Carotenoids Synthesized by UV-induced Mutants of a Non-acid-fast Strain of *Mycobacterium phlei*

A. KOLMANOVÁ, J. HOCHMANNOVÁ and I. MÁLEK

Department of Bacterial Genetics, Institute of Microbiology, Czechoslovak Academy of Sciences, Prague 4

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ABSTRACT. The composition of the cell pigment of three mutants (PN_1, PN_2, PN_3) of a non-acid-fast strain of *Mycobacterium phlei* (PN) induced with UV-radiation was investigated. It was found that the mutants contain carotenes typical for the original parent strain, but that the quantity of the colourless precursor, phytoene, is substantially lower, and, on the contrary, the amount of lycopene is increased (especially in mutants PN_2 and PN_3). In addition, all mutants synthesized xanthins in considerable quantities: the PN_1 mutant above all with the β-carotene carbon skeleton, whereas for the PN_2 and PN_3 mutants xanthins with the lycopene structure were typical. The hit of the genome of the original strain caused by the mutagen is thus manifested even in the synthesis of secondary metabolites.

Mutants with changed colour were isolated after the treatment of a number of pigmented microorganisms with UV-light. Griffiths and Stanier (1956) obtained a number of less pigmented mutants (brown, green, blue-green) from a purple wild strain of *Rhodopseudomonas spheroides* in this way. Mathews and Krinsky (1965) isolated colourless mutants after the UV-irradiation of *Sarcina lutea* and *Micrococcus* sp. Achromogenic mutants were induced in a number of photochromogenic and scotochromogenic mycobacteria by Tsukamura (1963). It is thus evident that the UV-treatment of microorganisms producing carotenoids resulted mostly in the inhibition of synthesis of coloured carotenoids and the accumulation of colourless precursors with a shorter chromophor.

A total of 46 mutants were isolated in this laboratory after the treatment of the photochromogenic non-acid-fast strain of *Mycobacterium phlei* (PN) with UV-radiation (Koníček & Málek, 1967, 1968). Thirty mutants were photochromogenic as the original strain, 11 mutants were achromogenic and 5 mutants were of scotochromogenic character. From them three intensively orange scotochromogenic mutants (PN_1, PN_2, PN_3) differing most considerably from the original light ochre strain when growing in the dark were chosen for the work to be described. It should be pointed out, however, that after the exposure of the PN strain to daylight its colour was similar to the colours of the PN_1, PN_2 and PN_3 mutants. It was the aim of the present study to establish what changes in the synthesis of carotenoids occurred in the selected UV-mutants and compare them with those caused by the effect of daylight on the original parent strain (Hochmannová *et al.*, 1970).

MATERIALS AND METHODS

Bacterial strains. A photochromogenic non-acid-fast strain of *Mycobacterium phlei* [No. 727 in the catalogue of *Mycobacteria* (Hauduroy, 1966); PN] isolated by Hubáček and Málek (1958) and three scotochromogenic mutants (PN_1, PN_2, PN_3) of this parent strain obtained after the UV-treatment (Koníček & Málek, 1967) were used throughout. All three mutants retain their non-acid-fast char-

acter, however, they are resistant to streptomycin (10 μg/ml) and colours of the cultures cultivated in the dark are much more intensive (orange) than that of the culture of the original strain grown under the same conditions (ochre). The PN_3 culture originally differed from remaining two by its ability to grow into a solid cultivation medium, but it lost this ability after two years of subcultivating. In this connection the mutant was further tested and it was found that its other characteristics including the formation and composition of the cell pigment were not changed. Therefore, possible differences in the character of growth of the culture were not taken into consideration when evaluating experiments with the PN_3 mutant.

Cultivation of mycobacteria. Inocula of all studied mycobacteria were prepared in a liquid Dubos medium with Tween 80 (0.05% final concentration). After three days the cultures were transferred to a solid agar medium containing tryptose (Bacto tryptose Difco 15.0 g; NaCl 0.2 g; K_2HPO_4 0.2 g; distilled water ad 1,000 ml; pH 7.2; agar 20.0 g; 10 ml of 50% glucose). Mycobacteria were grown in 2 liter Roux flasks for 7 days at 37 C in the dark. During the period when the PN_3 mutant grew into the agar, the cultivation was carried out in Petri dishes with cellophane membranes.

The extraction and separation of the pigments were described previously (Hochmannová *et al.*, 1970). The separation of the crude extract to hexane (carotenes) and methanolic (xanthins) parts was avoided in the present work as the original strain produced only hydrocarbons and the same isolation procedure was preferred even with the mutants. Xanthins were separated chromatographically in these cases.

Colourless precursors were separated first from the obtained mixtures of carotenoids by thin layer chromatography (TLC) on silica gel in petroleum etherbenzene (92 : 8). These compounds were detected according to their fluorescence under short wave UV light (Mineralight UVS-11 served as the source of UV radiation).

The coloured components were separated using TLC in more polar solvent systems, xanthins produced by UV-mutants in a strongly polar mixture benzene--ethanol (90 : 10).

Acetone-ethanolic (1 : 1) eluates o individual chromatographic bands were filtered and evaporated to dryness under reduced pressure, the residues were determined gravimetrically. Trace amounts of carotenoids that were not exactly identified and of products of polymerization that could not be separated by chromatography and that are not listed in Table 1 were considered when calculating the relative occurrence of individual carotenoids in a given strain of *Mycobacterium.*

The homogeneity of the obtained chromatographic fractions was verified (on Siligram foils) and the impure fractions were rechromatographed. Pure compounds were dissolved in suitable spectrally pure solvents and their visible and UV spectra measured in the Unicam SP-700 spectrophotometer. Spectroscopic data were in some cases supplemented by co-chromatography with pure standard compounds (lycopene and rhodoxanthin were obtained from F. Hoffmann — La Roche and Co., Basel, Switzerland; α- and β-carotenes were purchased from Mann Research Laboratories, New York, USA).

RESULTS

The original PN strain and its three UV-mutants produced roughly the same amounts of pigments, i.e. 1.5% as calculated per weight of wet packed cells when growing in the dark in the medium with tryptose. Table 1 summarizes the results of the analyses of the cell pigments from the studied mycobacteria. Carotenoids are listed according to their biogenetic sequence.

Table 1. Carotenoids isolated from UV-induced mutants (PN_1, PN_2, PN_3) of a non-acid-fast strain *Mycobacterium phlei* (PN)

Colour of the chromatographic fraction	λ_{max} in nm (solvent)	Carotenoid	Carotenoid content (%)			
			PN_1	PN_2	PN_3	PN
Colourless UV-fluorescence	273, **283***, 294 (isooctane)	phytoene	18.9	12.3	4.6	37.9
Yellow	415, **440**, 476 (hexane)	neurosporene	5.9	2.9	6.2	—
Brick-red	447, **476**, 505 (hexane)	lycopene+	7.8	22.8	13.4	3.4
Yellow	437, **462**, 493 (hexane)	γ-carotene	27.6	20.0	10.8	24.2
Yellow	425, **450**, 476 (hexane)	β-carotene+	5.8	0.5	—	20.9
Yellow	422, **444**, 473 (hexane)	α-carotene+	—	—	—	2.0
Yellow	427, **454**, 474 (hexane)	leprotene	5.8	4.9	—	—
Orange-pink	455, **482**, 514 (hexane) 470, **500**, 531 (benzene) 467, **500**, 526 (chloroform)	P-481	1.0	22.0	6.1	—
Orange	455, **483**, 515 (hexane) 467, **495**, 526 (benzene) 465, **490**, 524 (chloroform)	HO-P-481	0.5	traces	traces	—
Orange-red	463, **493**, 528 (hexane) 480, **510**, 547 (benzene) 476, **505**, 538 (chloroform)	spirilloxanthin	traces	3.8	19.2	—
Pink	461, **490**, 518 (hexane) 470, **502**, 529 (benzene) 467, **502**, 536 (chloroform)	torulene	6.3	traces	18.5	—
Carmine	455, **486**, 523 (hexane) 476, **505**, 543 (benzene) 482, **509**, 546 (chloroform)	rhodoxanthin+	13.3	2.5	—	—
Yellow-orange	385, **407**, 431 (hexane)	mycoxanthin	traces	traces	5.1	—

* The boldfaced values correspond to the main maxima;
\+ isolated carotenoid was co-chromatographed with authentic sample;
% weight per cent of individual fractions referred to the sum of carotenoids obtained after the first chromatography.

The photochromogenic PN strain produced considerable quantities of the colourless precursor, phytoene, under the described growth conditions, smaller quantities of phytoene were found in scotochromogenic mutants, in the PN_3 mutant in particular. Hydrocarbons γ- and β-carotenes, highly represented in the PN strain determine to a considerable extent its overall ochre colour. A dominant occurrence of γ-carotene in this strain has already been detected during its growth on the Sauton medium (Hochmannová *et al.*, 1968).

Also UV-mutants were found to be rich in γ-carotene. Furthermore, neuro-

sporene, a first coloured hydrocarbon on the biosynthetic pathway of carotenoids was detected. The above carotene was not detected in the original parent PN strain. The concentration of lycopene was found to be higher in mutants (in the PN_2 strain in particular) as compared with the parent strain. On the contrary, only little β-carotene was found in the mutants; α-carotene was not detected at all. Leprotene, found previously in the PN strain after a 20-hours exposure of the culture to daylight (Hochmannová *et al.*, 1970) was isolated in the PN_1 and PN_2 mutants. Xanthins, oxygen-containing derivatives of carotenes, were detected as typical components of the cellular pigment of the studied UV-mutants. Of a total of isolated carotenoids 21.1%, 28.3% and 48.9% xanthins were found in the PN_1, PN_2 and PN_3 mutants, respectively. In this respect UV-mutants resemble the PN strain containing 26.4% xanthins after a 20-h exposure to daylight (Hochmannová *et al.*, 1970). The occurrence of individual xanthins varied in the studied mutants. High quantities of rhodoxanthin (derived from β-carotene) were obtained from the PN_1 culture, P-481 carotenoid (a derivative of lycopene) dominated in the PN_2 mutant. Spirilloxanthin (belonging to the lycopene group) and torulene (containing γ-carotene carbon skeleton) were found as characteristic carotenoids of the PN_3 mutant. In addition, mycoxanthin, a compound typical for mycobacteria, was found in the PN_3 mutant; only trace quantities of this compound were detected in the remaining two mutants. The above carotenoid has been found in this laboratory only in the acid-fast strain of *Mycobacterium phlei* so far (Hochmannová *et al.*, 1968).

DISCUSSION

A similarity of the final effects of the two types of radiation on the biosynthesis of carotenoids may be observed when comparing results of analysis of carotenoids in the UV-mutants with data obtained in a study concerning the effect of daylight on the synthesis of carotenoids in the original photochromogenic strain (Hochmannová *et al.*, 1970), in spite of the fact that their mechanisms of action are different.

Ultraviolet radiation affects above all the genetic material of a cell directly by bringing about changes in DNA (formation of thymine dimers, hydration of cytosine and uracil, impairment of hydrogen bonds between DNA strands). In addition, further changes (e.g. formation of peroxides and their subsequent effect on cellular components) take place in the irradiated cells. As previously isolated mutants were used in the present work, the observed changes in the biosynthesis of carotenoids were apparently a result of genetically fixed hits. It may be assumed that genomes of the studied UV-mutants control the synthesis of carotenoids in such a way that enzymes required for the synthesis of xanthins (oxidases and methylases in particular) play a very important role. A lower activity of cyclases may be expected in the mutant PN_2, and also in the PN_3 mutant, rich in lycopene and its oxygen-containing derivatives as compared with the original parent strain characterized by high quantities of γ- and β-carotenes.

The formation of xanthins is also responsible for the pigmentation change of the original photochromogenic strain after exposure to daylight (Hochmannová *et al.*, 1970) but this property is not hereditary. In this case the light energy facilitates the oxidation of carotenes by air oxygen (photochemical effect) or possibly activates some enzymes responsible for the synthesis of carotenoids.

It follows from the quantitative balance of phytoene in the studied mycobacteria that both the mutagenic effect of UV-radiation and the daylight accelerated the conversion of this colourless precursor to coloured carotenoids.

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