**Knockout of three-component regulatory systems reveals that the apparently
constitutive plantaricin-production phenotype shown by *Lactobacillus plantarum*
on solid medium is regulated via quorum sensing**

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Abstract

It has been found that many bacteriocins from lactic acid bacteria (LAB) are only produced in broth cultures when specific growth conditions are achieved and a dedicated three-component regulatory system, involved in a quorum sensing (QS) mechanism, is switched on. Surprisingly, bacteriocin production in LAB occurs in an apparently constitutive manner on solid media. This study addresses the question of constitutive *versus* regulated bacteriocin production on solid media in two different QS-regulated plantaricin-producing strains: *Lactobacillus plantarum* NC8 and *L. plantarum* WCFS1. Construction of knockout mutants for their respective regulatory operons revealed that bacteriocin production is controlled through a QS mechanism in both strains, on solid as well as in liquid media. These results could be extensible to other bacteriocins from LAB which are only produced on agar plates and not in broth cultures. Our findings suggest that QS-regulated bacteriocin production in LAB has evolved for competing on solid supports rather than in liquid media. In practice, this could be of major importance in vegetable fermentations, where the solid substrate itself provides an enormous surface where bacteria can attach to and produce biofilms. Therefore, QS-regulated bacteriocinogenic LAB growing in biofilms are under the optimum conditions to produce bacteriocins. Selection of strains to be used as starter cultures for vegetable fermentations should take into account these facts.

1. Introduction

Bacterial communities produce antimicrobial compounds in to compete with other similar micro organisms. Among these, the proteinaceous compounds called bacteriocins seem to be directed to compete against related species or other bacteria sharing the same ecological niche (Tagg et al., 1976; Klaenhammer, 1993; Jack et al., 1995). Although the synthesis of most bacteriocins reported until present appears to be constitutive (Quadri, 2002), the production of these antimicrobial compounds can be an unstable trait in some cases, indicating the existence of regulatory mechanisms (Nes and Eijsink, 1999). On that account, differences in bacteriocin production between solid and liquid media have been observed since early studies in both Gram-negative colicinogenic strains (Reeves, 1965), and Gram-positive bacteria (Tagg et al., 1976). In lactic acid bacteria (LAB), bacteriocins have been a major focus of research because of their potential use as natural food preservatives (Daeschel, 1993; de Vuyst and Vandamme, 1994; Cotter et al., 2005). In this group of bacteria, production of these antimicrobial compounds on solid but not in liquid media has been claimed in several studies. Cintas et al. (1995) found that out of 55 isolates of LAB exhibiting antimicrobial activity on agar media only 12 of them produced an inhibitory substance in liquid media. Similar frequencies have been reported by Schillinger and Lücke (1989) for lactobacilli, and by Geis et al. (1983) for lactococci. Bacteriocins lactacin B (Barefoot and Klaenhammer, 1983), plantacin B (West and Warner, 1988), plantaricin F (Fricourt et al., 1994), and more recently enterolysin A (Nilsen et al., 2003) and streptin (Wescombe and Tagg, 2003) were found to be produced only on solid media. However, further investigations showed that most of these bacteriocins could be produced also in liquid media under appropriate conditions (Barefoot and Klaenhammer, 1984; Paynter

et al., 1997; Wescombe and Tagg, 2003). In addition, some LAB lose the ability to produce bacteriocins when inoculated in liquid media below a specific inoculum size (Diep et al., 1995; Saucier et al., 1995; Eijsink et al., 1996; Brurberg et al., 1997; Nilsen et al., 1998; O' Keeffe et al., 1999). In these cases, the bacteriocin-producing (Bac⁺) phenotype could only be restored when the culture was streaked onto solid media or by the addition of the cell-free supernatant (CFS) from a previous Bac⁺ culture. Further research revealed that bacteriocin production in these strains is regulated by a three-component regulatory system composed by an autoinducer peptide (AIP), a histidine-kinase protein (HK) and a response regulator (RR). Such AIP acts as an indicator of the cell density which is sensed by the corresponding HK, resulting in activation of the RR, which finally activates the expression of all operons necessary for bacteriocin synthesis, transport and regulation (Kleerebezem et al., 1997; Nes and Eijsink, 1999). This quorum-sensing (QS) or autoinduction mechanism mediated by AIPs was found in *Carnobacterium piscicola* (Axelsson and Holck, 1995; Quadri et al. 1997; Saucier et al., 1997; Kleerebezem et al., 2001), *Lactobacillus plantarum* (Diep et al., 1996; Brurberg et al., 1997; Maldonado et al., 2004b), *Lactobacillus salivarius* (Flynn et al., 2002), *Lactobacillus sake* (Brurberg et al., 1997; Diep et al., 2000) and *Enterococcus faecium* (Nilsen et al., 1998; O'Keeffe et al., 1999). Saucier et al. (1995) suggested that the differences observed in bacteriocin production between solid and liquid media could be attributable to differences in the rate of diffusion of the corresponding AIP: the AIP did not diffuse in agar as readily as in solution, allowing the cells on the agar surface to be in closer contact with the secreted AIP than in liquid medium. However, the functionality of the autoinduction mechanism on solid media has not been addressed yet.

In previous works, we have shown that *L. plantarum* NC8 is unable to produce bacteriocins when inoculated as a pure culture in liquid medium, regardless of the inoculum size and growth conditions (Maldonado et al., 2003 and 2004a). However, coculture of *L. plantarum* NC8 with specific Gram-positive bacteria or the addition of its specific autoinducer peptide PLNC8IF to broth cultures resulted in bacteriocin production by this strain (Maldonado et al., 2003, 2004a and 2004b). Moreover, addition of PLNC8IF induced not only the expression of the genes encoding the three two-peptide bacteriocins identified in NC8 (plantaricins NC8, EF and JK), but also the *plNC8If-plNC8Hk-plnD* regulatory operon (Fig. 1A), thereby demonstrating autoinduction (Maldonado et al., 2004b). In contrast, we observed that isolated colonies of *L. plantarum* NC8 growing on MRS agar always showed bacteriocin activity, thus indicating that bacteriocin production on solid medium appears as a constitutive trait.

Here we report that constitutive bacteriocin production by *L. plantarum* NC8 on solid medium is only apparent and it is in fact regulated by the same autoinduction mechanism as in broth cultures, i.e. requiring the expression of the operon encoding the three-component regulatory system *plNC8If-plNC8Hk-plnD*. Construction of a knockout (KO) for this operon in *L. plantarum* NC8 has demonstrated that functionality of such operon is fully indispensable for bacteriocin production both on solid and in liquid media. In addition, we have studied bacteriocin production by *L. plantarum* WCFS1, a strain from human origin whose complete genome sequence has been recently reported (Kleerebezem et al., 2003). This strain has a plantaricin biosynthesis cluster containing a regulatory operon (*plnABCD*) encoding an atypical three-component regulatory system consisting of an AIP (plantaricin A [PlnA]), a histidine kinase (PlnB) and two response regulators (PlnC and PlnD) (Fig. 1B). This plantaricin cluster was first discovered in *L. plantarum* C11, where production of plantaricins EF

and JK has been shown to be regulated by an autoinduction mechanism which depends on the expression of *plnABCD* (Diep et al., 1994 and 1996). Bacteriocin production by the WCFS1 strain had been supposed since its complete genome sequence was available, but an in-depth study had not been addressed before. Comparative bacteriocin production studies in the wild-type WCFS1 and a derivative, KO mutant strain lacking the operon *plnABCD* has reinforced the results and conclusions obtained with *L. plantarum* NC8.

2. Materials and methods

2.1. Bacterial strains, media and growth conditions

Bacterial strains are described in Table 1. *L. plantarum*, *L. pentosus* and *P. pentosaceus* strains were propagated in De Man-Rogosa-Sharpe (MRS) broth or agar (Oxoid) at 30°C. Where appropriate, erythromycin (Fluka) was added to the culture medium at 10 µg/ml. *Enterococcus faecalis* and *Listeria innocua* strains were propagated in Brain Heart Infusion (BHI) broth or agar (Oxoid) at 30°C. *L. lactis* MG1363 was grown in M17 broth (Oxoid) plus 1% (wt/vol) glucose (GM17), at 30°C. *L. lactis* MG1363 (pSIG308) was grown in GM17 containing 10 µg/ml of erythromycin. However, erythromycin was omitted when the CFS of the *L. lactis* MG1363 (pSIG308) culture was collected as a source of PLNC8IF. *Escherichia coli* DH5α was grown in Luria-Bertani (LB) broth or agar at 37°C with vigorous agitation. *E. coli* DH5α transformant cells harbouring recombinant plasmids were selected on LB agar plates supplemented with 150 µg of ampicillin (Fluka) or 200 µg of erythromycin per ml, respectively, 16 µl of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-

galactopyranoside) (50 mg/ml, Promega) per plate, and 4 µl of IPTG (isopropyl β-D-thiogalactoside) (200 mg/ml; Gibco BRL) per plate.

2.2. Bacteriocin and autoinduction assays.

To check for bacteriocin production by isolated colonies, the direct method described by Tagg et al. (1976) was used. Briefly, overnight cultures of *L. plantarum* NC8 or WCFS1 were serially diluted in sterile saline and plated onto MRS agar plates to obtain ca. 30 colonies per plate. Plates were incubated at 30°C for 24 h and overlaid with 4.5 ml soft agar inoculated with ca. 10⁵ CFU/ml of the selected indicator bacterial strains shown in Table 1. The appropriate culture medium was used to make the soft agar according to the indicator bacteria to be used. Plates were further incubated at 30°C 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, cells of *L. plantarum* NC8 or WCFS1 from either 24 to 72-h-old colonies on MRS agar plates or overnight broth cultures were inoculated into fresh MRS broth at inoculum sizes ranging from 10² to 10⁸ CFU/ml. Cultures were incubated at 30°C and samples were withdrawn at the late exponential phase of growth, i.e. O.D._{600nm} of 2.0, centrifuged and the CFSs checked for bacteriocin activity by the spot-on-lawn method as described previously (Jiménez-Díaz et al., 1993), using *P. pentosaceus* FBB63 as the indicator strain. 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), a recombinant strain which expresses PLNC8IF heterologously (Maldonado et al., 2004b). For this purpose, a 2-litre culture of this strain was processed by a protocol similar to that described previously for the

purification of plantaricin S (Jiménez-Díaz et al., 1995), but selecting those fractions that exhibited induction of bacteriocin production in NC8. Briefly, the CFS was precipitated with ammonium sulfate, desalted, and consecutively applied to cation-exchange (SP Sepharose fast-flow, Pharmacia) and hydrophobic-interaction (phenyl-Sepharose CL4B, Pharmacia) columns. The presence of PLNC8IF was verified by matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometry as described before (Maldonado et al., 2004b). As a source of the autoinducer peptide PlnA we used a semi-purified sample of this peptide obtained from the CFS of a 2-litre Bac⁺ *L. plantarum* WCFS1 broth culture at its exponential phase of growth and processed as described above for PLNC8IF. To verify that PlnA was present in this sample, MALDI-TOF mass spectrometric analysis was carried out by Dr. S. Ogueta, Unidad de Proteómica - S.C.A.I., Universidad de Córdoba, Córdoba, Spain. For autoinduction, 50-μl aliquots of the relevant semi-purified AIP (PLNC8IF or PlnA) were added to 1 ml of MRS inoculated with *ca.* 10⁸ cells from an overnight culture of the *L. plantarum* strain to be tested, incubated for 6 h at 30 °C and then the resulting CFS examined for bacteriocin activity. In all autoinduction experiments, pure *L. plantarum* NC8 or WCFS1 cultures were used as controls of both bacteriocin and autoinducer activities. To test for plantaricin production of *L. plantarum* NC8-KO1 and WCFS1-KO1 isolated colonies on solid medium in the presence of the corresponding AIP, a protocol similar to that used by Diep et al. (1995) was used. Briefly, overnight cultures of each KO mutant strain were serially diluted in saline and plated onto MRS agar plates containing PLNC8IF or PlnA to obtain *ca.* 30 colonies per plate. These AIP-containing agar plates were prepared by spreading 100-μl aliquots of semi-purified PLNC8IF or PlnA over the surface of the plates immediately before plating the diluted

cultures. These plates were incubated for 24 h at 30 °C and overlaid with 4.5 ml of MRS soft-agar containing ca. 10⁵ CFU/ml of the indicator strain *P. pentosaceus* FBB63.

2.3. DNA isolation and transformation procedures

Total genomic DNA from wild-type and derivative *L. plantarum* strains was isolated by the method of Cathcart (1995). Plasmid DNA from *E. coli* was extracted as described previously (Sambrook et al., 1989). Electroporation of *L. plantarum* NC8 and WCFS1 was carried out according to the method of Aukrust and Blom (1992). *E. coli* DH5 α was electroporated by the method of Dower et al. (1998).

2.4. Southern blot and hybridization

Genomic DNA from *L. plantarum* was digested with *Xma*I, the resulting fragments were electrophoretically separated by size on a 0.7 % agarose gel and then blotted onto a Genebind 45 nylon membrane (Amersham). The *ermAM* gene harboured by pIL252 was amplified by PCR as described below, labelled with fluorescein-11-dUTP in the same reaction and used as a probe in Southern hybridization experiments. Hybridization, washing and detection were performed using the ECL Labelling and Detection System in the conditions recommended by the manufacturer (Amersham).

2.5. Oligonucleotides, PCR and DNA sequencing

Oligonucleotides used as primers in PCR reactions (Table 2) were synthesized by MWG Biotech (Ebersberg, Germany). The relative position of those related to the

plantaricin cluster in NC8 and WCFS1 is depicted in Fig. 1. Primers PlnM-for (Maldonado et al., 2004b) and Eco-Del1 were used to amplify a 974-bp *L. plantarum* NC8 DNA fragment containing the *plnM* and *plnP* genes located upstream of the regulatory operon *plNC8If-plNC8Hk-plnD*. Primers Sal-Del2 and KpnPlnF-rev were used to amplify a 1,279-bp *L. plantarum* NC8 DNA fragment containing the *plnF* and *plnI* genes located downstream of *plNC8If-plNC8Hk-plnD*. The remaining primers related to the plantaricin cluster were used for diagnostic purposes. To amplify a DNA fragment containing the gene *ermAM*, primers erm-*EcoRI* and erm-*HindIII* were designed based on the published DNA sequence of the plasmid pIL252 (Genebank accession number AF039139), which encodes an adenine methylase conferring resistance to erythromycin and lincomycin. To facilitate subsequent cloning, *EcoRI*, *HindIII* and *SalI* sites were introduced at the ends of primers Eco-Del1 and erm-*EcoRI*, erm-*HindIII*, and Sal-Del2 respectively.

For amplification of DNA fragments up to 3 kb, 100- μ l reaction mixtures containing 2.5 mM MgCl₂, 1 $\times$ reaction buffer, 100 μ M 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 with a GeneAmp[®] PCR System 2400 thermal cycler (Perkin-Elmer). Amplification included denaturation at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 1 min, and polymerization at 72°C for 1 min. For amplification of DNA fragments larger than 3 kb we used the Expand Long Template PCR system (Roche Applied Science, Barcelona, Spain) under the conditions recommended by the manufacturer. PCR amplifications of DNA fragments used for cloning or sequencing were performed using the High-Fidelity PCR System (Roche) under the conditions recommended by the manufacturer. For screening purposes, DNA

extractions from *L. plantarum* and *E. coli* colonies to be used as the template for PCR were carried out according to the method of Ruiz-Barba et al. (2005).

DNA sequencing was performed by the Servicio de Secuenciación Automática de DNA (SSAD), CIB-CSIC, Madrid, Spain, with an ABI PRISM 377 DNA sequencer (Applied Biosystems, Perkin-Elmer).

2.6. Construction of a *plNC8If-plNC8Hk-plnD* knockout mutant of *L. plantarum* NC8

Restriction enzymes, T4 DNA ligase, and other DNA-modifying enzymes were used as recommended by the manufacturer (Boehringer Mannheim). To delete the regulatory operon *plNC8If-plNC8Hk-plnD* from the chromosome of *L. plantarum* NC8 by homologous recombination, plasmid pSIG316 (Table 2) was constructed. This suicide plasmid, which is unable to replicate in Gram-positive bacteria, contains a 906-bp sequence carrying the *plnP* gene and a 1,034-bp sequence carrying the *plnI* gene (Fig. 2). Both genes, which are flanking the *ermAM* gene, served as homologous DNA for allelic exchange of the regulatory operon *plNC8If-plNC8Hk-plnD* from the chromosome of *L. plantarum* NC8 with the *ermAM* gene of pSIG316 (Fig. 2A). The plasmid pSIG316 was introduced by electroporation in *L. plantarum* NC8 in its native circular state or previously linearized with *SacI* or *XbaI*. KO mutants were selected by plating out appropriate dilutions on MRS agar plus erythromycin. *Erm*^R colonies were further analyzed by PCR and phenotypically characterized as described below.

2.7. Construction of a *plnABCD* knockout mutant of *L. plantarum* WCFS1

For deletion of the entire regulatory operon *plnABCD* of *L. plantarum* WCFS1 the suicide plasmid pSIG316 was used, since in this strain this operon is also located between *plnP* and *plnI*, as in NC8 (Fig. 1). The entire plasmid pSIG316 as well as a 3.5-kb *Hae*II-digested DNA fragment from this plasmid (containing the cassette *plnP-ermAM-plnI*, Fig. 2B) were introduced separately into *L. plantarum* WCFS1 by electroporation. Erm^R colonies were selected and characterized as described above for the NC8 KO mutants.

3. Results

3.1. Bacteriocin production by *L. plantarum* NC8 and WCFS1

When isolated colonies of *L. plantarum* NC8 growing on MRS agar were tested for bacteriocin production against the panel of selected Gram-positive indicator bacterial strains shown in Table 1, clear halos of inhibition in lawns of almost all of them were observed, indicating the existence of bacteriocin activity. The only exceptions were *L. pentosus* LPS5 and *L. lactis* MG1363, which showed to be resistant. However, bacteriocin production in broth cultures can not take place when *L. plantarum* NC8 is growing as a pure culture unless its specific autoinducer PLNC8IF is added (Table 3).

On the other hand, isolated colonies of *L. plantarum* WCFS1 showed bacteriocin production against five of the indicator bacteria used, although its spectrum of activity was narrower than that shown by the NC8 strain. In fact, *L. pentosus* strains 128/2, BOM1, LPC1 and LPS5, as well as *L. lactis* MG1363 were resistant to WCFS1 bacteriocins. However, when using CFSs from broth cultures we found that bacteriocin

production in WCFS1 was dependent on the inoculum size. We observed that the threshold concentration was ca. 10^5 CFU/ml. Hence, when the WCFS1 strain was inoculated in MRS broth below this concentration, it lost its ability to produce bacteriocins (Table 3). In addition, when these non-bacteriocin producing (Bac^-) cultures were used to inoculate fresh MRS broth, they remained Bac^- independently of the inoculum size, indicating that the autoinducing circuit had been switched off. The Bac^+ phenotype of these broth cultures could only be restored by the addition of the autoinducer peptide PlnA (Table 3). MALDI-TOF mass spectrometry analysis of a partially purified, Bac^+ *L. plantarum* WCFS1 CFS showed a peak corresponding to PlnA (not shown). These results indicate the existence of a functional autoinduction mechanism which is responsible for bacteriocin production in the WCFS1 strain, which is driven by the autoinducing peptide PlnA, as it has been described for *L. plantarum* C11 (Diep et al., 1994 and 1996). However, in contrast to the C11 strain, we observed that the Bac^+ phenotype in WCFS1 could also be restored by plating out a Bac^- broth culture onto solid medium to obtain isolated colonies and reinoculating these colonies into fresh broth at a concentration above the mentioned threshold (10^5 CFU/ml).

3.2. KO mutation of the plantaricin regulatory operons of *L. plantarum* NC8 and WCFS1

To gain insight into the regulation of bacteriocin production in *L. plantarum*, we obtained KO mutants lacking the regulatory operons involved in bacteriocin production by *L. plantarum* NC8 and WCFS1. For this purpose we constructed the suicide plasmid pSIG316, which contains the genes *plnP* and *plnI* of *L. plantarum* NC8 and WCFS1 flanking an erythromycin resistance cassette (Fig. 2). In the wild-type NC8 and WCFS1

strains, *plnP* and *plnI* are located up- and downstream, respectively, of the regulatory operons (Fig. 1).

Electroporation of *L. plantarum* NC8 with pSIG316 resulted in several Em^R colonies which, after PCR analysis, showed to harbour the entire pSIG316 plasmid integrated into the *L. plantarum* NC8 chromosome. This integration was the result of a single crossing-over event (Campbell-type integration) between the genes *plnP* or *plnI* of pSIG316 and the homologous genes in the chromosome of the NC8 strain. These derivative strains were named *L. plantarum* NC8-INT. On the other hand, electroporation with linearised pSIG316 resulted in one Em^R colony which, after the corresponding PCR analysis, showed that the entire regulatory operon *plnNC8If-plnNC8Hk-plnD* had been replaced by the erythromycin cassette from pSIG316. This KO mutant strain was named *L. plantarum* NC8-KO1. This replacement was further confirmed by DNA sequencing of a PCR-amplified fragment from the chromosome of the NC8-KO1 mutant with the primer pair PlnM-for/PlnE-rev. Hence, homologous recombination with double crossing-over (DCO) between the *plnP* and *plnI* genes from the chromosome of NC8 with these genes from the suicide plasmid pSIG316 took place in *L. plantarum* NC8-KO1, leading to the substitution of the regulatory operon by the *ermAM* cassette (Fig. 2A). In Southern-blot experiments, the *ermAM* labelled probe hybridized with a unique >15-kb *Xba*I *L. plantarum* NC8-KO1 chromosomal DNA fragment, indicating the existence of just one copy of the *ermAM* gene in the mutant strain (not shown). In the wild-type *L. plantarum* NC8 strain, however, no hybridization was observed.

On the other hand, transformation of *L. plantarum* WCFS1 with intact pSIG316 resulted also in several Em^R colonies. PCR analysis using the primer pair PlnM-for/PlnE-rev showed that all of these transformants had the entire plasmid pSIG316

integrated into the chromosome *via* a Campbell-type integration (not shown). These transformants were designated *L. plantarum* WCFS1-INT. Transformation with linearized pSIG316 resulted in one Em^R colony which, after PCR analysis showed that the operon *plnABCD* had been replaced by the *ermAM* cassette (Fig. 2B). This KO strain was named *L. plantarum* WCFS1-KO1, being the result of a DCO recombination as illustrated in Fig. 2B.

3.3. Comparative bacteriocin production studies in KO and INT mutants of *L. plantarum* NC8 and WCFS1

As in the wild-type *L. plantarum* NC8 strain, CFSs from broth cultures of *L. plantarum* NC8-KO1 did not show bacteriocin activity (Table 3). However, in contrast to NC8, addition of the autoinducer peptide PLNC8IF to broth cultures of *L. plantarum* NC8-KO1 did not result in bacteriocin production (Table 3). Differences in bacteriocin production due to different growth kinetics between the KO mutant and the wild-type strain in MRS broth were excluded, since both cultures grew at identical rates (not shown). Interestingly, the isolated colonies of *L. plantarum* NC8-KO1 on MRS agar were unable to produce any bacteriocin activity (Table 3 and Fig. 2A). This indicated that the *plNC8If-plNC8Hk-plnD* regulatory operon is also necessary for bacteriocin production on solid medium. This Bac⁻ phenotype remained unchanged even after the addition of PLNC8IF to the agar plates (Table 3). Morphology and growth of NC8-KO1 colonies were identical to the wild-type *L. plantarum* NC8. In contrast, all of the *L. plantarum* NC8-INT strains showed the same phenotype as the wild-type strain (Table 3), indicating that the lack of the ability to produce bacteriocins in the KO strain was not attributable to the integration and/or expression of the *ermAM* gene. In the same

manner, in control experiments with *L. plantarum* NC8 transformed with the plasmid vector pIL252 the bacteriocin-production phenotype was identical to that of the wild-type strain (Table 3).

On the other hand, in contrast to the wild-type *L. plantarum* WCFS1, *L. plantarum* WCFS1-KO1 was unable to produce bacteriocin neither in broth nor on solid medium (Table 3 and Fig. 2B). Addition of PlnA to WCFS1-KO1 broth or agar-plate cultures did not induce bacteriocin production in this mutant strain either (Table 3). As isolated colonies of WCFS1-KO1 did not show bacteriocin activity, this fact suggests that bacteriocin production by WCFS1 on solid medium is also regulated by an autoinduction mechanism as it appears to be the case in broth cultures. Morphology and growth of the strain WCFS1-KO1 were virtually identical to WCFS1 wild-type. Finally, *L. plantarum* WCFS1-INT strain as well as *L. plantarum* WCFS1 transformed with the plasmid vector pIL252 showed the same phenotype as the wild-type strain (Table 3).

4. Discussion

We have shown that the discrepancy in the way *L. plantarum* NC8 produces bacteriocins on agar plates (apparently constitutive) and in broth cultures (QS regulated) is only apparent. Our results demonstrate that such bacteriocin production is indeed regulated by QS in both situations, as demonstrated by the Bac⁻ phenotype of a KO mutant in the bacteriocin regulatory operon both on agar-plate and broth cultures (Table 3). Thus, the Bac⁺ phenotype could not be restored even after the addition of the corresponding AIP, i.e. PLNC8IF (Table 3).

To assess whether these findings were applicable to other QS-regulated bacteriocinogenic *L. plantarum* strains, we studied the phenotype of *L. plantarum*

WCFS1. For this strain, our results show that bacteriocins are produced on solid medium in an apparently constitutive manner, while production in broth cultures is dependent on the inoculum size (Table 3). This result disagrees with that obtained by Sturme (2005), who showed that the native state of WCFS1 was Bac⁻ unless synthetic PlnA or a PlnA-containing CFS was added to the cultures. However, these differences could be due to variations in the initial inoculum size of WCFS1 used to test for bacteriocin production, or the use of different, less-sensitive indicator strains. We observed that highly diluted WCFS1 broth cultures (below 10⁵ CFU/ml) were not able to produce bacteriocins, as it had been described previously for most QS-regulated class-II bacteriocins of LAB (Diep et al., 1995; Saucier et al., 1995; Eijsink et al., 1996; Brurberg et al., 1997; Nilsen et al., 1998; O' Keeffe et al., 1999). Restoration of the Bac⁺ phenotype could only be achieved after the addition of a PlnA or by plating the culture out on solid medium. These results suggest that an autoinduction mechanism for bacteriocin production is also functional in WCFS1. This point has been reinforced since a KO mutant in the regulatory operon *plnABCD* (*L. plantarum* WCFS1-KO1) is unable to produce bacteriocin, even after the addition of PlnA (Table 3). The absence of bacteriocin activity surrounding the isolated colonies of *L. plantarum* WCFS1-KO1 has confirmed that on solid medium bacteriocin production by WCFS1 is also regulated by an autoinduction mechanism dependent on *plnABCD* expression.

The fact of obtaining the same phenotype with both NC8 and WCFS1 KO-mutant strains is very significative, for major differences are found between their respective bacteriocin regulatory operons (Fig. 1). Apart from exhibiting different AIPs, the presence in the WCFS1 strain of the regulatory operon *plnABCD* is intriguing, especially because of the existence of two different RR proteins: PlnC and PlnD. Diep et al. (2003) showed that PlnC and PlnD antagonize to activate or downregulate,

respectively, bacteriocin production in *L. plantarum* C11. However, the NC8 strain manages with just PlnD. Also, the absence of the operon encoding PLNC8 and the presence of the *plnN* and *plnO* genes in *L. plantarum* WCFS1 emphasize the genotypic differences between both strains. Actually, some of these differences are translated to the respective phenotype, for the spectrum of activity of the strain NC8 is wider than that of WCFS1 (Table 3). This result is most probably due to the fact that both strains produce plantaricins EF and JK, while the strain NC8 produces also plantaricin NC8. Therefore, it is very probable that other LAB strains which have been described to produce bacteriocins on solid but not in liquid media are in fact regulated by similar QS mechanisms in both situations, regardless the specific AIP or regulatory operon used. The constitutive phenotype on agar plates could be only apparent in all these cases.

However, the most stimulating question is why bacteriocins are produced in a phenotypically constitutive manner on solid but not in liquid media. In nature, most bacteria appear to thrive attached to surfaces within biofilms, where they are substantially different from the same bacteria living as planktonic microorganisms (Korber et al., 1995). When growing as bacterial biofilms or colonies, cells are in close contact with their neighbours, thus enabling communication between them to make group decisions *via* QS mechanisms (Miller and Bassler, 2001; Henke and Bassler, 2004). Chao and Levin (1981) pointed out that, by killing sensitive strains in a zone around the bacteriocin-producing colony, they could increase the concentration of resources available for themselves in a manner not possible in broth cultures. To date, we do not know whether the attachment to a solid surface *per se* provokes changes in the expression of relevant genes (i.e., involved in bacteriocin regulation), or it just enables bacterial communication by simply limited diffusion of this AIP, as suggested by Saucier et al. (1995). The result, in both cases, is an increase in the AIP level and the

activation of the autoinduction (QS) mechanism and thereby bacteriocin production. However, in LAB class-II bacteriocins whose production depends of a QS mechanism, broth cultures of the producing strains never appear to reach a *quorum* unless inoculated at a concentration above a certain threshold or an external source of a specific AIP (synthetic AIP or an AIP-containing CFS) is added to the culture medium. This flaw in the QS mechanism for bacteriocin production has been attributable to the existence of other environmental factors which should be the truly responsible for switching the bacteriocin-production machinery on (Nes and Eijsink, 1999). Whatever be the case, most LAB bacteriocins which are regulated by a QS mechanism will most probably be produced in those culture conditions which better mimic their natural ecological niche, such as growing on a solid support or the presence of other (inducing) micro organisms (Maldonado et al., 2004a and 2004b). Actually, Eglund et al. (2004) proposed that juxtaposition is required for effective interspecies signalling in natural systems, emphasizing the relevance of signal transmission over very short distances. The mechanism for bacteriocin production in *L. plantarum* NC8 and WCFS1, and most probably other QS-regulated bacteriocin-producing LAB strains, seems to be designed for competing on solid supports, where the rate cost/benefit of producing their antimicrobial compounds appears to be more favourable than in liquid media (Dykes and Hastings, 1997). This could be of major importance in vegetable fermentations such as olive fermentations, where the solid substrate itself represents an enormous surface where bacteria can attach to and produce biofilms. QS-regulated bacteriocinogenic LAB strains able to produce and/or attach to these biofilms are in the optimum conditions to produce bacteriocins. In contrast, constitutively-produced bacteriocins, such as plantaricin S from *L. plantarum* LPCO10, do not face this constriction and are usually produced on solid as well as in liquid environments (Leal et al., 1998). Therefore,

selection of strains to be used as starter cultures for vegetable fermentations should take into account these features.

Finally, the successful construction of the KO mutant strains *L. plantarum* NC8-KO1 and *L. plantarum* WCFS1-KO1 provide us with a useful tool to further extend the study of gene regulation involved in bacteriocin production. Both mutant strains are suitable hosts for the study or development of new expression vectors based on other bacteriocin-related regulatory operons, since interference by cross-talk between similar three-component regulatory systems can be avoided. This study has also demonstrated for the first time that substitution by DCO homologous recombination of chromosomal DNA fragments as big as 3.5 kb is possible in *L. plantarum*.

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Legends of the Figures

Figure 1. Genetic map of the plantaricin cluster in *L. plantarum* NC8 (A) and *L. plantarum* WCFS1 (B). Their respective plantaricin regulatory operon is underlined. Open arrows represent genes that are different among both strains. Lollipops indicate the positions of putative promoter sequences. Numbered arrowheads represent the positions of primers used in this study (see Table 2), as follows: 1, PlnM-for; 2, EcoDEL-1; 3, SalDEL-2; 4, KpnPlnF-rev; 5, PlnI-rev; 6, PlnE-rev; 7, PlnP-for; 8, IFNC8-for; 9, PlnF-for; 10, RR-rev. The genetic map shown for *L. plantarum* NC8 is taken from Maldonado et al. (2004b), and that for *L. plantarum* WCFS1 is represented according to the sequence data at GenBank accession number AL935263.

Figure 2. Phenotypic and genetic characterization of the plantaricin regulatory operon knockout mutants *L. plantarum* NC8-KO1 and *L. plantarum* WCFS1-KO1. Upper panels, bacteriocin assay of isolated colonies of *L. plantarum* NC8 and *L. plantarum* NC8-KO1 (panel A), and of *L. plantarum* WCFS1 and *L. plantarum* WCFS1-KO1 (panel B) showing the presence and absence, respectively, of inhibition halos. *P. pentosaceus* FBB63 was used as the indicator strain. Lower panels, diagrams of the homologous recombination with double crossing-over between the *plnP* [*P*] and *plnI* [*I*] genes from the chromosome of *L. plantarum* NC8 and of *L. plantarum* WCFS1 with the same genes from the suicidal plasmid pSIG316, leading to the substitution of the plantaricin regulatory operon by the *ermAM* cassette in the mutant strains *L. plantarum* NC8-KO1 and *L. plantarum* WCFS1-KO1, respectively. The arrowheads indicate the position of relevant primers (see Table 2) used in the genetic characterization of the locus. Primer number key: 1, PlnM-for; 2, PlnE-rev; 3, erm-EcoRI; 4, erm-HindIII.

Table 1. Bacterial strains used.

Bacterial strain	Features	Reference
<i>Escherichia coli</i> DH5α	Host strain for recombinant plasmids	Invitrogen
¹ <i>Lactobacillus plantarum</i> NC8	Em ^S Bac ⁺ ; plasmid free strain isolated from grass silage; inducible plantaricin (PLNC8, PlnEF and PlnJK) producer	Shrago <i>et al.</i> , 1986 Maldonado <i>et al.</i> , 2004b
² <i>L. plantarum</i> WCFS1	Em ^S Bac ⁺ ; isolated from human saliva; complete genome sequenced	Kleerebezem <i>et al.</i> , 2003
<i>L. plantarum</i> NC8-KO1	Em ^R Bac ⁻ ; derivative of <i>L. plantarum</i> NC8 lacking the operon <i>plNC8IF-plNC8Hk-plnD</i>	This work
<i>L. plantarum</i> WCFS1-KO1	Em ^R Bac ⁻ ; derivative of <i>L. plantarum</i> WCFS1 lacking the operon <i>plnABCD</i>	This work
<i>L. plantarum</i> NC8-INT	Em ^R Bac ⁺ ; derivative of <i>L. plantarum</i> NC8 having the plasmid pSIG316 integrated into the chromosome	This work
<i>L. plantarum</i> WCFS1-INT	Em ^R Bac ⁺ ; derivative of <i>L. plantarum</i> WCFS1 having the plasmid pSIG316 integrated into the chromosome	This work
<i>Lactococcus lactis</i> MG1363	Indicator strain for bacteriocin activity	Maldonado <i>et al.</i> , 2004a
<i>L. lactis</i> MG1363 (pSIG308)	Em ^R Bac ⁻ ; heterologous producer of PLNC8IF	Maldonado <i>et al.</i> , 2004b
<i>Enterococcus faecalis</i> CNRZ135	Indicator strain for bacteriocin activity	Maldonado <i>et al.</i> , 2004a
³ <i>Lactobacillus pentosus</i> 128/2	Indicator strain for bacteriocin activity	Maldonado <i>et al.</i> , 2003
³ <i>L. pentosus</i> BOM1	Indicator strain for bacteriocin activity	Maldonado <i>et al.</i> , 2004a
<i>L. pentosus</i> CECT4023 ^T	Indicator strain for bacteriocin activity; equivalent to <i>L. pentosus</i> ATCC8041	CECT
³ <i>L. pentosus</i> LPC1	Indicator strain for bacteriocin activity	Maldonado <i>et al.</i> , 2004a
³ <i>L. pentosus</i> LPS5	Indicator strain for bacteriocin activity	Maldonado <i>et al.</i> , 2004a
<i>L. plantarum</i> CECT748 ^T	Indicator strain for bacteriocin activity; equivalent to <i>L. plantarum</i> ATCC14917	CECT
<i>Listeria innocua</i> BL86/26	Indicator strain for bacteriocin activity	Maldonado <i>et al.</i> , 2004a
<i>Pediococcus pentosaceus</i> FBB63	Indicator strain for bacteriocin activity	Maldonado <i>et al.</i> , 2003

¹ Kindly provided by Lars Axelsson from MATFORSK, Norwegian Food Research Institute, Osloveien, Norway.

² Kindly provided by Michiel Kleerebezem from Wageningen Centre for Food Sciences, NIZO Food Research, Wageningen, The Netherlands.

³ Previously cited as *L. plantarum*. Identified as *L. pentosus* according to the genetic criteria of Torriani *et al.* (2001).

^TType strain.

CECT: Colección Española de Cultivos Tipo (Spanish Type-Culture Collection), Burjassot, Spain.

Table 2. Primers and plasmids used.

Primer	Sequence ¹	Reference
IFNC8-for	5' ATGAAAAACATTAATAAGTACACTGAAC 3'	Maldonado <i>et al.</i> , 2004a
RR-rev	5' GAGTGAAAAGTATCGCGTTCC 3'	Maldonado <i>et al.</i> , 2004a
PlnE-rev	5' ATGCTACAGTTTGAGAAGTTACA 3'	Maldonado <i>et al.</i> , 2004a
PlnF-for	5' CTATCCGTGGATGAATCCTC 3'	Maldonado <i>et al.</i> , 2004a
PlnI-rev	5' CCCAACTCAATCACCCATTAAC 3'	Maldonado <i>et al.</i> , 2004a
PlnM-for	5' TAAACAGGTAAAGCAGGTTGG 3'	Maldonado <i>et al.</i> , 2004a
PlnP-for	5' TCTGAGCTTGTTACACCTACC 3'	Maldonado <i>et al.</i> , 2004a
EcoDEL-1	5' <u>CGCG</u> <i>GAATTC</i> GTCACACTATTCAATAC 3' (<i>EcoRI</i>)	This work
SalDEL-2	5' <u>CGCG</u> <i>GTCGAC</i> GATAGTTGGAGTAGGG 3' (<i>SalI</i>)	This work
KpnPlnF-rev	5' <u>CGCG</u> <i>GGTACC</i> GGGGGAGATCAACAATTATG 3' (<i>KpnI</i>)	This work
erm-EcoRI	5' <u>CGCG</u> <i>GAATTC</i> GAAACAGCAAAGAATGG 3' (<i>EcoRI</i>)	This work
erm-HindIII	5' <u>CGCG</u> <i>AAGCTT</i> TAGTAACGTGTAACCTTCC 3' (<i>HindIII</i>)	This work

Plasmid	Size; Marker	Features	Reference
pUC18	2.7 Kb; Ap ^R	<i>E. coli</i> cloning vector	Stratagene
pBSII-KS+	2.9 Kb; Ap ^R	<i>E. coli</i> cloning vector	Stratagene
pIL252	4.8 kb, Em ^R	Low-copy-number Gram-positive cloning vector	Simon and Chopin, 1988
pSIG227	4.1 Kb; Ap ^R Em ^R	pBSIIKS+ containing an 1.1 kb <i>Sau3A</i> insert obtained from pIL252 which includes the <i>ermAM</i> gene from pIL252	This laboratory (unpublished)
pSIG313	3.8 Kb; Ap ^R	pBSIIKS+ containing an 0.9 kb <i>BclI-EcoRI</i> insert including the <i>plnP</i> gene of <i>L. plantarum</i> NC8	This work
pSIG314	3.8 Kb; Ap ^R	pUC18 containing an 1 kb <i>SalI-PstI</i> insert including the <i>plnI</i> gene of <i>L. plantarum</i> NC8	This work
pSIG315	5.0 Kb; Ap ^R Em ^R	pSIG313 containing an 1.1 <i>EcoRI-SalI</i> insert from pSIG227 which includes the <i>ermAM</i> gene from pIL252	This work
pSIG316	5.8 Kb; Ap ^R Em ^R	pSIG314 containing an 2.1 kb <i>SacI-SalI</i> insert from pSIG315 This vector harbors the <i>plnP-ermAM-plnI</i> gene fusion.	This work

¹Nucleotide sequences introduced for the recognition of specific restriction enzymes (in brackets) are shown in italic letters. A "clamp" nucleotide sequence (underlined) was added to the 5' end to facilitate restriction enzyme digestion.

Table 3. Comparative bacteriocin production in wild type and derivative strains of *Lactobacillus plantarum* NC8 and WCFS1

<i>Lactobacillus plantarum</i> Strain ¹	CFS ²	colonies ³
NC8	-	+
NC8+PLNC8IF	+	+
NC8-KO1	-	-
NC8-KO1+PLNC8IF	-	-
NC8-INT	-	+
NC8 (pIL252)	-	+
WCFS1 (>10 ⁵ CFU/ml) ⁴	+	+
WCFS1 (<10 ⁵ CFU/ml) ⁴	-	+
WCFS1 (<10 ⁵ CFU/ml) ⁴ + PlnA	+	+
WCFS1-KO1	-	-
WCFS1-KO1+ PlnA	-	-
WCFS1-INT (>10 ⁵ CFU/ml) ⁴	+	+
WCFS1-INT (<10 ⁵ CFU/ml) ⁴	-	+
WCFS1(pIL252) (>10 ⁵ CFU/ml) ⁴	+	+
WCFS1(pIL252) (<10 ⁵ CFU/ml) ⁴	-	+

¹For a description of the strains, see Table 1. ² Bacteriocin activity was assayed by the agar spot-on-lawn method using cell free supernatants (CFS) from MRS broth cultures of the *L. plantarum* strains obtained at the late exponential phase of growth, i.e. an O.D._{600nm} of 2.0. ³ Bacteriocin activity of isolated colonies of the *L. plantarum* strains on solid medium. *Pediococcus pentosaceus* FBB63 was used as the indicator strain. +: bacteriocin activity; -: no bacteriocin activity. ⁴Inoculum size for the broth culture used to obtain the corresponding CFS.

Figure 1, Maldonado-Barragán, Ruiz-Barba, and Jiménez-Díaz