Deuterium-labelled isotopomers of 2-*C*-methyl-D-erythritol as tools for the elucidation of the 2-*C*-methyl-D-erythritol 4-phosphate pathway for isoprenoid biosynthesis

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Escherichia coli synthesizes its isoprenoids via the mevalonate-independent 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway. The MC4100*dxs*::CAT strain, defective in deoxyxylulose-5-phosphate synthase, which is the first enzyme in this metabolic route, exclusively synthesizes its isoprenoids from exogenous 2-*C*-methyl-D-erythritol (ME) added to the culture medium. The fate of the hydrogen atoms in the MEP pathway was followed by the incorporation of [1,1-$^{2}H_{2}$]ME and [3,5,5,5-$^{2}H_{4}$]ME. The two C-1 hydrogen atoms of ME were found without any loss in the prenyl chain of menaquinone and/or ubiquinone on the carbon atoms derived from C-4 of isopentenyl diphosphate (IPP) and on the *E*-methyl group of dimethylallyl diphosphate (DMAPP), the C-5 hydrogen atoms on the methyl groups derived from IPP C-5 methyl group and the *Z*-methyl group of DMAPP. This showed that no changes in the oxidation state of these carbon atoms occurred in the reaction sequence between MEP and IPP. Furthermore, no deuterium scrambling was observed between the carbon atoms derived from C-4 and C-5 of IPP or DMAPP, suggesting a completely stereoselective IPP isomerase or no significant activity of this enzyme. The C-3 deuterium atom of [3,5,5,5-$^{2}H_{4}$]ME was preserved only in the DMAPP starter unit and was completely missing from all those derived from IPP. This finding, aided by the non-essential role of the IPP isomerase gene, suggests the presence in *E. coli* of two different routes towards IPP and DMAPP, starting from a common intermediate derived from MEP.

Key words: 1-deoxy-D-xylulose 5-phosphate, dimethylallyl diphosphate, *Escherichia coli*, isopentenyl diphosphate, ubiquinone.

INTRODUCTION

During the past decades, acetate and mevalonate have been considered to be the unique precursors of isoprenoid compounds in all living organisms [1]. However, a novel metabolic pathway was discovered in the late 1980s for the biosynthesis of isoprenoids in bacteria [2,3], green algae [4] and plants [5–8]. In this alternative biosynthetic route, a condensation step between (hydroxyethyl)thiamin diphosphate, resulting from the decarboxylation of pyruvate (**1**), and glyceraldehyde 3-phosphate [9] (**2**) yields 1-deoxy-D-xylulose 5-phosphate (DXP) (**3**) [10]. A rearrangement [4] followed by a reduction affords 2-*C*-methyl-D-erythritol 4-phosphate (MEP) (**4**) [9,11,12], which already possesses the branched C_5 isoprene carbon skeleton (Scheme 1). Because DXP (**3**) is involved in other metabolic routes than that leading to isoprenoids, such as the biosynthesis of pyridoxol phosphate and thiamin diphosphate [13–15], MEP (**4**) rather than DXP should be considered to be the first committed precursor of the mevalonate-independent pathway. Only two enzymes of the MEP pathway are known: DXP synthase (DXS), catalysing the condensation of (hydroxyethyl)thiamin diphosphate and glyceraldehyde 3-phosphate into DXP [16–18], and DXP reducto-isomerase, yielding MEP from DXP by a rearrangement followed by a reduction (Scheme 1) [19–21]. The steps between MEP (**4**) and isopentenyl diphosphate (**5**) (IPP) and/or its isomer dimethylallyl diphosphate (DMAPP) (**6**) are still unknown.

The origin and the fate of the carbon atoms finally found in the isoprene units derived from the MEP pathway are now well established. In contrast, the origin of the hydrogen atoms is not as clear. Incorporation experiments with deuterium-labelled 1-deoxy-D-xylulose (DX) or 2-*C*-methyl-D-erythritol (ME) have afforded interesting clues to its elucidation in the bacterium *Escherichia coli* and in higher plants [12,22–24]. [1,1-$^{2}H_{2}$]ME (**4a**) was previously incubated with a wild-type strain of *E. coli* [12]. However, the incorporation yield into the polyprenyl side chain of ubiquinone Q8 was very low. To improve the incorporation yield of ME, an *E. coli* mutant was constructed in which the gene of DXP synthase was disrupted. Such a mutant had to be complemented with ME, which served as the only isoprenoid precursor. With such a mutant, a quantitative ME incorporation into the prenyl chains of ubiquinone and menaquinone was expected. It therefore represented the best tool for the incorporation of the two deuterium-labelled [1,1-$^{2}H_{2}$]ME (**4a**) and [3,5,5,5-$^{2}H_{4}$]ME (**4b**) isotopomers described here (Scheme 1).

EXPERIMENTAL

Insertional disruption of the *dxs* gene of *E. coli* by site-directed marker insertion mutagenesis

A 7.9 kb *Xho*I–*Pst*I fragment containing the *E. coli dxs* gene was excised from plasmid pLR1 and cloned into plasmid pBluescript

Abbreviations used: DMAPP, dimethylallyl diphosphate; DX, 1-deoxy-D-xylulose; DXP, 1-deoxy-D-xylulose 5-phosphate; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; FAB, fast atom bombardment; IPP, isopentenyl diphosphate; ME, 2-*C*-methyl-D-erythritol; MEP, 2-*C*-methyl-D-erythritol 4-phosphate.

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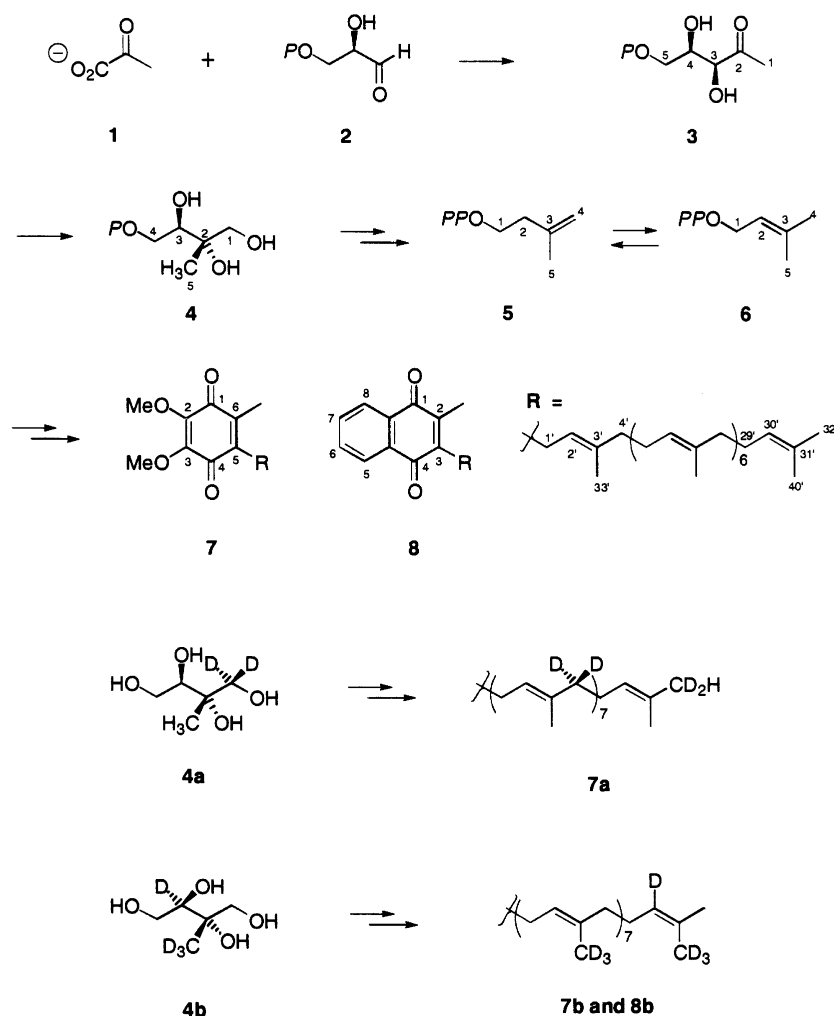

Scheme 1 MEP pathway for isoprenoid biosynthesis and incorporation of [1,1-$^2H_2$]ME (4a) and [3,5,5,5-$^2H_4$]ME (4b) into the prenyl chain of ubiquinone Q8 (7) and menaquinone MK8 (8)

to generate plasmid pLR5 (Figure 1A) [17]. The CAT (chloramphenicol acetyltransferase) gene present in plasmid pCAT19 [25] was excised by digestion with *Xba*I and *Pst*I, treated with T4 DNA polymerase and cloned between the two *Hin*dIII sites present in the *dxs* gene by blunt-end ligation (*Hin*dIII cohesive ends were previously converted to blunt ends by treatment with T4 DNA polymerase) resulting in plasmid pLR6 (Figure 1A). Restriction enzyme mapping was used to identify those clones in which the CAT gene was in the same orientation as the *dxs* gene. Plasmid pLR7 was constructed by subcloning the *Nhe*I–*Sph*I fragment excised from pLR6 into the corresponding sites of plasmid pBR322 (Figure 1A). Plasmid pLR7 was linearized by digestion with *Pst*I, incubated with calf intestine alkaline phosphatase and purified by agarose gel electrophoresis. The purified linear plasmid (250 ng) was used to transform strain JC7623 [26]. Transformed cells were plated on Luria–Bertani plates containing tryptone (10 g/l), yeast extract (5 g/l), NaCl (5 g/l), DX (2 mM) and chloramphenicol (17 μg/ml). Colonies showing both chloramphenicol resistance and DX auxotrophy were selected for further studies. The presence of the CAT insertion into the *dxs* gene was checked by PCR with primers Pr1 (5′-CCGTTTTATCGCCCCACTG-3′) and Pr2 (5′-GGAGTTCCAGTGCCTGAG-3′) (Figure 1B). Bacteriophage P1 lysates obtained after infection of one of the selected strains was used to transduce the CAT insertion into strain MC4100 [27]. The resulting strain was designated MC4100*dxs*::CAT. The presence of the CAT insertion into the *dxs* gene was checked by PCR with the above-mentioned primers. Although strain MC4100*dxs*::CAT showed good growth rates when the medium was supplemented with DX, its growth in the presence of ME (2 mM) was significantly lower. Derived strains with an improved capacity for ME utilization were selected by repeated plating on Luria–Bertani medium supplemented with ME.

Cell culture, labelling experiments and isolation of ubiquinone Q8 and menaquinone MK8

Escherichia coli MC4100*dxs*::CAT mutant was grown for 24 h in a medium containing tryptone (16 g/l), yeast extract (10 g/l), NaCl (5 g/l), chloramphenicol (17 mg/l), thiamin (1.5 μM), pyridoxol (1.5 μM) and ME (0.1 mM). The pH was adjusted to 7. Media were sterilized by heating at 120 °C for 30 min. Thiamin, pyridoxol and ME were sterilized separately by filtration (Millex-GS 0.22 μm Millipore filters). Incubation of [1,1-$^2H_2$]ME (**4a**) was performed with a 1-litre culture yielding ubiquinone Q8 (**7a**)

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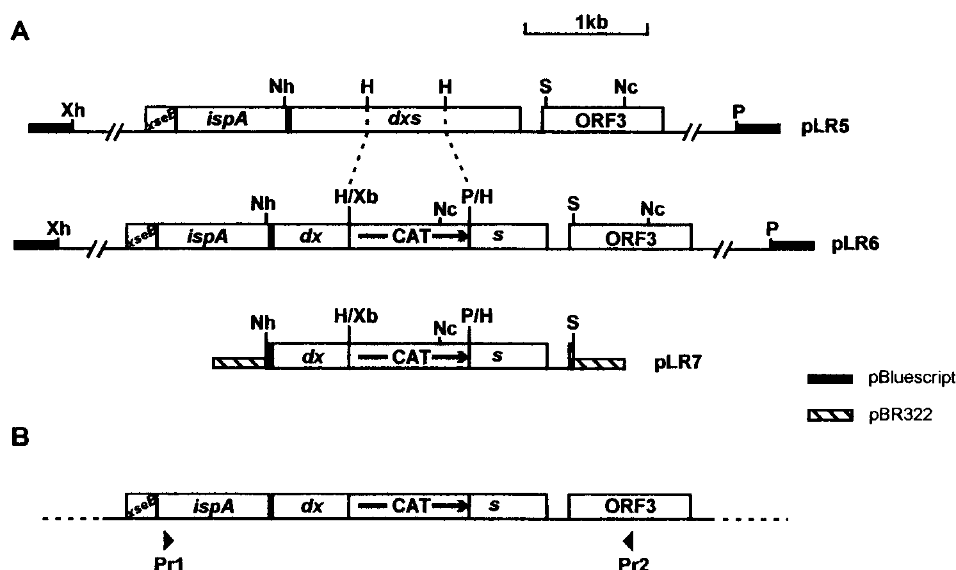

Figure 1 Insertional disruption of the *dxs* gene of *E. coli*

(**A**) Restriction map of the 7.9 kb *Xho*I–*Pst*I fragment containing the *dxs* gene. Only the genes contained in the same transcription unit as the *dxs* gene are indicated (open boxes). The CAT cassette is indicated by a shaded box. The restriction sites are as follows: H, *Hin*dIII; Nc, *Nco*I; Nh, *Nhe*I; P, *Pst*I; S, *Sph*I; Xb, *Xba*I; Xh, *Xho*I. (**B**) Schematic representation of the disrupted *dxs* gene in the strain MC4100*dxs*::CAT, showing the positions of primers Pr1 and Pr2 used for the PCR analysis.

(0.17 mg from 189 mg of freeze-dried cells), that of [3,5,5,5-$^2H_4$]ME with a larger (6-litre) culture affording ubiquinone Q8 (**7b**) (11.6 mg from 12 g of freeze-dried cells) and menaquinone MK8 (**8b**) (3.2 mg). The bacteria were harvested by centrifugation (10000 ***g***) at 4 °C and freeze-dried. The freeze-dried cells were extracted with chloroform/methanol (2:1) at 55 °C for 45 min. After filtration and evaporation of the solvents, the residue was washed with hexane. After evaporation of the hexane, the non-polar lipids were separated by TLC on silica gel (dichloromethane), yielding ubiquinone Q8 (**7**, R_F 0.60) and menaquinone MK8 (**8**, R_F 0.90), which were further purified by TLC [dichloromethane/cyclohexane (60:40, v/v), R_F 0.60].

Analytical methods

^{1}H-NMR spectra were measured either on a Bruker AV400 or on a Bruker ARX500 spectrometer, and ^{13}C-NMR spectra on a Bruker ARX500 spectrometer under the same conditions as those described previously [28]. ^{2}H-NMR spectra were recorded in chloroform solution on a Bruker AV400 spectrometer equipped with a Silicon Graphics station under the following conditions: 30° pulse, 4 s repetition time, 20.03 p.p.m. spectral width, 0.15 Hz fid resolution, digital acquisition mode, ^{1}H decoupling by WALTZ 16 during acquisition and relaxation. All NMR spectra were recorded at 300 K. Electron-impact MS was performed by direct inlet on a Finnigan-MAT TSQ 700 spectrometer with a 70 eV ionization energy; positive fast atom bombardment (FAB) MS was performed on a ZAB-HF spectrometer with an acceleration potential of 8 keV by using *m*-nitrobenzyl alcohol as matrix and xenon as ionization gas.

NMR data of deuterium-labelled ubiquinone and menaquinone

Ubiquinone Q8 (**7a**) resulting from the incubation of [1,1-$^2H_2$]ME. ^{1}H-NMR (C^2HCl_3, 400 MHz): δ (p.p.m.) = 1.57 (3H, broad s, 33′-CH_3), 1.59 (18H, broad s, 34′-, 35′-, 36′-, 37′-, 38′ and 39′-CH_3), 1.64 (1H, broad s, 32′-C^2H_2H) 1.74 (3H, broad s, 40′-CH_3), 2.01 (3H, s, 6-CH_3), 2.05 (14H, m, 5′-, 9′-, 13′-, 17′-, 21′-, 25′- and 29′-CH_2-), 3.18 (2H, d, J = 7.0 Hz, 1′-CH_2-), 3.98 (3H, s, -OCH_3), 3.99 (3H, s, -OCH_3), 4.93 (1H, tq, J = 1.5 Hz, J = 7.0 Hz, 2′-H), 5.02 (1H, tq, J = 1.5 Hz, J = 7 Hz, 6′-H), 5.11 (5H, tt, J = 1.5 Hz, J = 7.0 Hz, 10′-, 14′-, 18′-, 22′-, 26′ and 30′-H). ^{2}H-NMR without H–^{2}H decoupling ($CHCl_3$, 61 MHz): δ (p.p.m.) = 1.66 (2^2H, d, J = 2.2 Hz, 32′-C^2H_2H), 1.96 (14^2H, 4′-, 8′-, 12′-, 16′-, 20′-, 24′- and 28′-C^2H_2-). ^{2}H-NMR with H–^{2}H decoupling ($CHCl_3$, 61 MHz): δ (p.p.m.) = 1.66 (s, 32′-C^2H_2H), 1.96 (s, 4′-, 8′-, 12′-, 16′-, 20′-, 24′- and 28′-C^2H_2-).

Ubiquinone Q8 (**7b**) resulting from the incubation of [3,5,5,5-$^2H_4$]ME. ^{1}H-NMR (C^2HCl_3, 500 MHz): δ (p.p.m.) = 1.67 (3H, s, 32′-CH_3), 1.97 (14H, m, 4′-, 8′-, 12′-, 16′-, 20′-, 24′- and 28′-CH_2-), 2.01 (3H, s, 6-CH_3), 2.05 (14H, m, 5′-, 9′-, 13′-, 17′-, 21′-, 25′- and 29′-CH_2-), 3.18 (2H, d, J = 7.1 Hz, 1′-CH_2-), 3.98 (3H, s, OCH_3), 3.99 (3H, s, -OCH_3), 4.93 (1H, t, J = 7.1 Hz, 2′-H), 5.06 (1H, t, J = 6.9 Hz, 6′-H), 5.09 (1H, t, J = 6.9 Hz, 10′-H), 5.11 (4H, t, J = 6.9 Hz, 14′-, 18′-, 22′- and 26′-H). ^{2}H-NMR ($CHCl_3$, 61 MHz): δ (p.p.m.) = 1.57 (21^2H, 33′-, 34′-, 35′-, 36′, 37′-, 38′- and 39′-C^2H_3), 1.71 (3^2H, 40′-C^2H_3), 5.14 (1^2H, 30′-^{2}H). *Ubiquinone Q8* (*natural abundance*). ^{13}C-NMR (C^2HCl_3, 125 MHz): δ (p.p.m.) = 11.96 (methyl group at C-5), 16.04 (C-34′, C-35′, C-36′, C-37′, C-38′ and C-39′), 16.38 (C-33′), 17.70 (C-40′), 25.33 (C-1′), 25.71 (C-32′), 26.55, 26.71, 26.74 and 26.80 (C-5′, C-9′, C-13′, C-17′, C-21′, C-25′ and C-29′), 39.75 and 39.77 (C-4′, C-8′, C-12′, C-16′, C-20′, C-24′ and C-28′), 61.17 (-OCH_3), 118.88 (C-2′), 123.87 (C-6′), 124.18 (C-10′), 124.30 (C-14′, C-18′, C-22′ and C-26′), 124.44 (C-30′), 131.27 (C-31′), 134.91 and 134.96 (C-15′, C-19′, C-23′ and C-27′), 135.03 (C-11′), 135.28 (C-7′), 137.66 (C-3′), 138.89 and 141.73 (C-5 and C-6), 144.27 and 144.41 (C-2 and C-3), 183.94 and 184.80 (C-1 and C-4). *^{2}H-labelled ubiquinone Q8*. ^{13}C-NMR (C^2HCl_3, 125 MHz): δ (p.p.m.)/^{2}H-induced shift (p.p.b.) = 11.95 (methyl group at C-5), 15.22 (-C^2H_3, α-shift: −820 p.p.b., heptet, $J_{C,^2H}$

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= 20 Hz, C-34′, C-35′, C-36′, C-37′, C-38′ and C-39′), 15.55 (-C^2H_3, α-shift: −820 p.p.b., heptet, $J_{C,^2H}$ = 20 Hz, C-33′), 25.32 (C-1′), 25.57 (γ-shifts: −140 p.p.b., C-32′), 26.54, 26.69, 26.72 and 26.79 (C-5′, C-9′, C-13′, C-17′, C-21′, C-25′ and C-29′, β-shift for C-29′ not measurable), 39.70 and 39.72 (γ-shifts: −51 and −57 p.p.b., C-4′, C-8′, C-12′, C-16′, C-20′, C-24′ and C-28′), 61.15 (-OCH_3), 118.89 (γ-shift, +13, C-2′), 123.90 (γ-shift, +26 p.p.b., C-6′), 124.20 (γ-shift, +26 p.p.b., C-10′), 124.31 (γ-shift, +13 p.p.b., C-14′, C-18′, C-22′ and C-26′), 131.07 (β-shift: −204 p.p.b., C-31′), 134.87 (β-shift: −82 p.p.b., C-15′, C-19′, C-23′ and C-27′), 134.94 (β-shift, −96 p.p.b., C-11′), 135.19 (β-shift: −89 p.p.b., C-7′), 137.59 (β-shift: −77 p.p.b., C-3′), 138.87 and 141.70 (C-5 and C-6), 144.24 and 144.39 (C-2 and C-3), 183.94 and 184.80 (C-1 and C-4).

Menaquinone MK8 (**8b**) resulting from the incubation of [3,5,5,5-$^2H_4$]ME. ^{1}H-NMR (C^2HCl_3, 500 MHz): δ (p.p.m.) = 1.67 (3H, s, 32′-CH_3), 1.97 (14H, m, 4′-, 8′-, 12′-, 16′-, 20′-, 24′- and 28′-CH_2), 2.05 (14H, m, 5′-, 9′-, 13′-, 17′-, 21′-, 25′- and 29′-CH_2-), 2.18 (3H, s, 2-CH_3), 3.36 (2H, d, J = 7.0 Hz, 1′-CH_2-), 5.01 (1H, t, J = 7.0, 2′-H), 5.05 (1H, t, J = 6.8 Hz, 10′-H), 5.06 (1H, t, J = 6.7 Hz, 6′-H), 5.11 (4H, t, J = 6.5 Hz, 14′-, 18′-, 22′- and 26′-H), 7.68–7.69 (2H, m, aromatic protons), 8.07–8.09 (2H, m, aromatic protons). ^{2}H-NMR ($CHCl_3$, 61 MHz): δ (p.p.m.) = 1.56 (21^2H, 33′-, 34′-, 35′-, 36′-, 37′-, 38′- and 39′-C^2H_3), 1.77 (3^2H, 40′-C^2H_3), 5.14 (1^2H, 30′-^{2}H). *Menaquinone MK*8 (*natural abundance*). ^{13}C-NMR (C^2HCl_3, 125 MHz): δ (p.p.m.) = 12.70 (methyl group at C-2), 16.02, 16.04 and 16.06 (C-34′, C-35′, C-36′, C-37′, C-38′ and C-39′), 16.46 (C-33′), 17.70 (C-40′), 25.72 (C-32′), 26.03 (C-1′), 26.54, 26.69, 26.70, 26.74 and 26.80 (C-5′, C-9′, C-13′, C-17′, C-21′, C-25′ and C-29′), 39.71, 39.75 and 39.76 (C-4′, C-8′ C-12′, C-16′, C-20′, C-24′ and C-28′), 119.04 (C-2′), 123.87 (C-6′), 124.17 (C-10′), 124.28 and 124.31 (C-14′, C-18′, C-22′ and C-26′), 124.44 (C-30′), 126.22 (C-8), 126.34 (C-5), 131.27 (C-31′), 132.19 and 132.22 (C-4a and C-8a), 133.29 and 133.35 (C-6 and C-7), 134.92, 134.94, 134.96, 134.97, 134.99 and 135.26 (C-7′, C-11′, C-15′, C-19′, C-23′, C-27′), 137.60 (C-3′), 143.38 and 146.20 (C-2 and C-3), 184.55 and 185.50 (C-1 and C-4). *^{2}H-labelled menaquinone MK8*.^{13}C-NMR (C^2HCl_3, 125 MHz): δ (p.p.m.)/^{2}H induced shift (p.p.b.) = 12.69 (methyl group at C-2), 15.22 (-C^2H_3, α-shift: −820 p.p.b., heptet, $J_{C,^2H}$ = 19 Hz, C-34′, C-35′, C-36′, C-37′, C-38′ and C-39′), 25.58 (γ-shifts: −131 p.p.b., C-32′), 26.03 (C-1′), 26.53, 26.68 and 26.73 (C-5′, C-9′, C-13′, C-17′, C-21′, C-25′ and C-29′, β-shift for C-29′ not measurable), 39.71 (γ-shifts: −38 p.p.b. and −54 p.p.b., C-4′, C-8′, C-12′, C-16′, C-20′, C-24′ and C-28′), 119.11 (γ-shift: +13 p.p.b., C-2′), 123.89 (γ-shift: +22 p.p.b., C-6′), 124.20 (γ-shift: +22 p.p.b., C-10′), 124.31 (γ-shift: approx. 20–25 p.p.b., C-14′, C-18′, C-22′ and C-26′), 126.22 (C-8), 126.33 (C-5), 131.07 (β-shifts, −191 p.p.b., C-31′), 132.19 and 132.23 (C-4a and C-8a) 133.29 and 133.34 (C-6 and C-7), 134.86 and 135.17 (β-shifts: respectively −80 and −89 p.p.b., C-7′, C-11′, C-15′, C-19′, C-23′ and C-27′), 137.52 (β-shift: −73 p.p.b., C-3′), 143.37 and 146.20 (C-2 and C-3), 184.55 and 185.49 (C-1 and C-4).

RESULTS

Incorporation of [1,1-$^2H_2$]ME

[1,1-$^2H_2$]ME (**4a**) was synthesized from 3-methylfuran-2(5H)-furanone as described previously [12]. Ubiquinone Q8 (**7a**) resulting from the incorporation of [1,1-$^2H_2$]ME (**4a**) was analysed by NMR spectroscopy and MS. All analytical data showed that the quinone synthesized *de novo* was synthesized solely from deuterium-labelled precursor added to the culture medium. Indeed, only one ubiquinone isotopomer with 16 deuterium atoms synthesized from [1,1-$^2H_2$]ME (**4a**) (representing 92% of the total ubiquinone content) was detected next to small amounts of unlabelled ubiquinone (8%) derived from the cells of the preculture on ME of natural isotopic abundance. The ^{1}H-NMR spectrum displayed a broad signal at 1.64 p.p.m.; it corresponded to the C-32′ methyl group and integrated for one hydrogen atom in the place of the three hydrogen atoms found in the spectrum of the unlabelled ubiquinone. No signal corresponding to C-4′, C-8′, C-12′, C-16′, C-20′, C-24′ and C-28′ protons was detected at 1.98 p.p.m. The ^{2}H-NMR spectrum was recorded with H–^{2}H-coupling as well as in H–^{2}H-decoupling conditions, the former allowing quantitative integration of the signals. It displayed only two signals: a doublet at 1.69 p.p.m. ($J_{H,^2H}$ = 2.2 Hz), integrating for two deuterium atoms and corresponding to the dideuterated C-32′ methyl group, and a second signal at 1.96 p.p.m., integrating for 14 deuterium atoms, representing the signature of the C-4′, C-8′, C-12′, C-16′, C-20′, C-24′ and C-28′ deuterium atoms of dideuterated methylene groups. Strong evidence for an isoprenoid biosynthesis solely from the deuterated ME was obtained by FAB–MS. With this ionization method, ubiquinone is efficiently reduced into ubiquinol in the source of the mass spectrometer [12,29]. The mass spectrum displayed an m/z 744 molecular ion in place of the m/z 728 ion for ubiquinol Q8 (**7a**) of natural isotopic abundance. This corresponded to the presence of 16 deuterium atoms in the eight isoprene units of the prenyl side chain. No other ubiquinone isotopomer with a lower number of deuterium atoms was detected.

Incorporation of [3,5,5,5-$^2H_4$]ME

[3,5,5,5-$^2H_4$]ME (**4b**) was synthesized from butyne-1,4-diol [30]. The steps allowing the introduction of deuterium were a palladium(II)-catalysed hydrostannation of the triple bond and a coupling reaction between a vinyl iodide and a methylcyanocuprate. Deuterated ubiquinone Q8 (**7b**) and menaquinone MK8 (**8b**) resulting from the incubation of [3,5,5,5-$^2H_4$]ME (**4b**) with the *E. coli* DXS-defective strain were analysed by MS and ^{1}H-, ^{2}H- and ^{13}C-NMR spectroscopy. The ^{2}H-NMR spectrum of ubiquinone Q8 (**7b**) displayed three signals at 1.57 p.p.m. (corresponding to the C-33′, C-34′, C-35′, C-36′, C-37′, C-38′ and C-39′ trideuterated methyl groups), 1.71 p.p.m. (corresponding to the C-40′ terminal trideuterated *Z*-methyl group) and 5.14 p.p.m. (corresponding to the C-30′ vinylic proton), respectively integrating for 21, 3 and 1 deuterium atoms. The ^{2}H-NMR spectrum of menaquinone MK8 (**8b**) also showed three signals at 1.56, 1.77 and 5.14 p.p.m. with the same 21:3:1 integrations, indicating the presence of deuterium atoms on the same carbon atoms. In addition, the ^{1}H-NMR spectra of both quinones showed in the 5.11 p.p.m. region a signal integrating for four vinylic protons in place of the five protons observed in the spectra of the reference compounds with natural isotopic abundance. A trideuterated methyl group was therefore present in all isoprene units, whether they were derived from IPP or from DMAPP, and a vinylic deuterium was found in only a single isoprene unit. Localization of the deuterium atoms was verified by ^{13}C-NMR spectroscopy. In the ^{13}C-NMR spectrum of ubiquinone Q8 (**7b**), upfield-shifted signals were observed for C-31′ (−204 p.p.b.) and C-32′ (−140 p.p.b.), corresponding respectively to the additive β- or γ-shifts induced by the deuterium atoms at C-30′ and C-40′. The ^{13}C-NMR spectrum of menaquinone Q8 (**8b**) showed similar features, with additive upfield β-shifts (−191 p.p.b.) for the C-31′ signal and γ-shifts (−131 p.p.b.) for the C-32′ signal, again due to the presence of deuterium atoms on the C-30 and C-40 carbon atoms. Both ^{13}C-NMR spectra displayed an upfield α-shifted heptet at 15.22 p.p.m.

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(−820 p.p.b., $J_{C,^2H} = 19$ Hz) which corresponded to the signals of the C-34′, C-35′, C-36′, C-37′, C-38′ and C-39′ trideuterated methyl groups. Only in the spectrum of the major ubiquinone Q8 did the C-33′ trideuterated methyl group appear as an upfield-shifted (−820 p.p.b.) heptet ($J = 20$ Hz). The signals of the C-33′ (at 16.46 p.p.m. for the minor menaquinone MK8) and the C-40′ (17.70 p.p.m. for both quinones) methyl groups were missing. This was in accord with a perdeuteration of these methyl groups. The intensity of their heptet lines was, however, too low to be detected. The presence of the trideuterated methyl groups also induced upfield γ-shifts for the signals of all carbon atoms derived from C-4 of IPP (C-4′, C-8′, C-12′, C-16′, C-20′, C-24′ and C-28′) and downfield γ-shifts for the vinylic carbon atoms derived from C-2 of IPP (C-2′, C-6′, C-10′, C-14′, C-18′, C-22′ and C-26′). The signal of the vinylic C-30′ carbon atom of ubiquinone Q8 and menaquinone MK8 (124.44 p.p.m.) was absent from the spectra of both quinones, indicating that a deuterium atom was present on this carbon atom in the prenyl chains from both quinones.

Mass spectrometry fully confirmed the conclusions of NMR spectrometry and permitted a precise determination of the number of the deuterium atoms incorporated from [3,5,5,5-$^2H_4$]ME (**4b**). The FAB mass spectrum of ubiquinone Q8 (**7b**) displayed an m/z 753 ubiquinol Q_8 molecular ion; the electron-impact mass spectrum of menaquinone MK8 (**8b**) an m/z 741 molecular ion. Accordingly, 25 deuterium atoms were incorporated into the eight isoprene units of the two quinone prenyl side chains. No other deuterated isotopomers were detected, indicating again that only the exogenous [3,5,5,5-$^2H_2$]ME (**4b**) served as isoprenoid precursor for the DXS defective strain. This labelling pattern was in accord with the presence of three deuterium atoms in each isoprene unit derived from IPP that were labelled only on the methyl group, and of four deuterium atoms in the DMAPP-derived ω-unit. Furthermore, in the electron-impact mass spectra of ubiquinone Q8 and menaquinone MK8 (**8b**), the signal of the m/z 69 ion, which corresponds to the DMAPP starter unit, was shifted by 4 Da to m/z 73, confirming the retention of the four deuterium atoms from the deuterated ME precursor in DMAPP. The presence of a trideuterated methyl group in the isoprene unit derived from IPP was also confirmed by MS. The fragment containing the α-isoprene unit at m/z 235 for ubiquinone Q8 or m/z 225 for menaquinone MK8 was shifted by 3 Da in the mass spectra of the labelled quinones [29].

DISCUSSION

The *E. coli* MC4100*dxs*::CAT mutant in which the gene of DXP synthase was disrupted proved very useful for the incorporation of free ME into isoprenoids. Indeed, all isoprene units had to be synthesized from the exogenous ME added to the culture medium. This feature greatly simplified the analysis of the MS and NMR spectra of the isoprenoids obtained after feeding with the labelled ME, because only one isotopomer of the labelled isoprenoid was present. It is striking that *E. coli* is so far the only known organism possessing the MEP pathway that is capable of using free ME. Indeed, ME was not incorporated into the isoprenoids synthesized from the MEP pathway by the bacterium *Zymomonas mobilis* (L. Charon and M. Rohmer, unpublished work), the green alga *Scenedesmus obliquus* [31] or by the higher plant *Catharanthus roseus* [32]. Because DXP and MEP, and not the corresponding free alcohols, are respectively the substrate and the reaction product of DXP isomero-reductase [19,20], this implies that a kinase capable of phosphorylating ME is present in *E. coli*.

Incubation of [1,1-$^2H_2$]ME (**4a**) into the ubiquinone of the *E. coli* DXS-defective strain shed light on the quantitative incorporation of the labelled substrate into the prenyl chain from ubiquinone and menaquinone. This was in striking contrast with the modest yields observed with a wild-type strain [12]. This experiment confirmed our previous results with this wild-type strain: the two C-1 protons of MEP were integrally preserved on the corresponding carbon atoms of IPP and DMAPP, without any loss. No change occurred in the oxidation state of this carbon atom when MEP was converted into IPP and DMAPP. This suggested a key role for the carbon atom corresponding to C-3 of IPP in the reaction sequence leading from MEP to IPP. Such a hypothesis has been corroborated by the incorporation of [4-^{2}H]DX into ubiquinone in a wild-type *E. coli* [22] and of [2-^{13}C, 4-^{2}H]DX into phytol and lutein in *Catharanthus roseus* cell cultures [24].

[3,5,5,5-$^2H_4$]ME (**4b**) was therefore incubated with the *E. coli* DXS-defective strain. This experiment confirmed former results obtained with [5,5,5-$^2H_3$]DX [23]. In *E. coli*, the methyl group of DXP or MEP is not modified and retains its three protons throughout the whole reaction sequence leading to IPP and DMAPP. Accordingly, the deuterated methyl group could serve as an internal reference for ^{2}H-NMR for following the fate of the C-3 deuterium when [3,5,5,5-$^2H_4$]ME (**4b**) was incorporated into the prenyl chains of ubiquinone and menaquinone. This C-3 deuterium was retained only in the terminal ω-isoprene unit derived from DMAPP, which served as starter for the prenyltransferase, and was completely absent from all other units derived from IPP. This result was in accord with a former incorporation of [4-^{2}H]DX into the ubiquinone of a wild-type *E. coli* that showed deuterium retention in the DMAPP-derived unit only [22].

The C-4 and C-5 carbon atoms of IPP and DMAPP were to some extent found equivalent in many fungi and plants, when label from C-2 of mevalonate was not stereospecifically incorporated into the *E*-methyl group of the DMAPP-derived isoprene units [33]. Extensive studies on the yeast IPP isomerase shed light on a significant lack of selectivity of this enzyme, at least *in vitro* [33]. No deuterium scrambling between C-4 and C-5 of IPP or DMAPP was detected by NMR spectroscopy in the *E. coli* isoprenoids after feeding of deuterium-labelled ME isotopomers labelled with deuterium at C-1 or C-5, i.e. on the two carbon atoms corresponding to the methyl groups of DMAPP. Deuterium labelling from [1,1-$^2H_2$]ME (**4a**) was found only on carbon atoms corresponding to the C-4 *E*-methyl group of the DMAPP starter unit (corresponding to the C-32′ methyl group of the ω-isoprene unit of ubiquinone) and on those corresponding to C-4 of IPP. No deuterium was found on the carbon atoms derived either from the *Z*-methyl group of DMAPP or from the C-5 methyl group of IPP. Reciprocally, after the incubation of [3,5,5,5-$^2H_4$]ME (**4b**), deuterium was found only on the carbon atoms corresponding to the *Z*-methyl group of DMAPP (i.e. the methyl group at C-31′) and to the C-5 methyl group of IPP. Furthermore, no deuterium loss was observed on the methyl groups, either by NMR spectroscopy or by the more sensitive MS after incubation of [1,1-$^2H_2$]ME or [3,5,5,5-$^2H_4$]ME. No deuterium/proton exchange occurred on any of the carbon atoms derived from C-4 of IPP or DMAPP, indicating that a reversible activity of the IPP isomerase was not significant in the cells under the growth conditions used. The absence of methyl scrambling in the isoprene units from *E. coli* implies either that the reaction catalysed by the IPP isomerase is fully stereoselective in this bacterium, at least *in vivo* or, in the hypothesis of an incompletely selective reaction, that the isomerase activity was not significant in the labelling conditions and that IPP and

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DMAPP were independently synthesized via two different reaction sequences starting from a common intermediate derived from MEP. The latter hypothesis is supported by the non-essential role in isoprenoid biosynthesis of the *idi* gene encoding the IPP isomerase in *E. coli* [34].

The retention of the 3-H proton of MEP (i.e. of the 4-H proton of DXP) only in the DMAPP-derived isoprene units from *E. coli* and its complete absence from those formed from IPP is in accord with such a branching in the MEP pathway in *E. coli*. Even the knowledge of the enantioselectivity of an *E. coli* IPP isomerase [35] and of a farnesyl diphosphate synthase [36] (i.e. both eliminating the pro-*R* proton at C-2 of IPP) did not permit the decipherment of this enigma [37]. In contrast with *E. coli*, the C-4 proton of DX (corresponding to the C-3 proton of ME) was not found in all isoprene units of phytol and lutein from *Catharanthus roseus* cell cultures [24], whether they were derived from DMAPP or from IPP. In the bacterium *Zymomonas mobilis*, a similar loss of the hydrogen corresponding to the C-4 hydrogen of DX was observed: it was replaced by a hydrogen atom derived from NAD(P)H on all carbon atoms derived from C-2 of IPP and DMAPP in the triterpenes of the hopane series [28]. The 4-H of DX (i.e. the 3-H of ME) is accordingly not eliminated in the isomerization of IPP into DMAPP or in the elongation process catalysed by the prenyltransferase but it is lost in *Z. mobilis* before the formation of IPP. This indicates that DMAPP might be obtained by two different routes in the MEP pathway, depending on the organism.

The incorporation of ^{13}C-labelled precursors allowed us to determine the formation of the carbon skeleton of isoprene units via the MEP pathway, including the transposition step, and permitted the identification of DX and ME as IPP precursors. The incorporation of deuterium-labelled glucose into the phytol of the cyanobacterium *Synechocystis* [37] or into the linalol of *Mentha citrata* [38] were characterized either by low yields and/or significant deuterium loss and scrambling resulting from glucose metabolism, yielding no clearcut data on the localization of labelling. In contrast, deuterium-labelled isotopomers of DX and ME proved to be useful tools for detailed investigations of the tentative identification of further intermediates and has thrown light on at least two different routes from MEP to IPP and DMAPP.

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