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Carotenoid production in *Lactobacillus plantarum*

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ABSTRACT

Eighteen strains of *Lactobacillus plantarum* from different origins were screened for carotenoid production, as many of them exhibited a deep yellow pigmentation when cultured as isolated colonies on MRS-agar plates. We found that most of them produced significative amounts of the yellow C_{30} carotenoid 4,4'-diaponeurosporene in the range 1.8 to 54 mg/kg of dry cell weight. Although some of the strains produced just trace amounts of this carotenoid, PCR studies showed that all of them harbored the genes *crtM* and *crtN* which, inferred from homology, had been predicted in the three *L. plantarum* complete genome sequences currently available. Our results suggest the full functionality of a C_{30} carotenoid biosynthesis pathway in this species, driven by the operon *crtNM*. DNA sequencing of the entire *crtNM* operon in the maximum carotenoid-producing strain found in this study, i.e. *L. plantarum* CECT7531, was accomplished. Genes *crtM* and *crtN* were annotated as dehydrosqualene synthase and dehydrosqualene desaturase, respectively, in this strain.

1. Introduction

Carotenoids are a group of colored terpenoids with antioxidant properties which are widespread in the plant and animal kingdoms, as well as in fungi and in photosynthetic and non-photosynthetic microorganisms (Weedon, 1971; Phadwal, 2005). In the latter, although not essential for growing as heterotrophic organisms, they accomplish important biological functions. In Gram-positive bacteria, for instance, carotenoids play an important role in protecting from oxidative stress by scavenging free radicals with their conjugated double bonds (Clauditz et al., 2006). Also, it has been demonstrated that there is a correlation between carotenoid production and decreased membrane fluidity, which provides resistance to oleic acid killing in *Staphylococcus aureus* (Chamberlain et al., 1991). In addition, carotenoids are used commercially as food colorants, animal feed supplements and, more recently, for nutraceuticals, cosmetic and pharmaceutical purposes (Lee and Schmidt-Dannert, 2002; Klein-Marcuschamer et al., 2007). A lot of effort has been made at selecting microorganisms that can provide a cost-effective source of carotenoids (Bhosale, 2004). At present, metabolic engineering of diverse microorganisms, including non-carotenogenic ones, is being exploited for the biotechnological production of large amounts of reasonably pure carotenoids as well as to synthesize novel carotenoid structures by using combinatorial and *in vitro* evolutionary strategies (Lee and Schmidt-Dannert, 2002; Umeno et al., 2005; Das et al., 2007; Klein-Marcuschamer et al., 2007; Wang et al., 2007).

Lactic acid bacteria (LAB) are Gram-positive, low-GC, microaerophilic, non-sporulating, rod or cocci that ferment sugars to produce primarily lactic acid and are associated by their common physiological characteristics (Makarova et al., 2006). These bacteria are historically linked with food and feed fermentations, and their industrial importance is further evidenced by their Generally Regarded as Safe (GRAS) status (Holzapfel et al., 1995). Among LAB, selected species of the *Lactobacillus* genus are widely used as probiotics primarily in dairy products and dietary supplements (Reid, 1999; Ouwehand et al., 2002; Klaenhammer et al., 2005). One of these species, *Lactobacillus plantarum*, is industrially important and is involved in many vegetable fermentations (Buckenhüskes, 1997), as well as being a frequent inhabitant of the human intestinal tract (Johansson et al., 1993) which is already being used as a probiotic microorganism (Rodgers, 2008). In our laboratory, we have isolated a number of *L. plantarum* strains from olive fermentations and used some of them as starter cultures (Ruiz-Barba et al., 1994; Leal et al., 1998). Intrigued by the eye-catching deep yellow pigmentation of some of these strains when growing as isolated colonies on MRS-agar plates, we decided to search for the real nature of such coloring. Preliminary characterization of some *L. plantarum* strains showed the involvement of carotenoid compounds in the yellow pigmentation. Actually, Breithaupt *et al.* (2001) had described one *L. plantarum* strain isolated from baker's yeast which was able to produce the triterpenoid 4,4′-diaponeurosporene and its isomers, being the only report on the matter up to date. In this report, we describe the widespread presence of carotenoid production among *L. plantarum* strains from a wide variety of environments. This result was reinforced by the finding of the genes *crtM* and *crtN*, first described in the annotated complete genome sequence of *L. plantarum* WCFS1 as inferred by homology (Kleerebezem et al., 2003), in all of

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the strains investigated. Our results suggest the functionality of these genes in *L. plantarum*, although the nomenclature and function of their predicted products should be updated.

2. Materials and methods

2.1. Bacterial strains, media and growth conditions

L. plantarum strains used in this study are described in Table 1. They were cultured in MRS agar (Oxoid, Basingstoke, UK) at 30 °C. For preliminary optimization experiments, MRS broths from different sources were used, including the commercially available from Oxoid, Difco (Detroit, USA), Biokar (Beauvais, France), and Merck (Darmstadt, Germany). Also, for this purpose, two different defined media were used: DM1, containing, per litre, glucose (Panreac, 20 g), peptone (Difco, 10 g), beef extract (Oxoid, 8 g), yeast extract (Oxoid, 4 g), K_2HPO_4 (Fluka, 2 g), sodium acetate·$3H_2O$ (Fluka, 5 g), tri-ammonium citrate (Merck, 2 g), $MgSO_4 \cdot 7H_2O$ (Merck, 0.2 g), $MnSO_4 \cdot 4H_2O$ (Merck, 0.05 g), and Tween 80 (Sigma, 1 ml); and DM2, containing, per litre, glucose (Panreac, 22 g), yeast extract (Oxoid, 10 g), $(NH_4)_2HPO_4$ (Fluka, 2.5 g), $MgSO_4 \cdot 7H_2O$ (Merck, 0.05 g), $MnSO_4 \cdot H_2O$ (Merck, 0.005 g), and Tween 80 (Sigma, 0.2 ml). In all cases, pH was adjusted to 6.5 with 10 N HCl and media were sterilised at 121 °C, 1 atm, for 15 min.

2.2. Cell culture for carotenoid production and extraction

Five hundred ml of each of the MRS broths used were inoculated with a single colony of the *L. plantarum* strain to be tested and the culture was incubated for 24, 48, 72 or 96 h at 30 °C without aeration. Cells were collected by centrifugation at 12,000 × *g*, at 4 °C for 15 min, washed with sterile distilled water, and centrifuged again to obtain a pellet, which was submitted to lyophilization. Samples were stored at −20 °C until carotenoid extraction and chromatographic analysis (within 24–48 h). Data obtained were expressed in terms of dry cell weight (DCW).

2.3. Carotenoid extraction and analysis by high performance liquid chromatography (HPLC)

One gram of each bacterial cell pellet was introduced in a 15 ml polypropylene tube and extracted with 10 ml of N,N-dimethylformamide, at 65 °C for 15 min. The cell debris was separated by centrifugation at 5000 rpm and the upper phase, containing the carotenoid pigments, was transferred to a separator funnel. The operation was repeated until the complete exhaustion of color (usually four extractions were enough). All extracts were pooled and shaken with 100 ml of diethyl ether. A sufficient quantity of 10% NaCl was added at the end of the process to aid in the efficient separation of the liquid phases. Subsequently, the organic phase was dried over anhydrous Na_2SO_4, evaporated in a rotary evaporator, and taken up to 1 ml of acetone. Samples were centrifuged at 12,000 × *g* and stored at −30 °C until analyzed.

Monitoring and quantification of the bacterial carotenoid pigments were carried out by reversed-phase HPLC (RP-HPLC) using a method previously developed in our laboratory (Mínguez-Mosquera and Hornero-Méndez, 1993). The method involves a C18 reversed-phase column (Waters Spherisorb ODS2 column; 250 × 4.6 mm I.D., particle size 5 μm; Waters Ltd., Hertsfordshire, UK) and a binary gradient elution system of acetone –H_2O at a flow rate of 1.5 ml/min. Injection volume was 5 μl and detection was carried out at 440 nm. Quantification was performed by using an external standard calibration curve prepared with β-carotene (Sigma Chemical Co., St.Louis, MO), a commercially available yellow carotenoid whose chromatic characteristics are similar to the carotenoids found in the *L. plantarum* strains of this study. HPLC analyses were performed with a Waters 600E quaternary pump equipped with a diode array detector (PDA 996, Waters) and controlled with a Empower2 data acquisition software (Waters Corporation, Milford, Massachusetts, USA). For the conditions used in this study, the limit of detection was 0.01 mg/kg DCW of carotenoid in the sample, while the limit of quantification was 0.15 mg/kg DCW. Carotenoids detected below the limit of quantification are indicated as "traces" in Table 1.

Table 1
Lactobacillus plantarum strains and carotenoid production.

Strain	Origin	Colour[a]	Carotenoid production[b] (mg/kg dry cell weight ± SD)[e]	PCR[c]	Source[d]
CECT7531	Olive fermentation	Y	54.55 ± 0.65	1,2	IG-CSIC
CECT4185	Silage	Y	29.79 ± 1.10	2	CECT
LPT70/3	Olive fermentation	Y	29.74 ± 1.20	1	IG-CSIC
LB6	Wine	Y	29.13 ± 0.75	1,2	UV
LPT57/1	Olive fermentation	Y	27.41 ± 0.83	1	IG-CSIC
WCFS1	Human pharynx	Y	22.53 ± 1.03	1,2	WCFS
LPT49/6	Olive fermentation	Y	19.18 ± 0.90	1,2	IG-CSIC
LPT44/1	Olive fermentation	Y	18.97 ± 0.70	1	IG-CSIC
LPJ10	Olive fermentation	Y	14.78 ± 0.25	1	IG-CSIC
LPT57/2	Olive fermentation	Y	9.19 ± 0.44	1	IG-CSIC
NC8	Grass silage	Y	8.83 ± 0.67	1,2	Matforsk
ATCC10241	Pickled cabbage	Y	6.33 ± 0.56	1	ATCC
RP1	Commercial inoculum	Y	4.95 ± 0.88	1	Rhône-Poulenc
ATCC14431	Grass silage	W	2.30 ± 0.78	1	ATCC
CECT 748[f]	Pickled cabbage	W	1.78 ± 0.20	1	CECT
ATCC8014	Corn silage	W	Traces[g]	1	ATCC
CECT 220	Corn silage	W	Traces	1	CECT
LL441	Cheese	W	Traces	1	IPLA-CSIC

[a] Color to the naked eye of cell pellets obtained after centrifugation: Y=yellow; W=white.
[b] The majoritary carotenoid produced is 4,4′-diaponeurosporene.
[c] Primer pair that amplified the crtN–crtM gene cluster in that particular strain: 1 = crtN-for/crtM-rev; 2 = rbs–crtN-for/crtM-rev.
[d] Sources: IG-CSIC: Instituto de la Grasa-CSIC, Sevilla, Spain; CECT: Colección Española de Cultivos Tipo (Spanish Type-Culture Collection), Burjassot, Spain; UV: Sergi Ferrer and Isabel Pardo, University of Valencia, Valencia, Spain; WCFS: Michiel Kleerebezem, Wageningen Centre for Food Sciences, Wageningen, The Netherlands; Matforsk, Lars Axelsson, Norwegian Food Research Institute, Osloveien, Norway; ATCC: American Type Culture Collection, Manassas, Virginia, USA; Rhône-Poulenc: Rhône-Poulenc Industries SA, Courbevoie, France; IPLA-CSIC: Baltasar Mayo, Instituto de Productos Lácteos de Asturias-CSIC, Asturias, Spain.
[e] Cells were collected from 500 ml 24 h cultures in DM1 medium (see text). Figures are mean values of three independent assays ± standard deviations.
[f] Type strain, it is equivalent to *L. plantarum* ATCC14917[T].
[g] Carotenoids detected below the limit of quantification (0.15 mg/kg).

2.4. Pigment isolation and identification

Routine procedures for the isolation and identification of carotenoid pigments, already described in detail in previous publications (Mínguez-Mosquera et al., 1990; Mínguez-Mosquera and Hornero-Méndez, 1993) were used. Briefly, this consisted of: separation and isolation of the pigments by thin layer chromatography on silicagel 60GF plates; acquisition of UV–visible spectra (Hewlett-Packard UV–vis diode array spectrophotometer model 8452A) in different solvents and comparison with the values reported in the literature (Foppen, 1971; Davies, 1976, 1988; Britton, 1991, 1995), as well as chemical tests for the examination of 5,6-epoxide groups investigated by addition of 2% HCl in ethanol, acetylation with acetic anhydride–pyridine to test for hydroxyl groups and reduction with $NaBH_4$ in ethanol to test for carbonyl groups (Eugster, 1995).

2.5. Liquid chromatography / mass spectrometry (LC/MS)

LC/MS was performed on a Waters 2695 XE separation module (Waters Corporation, Milford, USA) coupled with a Waters 2998 Photodiode Array detector and Micromass ZMD4000 (Manchester, UK) mass spectrometer equipped with an atmospheric pressure chemical ionization (APcI) interface. Chromatographic conditions and MS parameters were used according to Breithaupt and Schwack (2000). The MS system was operated in full scan mode (*m/z* 200–1200), and the absorbance of carotenoids was recorded at 440 nm. A C30 YMC analytical column (YMC Europe GMBH, Germany) with 5 µm particle size and 250×4.6 mm dimensions was used. The injection volume was 20 µl.

2.6. Genetic analyses

The presence of the operon *crtNM* in the different *L. plantarum* strains was detected by PCR using oligonucleotide primers designed from the published nucleotide sequence of these predicted genes in *L. plantarum* WCFS1 (GenBank accession number [acc. no.] AL935261; Kleerebezem et al., 2003). The primer pair crtN-for (*CGC*GGAATTCATGAAGCAAGTATC-GATTATTGGC) and crtM-rev (*GATC*GAATTCTTAAGCCTCCTTAAGGGC-TAGTTC) was used to amplify a 2379-bp DNA fragment including the coding sequences for both genes. Alternatively, in those cases when the first primer pair did not amplified any DNA fragment, the primer pair rbs-crtN-for (*CTAG*GGTACCAAGGGGGAGATTTACTGATGAAGC) and crtM-rev was used to amplify a 2396-bp DNA fragment which included part of the putative *crtN–crtM* promoter. *Eco*RI restriction sites were introduced at the 5′ ends of primers crtN-for and crtM-rev, and *Kpn*I site at the 5′ end of primer rbs-crtN-for to facilitate future cloning strategies (bold face in the primer sequences above). Also, hanging sequences at the 5′ ends of the primers were introduced to ensure proper restriction digestions (italics in the primer sequences above). Total DNA from isolated *L. plantarum* colonies was extracted with chloroform as previously described (Ruiz-Barba et al., 2005). Amplification of DNA fragments was performed in 25 µl reaction mixtures containing 2.5 mM MgCl, 1×reaction buffer, 100 µM each of the deoxynucleoside triphosphates, 100 pmol of each primer, 5 U of *Taq* DNA polymerase (Promega), and 5 µl of total DNA solution prepared as described above as the template. A GeneAmp PCR system 2400 thermal cycler (Perkin-Elmer) was used with the following conditions: denaturation at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 15 s, annealing at 60 °C for 30 s, and polymerization at 72 °C for 2 min, plus a final polymerization step at 72 °C for 4 min. Alternatively, when no amplification was obtained with the later conditions, 58 °C was used as the annealing temperature. Alternatively, when the amplicon was needed for sequencing purposes, the PCR Extender System (5Prime Gmbh, Hamburg, Germany) was used under the conditions recommended by the manufacturer for high fidelity performance. PCR-amplified DNA fragments were finally analyzed by agarose gel electrophoresis. Homology searches were carried out using the Blastn, Blastp and FASTA programmes, and sequence alignments were carried out using the EMBOSS alignment algorithm, all of them available at EMBL-EBI (http://www.ebi.ac.uk). DNA sequencing was carried out by the Servicio de Secuenciación Automática de DNA (SSAD), CIB–CSIC, Madrid, Spain, with an ABI PRISM 377 DNA sequencer (Applied Biosystems, Perkin-Elmer). Nucleotide sequences of *crtM* and *crtN* genes in *L. plantarum* CECT7531 have been assigned the GenBank acc. no. GU474811.

3. Results

3.1. Most strains of L. plantarum analyzed produce the triterpenoid 4,4′-diaponeurosporene

Chromatographic analysis of the carotenoids extracted from the *L. plantarum* cell pellets showed in virtually all of the cases a main chromatographic peak at a retention time of 14.8 min (peak 1, Fig. 1). The absorption spectrum showed maxima absorbance at 416, 438 and 468 nm (%III/II = 95), which is consistent with a chromophore containing nine double conjugated bonds. In principle these properties suggested the presence of neurosporene in the analyzed samples, however the chromatographic mobility for this peak was different, eluting at earlier retention times (about 1 min) than neurosporene (lower polarity), which was revealed after comparison with a Rose hips extract containing neurosporene (data not shown). None of the chemical tests carried out to investigate the presence of either 5,6-epoxide, hydroxyl or carbonyl groups was positive, showing the absence of oxygen in the molecules under study and therefore its structure should be that of a carotene. The mass spectra showed a quasimolecular ion $[M+H]^+$ at *m/z* 403 which is in accord with the formula $C_{30}H_{42}$ of 4,4′-diaponeurosporene (Fig. 2). This is in agreement with data reported by Breithaupt et al. (2001) for *L. plantarum* LTH4936. Other two minoritary peaks were also found at retention times 15.3 and 15.6 min, and whose absorption maxima were 380,402, 426 (%III/II = 93) and 333, 349, 368 (%III/II = 81), respectively (peaks 2 and 3 in Fig. 1). Mass spectra of these peaks showed $[M+H]^+$ at *m/z* 405 and 407 respectively, which are in agreement with the molecular formulas $C_{30}H_{44}$ and $C_{30}H_{46}$, corresponding to 4,4′-diapo-ξ-carotene and 4,4′-diapophytofluene (also referred as 4,4′-diapo-7,8,11,12-tetrahydrolicopene) respectively, and corresponding to intermediates in the biosynthetic pathway of 4,4′-diaponeurosporene in *S. aureus* (Wieland et al., 1994) (Fig. 2).

3.2. Quantification of carotenoid production by L. plantarum strains

Preliminary results in our laboratory showed wide differences in the final carotenoid production regarding the brand and composition of the media used. As an example, for *L. plantarum* CECT7531 we obtained up to

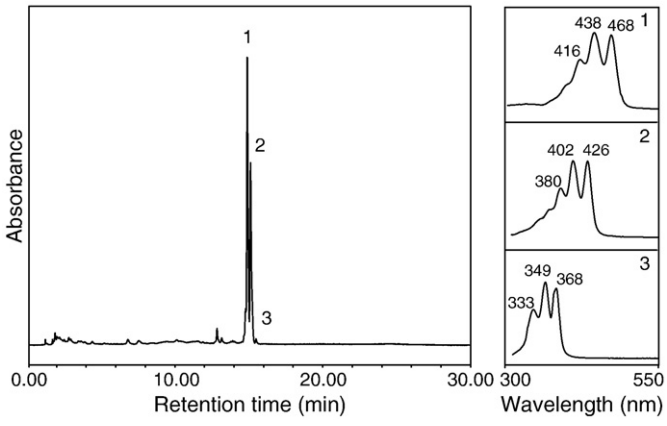

Fig. 1. Typical HPLC chromatographic analysis and UV–visible absorption spectra of the carotenoids extracted from *Lactobacillus plantarum* cell pellets. Peaks: 4,4′-diaponeurosporene (1), 4,4′-diapo-ξ-carotene (2) and 4,4′-diapophytofluene (3). Absorption maxima, expressed in nm, are indicated.

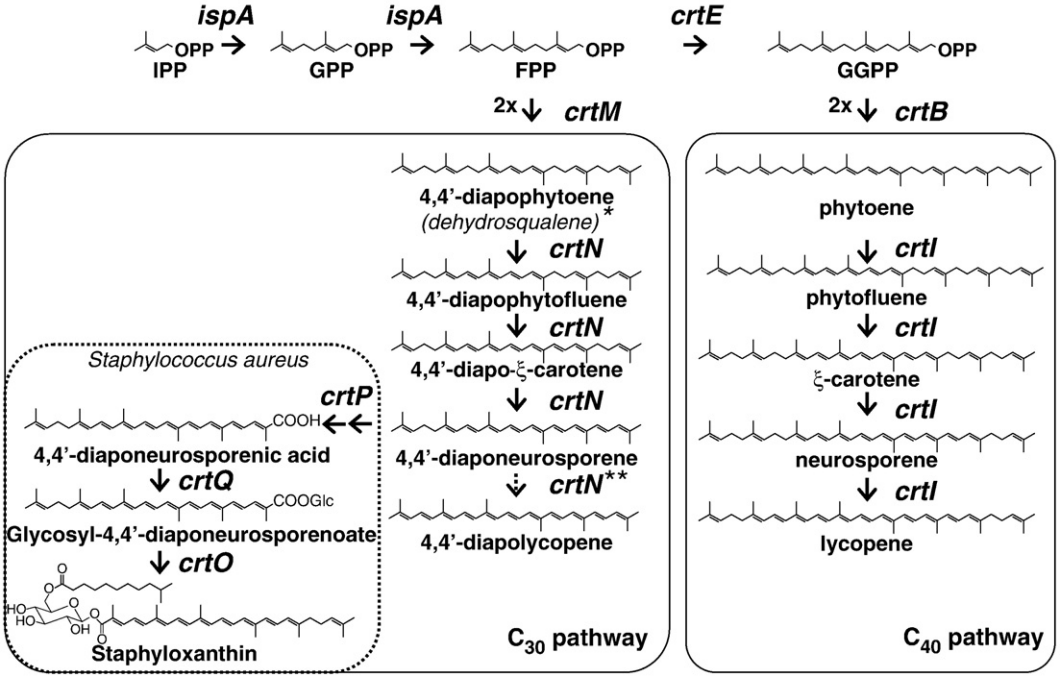

Fig. 2. Biosynthetic pathways of the triterpenoid (C_{30}) and tetraterpenoid (C_{40}) carotenoids in microorganisms. Abbreviations: IPP, isopentenyl pyrophosphate; GPP, geranyl pyrophosphate; FPP, farnesyl pyrophosphate; GGPP, geranyl geranyl pyrophosphate. Genes: (C_{30} and C_{40} pathways) *ispA*, geranyltranstransferase; (C_{30} pathway) *crtM*, dehydrosqualene synthase (also, diapophytoene synthase); *crtN*, dehydrosqualene desaturase (also, diapophytoene desaturase); *crtP*, diaponeurosporene oxidase; *crtQ*, glycosyl transferase; *crtO*, acyl transferase; (C_{40} pathway) *crtE*, GGPP synthase; *crtB*, phytoene synthase; *crtI*, phytoene desaturase. *Alternative common name.** Under specific conditions (see text), the desaturase coded by *crtN* in *S. aureus* is able to perform a fourth desaturation step, rendering 4,4′-diapolycopene.

54.55 mg/kg DCW of carotenoids in DM1 medium, 40.79 mg/kg in Oxoid MRS, 39.13 mg/kg in Difco MRS, 29.57 mg/kg in Biokar MRS, 26.62 mg/Kg in Merck MRS and just 18.79 mg/kg in DM2 medium. In all cases, cell counts after 24 h of incubation at 30 °C prior to carotenoid extraction was virtually identical (*ca.* 10^9 CFU/ml). As best results were obtained with DM1 medium, this was chosen as the standard medium for comparison among the strains tested. As it is shown in Table 1, most (72.2%) of the cell pellets of the screened *L. plantarum* strains looked fairly yellow. Broth cultures of selected strains incubated for 24, 48, 72 and 96 h showed that after the first 24 h of incubation the main carotenoid peak progressively disappeared. Thus, as an example, for *L. plantarum* CECT4185, we obtained 29.79 mg/kg DCW of carotenoids after 24 h, and only 19.29, 2.61 and 4.82 mg/kg after 48, 72 and 96 h, respectively. Therefore, broth cultures incubated at 30 °C for 24 h were chosen as the standard for maximum carotenoid production. Among the strains tested, carotenoid production ranged from traces (distinguishable characteristic carotenoid chromatographic peaks, but not reliably quantifiable) up to 54.55 mg/kg DCW produced by *L. plantarum* CECT7531 (Table 1). Yellow color of the pellets to the naked eye was observed for those strains producing at least 4.95 mg of carotenoids per kg of DCW (Table 1).

3.3. All *L. plantarum* strains tested harbor the operon crtNM described in *L. plantarum* WCFS1 genome sequence

Genetic analyses through PCR revealed that all of the *L. plantarum* strains tested contained the genes *crtN* and *crtM* arranged as an operon, as described in the annotated complete genome sequence of *L. plantarum* WCFS1 (acc. no. AL935261), a strain isolated from human pharynx (Kleerebezem et al., 2003). A 2379-bp amplicon was obtained from all but one of the strains tested when primers crtN-for and crtM-rev were used (Table 1), with either 58 or 60 °C as the annealing temperature. Although no amplification was obtained with *L. plantarum* CECT4185 using this primer pair, a 2396 bp amplicon was obtained from this strain when primers rbs-crtN-for and crtM-rev were used instead (Table 1). These results are in agreement with the expected sizes of the corresponding predicted amplicons. Only in five strains, amplification could be obtained with both primer pairs, suggesting a certain degree of genetic diversity in the promoter region among the *L. plantarum* strains used (Table 1). DNA sequence of the amplicon obtained when DNA from the maximum carotenoid-producing strain *L. plantarum* CECT7531 was used as the template showed 99.0% identity (id.) to the sequences of *crtM* and *crtN* genes reported for *L. plantarum* WCFS1. When translated, amino acid sequences of the putative proteins coded by *crtM* and *crtN* genes in the strain CECT7531 showed 99.0% and 99.6% id., respectively, to those proteins coded by homologous genes found in the strain WCFS1. The product of gene *crtM* in the strain CECT7531 has been annotated as dehydrosqualene synthase (also diapophytoene synthase; acc. no. GU474811) based on similarity (30.5% id.; 64% similarity [sim.]) to a homologous protein from *S. aureus* ATCC25904 (acc. no. O07854), where carotenoid biosynthesis has been extensively studied (Wieland et al., 1994; Pelz et al., 2005). In *S. aureus*, this enzyme catalyses the head-to-head condensation of two molecules of FPP into the colorless C_{30} carotenoid dehydrosqualene (diapophytoene) (Fig. 2). On the other hand, the product of gene *crtN* in the strain CECT7531 has been annotated as dehydrosqualene desaturase (also diapophytoene desaturase; acc. no. GU474811) based also on similarity (46% id.; 76% sim.) to a homologous protein from *S. aureus* ATCC25904 (acc. no. O07855). In *S. aureus*, this enzyme catalyses three successive dehydrogenation reactions that lead to the introduction of three double bonds into dehydrosqualene to render diaponeurosporene, with diapophytofluene and diapo-ξ-carotene as intermediates (Fig. 2).

4. Discussion

Although most carotenoids found in bacteria are tetraterpenoids (C_{40}) (Phadwal, 2005), triterpenoids (C_{30}) have been reported in three species of non-photosynthetic bacteria, namely *S. aureus* (Marshall and Wilmoth, 1981), *Enterococcus faecium* (Taylor and Davies, 1974) and *Methylobacterium rhodinum* (Raisig and Sandmann, 1999), as well as in all tested species of the photosynthetic heliobacteria (Takaichi

et al., 1997). Here we demonstrate that C_{30} carotenoids are also produced by most strains of *L. plantarum*. C_{30} and C_{40} carotenoid biosynthetic pathways are shown in Fig. 2. In the C_{30} pathway, condensation of two farnesyl pyrophosphate (FPP) molecules by diapophytoene synthase (coded by *crtM* gene) renders diapophytoene (also named dehydrosqualene; Fig. 2), the first colorless C_{30} carotenoid. Subsequently, successive desaturation reactions increase the number of conjugated double bonds in diapophytoene to produce colored carotenoids such as diaponeurosporene and diapolycopene (Lee and Schmidt-Dannert, 2002) (Fig. 2). Although desaturases are specific of the C_{30} or C_{40} routes, some interchangeability, either natural or induced through molecular engineering, have been reported (Raisig and Sandmann, 2001; Umeno et al., 2002, 2005). In the few microorganisms known to produce C_{30} carotenoids, the end product is either diaponeurosporene or compounds with further modifications, involving for instance oxidation and glycosilation steps, as it is in the case of staphyloxanthin in *S. aureus* (Pelz et al., 2005). The triterpenoid nature of these compounds avoids cyclation of end groups rendering ionone rings which can be further modified to diversify the number of carotenoid compounds with different colors and properties (Lee and Schmidt-Dannert, 2002; Umeno et al., 2005). Therefore, genetic manipulation of *L. plantarum* to increase the range of carotenoids being produced should start with the introduction of heterologous genes coding for geranylgeranyl pyrophosphate (GGPP) synthesis and appropriated desaturase and cyclase enzymes, so that C40 carotenoids could be obtained (see Fig. 2).

No strong correlation between carotenoid production and the origin of the different strains tested was observed, although all of the strains isolated from olive fermentations produced high amounts, including the maximum producer CECT7531 (Table 1). Reported carotenoid production by microorganisms is very diverse regarding net amounts produced. The different units and quantification methods used by different authors makes it very difficult to compare between species. On top of this, different authors have reported a number of environmental and cultural enhancers of carotenoid production by microorganisms (Bhosale, 2004). Thus, two-fold up to one thousand-fold increases have been obtained changing light irradiation or culture temperature, as well as addition of chemical compounds, intermediates of the tricarboxylic acid cycle, metal ions or salts to the culture medium (Bhosale, 2004). In our case, carotenoid production was found to be dependent on the actual culture medium composition, i.e. brand and actual chemical components. This result illustrates the importance of the actual composition and source of the different nutrients of the culture medium used in carotenoid production even when the basic formulation of the media used, i.e. MRS (de Man et al., 1960), is nominally identical. Optimization experiments with high carotenoid-producer strains to obtain higher carotenoid yields will be carried out considering different growth conditions and medium composition, using appropriated statistical factorial designs.

The ability to produce diaponeurosporene together with the presence of the genes *crtN* and *crtM* encoding the putative enzymes necessary for its synthesis, suggest the full functionality of the operon *crtNM* in *L. plantarum,* whose previously assigned function in the strain WCFS1 had been predicted based on similarity data. The ubiquitous presence of this operon in all of the strains tested and its possible functionality in most of them, suggests that this function plays a role in this species survival. In *S. aureus*, a species where the functions of the enzymes coded by the genes *crtM* and *crtN* have been well established, carotenoid biosynthesis has been associated to resistance to different stress conditions, especially the oxidative stress (Clauditz et al., 2006). Heat resistance (Cebrián et al., 2007), desiccation susceptibility (Wieland et al., 1994), oleic and linolenic acid resistance (Chamberlain et al., 1991; Wieland et al., 1994), and impairing of neutrophil killing (Liu et al., 2005) have also been demonstrated to be linked to the carotenoid content of *S. aureus* strains. In all cases, these effects have been related to either the antioxidant properties of carotenoids or their ability to stabilize bacterial cell membranes. Optimization experiments with trace-amount-producing strains are necessary to find out whether low production is due to defective *crtN–crtM* genes, in particular at the promoter regions, or other strain-specific metabolic characteristics are involved. Random and directed mutagenesis will be also very useful to increase the amount of carotenoids produced by a specific strain.

DNA sequence of the genes *crtM* and *crtN* from *L. plantarum* CECT7531, the maximum carotenoid producer strain in this study, showed that these genes are virtually identical to homologous genes reported in the three *L. plantarum* strains whose genome has been sequenced up to date: WCFS1, ATCC14917^T (isolated from pickled cabbage; acc. no. ACGZ00000000.1), and JDM1 (Zhang et al., 2009; used as a probiotic strain in China; acc. no. CP001617). *L. plantarum* ATCC14917^T is the type strain for the species *L. plantarum*, and it is equivalent to *L. plantarum* CECT748^T, used in this study (Table 1). Our results suggest that the operon *crtNM* is well conserved in *L. plantarum*, independently of the actual origin of the strain considered. In contrast to the C_{30} carotenoid biosynthetic pathway found in *L. plantarum*, in *S. aureus* diaponeurosporene is further converted to staphyloxanthin, the orange carotenoid present in most staphylococci strains (Pelz et al., 2005) (Fig. 2). For this, *S. aureus* harbors up to three extra enzymes coded by genes *crtO*, *crtP* and *crtQ*, which are located in the same operon as *crtM* and *crtN* (Pelz et al., 2005). Neither staphyloxanthin nor any of the intermediates in its biosynthesis (Fig. 2) was found in any of the *L. plantarum* strains tested in this study. Moreover, no gene sharing homology with *crtO*, *crtP* or *crtQ* could be found when we analyzed the *L. plantarum* WCFS1, ATCC14917^T or JDM1 complete genome sequences. Finally, under specific conditions, i.e. high heterologous expression levels and the effective concentration of substrates, the desaturase coded by *crtN* in *S. aureus* is able to perform a fourth desaturation step, rendering 4,4′-diapolycopene in amounts up to 50% of the total carotenoids produced (Umeno et al., 2002) (Fig. 2). This carotenoid was not found in any of the *L. plantarum* strains tested under the standard conditions used by us.

In conclusion, this study has shown the presence of C_{30} carotenoid biosynthesis in most of the *L. plantarum* strains studied, regardless of their origin. The ubiquitous presence of the genes *crtM* and *crtN*, probably involved in the biosynthesis of the yellow C_{30} carotenoid 4,4′-diaponeurosporene, in all of the strains tested as well as the actual carotenoid production by most of them suggests that the role of carotenoids in *L. plantarum* environmental fitness must be important. On the other hand, considering that *L. plantarum* is a bacterial species which is extensively used to ferment food and feed products while having a GRAS status, the use of selected high-carotenoid-producing strains could contribute to increase the total amount of antioxidants supplied in the human and animal diet. In addition, as *L. plantarum* is a recognized inhabitant of the gastrointestinal tract, the use of selected strains of *L. plantarum* as probiotics could provide with a regular supply of antioxidant molecules, such as carotenoids, in a place where their protective action is quite welcome. Therefore, carotenoid production should be considered as an important feature for the selection of novel probiotic *L. plantarum* strains.

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