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The *spxB* gene as a target to identify *Lactobacillus casei* group species in cheese

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ABSTRACT

This study focused on the *spxB* gene, which encodes for pyruvate oxidase. The presence of *spxB* in the genome and its transcription could be a way to produce energy and allow bacterial growth during carbohydrate starvation. In addition, the activity of pyruvate oxidase, which produces hydrogen peroxide, could be a mechanism for interspecies competition. Because this gene seems to provide advantages for the encoding species for adaptation in complex ecosystems, we studied *spxB* in a large set of cheese isolates belonging to the *Lactobacillus casei* group. Through this study, we demonstrated that this gene is widely found in the genomes of members of the *L. casei* group and shows variability useful for taxonomic studies. In particular, the HRM analysis method allowed for a specific discrimination between *Lactobacillus rhamnosus*, *Lactobacillus paracasei* and *L. casei*. Regarding the coding region, the *spxB* functionality in cheese was shown for the first time by real-time PCR, and by exploiting the heterogeneity between the *L. casei* group species, we identified the bacterial communities encoding the *spxB* gene in this ecosystem. This study allowed for monitoring of the active bacterial community involved in different stages of ripening by following the POX pathway.

1. Introduction

Lactic acid bacteria (LAB) constitute a group of Gram-positive and strictly fermentative bacteria that produce lactic acid as the major end product from carbohydrates. The *Lactobacillus* genus, which currently contains 204 species (http://www.bacterio.net/lactobacillus.html), is the largest group in the family *Lactobacillaceae*. Within this genus, the facultative heterofermentative species *Lactobacillus casei*, *Lactobacillus paracasei* and *Lactobacillus rhamnosus*, which are closely phylogenetically and phenotypically related, are regarded together as the *Lactobacillus casei* group (Felis and Dellaglio, 2007). Despite the controversial nature of their classification and nomenclature (Acedo-Felix, 2003; Diancourt et al., 2007; Felis et al., 2001; Judicial Commission of the International Committee on Systematics of Prokaryotes, 2008; Toh et al., 2013), these species can be isolated from different environments, including plants, and in particular, they play a key role in different food products, including meat and cheese. Regarding dairy products, the presence of bacteria belonging to the *L. casei* group is due to their natural occurrence in milk, together with their ability of growing in curd and cheese. Indeed, they represent the major microbiota group during ripening, due to their metabolic activities that allow them to tolerate the environment of the cheese (Peterson and Marshall, 1990; Settanni and Moschetti, 2010). Recent studies performed on *L. rhamnosus* growing in a cheese model system (Bove et al., 2012; Lazzi et al., 2014) revealed a redirection of bacterial metabolism toward acetate, an important flavor compound. In particular, Lazzi et al. (2014) observed an upregulation of the *spxB* gene, which encodes for pyruvate oxidase (POX), an enzyme that catalyzes the oxidation of pyruvate to acetyl phosphate, which is then converted to acetate in the subsequent reaction, leading to ATP production. Although *spxB* is present in the genome of different *Lactobacilli* (Lazzi et al., 2014), this metabolic pathway has been deepen studied only in *Lactobacillus plantarum* (Goffin et al., 2006; Lorquet et al., 2004; Zotta et al., 2012). A more in-depth knowledge regarding the presence of genes encoding for the POX pathway in bacterial genomes and their transcription is required,

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as it could indicate a way to produce energy in different habitats and permit bacterial growth in specific nutritional conditions. Furthermore, the activity of POX, due to hydrogen peroxide production, could be a mechanism involved in interspecies competition, as different authors have described for the *Streptococcus* community that inhabits the oral cavity (Zheng et al., 2011; Zhu et al., 2014). Complete genome sequencing of different *L. rhamnosus* and *L. paracasei* show the occurrence of multiple copies of genes encoding putative POXs, and it is known that gene duplication is an important evolutionary force that provides an organism with an opportunity to evolve new functions. It is well known how different bacterial species grow in succession during cheese ripening, and as reviewed by Gatti et al. (2014), this is especially extensive for long-ripened cheeses. The presence of different strains that mark specific moments in cheese ripening or that can develop differently throughout the ripening process are linked to the strains' abilities to adapt to specific environmental and technological conditions and could influence the features of cheese. All of these findings stress the importance of following the dynamics of technologically relevant bacteria during ripening: culture-independent methods, based on bacterial DNA or, even better, on RNA that identify active members of the microbial population, represent a significant tool to monitor species. The sequence of 16S rRNA has been extensively used to identify bacterial species and to fingerprint complex communities by different methods. Recently, certain LAB studies that were all based on 16S rRNA reported the use of High Resolution Melting (HRM), a post-PCR method that can be used to study sequence variation due to a single nucleotide polymorphism leading to specific melting temperatures and specific melting profiles (Patel, 2009; Reed et al., 2007). Porcellato et al. (2012) proposed the first application of this technique to characterize isolates from Norwegian cheese; subsequently, Iacumin et al. (2015) extended the method to a wider collection of bacteria belonging to the *L. casei* group, proving its effectiveness in comparison with other molecular approaches. Recently, the *groEL* gene, which encodes a heat shock protein, has been used as a marker for the genetic typing of the *L. casei* group species (Koirala et al., 2015), confirming that taxonomic assignment deserves attention from the scientific community and that the HRM technique is a promising technique.

Given the potential role of *spxB* in bacterial growth during cheese ripening, the aim of this study was to propose this gene as a possible target of the metabolically active microflora for the first time. For this purpose, the *spxB* gene was examined within the *L. casei* group species: 74 isolates from Parmigiano Reggiano cheese at different ripening stages were screened for the presence and the sequence heterogeneity of this gene. In addition, HRM and transcription analysis of *spxB* in cheese was investigated to target the metabolically active microflora directly in the cheese matrix.

2. Materials and methods

2.1. Strains and culture conditions

Seventy-four strains belonging to the species *L. casei*, *L. paracasei* and *L. rhamnosus*, isolated from Parmigiano Reggiano (Table 1A) and previously identified by Ward and Timmins (1999), were used in this study. All of these strains have been deposited in the bioresource collection of the Department of Food Science (University of Parma). The twelve reference strains used for the PCR specificity study were reported in Table 1B. All bacterial strains were maintained as frozen stocks (−80 °C) in MRS (*Lactobacillus*) or M17 (*Streptococcus*, *Lactococcus* and *Enterococcus*) broth (Oxoid, Milan, Italy) supplemented with 15% glycerol (w/v). Before use, the cultures were propagated twice with a 2% (v/v) inoculum into the appropriate media and incubated for 18 h at 37 °C anaerobically (AnaeroGen, Oxoid).

2.2. Nucleic acid preparation

Genomic DNA was extracted from bacterial cultures using the DNeasy Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. The DNA concentration was measured using an Eppendorf BioPhotometer Plus instrument and checked by agarose gel electrophoresis. The genomic DNA was diluted to 20 ng μL^{-1} for PCR. RNA was extracted by TRIzol (Invitrogen, Milano, Italy) from 6- and 24-month-old Parmigiano Reggiano cheeses and their respective raw milk sources. After the extraction, cheese samples were prepared following the protocol of Monnet et al. (2008) with a few modifications. Briefly, 150 mg of grated cheese was placed in a 2 ml screw cap tube containing 500 μL of zirconia/glass beads (diameter, 0.1 mm; BioSpec Products, Bartlesville, OK), followed by the immediate addition of 1.5 ml of TRIzol. The tubes were processed with a Mini-BeadBeater 8 (BioSpec Products, Bartlesville, OK) by three 60 s mixing sequences at maximum speed, interspersed with 60 s pauses at room temperature. Homogenized cheese samples and raw milk were processed following the TRIzol manufacturer's instruction. After extraction, RNA was quantified using a spectrophotometer (Jasco, Japan) and checked by agarose gel electrophoresis.

RNA was reverse transcribed into cDNA with Quantiscript Reverse Transcriptase (QuantiTect Reverse Transcription Kit, Qiagen, Milan, Italy) using random hexamer primers according to the manufacturer's instructions.

2.3. In silico analysis of spxB gene

The partially transcribed RNA sequence of the *spxB* gene of *L. rhamnosus*, accession number AB896775 (Lazzi et al., 2014), was used to find homologous pyruvate oxidase sequences in the NCBI database. Fourteen sequences from different species (Table 1C) were aligned and used to design primers for *spxB* gene region amplification within the *L. casei* group strains. A set of degenerate primers was designed (Table 2): the forward primer *poxPromFw* was located in the promoter region of the gene, and the reverse primer *poxPromRv* was in the coding region, which yielded an amplification product of 260 bp.

2.4. PCR amplification of the target gene and nucleotide sequencing

The partial fragments of the *spxB* genes of the *L. casei* group strains were amplified and sequenced in 44 out of 74 strains originating from milk and cheese at different stages of ripening (13 *L. rhamnosus*, 10 *L. casei* and 21 *L. paracasei*) (Table 1A) using the primers *poxPromFw* and *poxPromRv* (Table 2). PCR reactions were composed of 7 μL of sterile MilliQ water, 10 μL of 2× PCR GoTaq Master Mix (Promega), 1 μL of forward primer (10 mM), 1 μL of reverse primer (10 mM) and 1 μL of template DNA (20 ng/μL). The following thermal cycling conditions were used: initial strand denaturation at 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min, and a final extension step at 72 °C for 7 min. The resulting amplicons were purified using the QIAquick PCR purification Kit (Qiagen), sequenced by MACROGEN Europe (Amsterdam, The Netherlands) and aligned using DNAman software (vers. 4.15, Lynnon Biosoft Company). This software was used to generate a neighbor-joining (NJ) dendrogram where relative branch length was indicated. The bootstrap values were based on 1000 replications.