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RESEARCH ARTICLE

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Isolation and taxonomic study of a new canthaxanthin-containing bacterium, *Gordonia jacobaea* MV-1 sp. nov.

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Summary This article describes the isolation and taxonomic study of a coryneform isolate of a new *Gordonia* species (*G. jacobaea*), strain MV-1, which accumulates several carotenoids, including the ketocarotenoid *trans*-canthaxanthin. Identification of this new isolate by morphobiochemical methods did not allow unambiguous taxon assignment, but sequencing of the 16S rRNA gene clearly pointed to the genus *Gordonia, Gordonia sputi* being the closest fit. Differences in certain trans-versions/transitions in otherwise very well-conserved sequences of the described *Gordonia* species supported the proposal of this new taxon. The fact that both the best growth and best pigmentation were obtained with glucose, an inexpensive carbon source and at an industrially suitable temperature, suggests that this new bacterial strain may have good potential for the industrial production of canthaxanthin.

Introduction

Astaxanthin (3,3´-dihydroxy-4,4´-diketo-β-carotene) is a highly-oxidized carotenoid commonly found in marine environments [17] and is responsible for the colors of some birds such as flamingoes; the pigment is also found in crustaceans and certain fish. In the case of salmonids, the carotenoid cannot be synthesized by the animal and must be included in the diet, this being particularly important in pen-reared salmonids [15] to provide flesh pigmentation as well as flavor [8]. Canthaxanthin (4,4´-diketo-β-carotene) is a ketocarotenoid previous to astaxanthin in the accepted carotenoid pathway [2]. It is used in fish and poultry feeding with the same purposes as astaxanthin, especially for pigmenting the muscle of the rainbow trout (*Oncorhynchus mykiss*) and also as a food additive [10]. All-*trans*-astaxanthin and canthaxanthin have been synthesized by Hoffmann-La Roche Ltd., and are currently being used as salmonid feed additives under approval by the Food and Drug Administration (FDA, USA). Since regulations concerning the safety of chemicals as food additives are becoming stricter, the search for new natural sources of carotenoids with potential industrial use is rapidly gaining in importance. *Brevibacterium* KY-4313 was described as a potential source of canthaxanthin, and the production of this carotenoid by this microorganism has been improved. However, it is still insufficient for industrial demands [10]. Here we describe both the isolation of a new Gram-positive, non-sporulating, catalase-positive pigmented eubacterium that readily accumulates canthaxanthin, among other pigments, and the taxonomic studies leading to the proposal of the new species *Gordonia jacobaea*.

Materials and methods

Strains, plasmid and culture conditions *Gordonia jacobaea* strain MV-1 was isolated in the surroundings of our laboratory at Santiago de Compostela in routine air-sampling screening for microorganisms producing pink colonies. It was grown aerobically in a rotary shaker at 200 rpm at 30°C. The normal growth medium was YM (malt extract, 0.3%; yeast extract, 0.3%; peptone, 0.5%; glucose, 1%; and agar, 2%, if solid medium was to be used). The ability of MV-1 to use carbon sources was tested by replacing glucose in the usual growth medium by other carbon sources such as D-fructose, L-arabinose, D-raffinose, glycerol, Tween-80 and xylose. All sugars were obtained from Merck (Darmstadt, Germany) whereas Tween-80 was from Sigma (St. Louis, Missouri, USA). Strain HB 101 of *Escherichia coli* was used as reference for the determination of mol% GC of MV-1. *E. coli* competent cells used for cloning the 16S rRNA gene of MV-1 were TOP10 from Invitrogen (Groningen, the Netherlands). *E. coli*

cultures were grown at 37°C in LB medium (tryptone, 1%; yeast extract, 0.5%; NaCl 1%; and agar, 2%, for solid medium) supplemented with 60 mg/ml of ampicillin. 5 ml IPTG (200 mg/ml) and 16 ml X-Gal (50 mg/ml), both from Promega (Madison, WI, USA), were added to each LB-ampicillin plates for recombinant selection. The plasmid used for the cloning of the MV-1 16S rRNA gene was pBluescript SK+ [12].

DNA manipulations and PCR Restriction-enzyme digestions, DNA ligations, transformation of *E. coli* with plasmid DNA and *E. coli* plasmid DNA isolation were performed according to standard procedures [12]. Chromosomal DNA from MV-1 was isolated according to the method described by Velázquez et al. for *Lactobacillus casei* and *Lactobacillus fermentum* [16]. Chromosomal DNA from *E. coli* was isolated using the Promega DNA extraction kit. Universal primers pA 5´-AGAGTTTGATC CTGGCTCAG and pH* 5´-AAGGAGGTGATCCAGCCGCA amplified the MV-1 1500-bp 16S rRNA gene by using the protocol described by Funke et al. [6].

Nucleotide sequence determination The nucleotide sequence from the PCR product was determined by the dideoxy-chain termination method [13], using a Sequenase PCR product sequencing kit and from cloned DNA using a T7 Sequenase sequencing kit, version 2.0, both from Amersham (Rainham, UK). As label, γ^{35}S-dATP was used.

Mol% G+C determination Mol% G+C determination was performed by the thermal hyperchromicity method [5] using a Perkin-Elmer automatic spectrophotometer linked to a PARADE 286-plus sensor (Arche).

Biochemical characterization of the bacterial isolate The Biolog system (Biolog Inc., Hayward, CA, USA) was used for biochemical identification of the MV-1 strain. The system was used according to the manufacturer's instructions.

Chemicals HPLC-grade acetone (Merck, Darmstadt, Germany), hexane fraction from petroleum, and ethyl acetate (Romil Chemicals, Loughborough, UK) were filtered through 0.22 mm teflon membranes before use. DMSO was obtained from Merck (Darmstadt, Germany).

Pigment extraction The MV-1 strain was grown in YM broth. After 5 days, cells were harvested by centrifugation (5000× *g*, 10 min), washed with distilled water, and dried at 70°C for 2 h. Then, the pigment was directly pellet-extracted with acetone. Following this, 1 ml of 0.1 M phosphate buffer (pH 7.0) and 3 ml of hexane fraction from petroleum were added to the tube and vortexed for 30 s. The two phases were then separated by centrifugation and the pigment-containing upper hexane phase was recovered. Finally, samples were filtered through teflon membranes and stored at –20°C until analysis.

Colorimetric determination of total carotenoid content and HPLC analysis Pigment-containing samples were prepared in the hexane fraction from petroleum as described above, and the total carotenoid content was determined by scanning in the 400–500 nm region, using a Beckman DU-40 spectrophotometer and applying the formula proposed by An et al. [1]. Individual carotenoid determinations were carried out by high-performance liquid chromatography (HPLC), according to Sedmak et al. [14] and Calo et al. [4]. Chromatographic separations were performed on a silica 5 mm, 250 × 4.6 mm high-performance column with a silica 5 mm guard column (Tracer Spherisorb 5 m, Teknokroma, Madrid, Spain). The eluting solvent was hexane fraction from petroleum:ethyl acetate (1:1, v/v), flow rate being 1 ml/min and pressure 0.30 psi [4]. The eluant was monitored at 480 nm in the spectrophotometer. The results are given as mg carotenoid/g dry weight. The standards were β-carotene, α-carotene, γ-carotene, lycopene and xanthophyll, obtained from Sigma. Astaxanthin and canthaxanthin were obtained from Hoffmann-La Roche Ltd. (Basel, Switzerland).

Results

Identification of the isolate The isolated strain contained Gram-positive, non-sporing, rod-shaped cells with tendency to bipolar staining, catalase +, and with a mol% G+C of 61. Table 1 shows the results of the biochemical tests used to identify the bacterial isolate as recommended by the Biolog System. These results suggested that the isolated strain was of the genus *Corynebacterium*, *C. jeikeium* being the closest fit (84% similarity). However, some features of MV-1, such as the presence of carotenoids and the ability to use certain carbon sources, did not match the reported data for the type species of *C. jeikeium* [7]. As further studies on the strain were needed to identify the isolate, the 16S rRNA gene was sequenced after being amplified by PCR. Sequence analysis using data from the GenBank® revealed that the isolate unambiguously belongs to the genus *Gordonia*, *G. sputi* being the closest fit (99.5% similarity). The isolate was proposed as *G. jacobaea* MV-1 CECT 5282 (Spanish Type Culture Collection), and the accession number for its 16S rRNA gene in the GenBank® Nucleotide Sequence Database is AF251791.

Carotenoid analysis The total carotenoid content as measured spectrophotometrically was 227 μg/g dry weight. Individual carotenoids were investigated by HPLC analysis. This revealed the presence of several peaks corresponding to different carotenoids (Fig. 1A), one of which was identified as *trans*-canthaxanthin. For comparative purposes, a typical HPLC chromatogram of the carotenoids present in MV-1 along with some standards, are shown in Fig. 1B. The results obtained from the HPLC analyses, concerning the unambiguous identification of canthaxanthin, were confirmed by mass spectrometry (data not shown).

Table 1 Results of the characterization of the MV-1 strain of *Gordonia jacobaea* using the Biolog tests

Carbon source	Growth after 24 h at 22°C	Carbon source	Growth after 24 h at 22°C
Water	–	D-Tagatose	V
α-Cyclodextrin	–	D-Trehalose acid	–
β-Cyclodextrin	–	Turanose	–
Dextrin	–	Xylitol	–
Glycogen	–	D-Xylose	V
Inulin	–	Acetic acid	V
Mannan	–	α-Hydroxy-butyric acid	–
Tween-40	+	β-Hydroxy-butyric acid	–
Tween-80	+	γ-Hydroxy-butyric acid	–
N-Acetyl-D-glucosamine	–	p-Hydroxy-phenylacetic acid	–
N-Acetyl-D-mannosamine	–	α-Ketoglutaric acid	–
Amygdalin	–	α-Ketovaleric acid	V
L-Arabinose	V	Lactamide	–
D-Arabitol	–	D-Lactic acid methyl ester	–
Arbutin	–	L-Lactic acid	–
Cellobiose	–	D-Malic acid	–
D-Fructose	V	L-Malic acid	–
L-Fucose	–	Methyl pyruvate	+
D-Galactose	–	Mono-methyl succinate	–
D-Galacturonic acid	–	Propionic acid	–
Gentiobiose	–	Pyruvic acid	V
D-Gluconic acid	–	Succinamic acid	–
α-D-Glucose	V	Succinic acid	–
m-Inositol	–	N-Acetyl-L-glutamic acid	V
α-D-Lactose	–	Alaninamide	V
Lactulose	–	D-Alanine	–
Maltose	–	L-Alanine	–
Maltotriose	–	L-Alanyl-glycine	–
D-Mannitol	–	L-Asparagine	–
D-Mannose	V	L-Glutamic acid	–
D-Melezitose	–	Glycyl-L-glutamic acid	–
D-Melibiose	–	L-Pyroglutamic acid	–
α-Methyl-D-galactoside	–	L-Serine	–
β-Methyl-D-galactoside	–	Putrescine	–
3-Methyl-glucose	–	2,3-Butanediol	–
α-Methyl-D-glucoside	–	Glycerol	–
β-Methyl-D-glucoside	–	Adenosine	–
α-Methyl-D-mannoside	–	2´-Deoxyadenosine	V
Palatinose	–	Inosine	–
D-Psycose	V	Thymidine	–
D-Raffinose	–	Uridine	–
D-Rhamnose	–	Adenosine-5´-monophosphate	–
D-Ribose	–	Thymidine-5´-monophosphate	–
Salicin	–	Uridine-5´-monophosphate	–
Sedoheptulosan	–	Fructose-6-phosphate	–
D-Sorbitol	–	Glucose-1-phosphate	–
Stachyose	–	Glucose-6-phosphate	–
Sucrose	–	D-L-α-Glycerol-phosphate	–

Symbols: +, positive, –, negative, V, borderline.

Optimal culture temperature The isolate was grown at different temperatures to determine the optimal value for growth and carotenogenesis. After culture of MV-1 at 25, 30 and 35°C, and evaluation of the canthaxanthin contents, the temperature chosen as the possible optimal one from the industrial point of view was 30°C. At this temperature, maximum growth was attained at 50 h, and maximum carotenogenesis at 75 h. Figure 2 shows the typical one-step growth of MV-1 and onset of carotenogenesis at 30°C.

Growth and pigment formation with different carbon sources Table 2 shows the effect of different carbon sources on the growth and pigmentation of MV-1 strain. The isolate tended to clump, hindering cell manipulation, counting, pigment extraction and so forth. The presence of Tween-80, however, avoids clumping [11] and allows normal manipulation and counting of MV-1. Note that when Tween-80 was used as the sole source of carbon and energy, pigment accumulation was much lower than when glucose was the energy source.

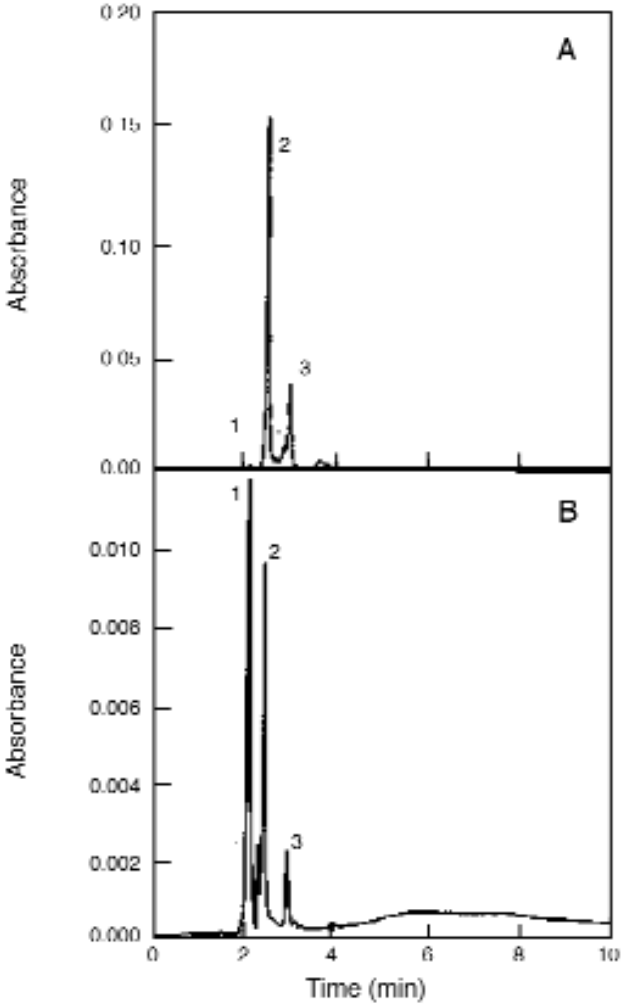

Fig. 1 HPLC-carotenoid profiles of an intracellular extract from *Gordonia jacobaea* MV-1 (A), and a mixture of standard carotenoids (B). Peaks: 1, β-carotene; 2, *trans*-canthaxanthin; 3, *trans*-astaxanthin

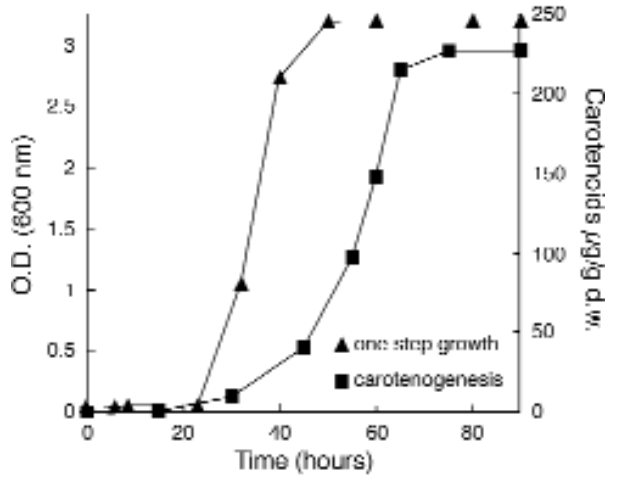

Fig. 2 Typical one-step growth of *Gordonia jacobaea* MV-1 with glucose as the carbon source and its onset of carotenogenesis

Table 2 Effect of different carbon sources on the growth and pigmentation of *Gordonia jacobaea* MV-1. Growth is referred in mg of dry weight/ml of liquid culture and pigmentation in μg of total carotenoids/g of dry weight

Carbon source	mg d.w./ml liquid culture	Total carot. (μg/g d.w.)
Glycerol	12.2	61.8
D-Fructose	6.3	75.56
D-Raffinose	1	113
D-Glucose	3.2	227
L-Arabinose	2.9	24.14
Xylose	3.9	24.87

Discussion

The coryneform bacteria form a heterogeneous group, which includes saprophytic species such as *Corynebacterium glutamicum* and *Brevibacterium lactofermentum.* These two species are of special interest, in fact for over forty years they have been extensively used for the industrial production of amino acids and nucleotides. They are Gram-positive, with a high mol% G+C and a complex cell surface structure with an effective permeability barrier outside the peptidoglycan formed by corynemycolic acids and other lipids [9]. Natural isolates of members of Corynebacteriaceae that contain ketocarotenoids such as *trans*-canthaxanthin are of great interest because much of the genetic background currently employed in *C. glutamicum* or in *B. lactofermentum* may be easily adapted to the new isolate to clone the genes involved in the synthesis of C_{40} compounds, or simply to obtain new hyperpigmented strains. In the case of the isolate MV-1, the carotenoid content in the wild type is comparable to that found in wild-type strains of the yeast *Phaffia rhodozyma* and should be easily increased by simple classical mutations. The optimal temperature for growth and carotenogenesis is ca. 30°C, which is normal in industrial fermenters. The best growth and accumulation of carotenoids were obtained when the culture medium was supplemented with glucose, an inexpensive carbon source. Also, it had been previously reported that, by adding certain precursors, such as mevalonic acid, to the medium the carotenoid content could be increased four-fold [3]. Both of these facts suggest that this new bacterium may have interesting potential for industrial ketocarotenoid preparation.

The results of the biochemical tests confirm the isolate as a new species, *Gordonia jacobaea*. In fact, some major features for taxonomic determination, such as the use of galactose or inositol as carbon sources, are different from those observed in other described species of the genus *Gordonia*. Table 3 shows the ability of the different species of the genus *Gordonia*, including *G. jacobaea*, to grow on different carbon sources.

Alignment of the 16S rRNA sequences reveals a similarity of 99.5% upon comparison of *G. jacobaea* with *G. sputi;* 96.0% with *G. rubropertincta;* 95.8% with *G. bronchialis* and 95.1%

Table 3 Ability for growth on different carbon sources of the species of the genus Gordonia

	*G. bronchialis**	*G. rubropertincta*	*G. sputi*	*G. terrae*	*G. jacobaea*
D-Cellobiose	+	–	+	+	–
D-Galactose	–	+	+	+	–
meso-Inositol	+	–	–	–	–
Raffinose	–	–	+	+	–
L-Rhamnose	–	–	–	+	–
Butane-2,3-diol	–	–	+	+	–
Butyric acid	–	+	–	–	–
L-Serine	–	+	–	+	–

* Type species.

with *G. terrae*. The analysis of the sequence of *G. jacobaea* shows variability in two major points of the sequence, which are highly conserved in the four previously described species. These points are a transversion in base 28 (G→C) and a transition in base 601 (A→G). A further transition between the sequences of *G. jacobaea* and *G. sputi* is present in base 157 (A→G), *G. jacobaea* being more similar in this point to the rest of the species in the genus. A noteworthy characteristic of the new species is an adenine insertion at position 17, which is absent in the other species. Additionally, there are two deletions in base 1414 and base 1421, which are also found in *G. sputi* and *G. rubropertincta*. All these variations in the 16S rRNA sequence, together with the phenotypic differences shown in table 3, support the proposal of a new species, *Gordonia jacobaea*, for the isolate.

Patented strain This new bacterial species is as from February 16th, 2000 protected by the Spanish patent No. P200000373 (owned by the University of Santiago de Compostela) for use as a source of both canthaxanthin and astaxanthin.

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