Response of *Escherichia coli* containing mycobacterial carotene genes to UV radiation

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The plasmid pC5, which encodes biosynthesis of lycopene in *Mycobacterium aurum A*+, was partially digested by restriction endonucleases and generated fragments were cloned. After transformation of *Escherichia coli* (colorless bacteria) with the plasmids so constructed, seven orange clones were detected and found to carry the same recombinant plasmid (pC51). *E. coli* cells containing this plasmid synthesize neurosporene and lycopene, and were more resistant to ultraviolet irradiation than nonpigmented strain.

INTRODUCTION

Carotenoid pigments are largely distributed in nature. They are present in all of the photosynthetic organisms as well as in some bacteria, fungi, and yeast [1]. Two major biological roles have been assigned to carotenoids in plants and prokaryotes. In photosynthetic organisms, these pigments are involved in trapping light energy. A more general role applicable to both photosynthetic and non-photosynthetic cell, is protection from photodynamic action [1]. Genes controlling the synthesis of these pigments have been studied in several organisms such as *Erwinia* species [2–4], *Mycobacterium aurum A*+ [5, 6], *Arabidopsis* [7, 8], *Xanthophyllomyces dendrorhous* [9] and *Brevibacterium linens* [10].

The synthesis of lycopene can be divided into two stages: the synthesis of phytoene and its desaturation (dehydrogenation) [11]. The precursor of carotene is isopentenyl pyrophosphate (IPP, C5) obtained from mevalonic acid. IPP is converted to farnesyl pyrophosphate (FPP, C15). The condensation of IPP and FPP are required for the synthesis of the geranylgeranyl pyrophosphate (GGPP, C20). This reaction is catalyzed by GGPP synthase. The gene coding for this enzyme has been cloned in several species [2, 4, 6, 12]. The dimerization of GGPP produces prephytoene pyrophosphate (PPPP, C40) that is converted into phytoene (C40 colorless hydrocarbon). These reactions are catalyzed by phytoene synthase [13]. The gene coding for this enzyme was cloned in *Erwinia herbicola* and leads to the synthesis phytoene from GGPP in *Escherichia coli* [2, 4, 14]. Four sequential desaturations are required to convert phytoene into lycopene by phytoene desaturase (dehydrogenase); the intermediate precursors are successively phytofluene, ζ-carotene and neurosporene (Figure 1) [11, 13]. In non-photosynthetic organisms such as *Erwinia herbicola* and *Erwinia uredorora*, phytoene desaturase is the product of a single gene that is capable to express in *E. coli* [2, 4, 13, 15].

Oxidative stress is recognized as one of the major contributors of increased risk of cancer. Considerable evidence suggests that lycopene has significant antioxidant potential and may play a role in preventing cancer and coronary heart disease [16, 17]. For the purpose of investigating beneficial
effect of this pigment, we decided to express in E. coli the mycobacterial genes responsible for its synthesis, and to study its evaluation as a protectant against UV radiation.

The pC5 plasmid was previously isolated from the wild-type Mycobacterium aurum A′ (yellow-orange phenotype) genomic bank. This vector which in addition to pHLD69 vector was partially digested by the restriction endonucleases Sau 3A, Sph I, or Pst I. The fragments generated were used to transform E. coli DH5α and the transformants selected on LB agar containing kanamycin and 150 µg/ml of kanamycin. This bacterium was grown in Luria Bertoni (LB), and their transformants were selected with 50 µg/ml of kanamycin.

Isolation of pC51 plasmid coding for lycopene in E. coli

The plasmid pC5 was partially digested by the restriction endonucleases Sau 3A, Sph I, or Pst I. The generated fragments were ligated respectively in the pHLD69 vector digested with Bam HI, Sph I, or Pst I. The ligation mixtures were used to transform E. coli DH5α and the transformants selected on LB agar containing 50 µg/ml of kanamycin and 0.4 mM of isopropyl-β-D-galacto-pyranoside (IPTG). Seven pigmented transformants were cloned, and their plasmid DNAs were extracted and analysed by some restriction endonucleases (Figure 2) did not form any carotene [5, 18]. This plasmid was partially digested by the restriction endonucleases Sau 3A, Sph I, or Pst I. The fragments generated were cloned, respectively, in the pHLD69 vector digested with Bam HI, Sph I, or Pst I. The cloning vector used was pHLD69 (6.3 kb), containing pUC19, the Ori R of pAL5000, and the Kan gene [5]. The second plasmid used in this work was pC5 that was previously isolated from the Mycobacterium aurum A′ genomic bank. This vector which in addition to pHLD69 vector (6.3 kb) contains an insert of about 8.8 kb controlling the biogenesis of lycopene in colorless M. aurum A11 and the buff-colored Mycobacterium smegmatis MTC-155 [18]. E. coli DH5α (kan', lac') was used as a host for the plasmids.

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Identification and quantitative determination of pigments

The pC51 vector was used to transform E. coli. The transformants were selected on LB-agar containing kanamycin and IPTG as described above. The harvest cells were extracted several times with chloroform/methanol (1:1 v/v). The extracts were dried in vacuo and the colored residues were dissolved in pure acetone. The cloudy acetone extracts were refrigerated at 4°C for several hours and then clarified by centrifugation.

Ultraviolet irradiation

Ten orange colonies of E. coli containing pC51 and cultivated in presence of IPTG and kanamycin on LB-agar at 37°C for 60 h were suspended in LB medium and then diluted to have 10^6 cells/ml. For each time of exposure to ultraviolet light, 100 µl of diluted culture were plated into Petri dishes containing 25 ml of LB-agar medium and then exposed to UV radiation. Count of colonies was made after 20 h of incubation. The same method was used for the control: E. coli and E. coli transformed by pHLD69. For all strains used and for each time of exposure, the given value represents the mean of two or three experiments. Irradiation was made by an ultraviolet lamp (6 W-254 nm tube; Vilber Lourmat, French) from the distance of 14, 50, or 77 cm. Time of exposure for these distances was respectively: 0, 2, 4, 5, 6, 7, 8, 9, 10, 11 seconds; 0, 5, 10, 15, 25, 35, 45, 50, 55 seconds; and 0, 5, 10, 15, 25, 35, 45, 55, 65, 75, 90, 110, 120, 130 seconds. A petri dish containing LB-agar medium was exposed to UV light for 15 min at a distance of 50 cm, and then inoculated with the E. coli strain. After incubation, the number of colonies was found to be identical to that found in parallel controls not exposed to UV light, showing that the medium did not deteriorate following UV exposition.

RESULTS

Obtaining of orange-pigmented colonies of E. coli

Clones of E. coli containing plasmid pC51 (Figure 2) did not form any carotene [5, 18]. This plasmid was partially digested by the restriction endonucleases Sau 3A, Sph I, or Pst I. The fragments generated were cloned, respectively, in the pHLD69 vector digested with Bam HI or Sph I or Pst I. The three ligation mixtures were used to transform E. coli (nonpigmented bacterium). After transformation, bacteria were transferred to solid media containing IPTG. All the clones obtained by the cloning with the enzymes Bam HI or Sph I were nonpigmented. Amongst those obtained...
by the cloning with Pst I, seven orange clones were detected and cloned. Plasmids DNAs were prepared from these orange colonies and subjected to Pst I, Sph I, Bam HI digestion. This experiment (results not shown) showed that all the clones harbored the same recombinant plasmid designated pC51 containing an insert of 4.42 kb (Figure 2). Further, E. coli was transformed by pC51, and all the clones obtained after 60 h of incubation at 37°C were orange-colored. It was therefore concluded that the plasmid pC51 is responsible for the orange pigmentation of the bacteria.

**Analysis of carotenoids**

Carotenoid pigments from E. coli containing pC51 were extracted and separated by preparative thin-layer chromatography (TLC) and two carotenoids were detected; the major red carotenoid was purified and proved to be identical to lycopene by its visible spectrum.

Indeed, this spectrum was similar(581,491),(938,520) to both the commercial lycopene and also the lycopene extracted from the wild-type M. aurum A+ (Figure 3). The identity of this major pigment was also verified by cochromatography with authentic sample. E. coli harboring plasmid pC51 accumulated large amounts of lycopene; its yield was 0.25 μg/mg dry wt after 60 h of incubation at 37°C (Figure 3). Furthermore, a yellow pigment was isolated and was confirmed to be neurosporene by its spectrum (Figure 3). This pigment is present in similar amount as compared to lycopene as it is converted to lycopene (Figure 3). However, phytoene, phytofluene and ζ-carotene (Figure 1) were not detected in extract of E. coli transformed by pC51.

**Resistance to ultraviolet irradiation of E. coli (pC51)**

To analyze the role of the carotenoids in protection against UV irradiation, E. coli (pC51) was exposed to UV rays as described in materials and methods section. Briefly, strains were incubated at 37°C for 60 h prior to UV exposure. As indicated in Figure 3, this incubation time was intentionally chosen as it permits the maximal synthesis of the carotenes. At a distance of 14 or 50 cm from the UV lamp, all strains had the same sensitivity to the UV light (Figure 4). However, at a distance of 77 cm, cells of E. coli (pC51) were more resistant than the parallel controls (E. coli and E. coli containing pHLD69; Figure 4). This result indicates that the carotenoid pigments are able
to prevent the lethal action of UV irradiation on bacterial cells.

**DISCUSSION**

The plasmid pC51 was isolated by subcloning the vector pC5 into the plasmid pHLD69. Clones of *E. coli* containing pC51 were orange, whereas *E. coli* harboring pC5 was not pigmented and did not form any carotene. This result suggests that the insert of pC51 was transcribed from the lacZ promoter (Figure 2). This insertion permits the development of a new colored marker for use in *E. coli* and mycobacteria.

It is established that the synthesis of lycopene is the result of stepwise desaturation of phytoene resulting successively in the formation of phytofluene, ζ-carotene, neurosporene, and lycopene (Figure 1) [11, 13]. We previously showed that plasmid pC5 controls the biogenesis of lycopene [18, 19]. This result was determined by complementation experiments that showed the formation of lycopene by colorless *Mycobacterium aurum* A11, which synthesizes phytoene [19]. However, these experiences were not conclusive as to whether the pC5 plasmid contained the genes responsible for GGPP and phytoene production (Figure 1). The use of a heterologous system such as *E. coli* (no mycobacterial host) may give an answer to this question. Indeed, cells of *E. coli* do not normally synthesize phytoene or other carotenoids, but they do produce FPP [14, 22], which is the precursor of GGPP (Figure 1). *E. coli* harboring pC51 produced lycopene, demonstrating that this plasmid codes for three enzymes: GGPP synthase, phytoene synthase, and phytoene desaturase. These enzymes are required to synthesize the lycopene from FPP in this bacteria (Figure 1). These results are in agreement with recent investigations concerning the sequencing of a DNA fragment of the plasmid pC1 [5, 18, 19], that contains the eight genes that intervene in synthesis of carotenoids in *M. aurum* A+ [6]. The plasmids pC1 and pC5 share a homologous sequence of 6.5 kb [18], and its sequencing showed that it did contain the genes coding for GGPP synthase, phytoene synthase, and phytoene desaturase. These genes were identified on the basis of sequence homologies [6]. A comparison of the results from this investigation with those obtained in the present study shows that the plasmid pC51 contains the three genes mentioned above (Figure 2).

Phytoene, phytofluene, and ζ-carotene were not detected in extracts of *E. coli* transformed by pC51, suggesting that these carotenoids were rapidly converted into lycopene (Figure 1). This result was in line with previous findings with extracts of *M. aurum* A+ and *M. aurum* NGR9, which only contain small amounts of neurosporene but not other precursors of lycopene [23]. It is now established that the intermediate precursors in the biosynthesis of the carotenoids are rapidly and sequentially converted. This is based on kinetic data in mycobacteria that form the carotenoids by induction, and from kinetic data on the accumulation of the carotenoids after removal of chemical inhibitors [24]. In all cases, the end-products of carotenogenesis appear in the extracts within the first hour, and the intermediate precursors

**Figure 4:** Susceptibility of various *E. coli* strains to UV radiations. Symbols: 1, *E. coli*; 2, *E. coli* containing pC51; 3, *E. coli* containing pHLD69; d, distance separating the bacterial culture from the UV lamp.
do not accumulate. This is explained by the intermediate may be difficult to find in bacterial extracts.

Exposure of pigmented strain [E. coli (pC51)] and colorless strains of E. coli [E. coli and E. coli (pHLDe99)] to UV light resulted in significant differences in the rate of killing of the colorless strains as compared with the colored strain, which was more resistant to UV radiation (Figure 4). The plasmid pC51, which in addition to pHLD0 vector, contains an insert of 4.42 kb (Figure 2). Nucleotide sequence analysis of pC1 (see above) reveals that the insert of pC51 only contains the three genes coding for GGPP synthase, phytoene synthase, and phytoene desaturase [6]. This result indicates that the showed differences between E. coli (pC51) and E. coli (pHLDe99) (Figure 4, d = 77 cm) was due to these genes, which mediate the biosynthesis of lycopene in E. coli. Thus, carotenoid pigments are able to diminish the lethal action of UV light. This function can be ascribed to the one or all carotenoids (neurosporene and lycopene) present in extract of E. coli carrying pC51. However, the neurosporene was present in too small amounts to be responsible for the showed effect. This is very probably due to lycopene, which is formed in larger quantities (Figure 3). Other scientific data sug-
gest that lycopene maybe an important defense mechanism against adverse effects of UV irradiation on the skin [25]. It is now well established that at least some of the adverse effects of UV exposure is mediated by free radicals that can dam-
age (the same as that produced by oxidants of metabolism) to biological molecules, especially to DNA, lipids, and pro-	ein. This damage significantly contributes to the develop-
ment of degenerative diseases such as cancer, immune-system decline, and cardiovascular disease [26]. Some previous stud-
ies showed that lycopene is a potent antioxidant that protects cells against free radicals, and may reduce the risk of chronic diseases such as cancer and coronary heart disease [27–29]. In addition to these therapeutic interests, this carotenone can be used equally in food industry as coloring. Lycopene is present in human blood, adipose tissue, adrenals, and testes, its major source for humans being the tomatoes [28]. E. coli containing pC51 produces a significant quantity of lycopene. This production may improve by transcribing the pC51 inserted fragment from a strong promoter, and eventually this system could be used as a source of production of this carotenoid.

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