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Isoprenoid biosynthesis via the methylerythritol phosphate pathway: accumulation of 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate in a *gcpE* deficient mutant of *Escherichia coli*

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Abstract—In the bacterium *Escherichia coli*, *gcpE* is an essential gene in the methylerythritol phosphate pathway for isoprenoid biosynthesis. Incubation of [1-^{3}H]methylerythritol with an *E. coli* mutant defective in the *gcpE* gene resulted in the accumulation of [1-^{3}H]methylerythritol 2,4-cyclodiphosphate. This suggests that the GCPE protein is involved in the further conversion of methylerythritol cyclodiphosphate into isoprenoids.

In most bacteria and in all plant plastids, isoprenoids are not synthesized via the classical mevalonate (MVA) pathway, but via the alternative MVA-independent 2-*C*-methyl-D-erythritol phosphate (MEP) route (Fig. 1).[1] In the latter metabolic pathway, isoprenoids are derived from pyruvate **1** and glyceraldehyde 3-phosphate **2**, which are converted into 1-deoxy-D-xylulose 5-phosphate (DXP) **3** by the thiamin diphosphate dependent enzyme DXP synthase. Four additional intermediates are known: MEP **4**, which results from the rearrangement of DXP followed by a reduction, 4-diphosphocytidyl methylerythritol **5** and its 2-phosphate **6**, as well as methylerythritol 2,4-cyclodiphosphate **7**. It is striking that the last four intermediates are characterized by the same oxidation state as 2-*C*-methyl-D-erythritol (ME). The discovery of the MEP pathway was the result of intense labeling experiments on bacterial triterpenoids of the hopane series[2] or on diterpenoids from ginkgo embryos,[3] followed by diverse genetic and bioinformatic approaches to identify the genes encoding DXP synthase (*dxs*),[4] DXP reductoisomerase (*dxr*)[5] and the additional known enzymes of the pathway (*ispD*/*ygbP*, *ispE*/*ych*B and *ispF*/*ygbB*).[6] Both labeling and genetic approaches also showed that in *E. coli* the MEP pathway branches at an unknown step, leading to the independent synthesis of isopentenyl diphosphate **8** (IPP) and dimethylallyl diphosphate **9** (DMAPP), the universal precursors of all isoprenoids.[7] In addition, there is strong genetic evidence for the involvement of two more genes, *gcpE* and *lytB*, in the main trunk of the pathway.[8,9] The biochemical function of the proteins encoded by those genes, however, remains to be established. ME cyclodiphosphate **7** is the last intermediate of the pathway described to date. The conversion of a ME cyclodiphosphate derivative into IPP and DMAPP, i.e. into the diphosphates of an allylic or a homoallylic alcohol is not obvious. Formally, it requires two reduction steps and one elimination. In this work, we report the accumulation of ME cyclodiphosphate **7** in *E. coli* cells defective in the *gcpE* gene, and we discuss the role of the *gcpE* gene product in the metabolism of ME cyclodiphosphate and the biosynthesis of isoprenoids.

Accumulation of methylerythritol cyclodiphosphate by an *E. coli* strain defective in the *gcpE* gene

For the identification of unknown genes of the MEP pathway, the *E. coli* EcAB3-1 strain possessing the enzymes required for mevalonate metabolism (meval-

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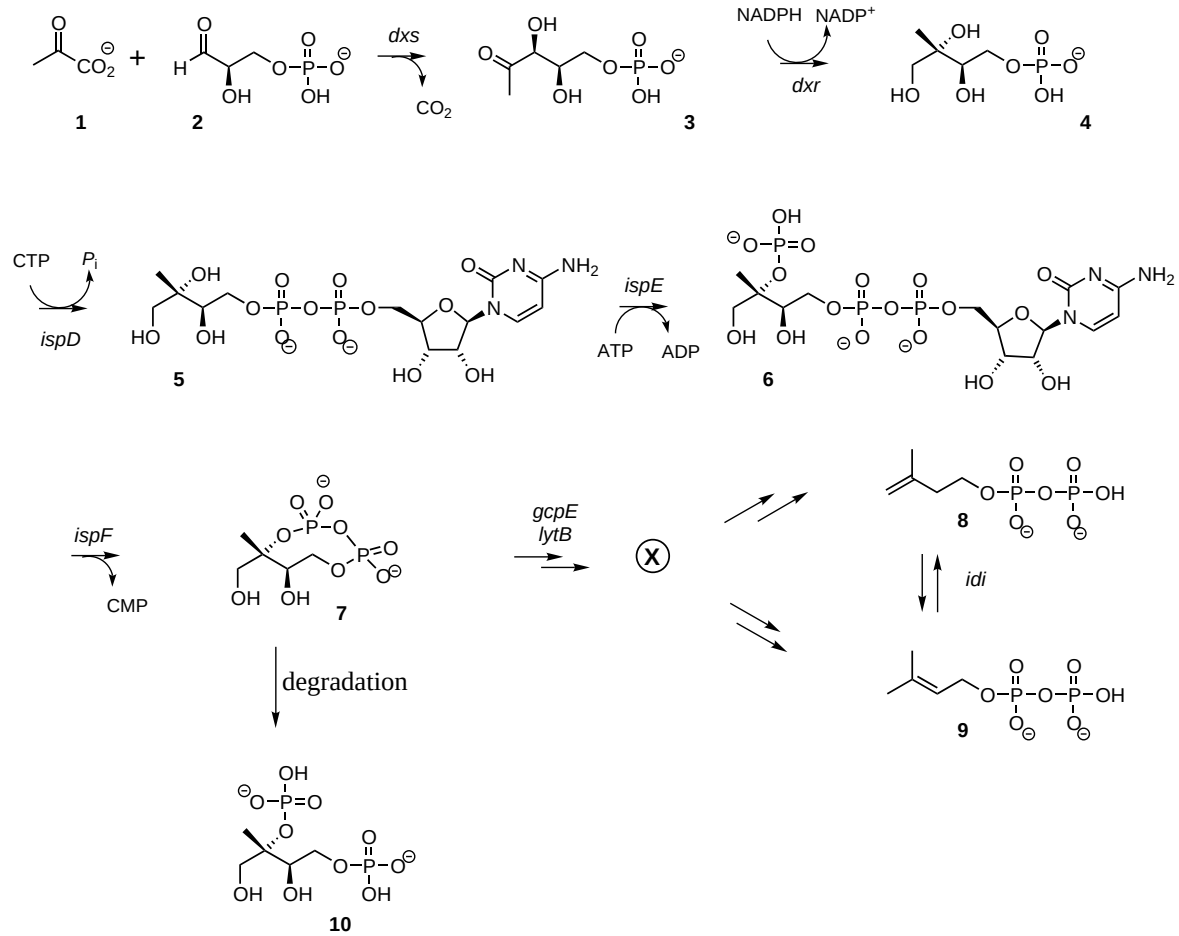

Figure 1. Methylerythritol phosphate (MEP) pathway for isoprenoid biosynthesis in *Escherichia coli*.

onate kinase, diphosphomevalonate kinase, diphosphomevalonate decarboxylase and IPP isomerase) was generated. Such a strain is capable of utilizing exogenous mevalonate added to the culture medium to build its isoprenoids and was utilized as parent strain for the generation of mutants of the MEP pathway.[10] The *E. coli* EcAB3-3 strain, lacking the *gcpE* gene and capable of utilizing MVA, was only growing when MVA was added to the culture medium. This showed that *gcpE* was an essential gene in the MEP pathway.[8]

E. coli is the only known organism, which is capable of utilizing exogenous free ME. After disruption of the *dxs* or the *dxr* gene, growth can be restored by the addition of free ME.[11] This implies that an unknown kinase is capable of converting the free tetrol into the corresponding 4-phosphate, which is the normal intermediate in the MEP pathway. The strategy used for the tentative identification of the GCPE protein substrate was thus based on the construction of the *E. coli* strain EcAB4-5 capable of utilizing MVA, and harboring simultaneously a disruption of the *dxs* gene, in order to enhance the incorporation of free ME, as well as a deletion of the *gcpE* gene, in order to induce the accumulation of the substrate of the GCPE protein. This mutant was grown on LB medium containing MVA as well as [1-^{3}H]ME[12] and analyzed for radioactive metabolites derived from the tritium labeled substrate.[13,14]

After TLC of the ethanol/water extract of the bacterial cells, a single major radioactive compound was detected using a radioactivity linear detector, isolated and identified by radiochemical methods. The amounts were too low for a direct spectroscopic identification. The radioactive metabolite coeluted on silica gel TLC plates and on a Sephadex® QAE A-25 ion exchange column chromatography with ME cyclodiphosphate **7**, which was prepared from benzylviologen treated *Corynebacterium ammoniagenes* and used as a carrier.[15,16] Upon HF hydrolysis, it released, like ME cyclodiphosphate, free ME, which was identified by TLC coelution with synthetic ME. On ion exchange chromatography, the radioactive ME cyclodiphosphate decomposed, like the carrier, into a 2:1 mixture (according to ^{31}P NMR) of ME cyclodiphosphate and of a compound eluting like ME 2,4-biphosphate **10**. The latter ME derivative **10** was also obtained after purification of ME cyclodiphosphate from *Corynebacterium ammoniagenes* using the same procedure and was identified by spectroscopic methods.[14] Negative ion mode electrospray mass spectrometry showed that the cyclic diphosphate structure was opened, and that two phosphate groups were present. Opening of the cyclic diphosphate was also

pointed out by the absence of a phosphorus/phosphorus coupling, which characterizes the ^{31}P NMR spectrum of ME cyclodiphosphate ($^2J = 22$ Hz):[15] the phosphorus signals appeared as two singlets, which were downfield shifted as compared to the two doublets observed in the spectrum of the corresponding cyclodiphosphate.

A control experiment was performed with the *E. coli* strain EcAB4-3, which corresponded to the previous strain, but harboring an intact *gcpE* gene. The same [1-^{3}H]ME incorporation experiment was performed. No accumulation of tritium labeled ME cyclodiphosphate was observed. In contrast, two apolar compounds were detected. They coeluted on TLC plates with ubiquinone-9 and menaquinone-9, which correspond to the only easily detectable final products of the isoprenoid metabolism in *E. coli*.

On the role of the *gcpE* gene product in the MEP pathway

Disruption of the *gcpE* gene induces in the *E. coli* cells the accumulation of ME cyclodiphosphate **7**, the last identified intermediate in the MEP pathway. This shows that the GCPE protein is required for the conversion of ME cyclodiphosphate **7** into isoprenoids. Such biochemical evidence confirms previous work, which only provided genetic evidence demonstrating that the *gcpE* gene is involved in the trunk line of the MEP pathway.[8a] In addition, conversion of [1-^{3}H]ME into ubiquinone-9 and menaquinone-9, the final products of the isoprenoid metabolism, when the *gcpE* gene is intact, and failure of incorporation, when this gene is disrupted, are also in agreement with the involvement of the GCPE protein in the MEP pathway. Although such labeling experiments confirmed that the GCPE protein is essential in the MEP pathway, they did not shed light on the precise role of this protein. It seems very likely that the *gcpE* gene product possesses an enzymatic activity, and that ME cyclodiphosphate **7** is its putative substrate, but it cannot be excluded that the GCPE protein could have a structural or regulatory role.

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12. The chromosomal MVA$^+$ operon of strain EcAB3-1[10] was incorporated in the genome of *E. coli* strain MG1655 by transduction with phage P1, to obtain strain EcAB4-1. The *E. coli* strain ECAB4-3 (*dxs::Tn10*) was generated in strain EcAB4-1 by insertion of '*ptac*-transposase mini-tet'

element of phage λ1098.[17] The *gcpE::CAT* genomic region of strain EcAB3-3[8a] was transferred to the genome of the strain EcAB4-3 by transduction with phage P1, to obtain the *E. coli dxs::Tn10 gcpE::CAT* double mutant EcAB4-5.

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14. *E. coli* strain EcAB4-5 was grown at 37°C in LB medium (500 mL) containing chloramphenicol (17 mg L^{-1}), tetracycline (7.5 mg L^{-1}), kanamycine (25 mg L^{-1}), L-arabinose (2 g L^{-1}), thiamine (5 mg L^{-1}), pyridoxol (3 mg L^{-1}) and MVA (0.1 mM). When a $OD_{600}=0.7$ was reached, the cells were harvested by centrifugation (6000 rpm, 10 min), resuspended in LB medium (30 mL) and added to the previous medium (500 mL), lacking L-arabinose and MVA, but containing [1-^{3}H]ME (1 mCi, 0.02 mM). After further growth at 37°C for 18 h, the cells were harvested by centrifugation (7500 rpm, 10 min), washed twice with water (50 mL) and extracted twice with 70% ethanol (60 mL) at 4°C for 2 h. Ethanol was evaporated under vacuum, and water removed by lyophilization, yielding a radioactive residue (1 μCi). An aliquot (1/10) was analyzed by silica gel TLC (isopropanol/water/EtOAc, 6:3:1). Radioactivity was monitored with a Berthold LB2832 linear detector. Two radioactive compounds were detected. The first one ($R_f=0.74$) corresponded to remaining [1-^{3}H]ME, and the second one ($R_f=0.62$) had a polarity comprised between those of MEP **4** and free ME. The latter compound coeluted on silica gel TLC plates with ME 2,4-cyclodiphosphate **7** (isopropanol/water/EtOAc, 6:3:1, $R_f=0.62$, or isopropanol/water/EtOAc, 6:1:3, $R_f=0.05$). HF hydrolysis (48%, 200 μl) of an aliquot (1/10) for 24 h at rt, followed by neutralization with $CaCO_3$ and centrifugation to remove CaF_2 and the excess of $CaCO_3$, released a radioactive compound coeluting with ME ($CHCl_3/CH_3OH$, 8:2, $R_f=0.18$). It showed the same chromatographic behavior on ion exchange columns as ME cyclodiphosphate. The remaining of the crude extract (0.8 μCi) was purified at 4°C on a QAE-A25 Sephadex® column using an ammonium acetate linear gradient (0.25 to 1.5 M) affording [1-^{3}H]ME (0.50 μCi) and a tritium labeled metabolite with the same retention time as ME cyclodiphosphate (0.30 μCi). The latter compound was run on a second QAE-A25 Sephadex® column and eluted with a 0.3–1 M $NaHCO_3$ gradient. The radioactive fractions were pooled, and Na^+ was exchanged with H^+ using a Dowex 50 W-8, prior to neutralization to pH 6 with NaOH (0.01 M) and lyophilization. Upon storage, the radioactive compound was characterized by the same degradation pattern as that of ME 2,4-cyclodiphosphate. After silica gel TLC (isopropanol/water/EtOAc, 6:3:1), two radioactive compounds ($R_f=0.60$ and $R_f=0.40$) were detected, respectively comigrating with ME 2,4-cyclodiphosphate **7** and ME 2,4-biphosphate **10**. Carriers **7** and **10** were isolated from benzylviologen treated *Corynebacterium ammoniagenes*[15] and purified.[16] Their structures were determined by spectroscopic methods. NMR data were obtained on the 2:1 mixture of ME 2,4-cyclodiphosphate **7** and ME 2,4-biphosphate **10**. ^{13}C NMR spectra were recorded in ^2H_2O containing a drop of C^2H_3CN using the $-C^2H_3$ signal as internal reference ($\delta=1.24$ ppm). ^{31}P NMR spectra were recorded in ^2H_2O using 85% H_3PO_4 as external reference ($\delta=0.00$ ppm).
ME 2,4-cyclodiphosphate 7. ^{13}C NMR (100.8 MHz): δ (ppm) = 84.6 (d, $J=8.5$ Hz, C-2), 69.1 (t, $J=2$ Hz, C-3), 67.7 (d, $J=5$ Hz, C-1), 66.4 (d, $J=6.5$ Hz, C-4), 17.0 (d, $J=5.5$ Hz, 2-Me). ^{31}P NMR (121.5 MHz): $\delta=-10.5$ (d, $J=22$ Hz), −14.6 (d, $J=22$ Hz). ESI MS (negative ion detection mode, H_2O/CH_3CN, 2:1): $m/z=321$ ($M-3H^+ + 2Na^+$), 299 ($M-2H^+ + Na^+$), 277 ($M-H^+$), 138 ($M-2H^+$).
ME 2,4-biphosphate 10. ^{13}C NMR (100.8 MHz): δ (ppm) = 84.4 (d, $J=2$ Hz, C-2), 74.9 (t, $J=5.5$ Hz, C-3), 72.4 (broad s, C-1), 64.8 (d, $J=4$ Hz, C-4), 21.1 (d, $J=2.5$ Hz, 2-Me). ^{31}P NMR (121.5 MHz): δ (ppm) = 17.4 (s), 4.7 (s). ESI MS (negative ion detection mode, H_2O/CH_3CN, 2:1): $m/z=339$ ($M-3H^+ + 2Na^+$), 169 ($M-4H^+ + 2Na^+$).
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