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PCR of *crtNM* combined with analytical biochemistry: An efficient way to identify carotenoid producing lactic acid bacteria

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ABSTRACT

Lactic acid bacteria (LAB) synthesize a wide variety of biochemical compounds during food fermentation. Carotenoids provide important biological functions for bacteria, and their consumption by humans has many beneficial effects. In this study, the presence of several genes involved in the production of carotenoids was determined by BLAST analysis and PCR in a collection of 156 LAB isolated from traditional amylaceous African fermented foods. Only the *crtE* gene and the *crtNM* operon were present and detected in *Lactobacillaceae*. Most of the strains with positive PCR detection of the operon *crtNM* produced carotenoid-like compounds when grown in MRS broth. The carotenoids produced differed from compounds previously identified in other LAB except for one peak, which was closely related to 4,4′-diaponeurosporene already reported in the literature in *Lactobacillus plantarum* species. Most producing strains belonged to *Lactobacillus fermentum* and *L. plantarum* species but a few *Pediococcus acidilactici* were also producers. Furthermore, the most efficient *L. plantarum* was able to synthesize carotenoids in a cereal fermented food. Genetic screening was shown to be efficient since, in all cases, it eliminated the need for biochemical analysis of strains in which no amplicons of the operon *crtNM* were obtained.

Introduction

Fermented foods are essential components of the human diet in many countries, and fermentation is carried out by microorganisms that play an essential role in the physical, nutritional, and organoleptic modification of the raw material. These microorganisms are responsible for the increase in the duration of conservation of food through acidification, but some are also known to be probiotic organisms with a wide range of health promoting effects [18]. From a nutritional point of view, several microbial functions can improve the quality of the food matrix, for example, by producing molecules of interest, such as vitamin B [13].

Some *Lactobacillus plantarum* strains have been shown to exhibit a deep yellow pigmentation when cultured as isolated colonies [7]. *L. plantarum* isolated from different origins (olive, silage, wine, cabbage or cheese) or from commercial inoculums were shown to be capable of synthesizing the yellow C_{30} carotenoid 4,4′-diaponeurosporene [5,7]. In most bacteria, carotenoids are not essential for growth but they have important biological functions, such as protection from oxidative stress by scavenging free radicals with their conjugated double bonds [6,24]. When consumed in the diet, they also act as antioxidants and many beneficial effects of carotenoids for humans have been reported. For example, they prevent cancer and reduce the risk of coronary heart disease [22]. Several carotenoids are used by the food industry as colorants. All these properties explain why the demand for natural and cost-effective sources of these compounds from the food, feed, cosmetics and pharmaceutical industries is increasing. Metabolic engineering has been used to produce carotenoids in initially low- or non-producing bacteria or to synthesize novel carotenoid structures [30]. On the other hand, inherently efficient carotenoid producing strains could be used to produce carotenoid-rich fermented foods naturally.

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Niche-specific adaptation has played a key role in the evolution of LAB [14], therefore, working on collections of bacteria isolated from original fermented foods may enable identification of specific functions that differ from those of LAB isolated from other widely studied products, such as dairy products. Our bacterial collection was isolated from traditional fermented pearl millet slurry and other amylaceous fermented foods. Some of the strains have interesting properties that have been demonstrated experimentally and some of them, for example, are able to: (i) survive simulated gastrointestinal conditions *in vitro* (low pH, and presence of bile salts), (ii) survive the rat digestive system, (iii) cause changes in the intestinal epithelium, and (iv) synthesize bacteriocin. Some of the strains were also able to bind to intestinal cell lines with a much higher binding capacity than other LAB currently known to be probiotics [3,27–29].

The increasing quantity of genetic information makes it possible to study the functional properties of LAB at the molecular level. Genetic screening is increasingly used to detect a potential function in a large collection of bacteria and has been used to screen very distinct functions, such as enzymes involved in the development of wine aroma, probiotic traits, and nutritional functions [11,16].

Bacteria that combine different functions could be useful for developing improved or new foods made from local raw materials with specific nutritional properties or that target specific health issues. In the present study, PCR screening was used to detect genes involved in the production of carotenoids. The collection of 158 *Lactobacillaceae* included 152 bacteria isolated from a traditional African pearl millet-based fermented slurry (*ben-saalga*) [27], four strains isolated from other traditional amylaceous fermented foods, and two strains from a collection used as controls. PCR-based screening was validated by measuring the actual production of carotenoids when the strains were grown in culture media. Finally, one of the most productive strains was inoculated into pearl millet slurry to check if it could naturally enrich the food with carotenoids.

Materials and methods

Materials

Whole-grain pearl millet flour was purchased in Ouagadougou (Burkina Faso) in February 2012. HPLC standards (α-carotene, β-carotene, β-cryptoxanthin, and lutein) were purchased from Sigma–Aldrich (Saint Quentin Fallavier, France). (E/Z)-phytoene, (E/Z)-phytofluene, and lycopene were purchased from Carotenature GmbH (Lupsingen, Switzerland).

Fermented samples, bacteria and culture conditions

Bacteria ($n = 156$) were isolated from amylaceous African fermented foods [27]. The positive control used for gene screening was *L. plantarum* WCFS1 [23], and the negative control was *Lactobacillus fermentum* IFO 3956 [15]. All 158 LAB were routinely cultured at 30 °C in de Man Rogosa and Sharpe (MRS) broth (Difco, Le Pont de Claix, France).

For the screening of carotenoid production in culture media, 50 mL of MRS broth were inoculated with a single colony of the bacterial strain to be tested, and the culture was incubated for 48 h at 30 °C without stirring. Cells were collected by centrifugation at ambient temperature (10,000 *g* for 10 min), washed with sterile 0.9% NaCl, and centrifuged again to obtain a pellet that was then lyophilized and stored at −20 °C.

The *L. plantarum* strain that proved to be the most efficient at producing a carotenoid in MRS broth, *L. plantarum* 2.4.1, was used to assess carotenoid biosynthesis in the fermented slurry. The whole-grain pearl millet flour was mixed with water (10%; w/v), and cooked at 80 °C for 10 min to limit the growth of endogenous bacteria. The slurry was cooled to 30 °C and inoculated (1%; v/w) with an overnight culture of the selected bacteria, which had previously been washed in 0.9% NaCl. The product was incubated for 48 h at 30 °C, lyophilized and then stored at −20 °C. The negative *L. fermentum* IFO 3956 strain was used under the same conditions in order to check for the absence of carotenoids in the slurry and ensure that the detection of carotenoids in the previous experiments was due to bacterial synthesis. Carotenoid extraction and chromatographic analysis were performed within 24 h.

Genetic analysis

DNA was extracted from the bacterial pellets of overnight pure cultures using the Wizard® genomic DNA purification kit (Promega, Charbonnières, France) with an additional lysis step using an amalgamator with zirconium beads (VWR, Fontenay-sous-Bois, France) [27]. The presence of the operon *crtNM* in the different LAB strains was detected by PCR using primers crtN-for (5′-CGCGGAATTCATGAAGCAAGTATCGATTATTGGC-3′) or rbscrtN-for (5′-CTAGGGTACCAAGGGGGAGATTTACTGATGAAGC-3′) and crtM-rev (5′-GATCGAATTCTTAAGCCTCCTTAAGGGCTAGTTC-3′) designed by another research team using the same PCR conditions and separation [7].

Sequences of the other carotenoid related genes were selected based on the functional analysis of various organisms: *crtO*, *crtP* and *crtQ* were selected from *Staphylococcus aureus*, and *crtE*, *crtB* and *crtI* from *Pantoea ananatis* and *Halomonas elongata* [1,17,20]. Using BLASTn, BLASTp and BLASTx algorithms (from April 2010), these genes were then searched for *in silico* in *Lactobacillaceae* in the genomic and protein database (NCBI). Once identified in *Lactobacillaceae*, sequences from species present in the collection of bacteria were used to design primers. Nucleotide sequences were aligned using the ClustalW program [26] in order to generate a single consensus sequence [8], which was used to design the primers with Primer3 software [21]. All primers were synthesized by Eurogentec (Angers, France).

The only gene found *in silico* in *Lactobacillaceae* was *crtE*, therefore, PCR was used to search for it. The PCR mixture (20 μL) contained a reaction cocktail of 200 μM (each) of deoxynucleoside triphosphate, 0.5 μM of each primer, 3.5 mM $MgCl_2$, 0.5 U *Taq* DNA polymerase (Promega), 10X *Taq* buffers, and 150 ng of DNA template. For *crtE*, the primers used were crtEf (5′-ACGCCATGCGTTATTCGGTA-3′) and crtEr (5′-GGCAACGTCTTCCCCAAACT-3′). The PCR conditions were one cycle at 95 °C for 5 min, 40 cycles at 95 °C for 30 s, at 60 °C for 10 s, and at 72 °C for 15 s, followed by one cycle at 72 °C for 5 min using a thermal cycler (Applied Biosystems Veriti™ VWR, Strasbourg, France). For *crtN* and *ctrM*, the PCR conditions were one cycle at 95 °C for 5 min, 40 cycles at 95 °C for 30 s, at 60 °C for 30 s, and at 72 °C for 120 s, followed by one cycle at 72 °C for 5 min, as described previously [7].

All the PCR products were separated in agarose gel and stained with ethidium bromide to check for the presence of a single amplicon. Amplicons obtained from one strain of *Pediococcus pentosaceus*, one strain of *Pediococcus acidilactici* and one strain of *L. fermentum* were purified and sequenced by MWG Operon (Germany, Ebersberg). The primers used for the sequence reaction were the same as those used for gene screening. Sequences of the gene amplicons were deposited in GenBank with accession numbers HF546054 to 546060.

Carotenoid extraction and analysis

Lyophilized samples (bacteria $n = 158$, or slurries ~150 mg) were homogenized with an Ultra-Turrax® (IKA, France) for 1 min in a