Carotenoid Pigments of Mycobacterium kansasii

HUGO L. DAVID

Center for Disease Control, Atlanta, Georgia 30333

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Partitioned between aqueous methanol and petroleum ether, the unsaponifiable pigments of Mycobacterium kansasii were all epiphatic. Thin-layer chromatography of these carotenoids showed that M. kansasii formed at least nine pigments. These pigments were identified by their chromatographic properties and spectral characteristics as phytoene, \( \alpha \)-carotene, neurosporene, lycopene, leproten, \( \gamma \)-carotene, \( \delta \)-carotene, \( \alpha \)-carotene, and \( \beta \)-carotene. Three additional pigmented spots on thin-layer chromatography found in trace amounts were possibly degradation products of the major carotenoids.

The cell mass of Mycobacterium kansasii becomes a deep orange-yellow when exposed to visible light under appropriate conditions (13). This color was attributed to the accumulation of \( \beta \)-carotene (3), and carotenogenesis was reported to be an induction reaction (2). Information about the carotenoids in these bacteria is fragmentary. Thus, Ebina and his associates (3) found \( \alpha \)- and \( \beta \)-carotene and phytofluene in Runyon strain P-6 and P-8, and Tsukamura (12) reported that strain Forbes 84 formed lycopene and \( \beta \)-carotene, whereas strain Bostrom D-35 formed only \( \beta \)-carotene. Lycopene was also found to be the pigment that accumulates in mutants that become pink rather than orange-yellow on photoinduction (11).

In this report I describe the isolation and characterization of nine carotenoids from M. kansasii; three additional pigments were not identified because they occurred in trace amounts in the strains analyzed and were possibly degradation products of the major carotenoids.

MATERIALS AND METHODS

Bacteria. Three strains of M. kansasii were used. Strain 37 (Runyon strain P-16 and Pollak “Bostrom” strain, which is the type strain of M. kansasii) is the parent strain; strain 2486 is a mutant that acquires a pink color on photoinduction; and strain 336 is a constitutive mutant. These strains are maintained in our culture collection.

Growth conditions. The bacteria were grown in 100 ml of a modified Proskauer and Boeck medium, the composition of which was described previously (2). Two-week-old cultures in 250-ml screw-cap Erlenmeyer flasks were illuminated with fluorescent light from two 40-W cool white Westinghouse tubes located at a distance of 30 cm from the growth flasks. Because the photochemical reaction is dependent on oxygen and the induction reaction is dependent on both oxygen and temperature (1, 2), illumination was carried out with the cultures in flasks, with loosened caps, in a water bath at 36 C. Illumination was continuous for 48 or 72 h. Strain 336 did not require illumination for pigment synthesis and therefore was not exposed to light.

Lipid extraction. The wet cell mass was extracted with an equal mixture of chloroform-methanol (1:1, vol/vol) until no more pigment was visible in the cell mass. The organic solvent layer was removed and then evaporated to dryness in a rotary evaporator (Büchi Instruments, Fort Lee, N.J.). The residue was dissolved in a 4% solution of potassium hydroxide in methanol, and the lipids were saponified by stirring at room temperature for 1 h. The mixture was then extracted with petroleum ether (30 to 60 C). The petroleum ether extract was washed once with distilled water and then evaporated to dryness.

Column and TLC. The pigments were dissolved in hexane and separated by thin-layer chromatography (TLC) with ALOX-25 precoated TLC plates (Brinkmann Instruments Co., Westbury, N.Y.). The chromatograms were developed in 1 or 2% acetone in hexane; the less-polar pigments were separated in 1% acetone in hexane, and the more-polar pigments were separated in 2% acetone in hexane. For purification, initial separation on alumina (Merck & Co., Inc., Rahway, N.J.) columns was followed by further purification on TLC plates. The columns were eluted with hexane and increasing concentrations of acetone in hexane. The pigments were recovered from TLC plates by extracting the scrapings with acetone.

Absorption spectra. The absorption spectra of the purified carotenoids were measured with a Beckman DB spectrophotometer.

Chemicals. Commercially available organic solvents were used without further purification. Nicotine was purchased from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Extraction and purification of the carotenoids. All pigments from M. kansasii were found to be epiphatic, which is in agreement with an earlier report (2). In the parent organism, \( \beta \)-
carotene and lycopene amounted to, respectively, about 85% and 15% of the total carotenoids estimated, as reported by Liaaen-Jensen and Jensen (9). Because the precursors of β-carotene are rapidly converted (2), they (with the exception of lycopene) occur in trace amounts in the extracts. However, the β-carotene precursors could be isolated from mutants or by blocking β-carotene synthesis with nicotine, which is known (6) to inhibit the cyclization of lycopene.

Spectra of the carotenoids. Because the carotenoids are unstable to light and oxidation (9), the spectra were always obtained on the day of preparation. Twelve spots were observed on TLC plates. Three of these compounds (R, 0.0, R, 0.06, and R, 0.12 in 2% acetone in hexane) were not identified because they were present in trace amounts in the extracts of the strains used. The identification of the remaining nine carotenoids is indicated in Table 1, and their spectra are shown in Fig. 1, 2, and 3.

Phenotype of the M. kansasi strains. As indicated, the various carotenoids were present in different amounts in the extracts of the three strains. For example, neurosporene was found in trace amounts in the parent strain 37 and in strain 335, but it was present in amounts sufficient for spectral analysis in extracts from strain 2486. The presence of the carotenoids in

The extracts of the three strains could, however, be ascertained by co-chromatography with the purified compounds (Table 2).

Inhibition of lycopene cyclization. Howes and Batra (6) reported that the alkaloid nico-

| Pigment no. | R, | Absorption maxima in hexane (nm) | Tentative identification |
|-------------|----|----------------------------------|-------------------------|
| 1           | 0.82 | (265-275), 285, (290-295)       | Phytoene                |
| 2           | 0.72 | (425), 445, 470                 | α-Carotene              |
| 3           | 0.70 | (425), 455, 485                 | β-Carotene              |
| 4           | 0.70 | 400, 420                       | γ-Carotene              |
| 5           | 0.65 | 430, 460, 490                  | Neuroporene            |
| 6           | 0.46 | 415, 435, 465                  | Leproteine              |
| 7           | 0.39 | (425), 455, 485                 | Lycopene               |
| 8           | 0.32 | 445, 475, 505                  | β-Carotene              |
| 9           | 0.23 | (425-430), 460, 485             | Unidentified            |
| 10          | 0.12 |                                | Unidentified            |
| 11          | 0.06 |                                | Unidentified            |
| 12          | 0   |                                | Unidentified            |

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### Table 1. Chromatographic and spectral properties of the carotenoids of M. kansasi

*The pigments were identified by comparison with published spectra (4, 5, 10); see Fig. 2, 3, and 4.

* R, determinations were made on activated ALOX-25 TLC plates developed in 2% acetone in hexane.

* Although β-carotene and γ-carotene have the same R, in the system shown, γ-carotene could be isolated from strain 2486, which does not synthesize β-carotene.

* Possibly degradation products of the major carotenoids.
Table 2. Phenotypes of M. kansasii strains 37, 2486, and 336

| Strain   | Conditions of carotenogenesis | Carotenoids*                                                                 |
|----------|-------------------------------|-----------------------------------------------------------------------------|
| Parent (37) | Inducible                     | Phytoene, (\(\gamma\)-carotene), (neuroporene), lycopene, (\(\gamma\)-carotene), (\(\delta\)-carotene), \(\alpha\)-carotene, \(\beta\)-carotene, leprotene |
| 2486     | Inducible                     | Phytoene, \(\gamma\)-carotene, neuroporene, lycopene, (\(\delta\)-carotene), (\(\alpha\)-carotene)            |
| 336      | Constitutive                  | Phytoene, (\(\gamma\)-carotene), (neuroporene), lycopene, \(\gamma\)-carotene, \(\delta\)-carotene, \(\alpha\)-carotene, \(\beta\)-carotene, leprotene |

*Carotenoids in parenthesis indicate that the compound was in trace amounts.

Ticine was an inhibitor of the cyclization of lycopene in M. marinum and in an unidentified Mycobacterium sp. The inhibition caused lycopene to accumulate. Nicotine also caused lycopene to accumulate in M. kansasii, and the cyclization of lycopene was completely inhibited at a concentration of 2.5 mM (Fig. 4). At this concentration, \(\alpha\)-, \(\beta\)-, \(\delta\)-, and \(\gamma\)-carotenes and leprotene were not detected on TLC plates.

**DISCUSSION**

Our study of the carotenoids of M. kansasii showed that these bacteria synthesize phytoene, \(\gamma\)-carotene, neuroporene, lycopene, \(\gamma\)-carotene, \(\delta\)-carotene, \(\alpha\)-carotene, \(\beta\)-carotene, and leprotene. The occurrence of phytofluene has been reported (3). Except for \(\beta\)-carotene and lycopene, which amounted to 85% and 15% of the total carotenoids, respectively, all others occurred in trace amounts or were undetectable in extracts of the wild-type parent strain. This finding is explained by the rapid conversion of the precursor carotenoids into the end products, which in M. kansasii are \(\alpha\)- and \(\beta\)-carotene and possibly leprotene. \(\beta\)-carotene was, however, the major end product in M. kansasii, as has been reported by others (3, 11, 12).

The various carotenoids were isolated from two mutants, one in which a mutation(s) affected the regulatory mechanisms (constitutive mutant) and one in which the mutation(s) affected the conversion of lycopene. The latter mutant accumulated phytoene, \(\gamma\)-carotene, and neuroporene in amounts sufficient for spectral analysis, and the pink color it acquired on photoinduction was caused by the accumulation of large amounts of lycopene.

These findings suggest that the biosynthesis of the carotenoids in M. kansasii is by step-by-step dehydrogenation of phytoene to form lycopene, which is then cyclized to the monocyclic and dicyclic carotenes.

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**FIG. 3.** Absorption spectra in hexane (-----) and in benzene (------) of carotenoid 7 (leprotene).

**FIG. 4.** Concentration of \(\beta\)-carotene and lycopene in cells treated with the indicated concentration of nicotine. The cells were exposed to nicotine immediately before illumination and were illuminated continuously overnight. The pigments were then purified by preparative TLC.
cyclization of lycopene (5). Thus, acyclic, monocyclic, and dicyclic carotenoids (adapted from reference 8). The pathway is inducible in M. kansasii; strain 336 is a constitutive mutant. If, Sites of nicotine action in M. kansasii; →, genetic block in strain 2486 of M. kansasii.

This study provides indirect evidence for the cyclization of lycopene and suggests that α- and β-carotene are synthesized separately (Fig. 5). Thus, I showed that a mutant that accumulated lycopene on photoinduction had a genetic block between lycopene and γ-carotene but formed δ- and α-carotene. Furthermore, nicotine, a known inhibitor of the cyclization of lycopene (6), blocked the synthesis of both α- and β-carotene as well as leprotene. The data currently available do not allow an interpretation concerning the immediate precursor of leprotene.

The enzymatic conversion of phytoene to acyclic, monocyclic, and dicyclic carotenoids was directly demonstrated recently (8). The fact that in M. marinum and M. kansasii carotenogenesis requires the synthesis of new proteins (1, 2) indicates that the synthesis of phytoene and its conversion is enzymatic, and therefore description of the photochromogenic reaction by the use of the expressions “photoactive” (7) or “photosynthesis” (11) seems inappropriate. On the basis of current understanding of the mechanism of photochromogeneity in M. marinum and M. kansasii, I suggest that pigmentation in the mycobacteria be designated as constitutive or inducible (photoinducible).

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