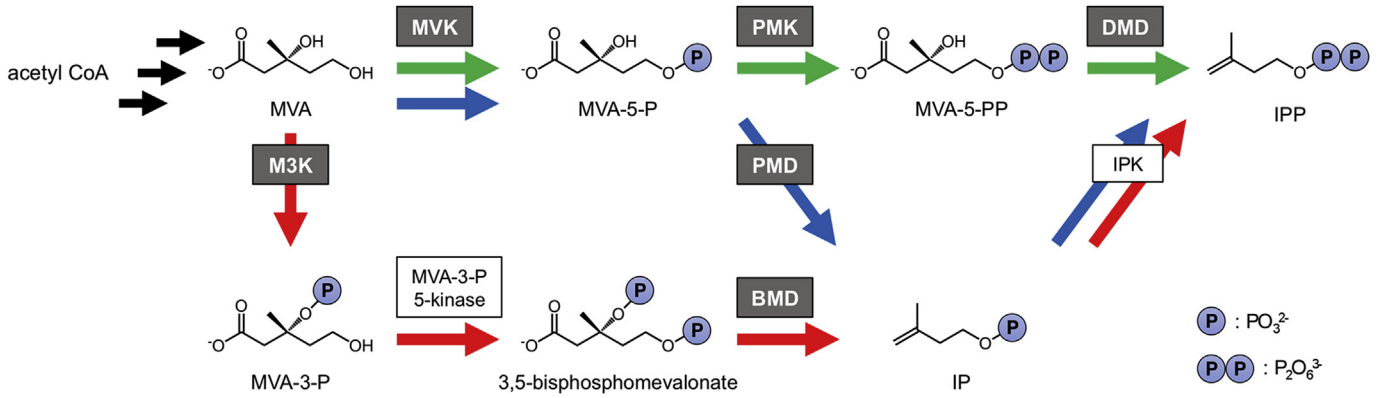

Fig. 1. GHMP kinase family enzymes in the classical and modified MVA pathways. The green, blue, and red arrows indicate the enzyme reactions of the classical pathway, modified pathway I, and modified pathway II, respectively. The black arrows indicate the upstream reactions that are held in common. Enzymes belonging to the GHMP kinase family are shown in the gray boxes, while non-GHMP family kinases appear in the open boxes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

genes of the remote homologs of DMD. The archaea utilize a relatively distant homolog of DMD, i.e., mevalonate 3-kinase (M3K), which converts MVA into 3-phosphomevalonate (MVA-3-P) [9,10]. MVA-3-P is phosphorylated by a non-GHMP kinase to give 3,5-bisphosphomevalonate, which is then converted into IP by the actions of another DMD homolog, bisphosphomevalonate decarboxylase (BMD) [11]. Finally, IPK converts IP into IPP [12]. The pathway found in *T. acidophilum* is referred to as the modified MVA pathway II.

The partial lack of the putative genes of the classical MVA pathway is reported to 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.

2. Materials and methods

Materials — Silica gel 60 normal-phase TLC plates and silica gel 60 RP-18 F_{254S} reverse-phase TLC plates were purchased from Merck Millipore, Darmstadt, Germany. The radiolabeled substrates, [2-^{14}C]MVA-5-P (55 Ci/mol) and [1-^{14}C]IPP (55 Ci/mol), were purchased from American radiolabeled chemicals, USA. Seiji Koike, ADEKA Corporation, Japan, donated the (*R*)-Mevalonolactone.

Information studies — A database search of the sets of putative orthologous genes was performed at the website of the Microbial Genome Database (MBGD; http://mbgd.genome.ad.jp/). Phylogenetic analysis of the MVA pathway-related enzymes of the GHMP kinase family was accomplished using the amino acid sequences of the previously identified enzymes registered in Brenda (http://brenda.enzyme.org/). The multiple alignments of the sequences containing those of Fjoh_1387, Fjoh_1417 and Fjoh_1389 were performed with the MAFFT server at the EBI website (http://www.ebi.ac.uk/Tools/msa/mafft/), and the phylogenetic tree was constructed based on the alignment data using CLC Sequence Viewer version 7.7 (Qiagen, USA). Amino acid sequence identities shared by enzymes were calculated using LALIGN (http://embnet.vital-it.ch/software/LALIGN_form.html) with a global-method mode.

Gene cloning and recombinant expressions of enzymes — The genes of Fjoh_1387, Fjoh_1417, and Fjoh_1389 were amplified using KOD DNA polymerase (TOYOBO) and the primer pairs listed below: 5′-cgcgcggcagccatatgaaaggaccactatttactc-3′ and 5′-ggatcctcgagcatattagaattggtaaacaacttctag-3′ for the Fjoh_1387 gene; 5′-cgcgcggcagccatatgtcaacaacctttacagtaacg-3′ and 5′-ggatcctcgagcatactactcctgcaaaatcatttcatc-3′ for the Fjoh_1417 gene; and, 5′-aaaaaacatatgttaacagcagctgattttatacc-3′ and 5′-aaaggatcctcaattatcaattaataatgcgccc-3′ for the Fjoh_1389 gene. The amplified gene of either Fjoh_1387 or Fjoh_1417 was cloned into an *Nde*I-cut pET15b plasmid (Promega) using an InFusion Cloning kit (TaKaRa) to construct pET15b-Fjoh_1387 and pET15b-Fjoh_1417, respectively. The amplified gene of Fjoh_1389 was cut with *Nde*I and *Bam*HI, and then ligated with an *Nde*I/*Bam*HI-cut pET15b plasmid to construct pET15b-Fjoh_1389. An *E. coli* Rosetta(DE3) strain transformed with either pET15b-Fjoh_1387 or pET15b-Fjoh_1417 was grown in LB medium containing 100 μg/mL ampicillin and 30 μg/mL chloramphenicol at 37 °C until the OD_{600} value of the culture medium reached 0.7. After induction with 1 mM IPTG, the culture was grown at 22 °C for 24 h with slower rotational shaking (at 90 rpm). An *E. coli* KRX strain transformed with pET15b-Fjoh_1389 was grown in LB medium containing 100 μg/mL ampicillin at 37 °C until OD_{600} reached 0.6. After induction with 0.1% L-rhamnose, the culture was grown at 37 °C for 24 h.

Purification of recombinant enzymes — The cells of transformed *E. coli* were harvested by centrifugation and then disrupted by sonication with a UP200S ultrasonic homogenizer (Hielscher Ultrasonics, Germany) in a buffer containing 20 mM sodium phosphate, pH7.4, 0.5 M NaCl, and 20 mM imidazole. The purification of polyhistidine-tagged recombinant proteins with a HisTrap FF crude column (GE Healthcare) was performed according to the manufacturer's protocol, using an elution buffer containing 20 mM sodium phosphate, pH 7.4, 0.5 M NaCl, and 500 mM imidazole. For the purification of Fjoh_1387, however, an elution buffer of pH8.3 was

Please cite this article in press as: H. Hayakawa, et al., Identification of enzymes involved in the mevalonate pathway of *Flavobacterium johnsoniae*, Biochemical and Biophysical Research Communications (2017), http://dx.doi.org/10.1016/j.bbrc.2017.04.120