Food Microbiology 76 (2018) 382–389

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Expression of genes involved in metabolism of phenolic compounds by *Lactobacillus pentosus* and its relevance for table-olive fermentations

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ARTICLE INFO

Keywords:
Lactobacillus pentosus
Tannase
Decarboxylase
Glucosidase
Olive fermentation
Phenolics

ABSTRACT

Genes with the potential to code for enzymes involved in phenolic compound metabolism were detected in the genome of *Lactobacillus pentosus* IG1, isolated from a green olive fermentation. Based on homology, these genes could code for a 6-P-β Glucosidase, two different Tannases, a Gallate decarboxylase and a *p*-Coumaric decarboxylase. Expression of up to seven of these genes was studied in *L. pentosus* IG1 (olive fermentation) and CECT4023^T (corn silage), including responses upon exposure to relevant phenolic compounds and different olive extracts. Genes potentially coding Tannase, Gallate decarboxylase and *p*-Coumaric acid decarboxylase significatively increased their expression upon exposure to such compounds and extracts, although it was strain dependent. In general, both the genetic organization and the characteristics of gene expression resembled very much those described for *Lactobacillus plantarum.* In accordance to the observed induced gene expression, metabolism of specific phenolic compounds was achieved by *L. pentosus*. Thus, methyl gallate, gallic acid and the hydroxycinamic acids *p*-coumaric, caffeic and ferulic were metabolized. In addition, the amount of phenolics such as tyrosol, oleuropein, rutin and verbascoside included in a minimal culture medium was noticeably reduced, again dependent on the strain considered.

1. Introduction

Lactobacillus pentosus is a lactic acid bacterium involved in a diverse range of food fermentations (Abriouel et al., 2011) that have a great economic impact within food industry. This is especially true for vegetable fermentations and, particularly, for table olive ones, where different strains have been extensively used as starter cultures to improve quality and safety of this product (Ruiz-Barba et al., 1994; de Castro et al., 2002; Panagou et al., 2008; Hurtado et al., 2010; Peres et al., 2008; Ruiz-Barba and Jiménez-Díaz, 2012). Olives are not edible unless somehow submitted to a debittering process which hydrolyzes their major phenolic compound: oleuropein. Different procedures have been traditionally used to achieve this debittering step but just three of them are used to produce economically important table olives: the Spanish-style, for green olives, and the natural and oxidation procedures, for black olives (Garrido Fernández et al., 1995; Sánchez et al., 2006; Rejano et al., 2010). Debittering of Spanish-style green olives is achieved through an alkali treatment involving dilute solutions of NaOH as well as a washing step with water (Sánchez et al., 2006; Rejano et al., 2010). Olives are finally covered with brine (10–12% [w/v] NaCl) and let to spontaneously ferment. Although at least three successive stages have been described, this fermentation is dominated by strains of *L. pentosus* (de Castro et al., 2002; Rejano et al., 2010; Ruiz-Barba and Jiménez-Díaz, 2012; Lucena-Padrós et al., 2014a, 2014b). Fermentation is completed after two-three months, when all fermentable material has been metabolized to, mainly, lactic acid and pH and free acidity are suitable for product preservation. As cited above, the utilization of appropriate starters based on strains of *L. pentosus* are being used to guarantee the results of this fermentation.

On the other hand, the phenolic content of the green olive fruits confers not only a bitter taste but also an inhibitory effect on a range of bacteria, especially lactic acid bacteria (LAB) (Ruiz-Barba and Jiménez-Díaz, 1989; Ruiz-Barba et al., 1990, 1991 and 1993; De Castro et al., 2005; Medina et al., 2007, 2008 and 2009). Therefore, the alkali treatment and the washing steps are critical for appropriate subsequent lactic acid fermentation. These steps are still carried out empirically in the production factories (called "*patios*", in the Spanish argot), being guided by the expertise of skilled personnel. However, the intrinsic variability of the fruits, their physical origin, their maturation degree and size, the use of irrigation in the olive groves and other unpredictable parameters make this task a somehow risky issue. In fact, errors at this point have been proposed as the major cause of "stuck"

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https://doi.org/10.1016/j.fm.2018.06.020
Received 13 February 2018; Received in revised form 7 May 2018; Accepted 30 June 2018
Available online 02 July 2018
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fermentations or even spoilage of these fermentations at early stages, what may give rise to defects such as "gas pockets" or "alambrado" (Borbolla y Alcalá et al., 1959, 1960; Rejano et al., 2010; Lanza, 2013). Gas pockets or "fish eye" are the result of the growth of gas-producing microorganisms, such as some yeast species and *Enterobacteriaceae*, under the olives cuticle (Vaughn et al., 1972; Lanza, 2013). Alambrado or "gated olives" result from the abnormal growth of microorganisms, especially Gram-negative bacteria, that produce an excess of CO_2 which accumulates within the olive pulp (Borbolla y Alcalá et al., 1959; Lanza, 2013).

Considering the above, it is clear that the use of starter cultures with increased capabilities to cope with the inhibitory phenolic compounds present in table olive fermentations would be an advantageous trait. In fact, several authors have described the selection of LAB, especially strains of *L. pentosus* and *Lactobacillus plantarum*, with enhanced capabilities to resist such inhibition in table olive fermentations (Ruiz-Barba and Jiménez-Díaz, 1989; Servili et al., 2006; Ghabbour et al., 2011; among others). The examination of the genome of a *L. pentosus* strain isolated from Spanish-style green olive fermentation (Maldonado-Barragán et al., 2011) revealed the presence of several potential phenolic compound-degrading genes as deduced by their DNA and protein sequence homology (Table 1). The aim of this work was to study the actual expression of these genes in two selected strains of *L. pentosus* from different origins. This has been done in the presence or absence of a set of phenolic compounds, some of them characteristic of this table olive fermentation, as well as with the addition of different types of olive extracts, prepared as in the Spanish-style procedure, in order to detect gene-expression changes. In addition, we have evaluated the capabilities of these strains to actually metabolize these phenolic compounds in a minimal culture medium. Finally, the presence of phenolic-compound degrading genes has been investigated in a range of wild-type *L. pentosus* strains isolated from Spanish-style green olive fermentations at different locations.

2. Materials and methods

2.1. Bacterial strains and culture media

Bacterial strains used in this study were *L. pentosus* IG1, isolated from a Spanish-style green olive fermentation (Maldonado-Barragán et al., 2011), and *L. pentosus* CECT 4023^T (=NCDO 363^T) isolated from corn silage (Zanoni et al., 1987). In order to examine the distribution of genes related to phenolic compounds metabolism, a set of 49 wild-type strains of *L. pentosus* isolated from Spanish-style green olive fermentations at five different locations were used (Table S1). All L. *pentosus* strains were routinely grown in MRS broth or agar (Biokar Diagnostics, Beauvais, France) aerobically at 30 °C. For expression experiments, a modified Basal Medium (MBM) was used as previously described by Rozès and Peres (1998), substituting glucose by galactose as described by Jiménez et al. (2014) to avoid possible catabolite repression. Briefly, per litre: D(+)-galactose 2 g (Panreac, Castellar del Vallès, Spain); tri-sodium citrate dihydrate 0.5 g (Sigma-Aldrich, St. Louis, USA); D-L malic acid 5 g (Sigma-Aldrich); casamino acids 1 g (casein hydrolysate, Difco, Sparks, USA); yeast nitrogen base without amino acids 6.7 g (Difco); pH 6.0.

2.2. Detection of genes related to metabolism of phenolic compounds in L. pentosus

The published sequence of the *L. pentosus* IG1 genome (acc. no. FR874854; Maldonado-Barragán et al., 2011) was used as a template. Search of homologies of the deduced protein sequences and comparative genomics were performed using the bioinformatic tools BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and those provided by the CoGe platform (https://genomevolution.org/coge/). Multiple sequence alignments were performed using Multalin (Corpet, 1988) at http://

Table 1
Homology of ORFs related to phenolic compound metabolism found in the chromosome of *Lactobacillus pentosus* IG1.

Locus tag[a]	Size (bp)	Predicted protein (aas)	Annotation[a]	% Protein identity (sim[b])	*L. plantarum* strain[c]	Predicted function	Locus tag (gene)	Accession no.
LPENT_00991	1509	502	β-glucosidase	83 (90)	WCFS1	β-glucosidase	lp_0906 (*pbg2*)	CCC78348
LPENT_01512	411	136	putative uncharacterized protein	85 (93)	WCFS1	Gallate decarboxylase (subunit D)	lp_0272 (*lpdD*)	CCC77799
LPENT_01513	564	187	3-octaprenyl-4-hydroxybenzoate carboxy-lyase	98 (98)	WCFS1	Gallate decarboxylase (subunit B)	lp_0271 (*lpdB*)	YP_004888312
LPENT_01783	537	178	phenolic acid decarboxylase	88 (96)	WCFS1	*p*-Coumaric acid decarboxylase	lp_3665 (*padA*)	YP_004891133
LPENT_01784	555	184	regulator of phenolic acid metabolism PadR	88 (93)	WCFS1	transcriptional regulator of phenolic acid metabolism	lp_3664 (*padR*)	YP_004891132
LPENT_02382	1413	470	tannase	73 (84)	WCFS1	Tannase	lp_2956 ($tanB_{Lp}$)	YP_004890536
LPENT_02396	1473	490	3-octaprenyl-4-hydroxybenzoate carboxy-lyase	98 (99)	WCFS1	Gallate decarboxylase (subunit C)	lp_2945 (*lpdC*)	YP_004890530
LPENT_02397	1380	459	transport protein	97 (99)	WCFS1	cation transport protein	lp_2943 (*gacP*)	YP_004890529
LPENT_02398	918	305	transcription regulator	86 (92)	WCFS1	transcriptional regulator (LysR family)	lp_2942 (*tanR*)	YP_004890528
LPENT_02868	1884	627	tannase	91 (95)	ATCC 14917^T	Tannase[d]	HMPREF0531_11477 ($tanA_{Lp}$)[e]	KRL35904

[a] According to *L. pentosus* IG1 genome (accession no. FR874854).
[b] In brackets, similarity.
[c] Highest homology scores among species for which work has been done on the actual expression and activity genes were found with *L. plantarum*.
[d] This tannase is not present in *L. plantarum* WCFS1.
[e] According to Jiménez et al. (2014).

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