Zeaxanthin and menaquinone-7 biosynthesis in *Sphingobacterium multivorum* via the methylerythritol phosphate pathway

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Abstract

Feeding of [1-13C]glucose, [U-13C6]glucose, [3-13C]alanine and [1-13C]acetate to *Sphingobacterium multivorum* showed that this bacterium utilizes the methylerythritol phosphate pathway for the biosynthesis of menaquinone-7 and zeaxanthin, a carotenoid of industrial importance. Differential incorporation of the labeled precursors gave some insight into the preferred carbon sources involved in isoprenoid biosynthesis. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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I. Introduction

Two distinct biosynthetic routes, the well-known mevalonate (MVA) pathway and the more recently identified methylerythritol phosphate (MEP) pathway, lead to isopentenyl diphosphate (IPP, 8) and dimethylallyl diphosphate (DMAPP, 9), the universal precursors of isoprenoids (Fig. 1) [1,2]. The latter biosynthetic route is widespread in Eubacteria, where it was discovered [3,4]. It is also present in the plastids of phototrophic organisms and in related organelles [5,6]. Starting material is pyruvate (1) and glyceraldehyde 3-phosphate (GAP, 2), and known intermediates include 1-deoxy-D-xylulose 5-phosphate (DXP, 3), 2-C-methyl-D-erythritol 4-phosphate (MEP, 4), 4-diphosphocytidyl methylerythritol (5), 4-diphosphocytidyl methylerythritol 2-phosphate (6) and methylerythritol 2,4-cyclodiphosphate (7) (for leading references on the latest identification of intermediates, see [7,8]).

*Sphingobacterium multivorum*, formerly referred to as *Flavobacterium multivorum*, is a non-photosynthetic bacterium synthesizing and accumulating natural carotenoids, which leads to a deep orange pigmentation. Independent of the species concerned, such isoprenoid pigments are essential for photoprotection or photosynthesis and represent an important target for the food industry. Recently, the major carotenoid pigments of *S. multivorum* were identified as zeaxanthin, β-cryptoxanthin, and β-carotene [9]. The AFB44-ATCC 55238 strain was constructed for overproduction of zeaxanthin in industrial quantities (see US patent 5,308,759). In order to understand how *S. multivorum* regulates zeaxanthin production, it was essential to identify the biosynthetic pathway leading to IPP in this bacterium. Thus, labeling experiments were performed in order to decipher the early steps involved in the biosynthesis of zeaxanthin (10) and menaquinone-7 (11), the two major isoprenoids of this bacterium (Fig. 2).
2. Materials and methods

2.1. Cultures of Sphingobacterium multivorum

*S. multivorum* ATCC 55238 (designation AFB44), initially deposited as *F. multivorum*, was grown at Applied Food Biotechnology (AFB) facilities (see US patent 5,308,759). After incorporation of isotope-labeled precursors (see below), cells were obtained as lyophilized samples, which were extracted for isoprenoid analyses. Growth conditions and composition of the culture medium were adapted according to the results obtained from preliminary labeling experiments. Bacteria were grown at 28°C in 3-l Erlenmeyer flasks on a rotary shaker for 24 h.

2.2. Incorporation of [1-13C]glucose

The culture medium contained high maltose corn syrup (5 g l⁻¹), NaCl (5 g l⁻¹), (NH₄)₂SO₄ (5 g l⁻¹), MgSO₄·7H₂O (1 g l⁻¹), casamino acids (5 g l⁻¹), FeSO₄·7H₂O (1 mg l⁻¹), thiamine hydrochloride (10 mg l⁻¹), deionized water to volume. Unlabeled glucose was filter sterilized and added to each flask (4 g l⁻¹) at 16 h post inoculation. [1-13C]glucose (99% isotopic abundance, 1 g l⁻¹) was added. Flasks were harvested 10 h after addition of labeled glucose.

2.3. Incorporation of [U-13C₆]glucose

Culture conditions were similar to those utilized for culture on [1-13C]glucose with the following changes. No high maltose corn syrup was utilized. [U-13C₆]Glucose diluted in a 1:9 ratio with unlabeled glucose was added to the medium from the beginning of the cultivation yielding a final glucose concentration of 5 g l⁻¹.

2.4. Incorporation of [1-13C]acetate

The culture medium contained yeast extract (0.25 g l⁻¹), casamino acids (1 g l⁻¹) and corn steep liquor (1 g l⁻¹) and mineral salts. [1-13C]Acetate (20% isotopic abundance, 1 g l⁻¹) was added from the beginning of the culture.

2.5. Incorporation of [3-13C]alanine

Growth conditions were similar to those of the culture on [1-13C]acetate. [3-13C]Alanine (20% isotopic abundance, 0.1 g l⁻¹) was added to the medium from the beginning of cultivation.

2.6. Zeaxanthin and menaquinone-7 isolation and characterization

Lyophilized cells were extracted three times with CHCl₃/CH₃OH (2:1) under reflux. Solvents were removed under reduced pressure, and the solid extract was washed with hexane in order to recover the menaquinone-7 (11). Menaquinone was further purified by TLC (CH₂Cl₂/hexane, 1:1, Rf = 0.40). The residue obtained after hexane washing was separated by TLC (CHCl₃/CH₃OH, 95:5) yielding pure zeaxanthin (10) (Rf = 0.52). Both isoprenoids were characterized by 1H- and 13C-nuclear magnetic resonance (NMR) spectroscopy and by mass spectrometry (direct inlet, electron impact ionization, 70 eV) and comparison of the spectra with data from the literature [10–12].

NMR spectra were recorded on a Bruker WP 400 spectrometer in [2H]chloroform solution. 13C Isotopic abundances were determined as reported previously [3,4]. In the case of zeaxanthin and menaquinone spectra, isotopic abundances were estimated in comparison with the signals of carbon atoms which were supposed to be unlabeled, i.e. the signal of carbon atoms derived from C-3 of IPP at 134.95 ppm (which is not labeled, either in the MVA pathway, or in the GAP/pyruvate route) for menaquinone and from C-4 of IPP at 138.49 ppm for zeaxanthin in the case of the incubation of [1-13C]glucose, from C-4 of IPP at 39.75 ppm for menaquinone and from C-2 of IPP for zeaxanthin at 42.59 ppm in the case of the incubation of [3-13C]alanine and finally from C-5 of IPP at 16.01 ppm for menaquinone in the case of the incubation of [1-13C]acetate (Table 1).

In the case of the incubation of [U-13C₆]glucose, 3J 13C/13C long-range couplings were observed in the 13C NMR spectrum of zeaxanthin (C-3/1b-CH₃ (3.2 Hz), C-4/C-7 (3.2 Hz), C-8/C-11 (6.4 Hz) and C-12/C-15 (9.3 Hz)), next to 1J 13C/13C couplings (C-3/C-2 (35 Hz), C-6/C-7 (56 Hz), C-10/C-11 (59 Hz), C-15/C-14 (61 Hz), C-1/1a-CH₃ (34 Hz), C-5/Me-C-5 (45 Hz), C-9/Me-C-9 (43 Hz), C-13/Me-C-13 (43 Hz)). 2J 13C/13C couplings were not detected.

### Table 1

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Isotope abundances of IPP carbon atoms (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C-1</td>
</tr>
<tr>
<td>[1-13C]Glucose</td>
<td></td>
</tr>
<tr>
<td>Menaquinone</td>
<td>4.2</td>
</tr>
<tr>
<td>Zeaxanthin</td>
<td>6.0</td>
</tr>
<tr>
<td>[3-13C]Alanine</td>
<td></td>
</tr>
<tr>
<td>Menaquinone</td>
<td>1.7</td>
</tr>
<tr>
<td>Zeaxanthin</td>
<td>1.1</td>
</tr>
<tr>
<td>[1-13C]Acetate</td>
<td></td>
</tr>
<tr>
<td>Menaquinone</td>
<td>0.8</td>
</tr>
<tr>
<td>Zeaxanthin</td>
<td>—</td>
</tr>
</tbody>
</table>

*All isoprene units were identically labeled in zeaxanthin as well as in menaquinone-7. For the sake of clarity, isotope abundances are given as mean values of those from all carbon atoms derived from the same carbon atom of IPP.
*Reference signal utilized for the evaluation of the isotope abundances.
*Only qualitative data were obtained for zeaxanthin in this feeding experiment. Signals of carbon atoms with natural isotope abundance were not observed.
2.7. Isolation of subcellular extracts from *S. multivorum* cells and hydroxymethylglutaryl-CoA reductase (HMGR) enzyme assay

Bacteria were grown in batch cultures (2 × 500 ml). Cells were harvested by centrifugation at 1000 × g for 10 min and were deep frozen in liquid N₂. For extraction, cells were ground in a mortar and cell debris was suspended in a phosphate buffer system (pH 7.5, 50 mM K₂PO₄, 300 mM sorbitol, 10 mM Na₂EDTA, 5 mM MgCl₂, to which 5 mM dithioerythritol was added fresh. The homogenate was centrifuged at 8000 × g for 30 min at 4°C. The pellet was discarded and the supernatant was recovered and used as a source of soluble proteins. HMGR activity was measured as previously described [13]. The radioactive test is based on the conversion of [3-¹⁴C]HMG-CoA into MVA in the presence of NADPH. After 10–30 min incubation at 37°C, a stop solution (12 N HCl/EtOH, 3:1, v/v) containing [5-³H]MVA as internal standard allowing estimation of the recovery yield was added. Silica gel TLC (benzene/acetone, 1:1, v/v) allowed the separation of mevalonolactone (Rf > 0.33) from unreacted HMG-CoA, which was converted under acidic conditions into hydroxymethylglutaric acid remaining on the start line. Radioactivity was quantified by liquid scintillation counting using suitable quenching correction methods for ¹⁴C and ³H.

3. Results

3.1. Labeling of isoprenic units in zeaxanthin and menaquinone after incorporation of [1-¹³C]glucose

The labeling experiment with [1-¹³C]glucose (12) (Fig. 3) was performed with *S. multivorum* cells grown on a com-
plex medium containing several carbon sources such as corn syrup with a high maltose concentration (5 g \(\text{l}^{-1}\)), casamino acids (5 g \(\text{l}^{-1}\)) and unlabeled glucose (4 g \(\text{l}^{-1}\)). [\(1\text{-}13\text{C}\)]Glucose (1 g \(\text{l}^{-1}\); isotopic abundance: 99%) was added after 12 h. This corresponded to a maximal isotopic abundance of 20% for glucose, if the dilution by the glucose formed by the hydrolysis of maltose is not taken into account. Labeling patterns were nearly identical in the isoprene units of zeaxanthin (10) and menaquinone (11) (Table 1). They corresponded to those expected from glucose catabolism via glycolysis yielding \([3\text{-}13\text{C}]\)glyceraldehyde phosphate and \([3\text{-}13\text{C}]\)pyruvate (Fig. 3) which after incorporation in the MVA-independent route yielded labeling on carbon atoms corresponding to C-1 and C-5 of IPP (Table 1). Identical labeling patterns were previously obtained from \([1\text{-}13\text{C}]\)glucose for isoprenoids of Escherichia coli and Alicyclobacillus acidoterrestris [4], the isoprenoids of green algae [14] and for the plastid isoprenoids from higher plants [5,6]. All these biological systems utilize glucose via glycolysis and synthesize their isoprenoid via the MVA-independent route. The values found for the isotopic abundances roughly corresponded to those expected from the uniform mixing of the two glucose pools derived either from free glucose or from glucose released by the hydrolysis of maltose. Slight differences were observed between the values found for the isoprene units of menaquinone and those of zeaxanthin. They correspond to some preferential synthesis of menaquinone from maltose and of zeaxanthin from free glucose. The biosynthesis of these two isoprenoids most probably did not occur (at least to some extent) at the same stage of the culture. Such differences of isotopic abundance between different isoprenoid series were previously detected in other bacteria and reflect the preferred use of a carbon source, depending on the growth conditions, when cells are grown on complex media [4].

3.2. Labeling of isoprenic units in zeaxanthin after incorporation of \([U\text{-}13\text{C}]\)glucose

In order to avoid the dilution effect by another carbon source, the labeling experiment with \([U\text{-}13\text{C}]\)glucose was performed using a culture medium containing no maltose. The labeled glucose was diluted in this case in a 1:9 ratio with unlabeled glucose. In the spectra of zeaxanthin 1J \(^{13}\text{C}/^{13}\text{C}\)couplings were observed between carbon atoms derived from C-1 and C-4 in each isoprenic unit (see Section 2). They indicated that C-1 and C-4 were derived from the same glucose molecule, i.e. from the same precursor, and represented the signature of the MVA-independent route and corresponded to the GAP (3)-derived moiety of IPP (8) [15]. Such small long-range coupling constants were not observed in the spectrum of menaquinone.
3.3. Labeling of isoprenic units in zeaxanthin and menaquinone after incorporation of [3-13C]alanine

As pyruvate and GAP are the first precursors in the MVA-independent route, it was tempting to verify whether L-alanine would be a suitable precursor for isoprenoids. Indeed, alanine can be readily converted into pyruvate (1) by a transaminase and consequently introduced in isoprenoid biosynthesis. A *S. multivorum* culture was therefore grown on a medium containing yeast extract, corn steep liquor and casamino acids in the presence of [3-13C]L-alanine (20% isotopic abundance). Only carbon atoms derived from C-5 of IPP were labeled in zeaxanthin and in menaquinone. Accordingly, only the pyruvate (1)-derived moiety was labeled in isoprenic units. No label was found in the GAP (2)-derived C3 subunit. This indicated that alanine was converted into pyruvate and incorporated into isoprenoid to a significant extent, as shown from the isotopic abundance (6.0–6.9%) found for the labeled positions (Table 1). This pyruvate was, however, not converted into GAP, as the GAP implicated in isoprenoid biosynthesis was unlabeled and therefore essentially synthesized from another carbon source than alanine. This raises a puzzling question concerning the origin of the triose phosphate, which must have been obtained from another carbon source, e.g. from maltose of the corn syrup.

3.4. Labeling of isoprenic units in zeaxanthin and menaquinone after incorporation of [1-13C]acetate

Acetate is the normal precursor of the MVA pathway where it is directly incorporated after conversion into acetyl-CoA. It can also be incorporated into the MVA-independent route, but this requires its insertion into the glyoxylate and tricarboxylic acid cycles to give phosphoenolpyruvate and GAP, two triose phosphate derivatives obtained from pyruvate and GAP, two triose phosphate derivatives obtained from pyruvate (and in menaquinone). Accordingly, only the pyruvate (1)-derived moiety was labeled in isoprenic units. No label was found in the GAP (2)-derived C3 subunit. This indicated that alanine was converted into pyruvate and incorporated into isoprenoid to a significant extent, as shown from the isotopic abundance (6.0–6.9%) found for the labeled positions (Table 1). This pyruvate was, however, not converted into GAP, as the GAP implicated in isoprenoid biosynthesis was unlabeled and therefore essentially synthesized from another carbon source than alanine. This raises a puzzling question concerning the origin of the triose phosphate, which must have been obtained from another carbon source, e.g. from maltose of the corn syrup.

3.5. HMGR assay

Though the presence of the alternative pathway in *Sphingobacterium* has been unequivocally proven by the above described experiments, the question remained whether enzymes of the classical MVA pathway are also or still present, as well as corresponding genes, similar to the situation in *Streptomyces*, where both pathways seem to co-exist, although the non-MVA pathway is active in early growth phase, whereas the MVA pathway is activated in stationary phase, giving rise to the synthesis of secondary products [16]. For that reason, a series of experiments were conducted.

A key regulatory reaction in the MVA pathway is catalyzed by HMGR, a membrane-bound, highly regulated enzyme in eukaryotes [17]. So far as is known, the enzyme catalyzes the only four-electron reduction of a substrate, dependent on two molecules of NADPH per unit of HMG-CoA, which is converted into MVA. When the most sensitive radiochemical assay method was applied, which would detect as little as a few pmol of substrate conversion, we could not observe any significant activity above background by using cell-free extracts of *S. multivorum*. There was also no NADH-dependent activity as was described for *Pseudomonas mevalonii*, a bacterial strain capable of growing on MVA as the sole carbon source [18]. The reliability of the test system was always checked in parallel by using microsomes isolated from yeast [19] and from plant cells, and by using a detergent-solubilized extract from radish membranes [13]. Those controls always gave the same positive results, and as HMGR is an enzyme that is quite tolerant to various conditions, except that dithioerythritol must be present to maintain thiol groups reduced, it can be excluded that, only because the assay conditions were not optimized, we were unable to find some activity significantly above background.

4. Discussion

The MVA and the MVA-independent pathways for isoprenoid biosynthesis can be easily distinguished by the labeling patterns observed in isoprenoids after incubation of a 13C-labeled precursor and examination of the 13C NMR spectra of the isoprenoids (Fig. 3). Such labeling experiments were therefore performed with *S. multivorum* in order to decipher the early steps involved in the biosynthesis of two major isoprenoids of this bacterium: zeaxanthin and menaquinone-7.

Feeding experiments were performed with *S. multivorum* using [1-13C]glucose, [U-13C6]glucose, [3-13C]alanine and [1-13C]acetate. Labeling patterns of isoprene units observed after feeding of each labeled precursor were identical in zeaxanthin and in menaquinone (Table 1). They corresponded exactly to those expected from the MVA-independent route. No evidence for the presence of the MVA pathway could be detected. This means that pyruvate and GAP, two triose phosphate derivatives obtained from glucose catabolism, are the primary carbon sources for this metabolic route. This is the first report of the operation of the MVA-independent pathway for the bio-
synthesis of a bacterial carotenoid in a microorganism naturally producing it, and which is already used at an industrial scale. Previous investigations showed that zeaxanthin was efficiently labeled with [2-14C]MVA by a bacterium described as a ‘Flavobacterium’ species [20]. However, this strain was later found to be unrelated to S. multivorum ATCC 55238, the strain we investigated. The differences in the metabolic pathways utilized by both strains for the formation of zeaxanthin and the apparent contradiction point out the problems related to the taxonomy of bacteria and our scarce knowledge on the biosynthesis of bacterial isoprenoids. The complementary enzyme measurements and PCR attempts also support the exclusive presence of the alternative pathway in this carotenogenic prokaryote. Indeed, HMGR enzyme tests were negative. In addition, we failed to amplify DNA from S. multivorum by using HMGR-specific probes recognizing the genes from many organisms [21], while amplification was possible from tobacco BY-2 cells and from Saccharomyces cerevisiae, organisms where the presence of the corresponding genes was proved earlier. The degenerate primers (forward: 5'-CCI ATG ACI GA(A) GG-3'; reverse: 5'-CAT (GA)TT CAT ICC CAT-3'), designed from two highly conserved sequences flanking amino acids 260–365 in the Arabidopsis thaliana HMGR protein [22] (revised SwissProt accession number P14891), amplify a fragment of about 300 bp under the used PCR conditions [21].

This was not necessarily expected as there is no absolute systematic survey of microorganisms beyond genome sequencing programs, in which S. multivorum has not been included yet. Lateral gene transfer has to be considered, when explaining the scattered distribution of genes composing either the MVA or the MEP pathway within the bacterial domain [23]. The pattern actually observed is incompatible with a simple scheme of vertical gene transmission.

In the meantime, considerable progress has been made in the identification of bacterial genes whose products are proven and/or suspected to be involved in the conversion of pyruvate and GAP into IPP and DMAPP. The access to more and more bacterial genomes will help to identify clusters and further orthologs in bacteria, cyanobacteria, diverse algae and plants. The biotechnologically most important task will be to identify rate-limiting steps in order to further enhance the production of valuable isoprenoids like zeaxanthin. Some stimulating effect has also been reported for E. coli strains overexpressing the deoxyxylulose phosphate synthase gene from Bacillus subtilis or Synecho-


cystis sp. 6803 [24], from Synchococcus leopoliensis [25] or from E. coli [26,27]. However, in contrast to those rather artificial systems tested, a bacterial strain like S. multivorum used in this study, which is already optimized in production of a nutritionally important isoprenoid like zeaxanthin and possibly transformable [28], could serve as the starting material for the generation of new strains over-producing novel carotenoids [29], or even more structurally distant isoprenoid derivatives of nutritional and pharmaceutical importance.

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