Isoprenoid biosynthesis in bacteria: a novel pathway for the early steps leading to isopentenyl diphosphate

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Incorporation of ^{13}C-labelled glucose, acetate, pyruvate or erythrose allowed the determination of the origin of the carbon atoms of triterpenoids of the hopane series and/or of the ubiquinones from several bacteria (*Zymomonas mobilis*, *Methylobacterium fujisawaense*, *Escherichia coli* and *Alicyclobacillus acidoterrestris*) confirmed our earlier results obtained by incorporation of ^{13}C-labelled acetate into the hopanoids of other bacteria and led to the identification of a novel biosynthetic route for the early steps of isoprenoid biosynthesis. The C_5 framework of isoprenic units results most probably (i) from the condensation of a C_2 unit derived from pyruvate decarboxylation (e.g. thiamine-activated acetaldehyde) on the C-2 carbonyl group of a triose phosphate derivative issued probably from dihydroxyacetone phosphate and not from pyruvate and (ii) from a transposition step. Although this hypothetical biosynthetic pathway resembles that of L-valine biosynthesis, this amino acid or its C_5 precursors could be excluded as intermediates in the formation of isoprenic units.

INTRODUCTION

The early steps of isoprenoid biosynthesis have been well documented in eukaryotes, especially through the pioneering work of Bloch, Lynen and Cornforth (see reviews [1–3]). The universally admitted precursor of isoprenoids, mevalonate (MVA), is synthesized by the condensation of three acetyl-CoA (Ac-CoA) units via aceto-Ac-CoA and 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) and affords after phosphorylation and decarboxylation isopentenyl diphosphate (IPP). Even if this biogenetic scheme has been now well established for many eukaryotes and some archaebacteria [4], little is known about isoprenoid biosynthesis in eubacteria.

Some results are even puzzling. Indeed, incorporation of ^{14}C- or ^{13}C-labelled acetate into the isoprenic side chain of ubiquinone from *Escherichia coli* was apparently in contradiction with the classical scheme [5,6]. Furthermore, incorporation of [1-^{13}C]- and [2-^{13}C]-acetate into the triterpenoids of the hopane series [7] from three Gram-negative bacteria (*Rhodopseudomonas palustris*, *Rhodopseudomonas acidophila* and *Methylobacterium organophilum*) grown on synthetic media containing only acetate as carbon source raised clearly unexpected problems concerning the early steps of isoprenoid biosynthesis [8]: (i) exogenous acetate was not, as expected, directly incorporated into this biosynthetic pathway; (ii) no scrambling of the isotopic enrichment occurred; (iii) the observed labelling patterns differed completely from those expected from the classical isoprenoid-biosynthetic route. They could be explained either in the frame of the former biogenetic scheme by a compartmentation of Ac-CoA metabolism with two non-interconvertible Ac-CoA pools (issued, e.g., from the glyoxylate cycle and from the Entner–Doudoroff catabolic pathway of glucose) or by an as-yet-unknown sequence of fully different enzymic reactions. In a preliminary experiment using [5-^{13}C]glucose, we also reported a labelling pattern for the hopanoids of *Zymomonas mobilis* equally incompatible with the classical scheme [9].

In the present paper we give now full evidence for the existence of a novel pathway concerning the early steps of isoprenoid biosynthesis toward IPP or dimethylallyl diphosphate (DMAPP). This could be achieved by the incorporation of ^{13}C-labelled precursors (glucose, acetate, erythrose and pyruvate) into polyterpenoids (hopanoids and ubiquinones; Figure 1) from several bacteria differing one from each other by the metabolic pathways implied in the utilization of the carbon source added to the culture medium. A novel hypothetical pathway could be thus proposed: the C_5 isoprenic units IPP or DMAPP would not be formed via HMG-CoA and MVA, but by direct condensation of thiamine-activated acetaldehyde arising from pyruvate decarboxylation on a C_3 unit derived from a triose phosphate (e.g. dihydroxyacetone phosphate), followed by a transposition step.

EXPERIMENTAL

Bacterial cultures and isolation of the polyterpenoids

^{13}C-labelled acetate was obtained from Aldrich, glucose was from Omicron Biochemical Inc. (South Bends, IN, U.S.A.) and pyruvate from Eurisotop (Saint Aubin, France).

Zymomonas mobilis [A.T.C.C. (American Type Culture Collection) 29191] was grown anaerobically at 30 °C on a minimal medium containing KH_2PO_4 (3.5 g·l^{-1}), $MgSO_4,7H_2O$ (2.0 g·l^{-1}), NH_4Cl (1.6 g·l^{-1}), citric acid (0.2 g·l^{-1}), $(NH_4)_2Fe(SO_4)_2,6H_2O$ (14 mg·l^{-1}), biotin (1 mg·l^{-1}), calcium pantothenate (1 mg·l^{-1}, sterilized by filtration of a 0.1 % solution) and glucose (20 g·l^{-1}, sterilized separately as a concentrated solution) [10]. The bacteria were harvested at the end of the exponential growth phase. The freeze-dried cells from a 1-litre culture (330 mg) were extracted with chloroform/methanol (2:1, v/v), and the

Abbreviations used: Ac-CoA, acetyl-CoA; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; MVA, mevalonate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate.

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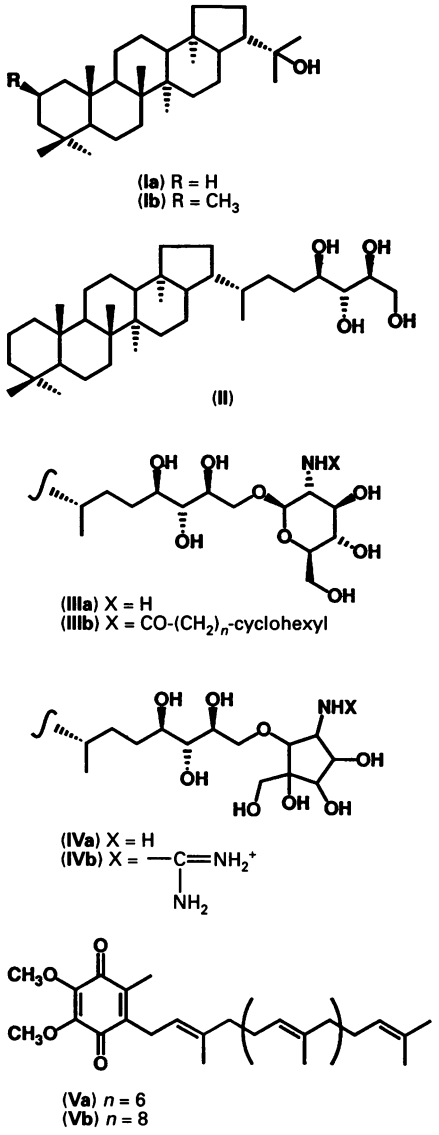


Figure 1 Polyterpenoids analysed for their isotopic enrichments after incorporation of ^{13}C-labelled glucose

The following polyterpenoids were analysed: *Z. mobilis*: bacteriohopanetetrol glycoside (**IIIa**) and bacteriohopanetetrol ether (**IVa**); *M. fujisawaense*: diplopterol (**Ia**), 2β-methyldiplopterol (**Ib**), bacteriohopanetetrol (**II**), tetrol ether (**IVb**) and ubiquinone-10 (**Vb**); *A. acidoterrestris*: bacteriohopanetetrol (**II**), bacteriohopanetetrol glycosides (**IIIa**) and (**IIIb**); *E. coli*: ubiquinone-8 (**Va**).

hopanoids isolated as previously described, yielding the hepta-acetates of (**IIIa**, Figure 1) (4 mg) and (**IVa**) (3 mg) [11,12].

Methylobacterium fujisawaense (strain SAL isolated by Corpe and Rheem [13] and identified by Green et al. [14] on the basis of phenotypic tests) was grown aerobically in 2-litre Erlenmeyer flasks on the ammonium/mineral-salt medium for methylotrophs [15] containing an additional carbon source (see Table 1) and vitamins (biotin, nicotinamide, riboflavin, calcium pantothenate and cyanocobalamin, all at a 0.6 mg·l^{-1} concentration). The freeze-dried cells (0.5–1 g, depending on the cultures) from a 3-litre culture were extracted with chloroform/methanol (2:1, v/v) [11]. This extract was evaporated to dryness and the residue extracted with n-hexane in order to recover the ubiquinone-10 (**Vb**). The hexane-insoluble part was acetylated and separated by t.l.c. as usual, giving the mixture of diplopterol (**Ia**) and 2β-methyldiplopterol (**Ib**), the tetra-acetate of (**II**) (1.1–1.4 mg·g^{-1}) and the octaacetate of (**IVb**) (traces or 2.1–3.5 mg·g^{-1}) [11]. The hexane extract was also separated by t.l.c. (dichloromethane) giving pure ubiquinone-10 (**Vb**) (0.6 to 1 mg·g^{-1}, R_F = 0.40) and a mixture of diplopterol and 2β-methyldiplopterol which was added to the corresponding compounds found in the hexane insoluble residue. (**Ia**) and (**Ib**) were separated one from each other by reverse-phase h.p.l.c. (C_{18} Zorbax ODS column; methanol/water, 48:2, v/v; flow rate 1 ml·min^{-1}) [11], silylated at room temperature for 15 min with a mixture of bis(trimethylsilyl)trifluoroacetamide and pyridine (1:1, v/v) and purified after elimination of the excess of reagent under a N_2 stream by t.l.c. (hexane/ethyl acetate, 49:1, v/v) to give the trimethylsilyl ethers of (**Ia**) (0.8 to 1.4 mg·g^{-1}) or (**Ib**) (1.2–2.4 mg·g^{-1}, R_F = 0.55 for both trimethylsilyl ethers). For unknown reasons, the ratio between the hopanoids changed dramatically, but slowly and reversibly, when the cells were transferred from a complex medium containing yeast extract and peptone to a minimal medium with acetate or glucose as sole carbon source (Table 1).

Alicyclobacillus acidoterrestris [strain GD3b; DSM (Deutsche Sammlung von Mikroorganismen) 3922] [16,17] was grown aerobically in 2-litre Erlenmeyer flasks at 40 °C for 48 h on the medium described by Deinhard et al. [16]. The carbon source was glucose (1 g·l^{-1}), and yeast extract was replaced by the following vitamins: biotin (0.1 mg·l^{-1}), nicotinamide (2 mg·l^{-1}), thiamine (0.15 mg·l^{-1}), folic acid (0.6 mg·l^{-1}), cyanocobalamin (0.1 mg·l^{-1}) and calcium pantothenate (5 mg·l^{-1}) [18]. The chloroform/methanol (2:1, v/v) extract of freeze-dried cells (0.45 g from a 3-litre culture) was acetylated and separated by t.l.c. (cyclohexane/ethyl acetate, 7:5, v/v) giving the polyacetates of (**II**) (1.8 mg, R_F = 0.76), (**IIIa**) (1.2 mg, R_F = 0.61) and (**IIIb**) (1.3 mg, R_F = 0.10).

Escherichia coli (DSM 30083) was grown on a synthetic medium containing a single carbon source (acetate, glucose; see Table 1) NH_4Cl (1 g·l^{-1}), $MgSO_4$,$7H_2O$ (0.2 g·l^{-1}), KH_2PO_4 (1 g·l^{-1}), $FeSO_4$,$7H_2O$ (50 mg·l^{-1}), $CaCl_2$ (20 mg·l^{-1}), $MnCl_2$ (20 mg·l^{-1}), $NaMoO_4$,$2H_2O$ (1 mg·l^{-1}) and the same vitamins at the same concentrations as those required for the growth of *M. fujisawaense*. Ubiquinone-8 (**Va**) (1.1 mg from 1.4 g of freeze-dried cells from a 4-litre culture) was obtained as reported for ubiquinone-10 (**Vb**) from *M. fujisawaense* by t.l.c. (dichloromethane, R_F = 0.62).

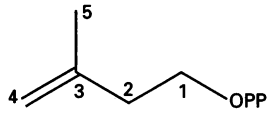
Polyterpenoid identification and evaluation of the isotopic abundances

Hopanoids from *Z. mobilis* and *M. fujisawaense* have been identified by ^{1}H- and ^{13}C-n.m.r. spectroscopy and by comparison with the corresponding hopanoids previously isolated in this laboratory [11,12], those of *Alicyclobacillus acidoterrestris* by comparison (^{1}H- and ^{13}C-n.m.r. spectroscopy) with hopanoids we have isolated from *A. acidocaldarius* and already described by Langworthy and co-workers [19,20]. Ubiquinones were identified by ^{1}H- and ^{13}C-n.m.r. spectroscopy and electron-impact direct-inlet m.s. and comparison with authentic standards from Hoffmann–La Roche (Basle, Switzerland).

Assignment of the ^{13}C-n.m.r. spectra was made according to data from the literature [21] or obtained in the laboratory [11,22–24]. Isotopic abundances were determined by ^{13}C-n.m.r. spectroscopy in [^{2}H]chloroform solution on Bruker WP 400 or Bruker AC 250 spectrometers as reported previously [8] and are indicated as mean values of the isotopic abundances of the equivalent carbon atoms from all isoprenic units (Table 1).

Table 1 Incorporation of ^{13}C-labelled precursors into bacterial polyterpenoids

As all isoprenic units from the analysed hopanoids and ubiquinones were always identically labelled, for the sake of clarity the labelling patterns are only indicated for the framework of IPP, whose numbering is given in Figure 2. Acetate and pyruvate were utilized as their sodium salts. Notes: [a] In the case of the [2-^{13}C]acetate experiments, as all carbon atoms from isoprenic units were labelled, it was difficult to evaluate correctly the isotopic abundances; only relative abundances are therefore given; [b] values of the ^2J $^{13}C/^{13}C$ coupling constants for the carbon atoms derived from C-4 and C-5 of glucose; ubiquinone (**Vb**): in all isoprenic units: ^2J = 2.5–3 Hz; diplopterol (**Ia**) trimethylsilyl ether: C-3/C-23 <0.5 Hz, C-1/C-5 1 Hz, C-7/C-9 0.9 Hz, C-13/C-15 1 Hz, C-17/C-19 2.5 Hz, C-21/C-29 1.5 Hz; 2β-methyldiplopterol (**Ib**) trimethylsilyl ether: C-3/C-23 <0.5 Hz, C-1/C-5 0.8 Hz, C-7/C-9 0.8 Hz, C-13/C-15 0.9 Hz, C-17/C-19 2.6 Hz, C-21/C-29 1.6 Hz; tetra-acetate of (**II**): C-3/C23 <0.5 Hz, C-1/C-5 0.9 Hz, C-7/C-9 0.8 Hz, C-13/C-15 0.8 Hz, C-17/C-19 3.1 Hz ,C-21/C-29 <0.5 Hz; [c] in the case of the [3-^{13}C]pyruvate incorporation, no values are indicated; indeed C-1 and C-5 were always labelled, but the isotopic abundances were different in all analysed polyterpenoids ranging from about 10% for C-1 as well as for C-5 in ubiquinone-8 (**Va**) and diplopterol (**Ia**) to 20% for 2β-methyldiplopterol (**Ib**) in the case of the culture grown in the presence of acetate, or from a non-significant level in bacteriohopanetetrol (**II**), to 2.0–2.5% (C-5) and 5–6% (C-1) in ubiquinone-8 (**Va**) and diplopterol (**Ia**) and 2.5% (C-5) and 10% (C-1) for 2β-methyldiplopterol (**Ib**) in the case of the culture grown in the presence of glucose. This reflects probably preferential consumption of one of the proposed carbon sources and different turnovers for each isoprenoid during bacterial growth; [d] As erythrose was only weakly incorporated in the chain of ubiquinone, no quantitative data are given. The structure of IPP is shown below:



Incorporation expt.	Isotopic abundance of the precursor (%)	Carbon source concentration ($g \cdot l^{-1}$)	Analysed polyterpenoids	Isotopic enrichments of polyterpenoids C-1	C-2	C-3	C-4	C-5
Z. mobilis								
[1-^{13}C]Glucose	10	20	(**IIIa**), (**IVa**)	–	–	–	–	–
[2-^{13}C]Glucose	10	20		–	–	6	–	–
[3-^{13}C]Glucose	10	20		–	–	–	–	5
[5-^{13}C]Glucose	10	20		–	8	4	–	–
[6-^{13}C]Glucose	8.5	20		10	–	–	–	5
M. fujisawaense								
[1-^{13}C]Acetate	10	1.5	(**Ia**), (**Ib**), (**II**), (**IIIb**), (**Vb**)	–	–	–	5	–
[2-^{13}C]Acetate[a]	10	1.5		*1*	*1*	*1*	*1/2*	*1*
[6-^{13}C]Glucose	10	1		10	–	–	–	5
[4,5-$^{13}C_2$]Glucose[b]	99	0.1 (+ Unlabelled glucose, 0.9)	(**Ia**), (**Ib**), (**II**), (**Vb**)	–	7	–	7	–
[3-^{13}C]Pyruvate[c]	99	0.1 (+ Acetate or glucose, 0.9)		+	–	–	–	+
	99							
[2-^{13}C]Erythrose	99	0.066 (+ glucose, 2.7)	(**Ia**), (**Ib**), (**II**), (**IIIb**), (**Vb**)	–	–	–	20	–
E. coli								
[1-^{13}C]Acetate	10	1	(**Va**)	–	–	–	7	–
[2-^{13}C]Acetae[a]	10	1		*1*	*1*	*1*	*1/2*	*1*
[1-^{13}C]Glucose	10	1		5	–	–	–	4
[6-^{13}C]Glucose	10	1		7	–	–	–	6
[2-^{13}C]Erythrose[d]	99	0.1 (+ acetate 0.9)		–	–	–	+	–
A. acidoterrestris								
[1-^{13}C]Glucse	10	1	(**II**), (**IIIa**), (**Vb**)	2	–	–	–	2
[6-^{13}C]Glucose	10	1		5	–	–	–	5

Signals utilized as internal references for the estimation of the isotopic abundances arose either from the carbonyl signals of the polyacetates of (**IIIa**) and (**IVa**) from *Z. mobilis* obtained after acetylation with [1,1′-$^{13}C_2$]acetic anhydride (20% isotopic abundance) [8,10], or from the silicon-linked methyl groups of the trimethylsilyl ethers of (**Ia**) and (**Ib**) [chemical shift (δ) 2.77 p.p.m.] or from the methyl signals of acetoxy groups [8]. In some cases the results were refined using mean values of the intensities of the signals from carbon atoms that were shown unlabelled by the preceding methods [10]. As no signals could be utilized as internal standards in the ^{13}C-n.m.r. spectra of ubiquinones, isotopic abundances were estimated in comparison with the signals of unlabelled carbon atoms.

RESULTS AND DISCUSSION

Incorporation of ^{13}C-labelled precursors into bacterial polyterpenoids

The bacteria utilized for our biosynthetic studies have been grown on synthetic mineral media containing usually one single carbon source. This avoided endogenous dilution by other carbon sources and facilitated the interpretation of the labelling patterns as only few anabolic and catabolic pathways were expected to be involved in these growth conditions. In *M. fujisawaense* and *E. coli*, acetate is thus incorporated into the glyoxylate and tricarboxylic acid cycles, leading to pyruvate and/or phosphoenolpyruvate. Glucose is utilized via the Entner–Doudoroff

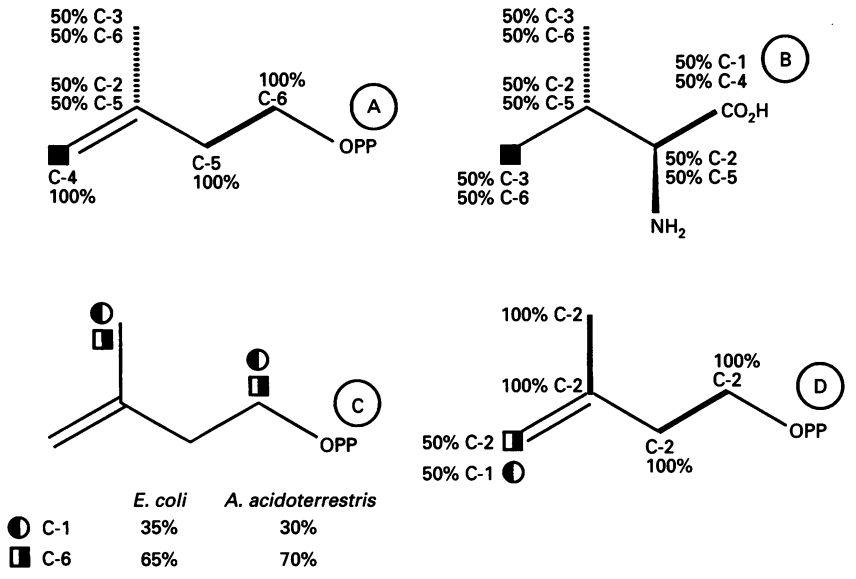


Figure 2 Origin of the carbon atoms as calculated from isotopic abundances (Table 1)

Origins are shown for (i) the isoprenoids (pattern A) and L-valine (pattern B) [10] from *Z. mobilis* after feeding with D-glucose, confirmed by the experiments performed on *M. fujisawaense*; (ii) the isoprenoids (pattern C) from *E. coli* and *A. acidoterrestris* after feeding with D-glucose; (iii) the isoprenoids (pattern D) from *M. fujisawaense, M. organophilum, R. palustris, R. acidophila* [8] and *E. coli* after feeding with acetate

pathway by *M. fujisawaense* and *Z. mobilis*. The latter bacterium grows only on glucose as carbon source, lacks other catabolic pathways of this hexose and has an incomplete tricarboxylic acid cycle [25–27]. Glucose catabolism occurs via glycolysis and the oxidative pentose phosphate pathway in *E. coli* and most probably also in *A. acidoterrestris* as in bacilli [27]. Labelling patterns observed on the polyhydroxylated side chain of the bacteriohopane derivatives and on the carbapseudopentose moiety of ethers (**IVa**) and (**IVb**) confirmed our earlier results and will not be discussed here. The acyclic C_5 chain corresponds to a D-pentose derivative linked through its C-5 carbon atom to the triterpenic moiety, and the latter five-membered ring results from the formation of a carbon–carbon bond between C-1 and C-5 from glucose [8,9].

All results concerning the incorporation of ^{13}C-labelled precursors into isoprenic units are summarized in Table 1 and Figure 2. In the case of the ethanologenic bacterium *Z. mobilis*, feeding with [1-^{13}C]-, [2-^{13}C]-, [3-^{13}C]-, [5-^{13}C]- or [6-^{13}C]-glucose allowed one to determine the origin of all carbon atoms of the isoprenic units from the hopanoids (Figure 2, pattern A). The C-4 carbon atom of isoprenic units was never labelled and could accordingly only arise from C-4 of glucose. The patterns observed on the hopanoids after feeding of each labelled glucose were again not consistent with the classical pathway (Figure 2, pattern A), as in this bacterium acetate units could only indistinctly derive either from C-2/C-3 of glucose (via pyruvate), or from C-5/C-6 (via glyceraldehyde 3-phosphate and pyruvate).

Our previous results obtained with ^{13}C-labelled acetate could not be directly correlated with those obtained with glucose, as most of the bacteria we used could be satisfactorily grown either on acetate (*Rhodopseudomonas palustris*, *R. acidophila*) or on glucose (*Z. mobilis*) as sole carbon source, but not on both substrates. This could be done with a strain of *M. fujisawaense*, a versatile bacterium possessing a rather simple hopanoid composition [28] [diplopterol (**Ia**), 2β-methyldiplopterol (**Ib**), small amounts of bacteriohopanetetrol (**II**) and, in most cultures, the bacteriohopanetetrol ether (**IVb**); Figure 1], growing on acetate or glucose and capable of utilizing many other carbon sources [14]. The labelling patterns of the isoprenoids (hopanoids and ubiquinone-10) from *M. fujisawaense* were identical after feeding of [1-^{13}C]acetate and [2-^{13}C]acetate (Table 1; Figure 2, pattern B) with those obtained using the two *Rhodopseudomonas* species or *M. organophilum* and after feeding of [6-^{13}C]glucose with that obtained with *Z. mobilis*.

Furthermore, similar incorporation experiments with [1-^{13}C]-acetate and [2-^{13}C]acetate as well as [6-^{13}C]glucose have been performed with *E. coli*, which does not synthesize hopanoids [7]. Labellings in this case of the isoprenic units from ubiquinone-8 were qualitatively identical with those observed for the isoprenoids of *M. fujisawaense* (Table 1). These experiments performed on *E. coli* were completed by an incorporation of [1-^{13}C]glucose. As C-1 of glucose is completely lost as CO_2 by the decarboxylation of pyruvate, no label could be incorporated into the hopanoids of *Z. mobilis* from this precursor. In *E. coli*, however, growth on [1-^{13}C]glucose afforded the same labelling pattern of isoprenoids as that obtained from [6-^{13}C]glucose (Table 1; Figure 2, pattern C). This corresponds to glucose degradation via the Embden–Meyerhof–Parnas pathway instead of the Entner–Doudoroff pathway [27]. Finally, as all tested bacteria were Gram-negative, a last set of experiments was performed with the Gram-positive *A. acidoterrestris*, which could be grown on glucose, but not on acetate. Incorporation of [1-^{13}C]- or [6-^{13}C]-glucose led qualitatively to labelling patterns of the bacteriohopane glycosides (**IIIa**) and (**IIIb**) similar to those obtained for the ubiquinone in *E. coli*. Differences were only found in the intensities of the isotopic enrichments and reflected different regulations of the catabolic pathways for glucose.

All results obtained with our labelling experiments must be thus most probably interpreted in the frame of a single biogenetic scheme, and the peculiarities we have observed for the early steps of isoprenoid biosynthesis could be a rather general feature valid for all polyterpenoids from numerous bacterial taxa. From the

origin of the carbon atoms as deduced from the labelling experiments performed with *Z. mobilis* (Table 1; Figure 2, pattern A), it was reasonable to postulate a new biogenetic scheme. The basic C_5 framework of isoprenic units derives from only two precursors corresponding respectively to carbon atoms C-3 and C-5 of isopentenol (which have each a dual origin) and to carbon atoms C-1, C-2 and C-4 (which derive each from a single carbon atom from glucose).

The precursor for C-3 and C-5 of IPP

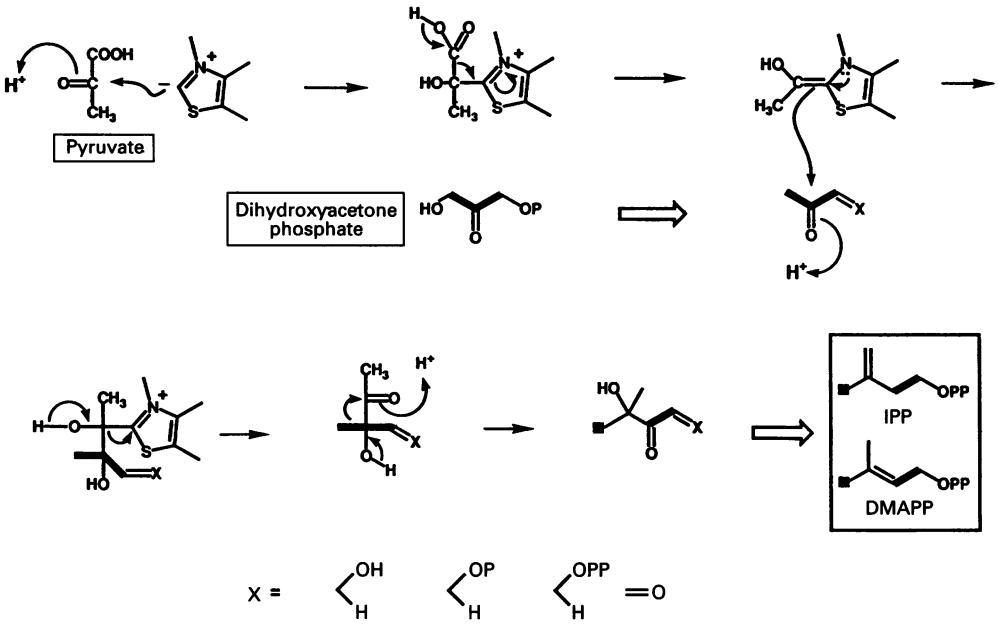
In *Z. mobilis* C-3 of IPP arises indistinctly from C-2 and C-5 of glucose and C-5 from C-3 or from C-6 of glucose (Figure 2, pattern A). This could correspond to the mixing of two equivalent pools of a same C_2 precursor of dual origin. Such a precursor could be obtained from the decarboxylation of pyruvate. Indeed, in *Z. mobilis*, glucose is oxidized into 2-oxo-3-deoxy-6-phosphogluconate. This oxo acid is cleaved into pyruvate (corresponding to C-1, C-2 and C-3 of glucose) and glyceraldehyde-3-phosphate, which can be converted into pyruvate (corresponding in this case to C-4, C-5 and C-6 of glucose). After mixing of the two preceding pyruvate pools and after decarboxylation, a C_2 precursor derived from thiamine-activated acetaldehyde is obtained, deriving equally from C-2 and C-3 as well as from C-5 and C-6 of glucose. This interpretation is, furthermore, in full accordance with the labelling patterns obtained in the isoprenoids from *M. fujisawaense*, *E. coli* and the bacteria we have previously examined by feeding of ^{13}C-labelled acetate [8] (Figure 2, pattern D). Indeed, after incorporation of the exogenous acetate from the culture medium into the glyoxylate cycle and the tricarboxylic acid cycle, C-2 and C-3 from pyruvate or phosphoenolpyruvate obtained from oxaloacetate derive solely from the acetate methyl group, whereas C-1 derives equally from carboxylate or from methyl group of acetate. Decarboxylation of such a pyruvate yields a C_2 acetyl unit whose carbon atoms derive solely from C-2 of acetate. When [2-^{13}C]acetate of high isotopic abundance (90%) was diluted with succinate (an intermediate of the glyoxylate cycle) in a 1:2 molar ratio and utilized as carbon source by *R. palustris* [8], one could even clearly observe the $^1J^{13}C/^{13}C$ coupling constants between all carbon atoms corresponding to C-3 and C-5 of IPP [8]. This shows that the two former carbon atoms are incorporated simultaneously into isoprenic units from a single C_2 precursor.

The precursor for C-1, C-2 and C-4 of IPP

Concerning the three remaining carbon atoms C-1, C-2 and C-4 of IPP which derive respectively from C-6, C-5 and C-4 of glucose, the most striking question is whether they arise from two precursor molecules as in the classical pathway or from one single precursor with insertion of the abovementioned C_2 unit between the two carbon atoms derived from C-5 and C-4 of glucose. After incubation of doubly labelled [4,5-$^{13}C_2$]glucose diluted with unlabelled glucose in a 1:9 ratio with *M. fujisawaense*, the coupling between the carbon atoms derived from C-4 and C-5 of glucose was still observed in all isoprenic units: all signals from ^{13}C enriched positions were doublets showing characteristic $^2J^{13}C/^{13}C$ coupling constants (Table 1). This unambiguously points out the following features: (i) C-4 and C-5 of glucose are simultaneously introduced into isoprenic units; (ii) the formerly described C_2 precursor is inserted between these two carbon atoms; (iii) a transposition step is required in order to obtain the isoprenic framework; (iv) all these conditions rule completely out the occurrence of the classical pathway and HMG-CoA as precursor for isoprenoids.

We postulated first that mevalonate could be the precursor of the polyterpenoids. In this case, assuming that the carbon atom lost by the decarboxylation of mevalonate diphosphate into isopentenyl diphosphate derived from C-3 of glucose, one could recognize the C_4 skeleton of a D-erythrose derivative as a possible precursor of mevalonate. Indeed, erythrose 4-phosphate is obtained via the non-oxidative pentose phosphate pathway and derives from carbon atoms C-3–C-6 of glucose. Feeding of [2-^{13}C]erythrose to *M. fujisawaense* or *E. coli* effectively led to the same labelling pattern in isoprenoids as that expected from an incorporation of [4-^{13}C]glucose (Table 1). This result is, however, ambiguous, and does not correctly prove the direct implication of erythrose as precursor for isoprenoids, as the free tetraose could be incorporated in the place of erythrose 4-phosphate into the non-oxidative pentose phosphate pathway. Through transketolase, [2-^{13}C]erythrose and xylulose-5-phosphate could be converted into unlabelled glyceraldehyde 3-phosphate and [4-^{13}C]fructose, which, in turn, could be incorporated into isoprenoid biosynthesis like glucose.

In fact erythrose can be eliminated as a precursor for isoprenic units by the results obtained with *E. coli* and *A. acidoterrestris*. These two bacteria incorporated similarly [1-^{13}C]- as well as [6-^{13}C]-glucose into their polyterpenoids, and there is no known metabolic pathway permitting the incorporation of C-1 of glucose into erythrose. We assumed therefore that mevalonate was not the precursor for isoprenoids in the bacteria we were working on. In this case, the C_5 isoprenic framework could directly be formed by the condensation of the C_2 unit derived from pyruvate decarboxylation on a C_3 unit the origin of which has still to be determined. Again the clue for a possible origin of the second precursor could be deduced from the origin of the carbon atoms as found after feeding *Z. mobilis* or *M. fujisawaense* with ^{13}C-labelled glucose. Indeed, C-1, C-2 and C-4 from isopentenol derive respectively from C-6, C-5 and C-4 of glucose. This corresponds exactly to the C_3 framework of the triose phosphates obtained in these bacteria by the glucose catabolism via the Entner–Doudoroff pathway. This catabolic sequence yields from one molecule of glucose on the one hand pyruvate (C-1–C-3 of glucose) and, on the other hand, glyceraldehyde-3-phosphate (C-4–C-6 of glucose). This hypothesis was in accordance with the incorporation of ^{13}C-labelled glucose into the isoprenoids from *E. coli* and *A. acidoterrestris*, which metabolize glucose via glycolysis and oxidative pentose phosphate pathway. Indeed in the glycolysis, fructose 1,6-diphosphate synthesized from glucose is cleaved into dihydroxyacetone phosphate and glyceraldehyde 3-phosphate, which can be isomerized one into the other: this allows the incorporation of C-1 as well as C-6 from a hexose. The lower incorporation of C-1 is due to partial glucose metabolism through the oxidative pentose phosphate pathway in which C-1 is lost by decarboxylation of 6-phosphogluconate. Experiments performed with ^{13}C-labelled acetate confirmed this interpretation: C-1 and C-2 of IPP derive uniquely from acetate methyl group, whereas C-4 derives equally from carboxylate or from methyl groups (Table 1; Figure 2, pattern D; [8]). This corresponds exactly to the origin of the carbon atoms of phosphoenolpyruvate issued from glyoxylate cycle and the tricarboxylic acid cycle, and hence of those of triose phosphates derived from them. Finally incorporation of [3-^{13}C]pyruvate into the polyterpenoids of *M. fujisawaense* was also in agreement with this interpretation. It labelled efficiently C-5 of IPP (Table 1), even in the presence of unlabelled acetate or glucose with a rather low isotopic dilution compatible with its postulated role of a



Scheme 1 Novel hypothetical biogenetic scheme for the early steps of isoprenoid biosynthesis in bacteria

direct precursor for the C_2 unit after decarboxylation. It was also incorporated as expected from the gluconeogenesis into the C-1 position of IPP, most probably via oxaloacetate and phosphoenolpyruvate.

Novel hypothetical biogenetic scheme

The following main feature for a hypothetical scheme for the early steps of isoprenoid biosynthesis could be proposed from the labelling experiments we have performed (Scheme 1). Formation of the C_5 isoprenic framework could proceed much like the biosynthesis of L-valine. Addition of thiamine-activated acetaldehyde resulting from pyruvate decarboxylation on the C-2 carbonyl group of a dihydroxyacetone derivative (e.g. dihydroxyacetone, monohydroxyacetone or their mono- or diphosphates, methylglyoxal...) is followed by a transposition step. The following steps (reduction, isomerization, water elimination) are not known, but could again in many aspects be similar to those found in L-valine biosynthesis. However, at least for *Z. mobilis*, L-valine or one of its C_5 precursors can be fully excluded as precursor for isoprenoids, as the labelling patterns of the amino acids have been determined for each experiment [10]. The origin of the carbon atoms of this amino acid arising only from pyruvate (Figure 2, pattern B) is different from those of the isoprenic units (Figure 2, pattern A). Indeed, the L-valine skeleton resulted, as expected, from the condensation of thiamine-activated acetaldehyde on the carbonyl group of pyruvate indifferently obtained from glucose by the Entner–Doudoroff pathway, either directly (C-1–C-3 of glucose) or via glyceraldehyde 3-phosphate (C-4–C-6 of glucose).

Isoprenoid biosynthesis in Eubacteria: novel pathway versus classical mevalonate pathway

The discovery of a novel biosynthetic pathway for the early steps of isoprenoid biosynthesis was in fact only made possible owing to the efforts we have invested during the last 12 years in the chemistry and biochemistry of the bacterial hopanoids. Indeed, in contrast with the other much more widespread bacterial polyterpenoids such as bactoprenol, the isoprenoid quinones or even the carotenoids, these triterpenoids are present in much higher concentrations (e.g. in *Z. mobilis* up to 30 mg/g dry wt.). They are stable and readily isolatable, once the proper methodology has been set up, and in their ^{13}C-n.m.r. spectra nearly all signals are clearly resolved and identified, making them admirably suited for stable-isotope incorporation.

A few papers indicated that the classical pathway is operative in some bacteria. Unquestionable evidence is for instance given by the normal incorporation of [2-^{13}C]- or [1,2-$^{13}C_2$]-acetate into the isoprenic moiety of antibiotics from several Actinomycetes [29–32]. Although the radioactivity has usually not been localized, the incorporation of ^{14}C and, in one case of ^{13}C labelled mevalonate into bacterial isoprenoids of different series (C_{30} and C_{40} carotenoids, acyclic polyprenol derivatives, isopentenyladenosine, sterols), is also in favour of the occurrence of the classical pathway [33–44]. This pathway is also present in the Archaebacteria examined so far, as shown by the incorporation of labelled acetate, mevalonate, glycerol or glucose into the lipids containing phytanyl chains of the thermoacidophilic *Caldariella acidophila* [4] or the halophilic *Halobacterium* species [45–48] and by the conversion of HMG-CoA into MVA by enzymic preparations from the latter bacteria [49]. In contrast, the negative results obtained after tentative incorporation of expected intermediates such as acetate or mevalonate or the poor incorporation rates are against the presence of the classical route in several other bacteria [50–53]. Furthermore ^{14}C-labelled mevalonate was not incorporated into isoprenoids in cell-free systems from *M. fujisawaense* (M. Knani and M. Rohmer, unpublished work). Neither ^{14}C-labelled mevalonate nor mevalonate phosphate or mevalonate diphosphate could be converted into isopentenyl diphosphate by cell-free systems from *Z. mobilis* and *E. coli*, whereas ^{14}C-labelled IPP was detected using those identically prepared from *Myxococcus fulvus*, *Lactobacillus plantarum*, *Staphylococcus carnosus* and *Halobacterium cutirubrum* [53a]. Although negative results are of ambiguous nature, indicating, for instance, eventually inappropriate conditions for the enzymic

test, the failure to incorporate mevalonate into the isoprenoids from bacteria, some of which have been clearly found to possess the novel pathway (e.g. *Z. mobilis* and *E. coli*), is in accordance with the presence of another biosynthetic route. Finally the enzymes of the early steps of isoprenoid biosynthesis leading to IPP have been almost entirely uncharacterized in bacteria. Only the HMG-CoA reductase activity has been thoroughly characterized, but this enzyme was, rather, involved in the catabolism of MVA, which could be utilized as carbon source by the investigated bacteria [54].

However, when the position of the labelled atoms could be determined after labelling experiments, discrepancies with the classical pathway could be clearly highlighted by other authors and do confirm our new hypothetical scheme. By feeding with [6-$^2H_2$]glucose a *Streptomyces* species which is capable of synthesizing sesquiterpenoids, the ^{2}H-n.m.r. spectra of the pentalenelactone derivatives showed unambiguously the retention of the ^{2}H atoms from C-6 of glucose on the carbon atoms derived from C-1 and C-5 of IPP [55], as expected from the origin of the carbon atoms according to the new scheme (Figure 2, pattern A). Furthermore [1-^{14}C]- or [2-^{14}C]-acetate was incorporated into the side chain of the ubiquinones of *E. coli*, *Azotobacter vinelandii*, *Pseudomonas sesami* and *Rhodopseudomonas capsulata* in a non-classical way: C-1 and C-2 from acetate were indifferently incorporated into C-1 of isopentenol, and this ratio was not in accordance with that expected from the known pathway via acetoacetyl- and hydroxymethylglutaryl-CoA [5]. Pandian et al. [5] proposed the formation of mevalonate via the acetolactate pathway, normally involved in the biosynthesis of L-valine, with pyruvate, acetaldehyde and carbon dioxide as precursors, but this pathway is not consistent with our labelling results. In a study on the biosynthesis of the isoprenic chain of ubiquinone in *E. coli*, Zhou and White [6] also excluded this pathway, as its postulated intermediates were not incorporated. [1,2-$^{13}C_2$]Acetate fed in the presence of unlabelled glucose labelled the fatty acids, but not the isoprenoids, whereas [U-^{13}C]glucose was efficiently incorporated in both lipid families. This feature is explained by our new scheme, as the precursors would be derived from a triose phosphate and pyruvate, both readily obtained from glucose, but synthesized from acetate via the much longer sequence of enzymic reactions of the glyoxylate cycle. These authors could finally bring evidence that a C_2 unit derived from C-2 and C-3 of pyruvate corresponded to C-3 and C-5 of IPP, which is in accordance with our results, but proposed only a modification of the classical pathway leading to mevalonate with enzyme-bound acetyl groups resulting from pyruvate decarboxylation. Definitive conclusions could not be drawn from these experiments, as the incorporation of the labelled precursors was probably hampered by other competing carbon sources from the culture medium.

What can be now retained from the isoprenoid biosynthetic pathway? [^{14}C]IPP, when tested, has been always successfully incorporated by the known way into bacterial isoprenoids, suggesting its universal role, shared with DMAPP, as precursor for polyterpenoids [56–67].

The novel biosynthetic pathway leading to the C_5 precursors of isoprenic units has been characterized now in eight different bacteria (*Z. mobilis*, *M. organophilum*, *M. fujisawaense*, *R. acidophilas*, *R. palustris*, *E. coli* and *A. acidoterrestris*) we have investigated ourselves and most probably the *Streptomyces* species studied by Cane and co-workers [55]. It is most probably of general significance: it concerns polyterpenoids from several series such as sesquiterpenoids (pentalenelactone), triterpenoids (hopanoids) and acyclic polyprenols (ubiquinones), and the choice of the micro-organisms was only dictated by the convenience of the isolation of their polyterpenoids and/or their metabolic capacities and not by any phylogenetic or taxonomic relationships. Our main target is now the full elucidation of this novel mode of formation of IPP and DMAPP and to try to find out the distribution and eventually the phylogenetic significance of this novel pathway for the early steps of isoprenoid biosynthesis.

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