Distribution of the plantaricin operons and co-culture inducibility among Lactobacillus plantarum strains isolated from olive fermentations

Antonio Maldonado Barragán, Belén Caballero Guerrero, Rufino Jiménez Díaz and José Luis Ruiz Barba
Departamento de Biotecnología de Alimentos, Instituto de la Grasa (C.S.I.C.)
Avda. Padre García Tejero, 4, Aptdo. 1078, 41012 Sevilla (✉ jlruiz@cica.es)

Introduction

Bacteriocins are proteinaceous compounds produced by certain bacteria exhibiting antimicrobial activity against a specific panel of other bacteria. Our research group has demonstrated in the past that bacteriocin production play an important role in *Lactobacillus plantarum* viability in olive fermentations (Ruiz-Barba et al., 1994 and 2009). Therefore, bacterial strains used as starter cultures for olive and other food fermentations will most probably benefit of being bacteriocin producers. Although bacteriocin production is **constitutive** in some strains, this is not always the case. Actually, bacteriocin production in many Lactic Acid Bacteria (LAB), a bacterial group which includes *L. plantarum* species, is controlled by specific peptides called autoinducers (**AIPs**) *via* a sophisticated mechanism which senses cell density of the producing culture and it is known as **"*quorum sensing*"** (**QS**). To date, up to three regulated bacteriocin operons have been fully described in this species, i.e. **plantaricin NC8, EF and JK**, each of them consisting of **two-peptide bacteriocins**. Biosynthesis of these bacteriocins has been shown to be switched on through specific AIPs and, in some strains, also by the **presence of specific competing bacteria** in the same culture medium.

Here we report how these bacteriocin and regulatory operons are distributed among *L. plantarum* strains isolated from olive fermentations, and compared with strains isolated from other environments. Also, we have investigated how co-culture with specific bacteria can promote bacteriocin production in certain *L. plantarum* strains. To accomplish these goals, we have proceeded as follows:

i) Strains have been characterized using a molecular approach, i.e. Randomly Amplified Polymorphic DNA (**RAPD**)
ii) The presence of six different operons involved either in bacteriocin production, regulation, maturation or transport have been investigated using **PCR** with specific oligonucleotide primers.
iii) Bacteriocin production both in solid and liquid culture media have been investigated.
iv) The **inducibilty** of bacteriocin production by two distinct AIPs, **PLNC8IF and plantaricin A**, has been checked in each strain.
v) The **inducibilty** of bacteriocin production by **co-culture** with specific Gram-positive bacteria has been studied.

Materials and methods

Bacterial strains and media. Bacterial strains used in this study are listed in **Table1**. They were cultured in MRS (Oxoid) broth or agar at 30ºC.

Bacteriocin production, induction and autoinduction assays.
To check for **bacteriocin production by isolated colonies**, overnight cultures of *L. plantarum* strains were serially diluted in sterile saline and plated onto MRS agar plates to obtain ca. 30 colonies per plate. Plates were incubated at 30ºC for 24 h and overlaid with 4.5 ml soft agar inoculated with ca. 105 CFU/ml of the indicator strains shown in Table 1. Plates were further incubated at 30ºC for 24 to 48 h and examined for clear halos in the lawns of the indicator bacteria around isolated colonies, indicating bacteriocin activity. To check for **bacteriocin production in broth cultures**, one isolated colonies of *L. plantarum* strains were inoculated into fresh MRS broth. Cultures were incubated at 30ºC and samples were withdrawn at the late exponential phase of growth, centrifuged and the cell free supernatant (CFS) checked for bacteriocin activity by the spot-on-lawn method. *Lactobacillus pentosus* 128/2 and *Pediococcus pentosaceus* FBB63 were used as indicator strains.
For the **autoinduction experiments**, as a source of the autoinducer peptide PLNC8IF we used a semi-purified sample of this peptide obtained from a CFS of *L. lactis* MG1363(pSIG308)(Maldonado et al., 2004b). As a source of the autoinducer peptide plantaricinA (PlnA) we used a semi-purified sample of this peptide obtained from a CFS from L. *plantarum* WCFS1. For autoinduction, 50-µl aliquots of the PLNC8IF or PlnA were added to 1 ml of MRS inoculated with *ca.* 10^8 cells from an overnight culture of the *L. plantarum* strain to be tested, incubated for 6 h at 30 ºC and then the resulting CFS examined for bacteriocin activity, as described above.
Bacteriocin induction by cocultivation was done as follows: a 1% inoculum of the *L. plantarum* strain to be tested was cultured with a 0.5% inoculum of the inducer strain *L. lactis* MG1363 in MRS broth, the mixed culture held at 30ºC for 6-8 h and then the inhibitory activity in the CFS assayed.

DNA amplification (PCR) techniques.
Several combinations of primers were used in order to check the presence/absence of target genes and their relative positions. As a reference we used the published DNA sequences of the plantaricin cluster in *L. plantarum* NC8 (acc.no.AF522077) and *L. plantarum* WCFS1 (acc.no.AL935263)(**Figure 1**). For amplification of DNA fragments up to 3 kb, 25-µl reaction mixtures containing 2.5 mM Mg Cl2, 1× reaction buffer, 100 µM concentrations of each of the deoxynucleotides triphosphates (dNTPs), 100 pmol of each of the primers, 5 U of Taq DNA polymerase (Promega) and 250 ng of genomic DNA as the template were used. Amplification included denaturation at 94ºC for 4 min, followed by 30 cycles of denaturation at 94ºC for 30 seg, annealing at 60ºC for 1 min, and polymerisation at 72ºC for 1 min. For amplification of DNA fragments larger than 3 kb, the Expand Long Template PCR System (Roche) was used according to the manufacturer instructions.
Randomly Amplified Polymorphic DNA (RAPD) from *L. plantarum* isolated colonies was done using the primer OPL5 (5'-ACGCAGGCAC-3') as described previously (Ruiz-Barba et al., 2005).

Table 1

Strain	Isolated from
Lactobacillus plantarum	
LPT70/3	green olives fermentation
LPT57/1	green olives fermentation
LPT44/1	green olives fermentation
LPT34/1	green olives fermentation
LPT49/6	green olives fermentation
37A	green olives fermentation
RP1	green olives fermentation
NC8	grass silage
NCDO1193	grass silage
ATCC 14431	grass silage
WCFS1	human saliva
LB6	wine fermentation
CECT 748	cabbage fermentation
indicator strains	
Pediococcus pentosaceus FBB63	vegetable fermentation
Lactobacillus pentosus 128/2	green olives fermentation
inducing strains	
Lactococcus lactis MG1363	cheese
Pediococcus pentosaceus FBB63	vegetable fermentation

Figure 1

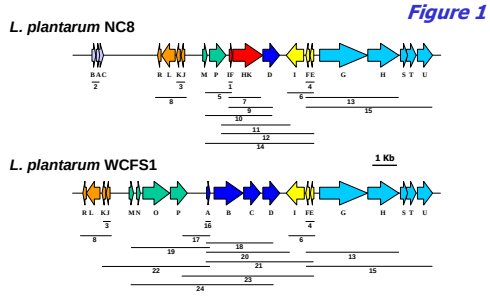

Figure 1. Sizes and relative positions of DNA fragments amplified by PCR with specific primer pairs corresponding to relevant genes, operon or regions in the plantaricin cluster of two *L. plantarum* strains used as references.

Results

Figure 2. RAPD analysis of L. plantarum strains

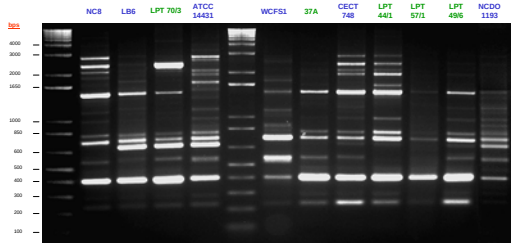

Figure 2. RAPD analysis of the strains used in this study clearly shows their genetic diversity. These results were subsequently confirmed by comparing their plasmid profiles (not shown) and several phenotyping tests involving bacteriocin production and induction.

Figure 3. PCR analysis of the plantaricin cluster

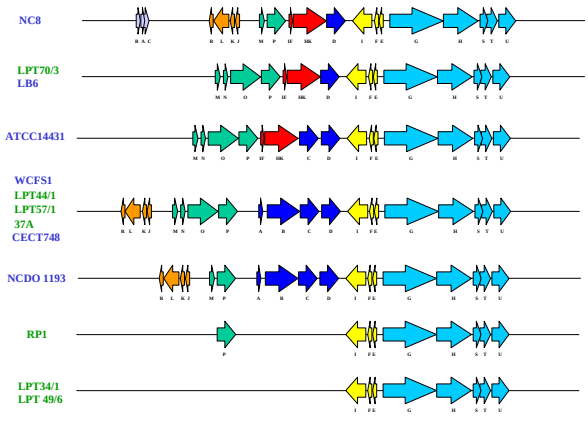

Figure 3. PCR analysis of the genes and operons harboured by the *L. plantarum* strains used in this study is shown in this schematic representation. Gene content, gene sizes and relative localisation has been well stablished for each strain.

Bacteriocin operons
The operon *plnEFI* which encodes the two-component bacteriocin plantaricin EF and its putative immunity protein (PlnI) was present in all *L. plantarum* strains studied. In contrast, the operon *plnJKRL* encoding the two-component bacteriocin plantaricin JK and its putative immunity proteins (PlnR and PlnL) was detected in all of the strains but *L. plantarum* ATCC 14431, LB6 and LPT70/3. The operon *plnNC8BAC* encoding the two-peptide plantaricin NC8 (PLNC8a+ PLNC8b) of *L. plantarum* NC8 was not detected in any *L. plantarum* strain but the original NC8.

Regulatory operons
With respect to the genes encoding the three-component regulatory system, we found at least three different gene combinations. *L. plantarum* LB6 and LPT70/3 showed the regulatory operon *plNC8IF-plNC8HK-PlnD* of NC8, encoding an autoinducer peptide (NC8IF), a histidine protein kinase (NC8HK) and a response regulator (PlnB). *L. plantarum* WCFS1, 37A, LPT44/1, LPT57/1 and CECT 748, however, harboured the regulatory operon *plnABCD* encoding the autoinducer peptide plantaricin A (PlnA), the histidine protein kinase PlnB and two response regulators, PlnC and PlnD. In addition, three strains presented anomalous regulatory operons. In *L. plantarum* ATCC 14431 an insertion of *ca.* 800 bp was observed with primers targeting the genes *plNC8If* and p*lNC8HK*.

Maturation, transport and accessory operons
All of the strains studied presented the operon *plnMNOP*, with the exception of *L. plantarum* NC8 that presented the operon *plnMP* instead. The gene cluster *plnGHSTU* encoding a dedicated ABC-transporter and their accessory proteins was found in all *L. plantarum* strains.

Table 2. Bacteriocin production and induction profiles

L. plantarum	Genotype	Bacteriocin production: Solid medium 128/2	Bacteriocin production: Solid medium FBB63	Bacteriocin production: Broth 128/2	Bacteriocin production: Broth FBB63	Bacteriocin induction: Coculture	Bacteriocin induction: NC8IF	Bacteriocin induction: PlnA
NC8	*plNC8ABC, plnJK, plnMO, plNC8IfHk-plnD, plnEFI, plnGHSTU*	+	+	-	-	+	+	-
LB6	*plnMNOP, plNC8IfHk-plnD plnEFI, plnGHSTU*	-	+	-	-	+	+	-
LPT70/3	*plnMNOP, plNC8IfHk-plnD plnEFI, plnGHSTU*	-	+	-	-	+	+	-
CECT748	*plnJK, plnMNOP plnABCD, plnEFI, plnGHSTU*	-	-	-	-	-	-	-
LPT44/1	*plnJK, plnMNOP plnABCD, plnEFI, plnGHSTU*	-	+	-	-	-	-	+
LPT57/1	*plnJK, plnMNOP plnABCD, plnEFI, plnGHSTU*	-	+	-	+	nd	nd	nd
37A	*plnJK, plnMNOP plnABCD, plnEFI, plnGHSTU*	-	+	-	-	-	-	+
NCDO1193	*plnJK, plnMP, plnABCD, plnEFI, plnGHSTU*	-	+	-	-	-	-	+
LPT34/1	*plnEFI, plnGHSTU*	-	-	-	-	-	-	-
LPT49/6	*plnEFI, plnGHSTU*	-	-	-	-	-	-	-
RP1	*plnEFI, plnGHSTU*	-	-	-	-	-	-	-

Table 2. As **all of the *L. plantarum* strains tested harboured genes related to bacteriocin production**, we studied their ability to produce bacteriocins, both in solid as well as in liquid cultures, as well as the existence of co-culture induction and/or autoinduction mechanisms.

Bacteriocin production: although harbouring genes for bacteriocin production, not all of the strains were able to produce bacteriocins. Significative differences could be observed regarding production on solid medium or liquid cultures. Also, the strain used as indicator was very important for this analysis.

Solid medium: most of the strains tested produced bacteriocins when assayed on solid medium. However, only slightly more than 50% of the strains isolated from olive fermentations produced bacteriocins. In addition, only the strain *L. plantarum* NC8, the only one harbouring plantaricin NC8, was active against *L. pentosus* 128/2.

Liquid cultures: only one strain, *L. plantarum* LPT57/1 isolated from an olive fermentation, produced bacteriocins when grown in liquid medium. This result suggests the existence of regulatory circuits dependent on quorum sensing mechanisms in most of the strains.

Coculture induction: Only the strains harbouring the regulatory operon *plNC8If-plNC8Hk-plnD* were able to produce bacteriocins when cocultured with the strain *Lactococcus lactis* MG1363 used as an inducer. Among these strains, *L. plantarum* LPT70/3 was isolated from an olive fermentation.

Autoinduction: our results indicate that there are two different regulatory operons controlling bacteriocin production in *L. plantarum*: ***plNC8If-plNC8HK-plnD*** and ***plnABCD***. The strains controlled by the first operon are only induced by the specific **autoinducer peptide NC8IF**, while those controlled by the latter operon are only induced by the specific **autoinducer peptide PlnA.** Therefore, **no interchangeability has been detected among these two regulatory systems**.

Conclusions

1.- All of the *Lactobacillus plantarum* strains isolated from Spanish-style green olive fermentations harboured, at least, the operon for plantaricin EF production/immunity (*plnEFI*) as well as that for bacteriocin maturation and transport (*plnGHSTU*).

2.- Although all of the strains tested harboured genes for bacteriocin production, only 57 % of them produced bacteriocins.

3.- The fact that none but one strain (*L. plantarum* 57/1) produced bacteriocins in liquid cultures suggested the presence of regulatory mechanisms dependent on "quorum sensing".

4.- Only two bacteriocin regulatory operons were found, one of them dependent on the autoinducer NC8IF and the other dependent on the autoinducer plantaricin A. These autoinducers were not interchangeable.

5.- Only one strain isolated from an olive fermentation, *L. plantarum* LPT70/3, was found to be induced in the presence of bacteriocin-inducing bacteria such as *Lactococcus lactis*.

***Final remark*: for *L. plantarum* strains isolated from olive fermentations, either coculture with a bacteriocin-inducing bacteria or the addition of a specific autoinducer peptide were necessary to produce bacteriocins in a liquid environment. Accordingly, this fact has to be considered when *L. plantarum* strains are going to be used as bacteriocin-producing starter cultures for olive fermentations.**

Resumen

*La producción de sustancias antimicrobianas denominadas **bacteriocinas** ha demostrado jugar un papel importante en la viabilidad de la especie bacteriana **Lactobacillus plantarum** en fermentaciones de aceitunas verdes al estilo español o sevillano. Tal producción de bacteriocinas ha mostrado estar regulada en la mayoría de los casos por un mecanismo de "**quorum sensing**" que implica un operón regulador específico. Hasta la fecha, tres diferentes operones de producción de bacteriocinas reguladas por este mecanismo han sido descritos en esta especie, a saber, **plantaricina NC8, plantaricina EF y plantaricina JK**, todos ellos consistentes en bacteriocinas de dos péptidos. Se ha demostrado que la biosíntesis de estas bacteriocinas se pone en marcha ante la presencia de péptidos específicos llamados **autoinductores** y, en algunas cepas, también mediante el **cocultivo** con cepas bacterianas específicas. En esta comunicación presentamos cómo estos operones, tanto de producción de bacteriocinas como de regulación de la misma, están distribuidos entre cepas de L. plantarum aisladas de fermentaciones de aceitunas, comparándolas con cepas de la misma especie aisladas de otros ambientes. Sólo el operón de la **plantaricina EF y aquellos genes implicados en la maduradción y transporte de las bacteriocinas están presentes en todas las cepas estudiadas**. En contraste, plantaricina NC8 no se encontró en ninguna de las cepas aisladas de fermentaciones de aceitunas. Se encontraron dos operones reguladores diferentes, que eran controlados por alguno de los dos **autoinductores específicos asociados: PLNC8IF o plantaricina A**. Aunque conteniendo genes de producción de bacteriocinas, algunas cepas no producían estos compuestos antimicrobianos, mientras que en otras cepas se encontró que la producción de bacteriocinas se podía inducir mediante el **cocultivo con cepas específicas de bacterias Gram positivas**.*

***Para que una cepa de L. plantarum produzca bacteriocinas en un medio de cultivo líquido**, tal como las salmueras de fermentación de aceitunas, es necesario que,*

***1.- La producción de bacteriocinas sea constitutiva**.*

***2.- En caso de ser regulada por mecanismos de "quorum sensing", que o bien se cultive en presencia del autoinductor específico o bien se cocultive junto con una cepa bacteriana inductora**.*

References

◇ Ruiz-Barba, J.L., D.P. Cathcart, P.J. Warner, and R. Jiménez-Díaz (1994). Use of *Lactobacillus plantarum* LPCO10, a bacteriocin producer, as a starter culture in Spanish-style green olive fermentations. Applied and Environmental Microbiology, 60: 2059- 2064.
◇ Maldonado, A. , J.L. Ruiz Barba, y R. Jiménez Díaz (2003). Purification and Genetic Characterization of Plantaricin NC8, a Novel Co-culture Inducible Two-Peptide Bacteriocin from *Lactobacillus plantarum* NC8. Applied and Environmental Microbiology, 69: 383-389.
◇ Maldonado, A. , J.L. Ruiz Barba, y R. Jiménez Díaz (2004a). Production of Plantaricin NC8 by *Lactobacillus plantarum* NC8 is induced in the Presence of Different Types of Gram-positive Bacteria. Archives of Microbiology, 181: 8-16.
◇ Maldonado, A., R. Jiménez Díaz, y J.L. Ruiz Barba (2004b). Induction of plantaricin production in *Lactobacillus plantarum* NC8 after coculture with specific Gram-positive bacteria is mediated by an autoinduction mechanism. Journal of Bacteriology, 186:1556-1564.
◇ Maldonado, A (2005). Regulación de la producción de bacteriocinas en *Lactobacillus plantarum*. Tesis Doctoral, Universidad de Sevilla.
◇Ruiz-Barba, J.L., Maldonado, A., Jiménez-Díaz, R., 2005. Small-scale total DNA extraction from bacteria and yeast for PCR applications. Analitical Biochemistry 347, 333-335.
◇ Ruiz-Barba, J.L., B. Caballero-Guerrero, A. Maldonado-Barragan, and R. Jimenez-Diaz (2009). Coculture with specific bacteria enhances survival of *Lactobacillus plantarum* NC8, an autoinducer-regulated bacteriocin producer, in olive fermentations. Food Microbiology, In Press, Corrected Proof, Available online 9 October 2009, ISSN 0740-0020, DOI: 10.1016/j.fm.2009.10.002.

Acknowledgements. *This work has been funded by the Spanish Ministry of Science and Technology (MCYT) and the Spanish Ministry of Education and Science (MEC) through the Projects AGL2003-00642 and AGL2006-00763, respectively.*