Analysis of Canthaxanthin and Related Pigments from *Gordonia jacobaea* Mutants

Trinidad de Miguel, Carmen Sieiro, Margarita Poza, and Tomás G. Villa*

Departamento de Microbiología y Parasitología, Facultad de Farmacia, Universidad de Santiago de Compostela, 15706 Santiago de Compostela, Spain

A collection of 43 mutant strains of the bacterium *Gordonia jacobaea* was obtained by means of ethyl methanesulfonate treatment, and the strains were selected for their different pigmentation with respect to the wild-type strain. None of the mutants showed auxotrophy. They all showed good genetic stability and a growth rate similar to that of the parental strain. Canthaxanthin and other carotenoids from these mutants were extracted with acetone and ethanol and separated by high-performance liquid chromatography (HPLC). These HPLC analyses, together with spectrophotometric detection at 480 nm, revealed variations in the pigment contents of the different mutant strains.

Keywords: *Gordonia jacobaea; canthaxanthin; EMS; HPLC*

INTRODUCTION

Canthaxanthin (*β*-*β*-carotene-4,4′-dione) is a ketocarotenoid widely distributed in nature. It was first isolated from the edible mushroom *Cantharellus cinnabarinus* and from flamingo feathers (*1*). It is commonly used in fish and poultry feeding, especially for pigmenting the muscle of the rainbow trout (*Oncorhynchus mykiss*), and also as a food additive (E-161). It must be included in chicken or fish diet to obtain an acceptable degree of flesh pigmentation because these pigments cannot be synthesized by the animal. The ketocarotenoid astaxanthin (3,3′-dihydroxy-*β*-*β*-carotene-4,4′-dione) is used for the same purposes as canthaxanthin, being particularly important in the diet of penreared salmonids (*2*) to provide both flesh pigmentation and flavor (*3*). *all-trans*-Canthaxanthin and astaxanthin have been synthesized by Hoffmann-La Roche Ltd. and are currently being used as salmonid feed additives under approval by the U.S. Food and Drug Administration (FDA). However, regulations concerning the safety of chemicals as food additives are becoming stricter, so the search for new natural sources of carotenoids of potential industrial use is rapidly gaining importance. *Brevibacterium* KY-4313 has been described as a potential source of canthaxanthin, and although the production of this carotenoid by this microorganism has been improved, it remains insufficient for industrial demands (*4*). The pigmented bacterium *Gordonia jacobaea* (CECT 5282) was isolated in our laboratory in routine air sampling during screening for microorganisms producing pink colonies (*5*). High-performance liquid chromatography (HPLC) and mass spectrometry (MS) analyses of the carotenoid extracts of *G. jacobaea* revealed that the main pigment present in this isolate is the ketocarotenoid canthaxanthin. However, the low level of production of this carotenoid by *G. jacobaea* (∼200 *μ*g/g of dry weight) is a handicap in its use for industrial purposes. The aim of this study was to obtain mutant strains from the canthaxanthin-producing bacterium *G. jacobaea* and to characterize the pigment extracted with acetone and ethanol from these mutant strains.

* Author to whom correspondence should be addressed (e-mail mpvilla@usc.es; telephone +34 81 563100, ext. 14949; fax +34 81 592490).

EXPERIMENTAL PROCEDURES

Strain and Media. The strain of *G. jacobaea* used was the natural isolate CECT 5282 (*5*), and the medium normally used for its growth was YPD (glucose, 20 g/L; yeast extract, 10 g/L; peptone, 20 g/L; and 3% agar for solid medium). For testing auxotrophies, minimal medium for corynebacteria (*6*) was used.

Reagents and Chemicals. Ethyl methanesulfonate (EMS) was obtained from Sigma Chemical Co. (St. Louis, MO). Hexane fraction from petroleum and ethyl acetate were of HPLC grade from Romil Chemicals (Leics, U.K.). Acetone and absolute ethanol were from Merk (Darmstadt, Germany).

Mutagenesis. The mutant strains of *G. jacobaea* were EMS-generated according to the procedure described by Fitzpatrick et al. (*7*) for *Corynebacterium glutamicum*. Mutagenesis was accomplished with cells grown in liquid YPD medium to an optical density (600 nm) of 0.3−0.4. EMS was dispensed into 1 mL samples of YPD medium at 200, 400, 600, 800, and 1000 *μ*g/mL final concentrations to determine the minimal inhibitory concentration (MIC). In the case of the highest concentrations, 4 mL of 0.1 M phosphate buffer (pH 7.0) was added to allow EMS to be in solution, maintaining the initial amount of the mutagen. After the MIC had been checked, the experiment was repeated, adding EMS at a concentration of 600 *μ*g/mL to 1 mL of liquid culture samples, and the cell suspensions were incubated for 10, 20, 30, 40, 50, and 60 min (48−94% kill, depending on the time of exposure to the mutagen). Cells were then washed three times in buffer and plated onto YPD plates.

Pigment Extraction. The wild-type strain of *G. jacobaea* and its mutants were grown in YPD broth. After 7 days had elapsed, cells from 5 mL of liquid culture were harvested by centrifugation (5000*g*, 10 min) and washed with distilled water, and the pigment was pellet-extracted with acetone. Five milliliters of acetone was added to the pellet, and the cells were resuspended by vortexing. After centrifugation, the supernatant was recovered and the remaining pigment was extracted again with 5 mL of acetone. The procedure was repeated until

10.1021/jf001169z CCC: $20.00 © 2001 American Chemical Society
Published on Web 02/10/2001

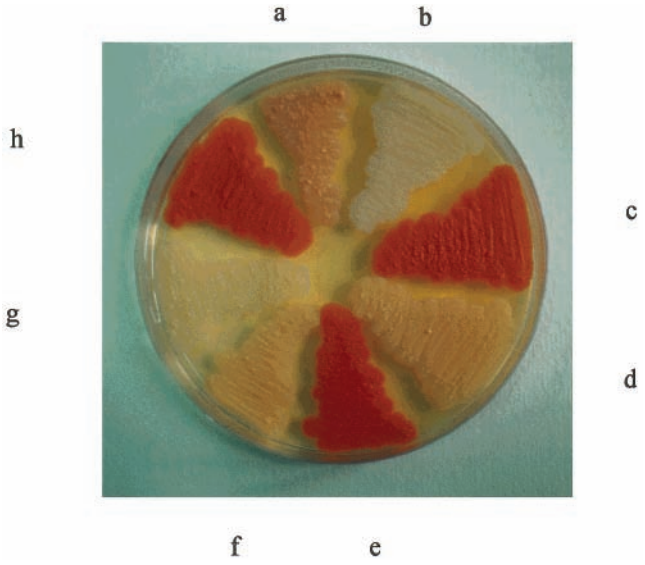

Figure 1. Several mutants of *G. jacobaea* growing on an agar plate: (a) wild-type strain; (b) mutant 1; (c) mutant 26; (d) mutant 30; (e) mutant 20; (f) mutant 12; (g) mutant 2; (h) mutant 22.

the cells remained colorless. When the samples were to be analyzed by HPLC, 1 mL of 0.1 M phosphate buffer (pH 7.0) and 3 mL of the hexane fraction from petroleum were added to the tube containing the pigment in acetone and vortexed for 30 s. The two phases were then separated by centrifugation, and the pigment-containing upper hexane phase was recovered. Finally, the samples were filtered through Teflon membranes and stored at −20 °C until analysis. To test the possibility of extracting the pigments directly in ethanol, this solvent was added instead of acetone during the first steps of the extraction.

Colorimetric Determination of Total Carotenoid Content and HPLC Analysis. Pigment-containing samples were prepared in the hexane fraction from petroleum as described above, and total carotenoid contents were determined by scanning in the 400−500 nm region using a Beckman DU-40 spectrophotometer and applying the formula proposed by An et al. (*8*) ($E^{1\%}_{1cm} = 1900$). The results are given as micrograms of carotenoids per gram of dry weight.

Individual carotenoid determinations were carried out by HPLC according to the method of Sedmak et al. (*9*) and Calo et al. (*10*), but using a different column. Chromatographic separations were performed on a Teknokroma silica 5 μm, 250 × 4.6 mm, high-performance column with a Teknokroma silica 5 μm guard column. The eluting solvent was 1:1 hexane fraction from petroleum/ethyl acetate (v/v), the flow rate being 1 mL min^{-1} and the pressure 0.70 psi. The eluant was monitored at 476 nm.

RESULTS AND DISCUSSION

After standard mutagenesis of *G. jacobaea* with EMS and screening of 8102 colonies, 43 mutant colonies growing on YPD agar plates were selected visually for their different degrees of pigmentation with respect to the wild-type strain (5.3×10^{-3} frequency of color mutants). The mutants thus obtained could be divided into several groups: albino mutants, hypopigmented mutants, hyperpigmented mutants, and yellow mutants (Figure 1). They all presented a fair genetic stability as far as the pigment content was concerned (reversion $<10^{-4}$) and displayed a growth rate similar to that of the wild-type strain. None of the mutant strains showed any auxotrophy when plated in minimum medium for corynebacteria. Spectrophotometric analyses of total carotenoid contents in acetone revealed large differences among the 43 mutants. Albino mutants, which were visually classified by their white color, showed no carotenoids. Hyperpigmented mutants had increased carotenoid contents up to 10-fold with respect to the wild-type strain, mutant 20 being the most pigmented strain with a production of 2500 μg of total carotenoids/g of dry weight. The mutants visually classified as hypopigmented and yellow mutants accumulated fewer total carotenoids than the wild-type strain (Table 1).

Table 1. Carotenoid Contents Obtained from HPLC Analyses of Wild-Type and Several Other *G. jacobaea* Strains

	retention time t_r						
strain	2.06 min	2.29 min	2.33 min	2.75 min	2.8 min	2.96 min	total
wild-type	6.13		199.5		21.27		227
1							0
2							0
12	1.34		46.66				48
13	1.67		57.72		5.61		65
15	0.55		31.03		3.42		35
17	2.64		71.24		11.12		85
19	67.38		834.66	629.50	140.11		1672
20	144.03	795.69	703.24	213.81	64.29	575.40	2496
21	21.31		307.73		323.86	48.10	701
22	52.99		896.77		288.24		1238
23	49.13		592.57		229.29		871
24	19.01		309.56		102.43		431
26	70.25		966.78		364.98		1402
27	8.88		177.74		30.39		217
28	6.34		98.19		21.47		126
29	17.65		188.61	147.70	40.04		394
30	3.4		80.85	60.65		35.1	180
31	4.71		47.91		12.38		65
33	11.94		142.55		31.51		186
42	13.49		217.23	179.39	59.89		470

[a] Values are given in micrograms of total carotenoids per gram of dry weight of bacteria. The retention time of 2.06 corresponds to β-carotene, 2.33 to canthaxanthin, and 2.8 to astaxanthin.

The qualitative results obtained from HPLC analyses were compared to those obtained in our previous studies of the wild-type strain of *G. jacobaea* (*5*), and this allowed us to classify the pigmented mutants according to two main carotenoid profiles, shown in Figure 2. Most of the hypopigmented and yellow mutants exhibited profiles similar to those from the wild-type strain, with a main canthaxanthin peak at a retention time of 2.33 min and two minor peaks at retention times of 2.06 and 2.8 min corresponding to β-carotene and astaxanthin, respectively. The other profile was the one displayed by some of the hyperpigmented mutants. In these, apart from the typical three peaks present in the wild-type strain, a peak was seen at a retention time of 2.75 min, corresponding to a carotenoid, which should be structurally very close to canthaxanthin, and another peak at a retention time of 2.96 min. In the case of mutant 20, there was a sixth peak at a retention time of 2.29 min, which was absent in the rest of the mutants. The occurrences of each compound are shown in Table 1.

When acetone was replaced by ethanol during the pigment extraction step, the results obtained from the spectrophotometric analyses showed that the amount of total carotenoids decreased slightly with respect to the acetone extraction. However, these results highlighted the relative efficiency of ethanol in carotenoid extraction, the percentage of extraction with this solvent being >90% in all cases, including the wild-type strain. This is an advantage with respect to other carotenoid-producing microorganisms such as *Phaffia rhodozyma*,

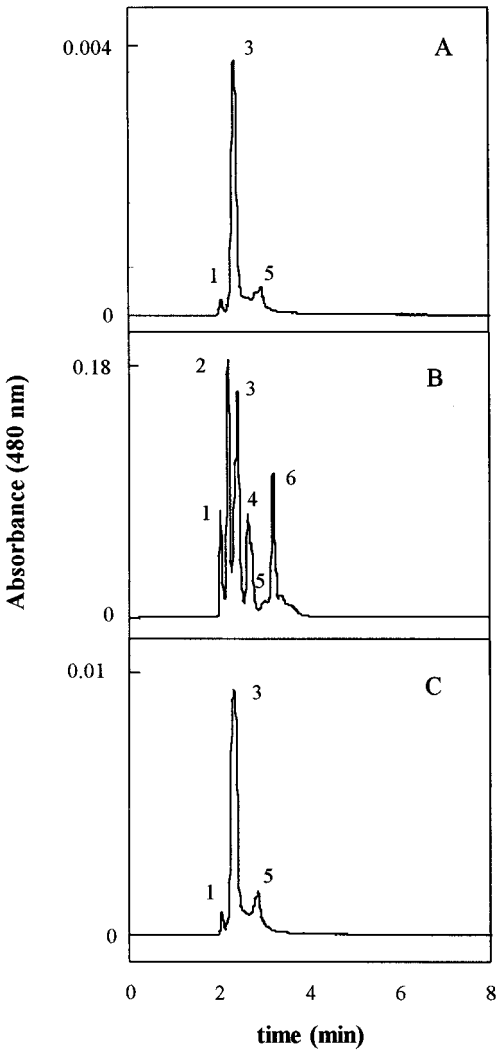

Figure 2. Typical chromatograms of a pigment extract from (A) the hypopigmented mutant strain 12, (B) the hyperpigmented mutant strain 20, and (C) the wild-type strain of *G. jacobaea*. Peaks: (1) β-carotene; (3) canthaxanthin; (5) astaxanthin; (2, 4, 6) unidentified carotenoids.

the pigments of which cannot be extracted directly with ethanol from the wild-type strain, making necessary the search for mutants having pigments that can be directly extracted with this nontoxic solvent.

The amount of canthaxanthin present in the hyperpigmented strains of *G. jacobaea*, especially in some of them (i.e., mutant 22), is high enough for them to be considered as alternative sources of this pigment for possible industrial applications. Apart from their high pigment production, the advantages of these mutants from the industrial point of view are (i) the optimal temperature for growth and carotenogenesis, 30 °C, which is normal in industrial fermenters; (ii) the use of glucose, an inexpensive carbon source, for optimal growth and pigmentation medium; and (iii) the fact that >90% of the total pigments can be extracted directly with ethanol, which is a nontoxic solvent allowed for human and animal feed. The use of ethanol in pigment extraction has several advantages, such as (i) its lower toxicity as compared to other solvents employed in carotenoid extraction, (ii) the prevention of toxic residue dumping (which would be derived from the use of acetone), and (iii) the prevention of the risk of animal intoxication due to residual acetone, which in turn may remain in the pigmented feeding.

In the future, albino mutants may also be very useful for genetic purposes. Their biosynthetic pathway can be arrested at the level of the *crtI* gene, which regulates the conversion of colorless *cis*-phytoene in neurosporene, the first colored compound in the carotenogenic pathway, or even at a previous step. Because all of the albino mutants were obtained from the *G. jacobaea* wild-type strain, which produces carotenoids, it is probable that they contain genes from the first steps of the pathway and the promoter for their regulation. This would allow the expression of such genes in cloning experiments and their visual detection.

The results reported here open the way to experimental approaches to *G. jacobaea* as a natural source of the pigment canthaxanthin or related carotenoids in the food industry. In this sense, investigations focused on the pigmentation of rainbow trout (*Oncorynchus mykiss*) are currently being developed at our laboratory.

LITERATURE CITED

(1) Weedon BCL Occurrence. In *Carotenoids*; Isler, O., Ed.; Birkhauser Verlag: Basel, Switzerland, 1971; pp 29−59.
(2) Sinnet, R. Fish pigmentation. *Trout News* **1988**, *7*, 8−11.
(3) Johnson, E. A.; Villa, T. G.; Lewis, M. J. *Phaffia rhodozyma* as an astaxanthin source in salmonid diets. *Aquaculture* **1980**, *20*, 123−134.
(4) Nelis, H. J.; De Leenher, A. P. Microbial sources of carotenoid pigments used in foods and feed. *J. Appl. Bacteriol.* **1991**, *70*, 181−191.
(5) de Miguel, T.; Sieiro, C.; Poza, M.; Villa, T. Isolation and taxonomic study of a new canthaxanthin-containing bacterium (*Gordonia jacobaea* MV-1 sp. nov.) *Int. Microbiol.* **2000**, *3*, 107−111.
(6) Kaneko, H.; Sakaguchi, K. Fusion of protoplasts and genetic recombination of *Brevibacterium flavum*. *Agric. Biol. Chem.* **1979**, *43*, 867−868.
(7) Fitzpatrick, R.; O'Donohue, M.; Joy, J.; Heery, D. M.; Dunica, L. K. Construction and characterization of *rec*A mutant strains of *Corynebacterium glutamicum* and *Brevibacterium lactofermentum*. *Appl. Microbiol. Biotechnol.* **1994**, *42*, 575−580.
(8) An, G. H.; Schuman, D. B.; Johnson, E. A. Isolation of *Phaffia rhodozyma* mutants with increased astaxanthin content. *Appl. Environ. Microbiol.* **1989**, *55*, 116−124.
(9) Sedmak, J. J.; Weerasinghe, D. K.; Jolly, S. O. Extraction and quantification of astaxanthin from *Phaffia rhodozyma*. *Biotechnol. Tech.* **1990**, *4*, 107−112.
(10) Calo, P.; Velázquez, J. B.; Sieiro, C.; Blanco, P.; Longo, E.; Villa, T. G. Analysis of astaxanthin and other carotenoids from several *Phaffia rhodozyma* mutants. *J. Agric. Food Chem.* **1995**, *43*, 1396−1399.

Received for review September 22, 2000. Revised manuscript received January 16, 2001. Accepted January 18, 2001. T.M. was a recipient of a fellowship from the Ramón Areces Foundation, Madrid.

JF001169Z