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

Antonio Maldonado-Barragán, José Luis Ruiz-Barba*, and Rufino Jiménez-Díaz

Departamento de Biotecnología de Alimentos, Instituto de la Grasa, Consejo Superior de Investigaciones Científicas, Avda. Padre García Tejero, 4; Aptdo. 1078; 41012 Seville, Spain

*For correspondence: Departamento de Biotecnología de Alimentos, Instituto de la Grasa (CSIC), Avda. Padre García Tejero, 4, Aptdo. 1078, 41012 Seville, Spain.

E-mail: jlruiz@cica.es.

Tel.: +34 54 69 08 50. Fax: +34 54 69 12 62.

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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.

92 In previous works, we have shown that *L. plantarum* NC8 is unable to produce
93 bacteriocins when inoculated as a pure culture in liquid medium, regardless of the
94 inoculum size and growth conditions (Maldonado et al., 2003 and 2004a). However,
95 coculture of *L. plantarum* NC8 with specific Gram-positive bacteria or the addition of
96 its specific autoinducer peptide PLNC8IF to broth cultures resulted in bacteriocin
97 production by this strain (Maldonado et al., 2003, 2004a and 2004b). Moreover,
98 addition of PLNC8IF induced not only the expression of the genes encoding the three
99 two-peptide bacteriocins identified in NC8 (plantaricins NC8, EF and JK), but also the
100 *plNC8If-plNC8Hk-plnD* regulatory operon (Fig. 1A), thereby demonstrating
101 autoinduction (Maldonado et al., 2004b). In contrast, we observed that isolated colonies
102 of *L. plantarum* NC8 growing on MRS agar always showed bacteriocin activity, thus
103 indicating that bacteriocin production on solid medium appears as a constitutive trait.
104 Here we report that constitutive bacteriocin production by *L. plantarum* NC8 on
105 solid medium is only apparent and it is in fact regulated by the same autoinduction
106 mechanism as in broth cultures, i.e. requiring the expression of the operon encoding the
107 three-component regulatory system *plNC8If-plNC8Hk-plnD*. Construction of a
108 knockout (KO) for this operon in *L. plantarum* NC8 has demonstrated that functionality
109 of such operon is fully indispensable for bacteriocin production both on solid and in
110 liquid media. In addition, we have studied bacteriocin production by *L. plantarum*
111 WCFS1, a strain from human origin whose complete genome sequence has been
112 recently reported (Kleerebezem et al., 2003). This strain has a plantaricin biosynthesis
113 cluster containing a regulatory operon (*plnABCD*) encoding an atypical three-
114 component regulatory system consisting of an AIP (plantaricin A [PlnA]), a histidine
115 kinase (PlnB) and two response regulators (PlnC and PlnD) (Fig. 1B). This plantaricin
116 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 30ºC. 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 30ºC. *L. lactis* MG1363 was grown in M17 broth (Oxoid) plus 1% (wt/vol) glucose (GM17), at 30ºC. *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 37ºC 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 erytromycin per ml, respectively, 16 µl of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-

142 galactopyranoside) (50 mg/ml, Promega) per plate, and 4 μl of IPTG (isopropyl β-D-
143 thiogalactoside) (200 mg/ml; Gibco BRL) per plate.
144
145 *2.2. Bacteriocin and autoinduction assays.*
146
147 To check for bacteriocin production by isolated colonies, the direct method
148 described by Tagg et al. (1976) was used. Briefly, overnight cultures of *L. plantarum*
149 NC8 or WCFS1 were serially diluted in sterile saline and plated onto MRS agar plates
150 to obtain ca. 30 colonies per plate. Plates were incubated at 30ºC for 24 h and overlaid
151 with 4.5 ml soft agar inoculated with ca. 10^5 CFU/ml of the selected indicator bacterial
152 strains shown in Table 1. The appropriate culture medium was used to make the soft
153 agar according to the indicator bacteria to be used. Plates were further incubated at 30ºC
154 for 24 to 48 h and examined for clear halos in the lawns of the indicator bacteria around
155 isolated colonies, indicating bacteriocin activity. To check for bacteriocin production in
156 broth cultures, cells of *L. plantarum* NC8 or WCFS1 from either 24 to 72-h-old colonies
157 on MRS agar plates or overnight broth cultures were inoculated into fresh MRS broth at
158 inoculum sizes ranging from 10^2 to 10^8 CFU/ml. Cultures were incubated at 30ºC and
159 samples were withdrawn at the late exponential phase of growth, i.e. $O.D._{600nm}$ of 2.0,
160 centrifuged and the CFSs checked for bacteriocin activity by the spot-on-lawn method
161 as described previously (Jiménez-Díaz et al., 1993), using *P. pentosaceus* FBB63 as the
162 indicator strain. For the autoinduction experiments, as a source of the autoinducer
163 peptide PLNC8IF we used a semi-purified sample of this peptide obtained from a CFS
164 of *L. lactis* MG1363(pSIG308), a recombinant strain which expresses PLNC8IF
165 heterologously (Maldonado et al., 2004b). For this purpose, a 2-litre culture of this
166 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.* 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. 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

191 cultures. These plates were incubated for 24 h at 30 ºC and overlaid with 4.5 ml of MRS
192 soft-agar containing ca. 10^5 CFU/ml of the indicator strain *P. pentosaceus* FBB63.
193
194 *2.3. DNA isolation and transformation procedures*
195
196 Total genomic DNA from wild-type and derivative *L. plantarum* strains was
197 isolated by the method of Cathcart (1995). Plasmid DNA from *E. coli* was extracted as
198 described previously (Sambrook et al., 1989). Electroporation of *L. plantarum* NC8 and
199 WCFS1 was carried out according to the method of Aukrust and Blom (1992). *E. coli*
200 DH5α was electroporated by the method of Dower et al. (1998).
201
202 *2.4. Southern blot and hybridization*
203
204 Genomic DNA from *L. plantarum* was digested with *Xma*I, the resulting
205 fragments were electrophoretically separated by size on a 0.7 % agarose gel and then
206 blotted onto a Genebind 45 nylon membrane (Amersham). The *ermAM* gene harboured
207 by pIL252 was amplified by PCR as described below, labelled with fluorescein-11-
208 dUTP in the same reaction and used as a probe in Southern hybridization experiments.
209 Hybridization, washing and detection were performed using the ECL Labelling and
210 Detection System in the conditions recommended by the manufacturer (Amersham).
211
212 *2.5. Oligonucleotides, PCR and DNA sequencing*
213
214 Oligonucleotides used as primers in PCR reactions (Table 2) were synthesized
215 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-*Eco*RI and erm-*Hind*III 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, *Eco*RI, *Hind*III and *Sal*I 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-µl reaction mixtures containing 2.5 mM $MgCl_2$, 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 with a GeneAmp® PCR System 2400 thermal cycler (Perkin-Elmer). Amplification included denaturation at 94ºC for 4 min, followed by 30 cycles of denaturation at 94ºC for 30 sec, annealing at 60ºC for 1 min, and polymerization at 72ºC 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

241 extractions from *L. plantarum* and *E. coli* colonies to be used as the template for PCR
242 were carried out according to the method of Ruiz-Barba et al. (2005).
243 DNA sequencing was performed by the Servicio de Secuenciación Automática
244 de DNA (SSAD), CIB-CSIC, Madrid, Spain, with an ABI PRISM 377 DNA sequencer
245 (Applied Biosystems, Perkin-Elmer).
246
247 *2.6. Construction of a plNC8If-plNC8Hk-plnD knockout mutant of L. plantarum NC8*
248
249 Restriction enzymes, T4 DNA ligase, and other DNA-modifying enzymes were
250 used as recommended by the manufacturer (Boehringer Mannheim). To delete the
251 regulatory operon *plNC8If-plNC8Hk-plnD* from the chromosome of *L. plantarum* NC8
252 by homologous recombination, plasmid pSIG316 (Table 2) was constructed. This
253 suicide plasmid, which is unable to replicate in Gram-positive bacteria, contains a 906-
254 bp sequence carrying the *plnP* gene and a 1,034-bp sequence carrying the *plnI* gene
255 (Fig. 2). Both genes, which are flanking the *ermAM* gene, served as homologous DNA
256 for allelic exchange of the regulatory operon *plNC8If-plNC8Hk-plnD* from the
257 chromosome of *L. plantarum* NC8 with the *ermAM* gene of pSIG316 (Fig. 2A). The
258 plasmid pSIG316 was introduced by electroporation in *L. plantarum* NC8 in its native
259 circular state or previously linearized with *Sac*I or *Xba*I. KO mutants were selected by
260 plating out appropriate dilutions on MRS agar plus erythromycin. Erm^R colonies were
261 further analyzed by PCR and phenotypically characterized as described below.
262
263 *2.7. Construction of a plnABCD knockout mutant of L. plantarum WCFS1*
264

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

290 production in WCFS1 was dependent on the inoculum size. We observed that the
291 threshold concentration was ca. 10^5 CFU/ml. Hence, when the WCFS1 strain was
292 inoculated in MRS broth below this concentration, it lost its ability to produce
293 bacteriocins (Table 3). In addition, when these non-bacteriocin producing (Bac^-)
294 cultures were used to inoculate fresh MRS broth, they remained Bac^- independently of
295 the inoculum size, indicating that the autoinducing circuit had been switched off. The
296 Bac^+ phenotype of these broth cultures could only be restored by the addition of the
297 autoinducer peptide PlnA (Table 3). MALDI-TOF mass spectrometry analysis of a
298 partially purified, Bac^+ *L. plantarum* WCFS1 CFS showed a peak corresponding to
299 PlnA (not shown). These results indicate the existence of a functional autoinduction
300 mechanism which is responsible for bacteriocin production in the WCFS1 strain, which
301 is driven by the autoinducing peptide PlnA, as it has been described for *L. plantarum*
302 C11 (Diep et al., 1994 and 1996). However, in contrast to the C11 strain, we observed
303 that the Bac^+ phenotype in WCFS1 could also be restored by plating out a Bac^- broth
304 culture onto solid medium to obtain isolated colonies and reinoculating these colonies
305 into fresh broth at a concentration above the mentioned threshold (10^5 CFU/ml).
306
307 *3.2. KO mutation of the plantaricin regulatory operons of L. plantarum NC8 and*
308 *WCFS1*
309
310 To gain insight into the regulation of bacteriocin production in *L. plantarum*, we
311 obtained KO mutants lacking the regulatory operons involved in bacteriocin production
312 by *L. plantarum* NC8 and WCFS1. For this purpose we constructed the suicide plasmid
313 pSIG316, which contains the genes *plnP* and *plnI* of *L. plantarum* NC8 and WCFS1
314 flanking an erythromycin resistance cassette (Fig. 2). In the wild-type NC8 and WCFS1

315 strains, *plnP* and *plnI* are located up- and downstream, respectively, of the regulatory
316 operons (Fig. 1).
317 Electroporation of *L. plantarum* NC8 with pSIG316 resulted in several Em^R
318 colonies which, after PCR analysis, showed to harbour the entire pSIG316 plasmid
319 integrated into the *L. plantarum* NC8 chromosome. This integration was the result of a
320 single crossing-over event (Campbell-type integration) between the genes *plnP* or *plnI*
321 of pSIG316 and the homologous genes in the chromosome of the NC8 strain. These
322 derivative strains were named *L. plantarum* NC8-INT. On the other hand,
323 electroporation with linearised pSIG316 resulted in one Em^R colony which, after the
324 corresponding PCR analysis, showed that the entire regulatory operon *plNC8If-*
325 *plNC8Hk-plnD* had been replaced by the erythromycin cassette from pSIG316. This KO
326 mutant strain was named *L. plantarum* NC8-KO1. This replacement was further
327 confirmed by DNA sequencing of a PCR-amplified fragment from the chromosome of
328 the NC8-KO1 mutant with the primer pair PlnM-for/PlnE-rev. Hence, homologous
329 recombination with double crossing-over (DCO) between the *plnP* and *plnI* genes from
330 the chromosome of NC8 with these genes from the suicide plasmid pSIG316 took place
331 in *L. plantarum* NC8-KO1, leading to the substitution of the regulatory operon by the
332 *ermAM* cassette (Fig. 2A). In Southern-blot experiments, the *ermAM* labelled probe
333 hybridized with a unique >15-kb *Xba*I *L. plantarum* NC8-KO1 chromosomal DNA
334 fragment, indicating the existence of just one copy of the *ermAM* gene in the mutant
335 strain (not shown). In the wild-type *L. plantarum* NC8 strain, however, no hybridization
336 was observed.
337 On the other hand, transformation of *L. plantarum* WCFS1 with intact pSIG316
338 resulted also in several Em^R colonies. PCR analysis using the primer pair PlnM-
339 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

365 manner, in control experiments with *L. plantarum* NC8 transformed with the plasmid
366 vector pIL252 the bacteriocin-production phenotype was identical to that of the wild-
367 type strain (Table 3).
368 On the other hand, in contrast to the wild-type *L. plantarum* WCFS1, *L.*
369 *plantarum* WCFS1-KO1 was unable to produce bacteriocin neither in broth nor on solid
370 medium (Table 3 and Fig. 2B). Addition of PlnA to WCFS1-KO1 broth or agar-plate
371 cultures did not induce bacteriocin production in this mutant strain either (Table 3). As
372 isolated colonies of WCFS1-KO1 did not show bacteriocin activity, this fact suggests
373 that bacteriocin production by WCFS1 on solid medium is also regulated by an
374 autoinduction mechanism as it appears to be the case in broth cultures. Morphology and
375 growth of the strain WCFS1-KO1 were virtually identical to WCFS1 wild-type. Finally,
376 *L. plantarum* WCFS1-INT strain as well as *L. plantarum* WCFS1 transformed with the
377 plasmid vector pIL252 showed the same phenotype as the wild-type strain (Table 3).
378
379 **4. Discussion**
380
381 We have shown that the discrepancy in the way *L. plantarum* NC8 produces
382 bacteriocins on agar plates (apparently constitutive) and in broth cultures (QS regulated)
383 is only apparent. Our results demonstrate that such bacteriocin production is indeed
384 regulated by QS in both situations, as demonstrated by the Bac^- phenotype of a KO
385 mutant in the bacteriocin regulatory operon both on agar-plate and broth cultures (Table
386 3). Thus, the Bac^+ phenotype could not be restored even after the addition of the
387 corresponding AIP, i.e. PLNC8IF (Table 3).
388 To assess whether these findings were applicable to other QS-regulated
389 bacteriocinogenic *L. plantarum* strains, we studied the phenotype of *L. plantarum*

390 WCFS1. For this strain, our results show that bacteriocins are produced on solid
391 medium in an apparently constitutive manner, while production in broth cultures is
392 dependent on the inoculum size (Table 3). This result disagrees with that obtained by
393 Sturme (2005), who showed that the native state of WCFS1 was Bac^- unless synthetic
394 PlnA or a PlnA-containing CFS was added to the cultures. However, these differences
395 could be due to variations in the initial inoculum size of WCFS1 used to test for
396 bacteriocin production, or the use of different, less-sensitive indicator strains. We
397 observed that highly diluted WCFS1 broth cultures (below 10^5 CFU/ml) were not able
398 to produce bacteriocins, as it had been described previously for most QS-regulated
399 class-II bacteriocins of LAB (Diep et al., 1995; Saucier et al., 1995; Eijsink et al., 1996;
400 Brurberg et al., 1997; Nilsen et al., 1998; O' Keeffe et al., 1999). Restoration of the
401 Bac^+ phenotype could only be achieved after the addition of a PlnA or by plating the
402 culture out on solid medium. These results suggest that an autoinduction mechanism for
403 bacteriocin production is also functional in WCFS1. This point has been reinforced
404 since a KO mutant in the regulatory operon *plnABCD* (*L. plantarum* WCFS1-KO1) is
405 unable to produce bacteriocin, even after the addition of PlnA (Table 3). The absence of
406 bacteriocin activity surrounding the isolated colonies of *L. plantarum* WCFS1-KO1 has
407 confirmed that on solid medium bacteriocin production by WCFS1 is also regulated by
408 an autoinduction mechanism dependent on *plnABCD* expression.
409 The fact of obtaining the same phenotype with both NC8 and WCFS1 KO-
410 mutant strains is very significative, for major differences are found between their
411 respective bacteriocin regulatory operons (Fig. 1). Apart from exhibiting different AIPs,
412 the presence in the WCFS1 strain of the regulatory operon *plnABCD* is intriguing,
413 especially because of the existence of two different RR proteins: PlnC and PlnD. Diep
414 et al. (2003) showed that PlnC and PlnD antagonize to activate or downregulate,

415 respectively, bacteriocin production in *L. plantarum* C11. However, the NC8 strain
416 manages with just PlnD. Also, the absence of the operon encoding PLNC8 and the
417 presence of the *plnN* and *plnO* genes in *L. plantarum* WCFS1 emphasize the genotypic
418 differences between both strains. Actually, some of these differences are translated to
419 the respective phenotype, for the spectrum of activity of the strain NC8 is wider than
420 that of WCFS1 (Table 3). This result is most probably due to the fact that both strains
421 produce plantaricins EF and JK, while the strain NC8 produces also plantaricin NC8.
422 Therefore, it is very probable that other LAB strains which have been described to
423 produce bacteriocins on solid but not in liquid media are in fact regulated by similar QS
424 mechanisms in both situations, regardless the specific AIP or regulatory operon used.
425 The constitutive phenotype on agar plates could be only apparent in all these cases.
426 However, the most stimulating question is why bacteriocins are produced in a
427 phenotypically constitutive manner on solid but not in liquid media. In nature, most
428 bacteria appear to thrive attached to surfaces within biofilms, where they are
429 substantially different from the same bacteria living as planktonic microorganisms
430 (Korber et al., 1995). When growing as bacterial biofilms or colonies, cells are in close
431 contact with their neighbours, thus enabling communication between them to make
432 group decisions *via* QS mechanisms (Miller and Bassler, 2001; Henke and Bassler,
433 2004). Chao and Levin (1981) pointed out that, by killing sensitive strains in a zone
434 around the bacteriocin-producing colony, they could increase the concentration of
435 resources available for themselves in a manner not possible in broth cultures. To date,
436 we do not know whether the attachment to a solid surface *per se* provokes changes in
437 the expression of relevant genes (i.e., involved in bacteriocin regulation), or it just
438 enables bacterial communication by simply limited diffusion of this AIP, as suggested
439 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, Egland 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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706 **Legends of the Figures**

707

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

717

718 **Figure 2**. Phenotypic and genetic characterization of the plantaricin regulatory operon
719 knockout mutants *L. plantarum* NC8-KO1 and *L. plantarum* WCFS1-KO1. Upper
720 panels, bacteriocin assay of isolated colonies of *L. plantarum* NC8 and *L. plantarum*
721 NC8-KO1 (panel A), and of *L. plantarum* WCFS1 and *L. plantarum* WCFS1-KO1
722 (panel B) showing the presence and absence, respectively, of inhibition halos. *P.*
723 *pentosaceus* FBB63 was used as the indicator strain. Lower panels, diagrams of the
724 homologous recombination with double crossing-over between the *plnP* [*P*] and *plnI* [*I*]
725 genes from the chromosome of *L. plantarum* NC8 and of *L. plantarum* WCFS1 with the
726 same genes from the suicidal plasmid pSIG316, leading to the substitution of the
727 plantaricin regulatory operon by the *ermAM* cassette in the mutant strains *L. plantarum*
728 NC8-KO1 and *L. plantarum* WCFS1-KO1, respectively. The arrowheads indicate the
729 position of relevant primers (see Table 2) used in the genetic characterization of the
730 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
Escherichia coli DH5α	Host strain for recombinant plasmids	Invitrogen
[1]*Lactobacillus plantarum* NC8	Em^S Bac^+; plasmid free strain isolated from grass silage; inducible plantaricin (PLNC8, PlnEF and PlnJK) producer	Shrago *et al.*, 1986 Maldonado *et al.*, 2004b
[2]*L. plantarum* WCFS1	Em^S Bac^+; isolated from human saliva; complete genome sequenced	Kleerebezem *et al.*, 2003
L. plantarum NC8-KO1	Em^R Bac^-, derivative of *L. plantarum* NC8 lacking the operon *plNC8If-plNC8Hk-plnD*	This work
L. plantarum WCFS1-KO1	Em^R Bac^-, derivative of *L. plantarum* WCFS1 lacking the operon *plnABCD*	This work
L. plantarum NC8-INT	Em^R Bac^+, derivative of *L. plantarum* NC8 having the plasmid pSIG316 integrated into the chromosome	This work
L. plantarum WCFS1-INT	Em^R Bac^+, derivative of *L. plantarum* WCFS1 having the plasmid pSIG316 integrated into the chromosome	This work
Lactococcus lactis MG1363	Indicator strain for bacteriocin activity	Maldonado *et al.*, 2004a
L. lactis MG1363 (pSIG308)	Em^R Bac^-; heterologous producer of PLNC8IF	Maldonado *et al.*, 2004b
Enterococcus faecalis CNRZ135	Indicator strain for bacteriocin activity	Maldonado *et al.*, 2004a
[3]*Lactobacillus pentosus* 128/2	Indicator strain for bacteriocin activity	Maldonado *et al.*, 2003
[3]*L. pentosus* BOM1	Indicator strain for bacteriocin activity	Maldonado *et al.*, 2004a
L. pentosus CECT4023^T	Indicator strain for bacteriocin activity; equivalent to *L. pentosus* ATCC8041	CECT
[3]*L. pentosus* LPC1	Indicator strain for bacteriocin activity	Maldonado *et al.*, 2004a
[3]*L. pentosus* LPS5	Indicator strain for bacteriocin activity	Maldonado *et al.*, 2004a
L. plantarum CECT748^T	Indicator strain for bacteriocin activity; equivalent to *L. plantarum* ATCC14917	CECT
Listeria innocua BL86/26	Indicator strain for bacteriocin activity	Maldonado *et al.*, 2004a
Pediococcus pentosaceus FBB63	Indicator strain for bacteriocin activity	Maldonado *et al.*, 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).
^{T}Type 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 *et al.*, 2004a
RR-rev	5' GAGTGAAAAGTATCGCGTTCC 3'	Maldonado *et al.*, 2004a
PlnE-rev	5' ATGCTACAGTTTGAGAAGTTACA 3'	Maldonado *et al.*, 2004a
PlnF-for	5' CTATCCGTGGATGAATCCTC 3'	Maldonado *et al.*, 2004a
PlnI-rev	5' CCCAACTCAATCACCCATTAAC 3'	Maldonado *et al.*, 2004a
PlnM-for	5' TAAACAGGTAAAGCAGGTTGG 3'	Maldonado *et al.*, 2004a
PlnP-for	5' TCTGAGCTTGTTACACCTACC 3'	Maldonado *et al.*, 2004a
EcoDEL-1	5' CGCG *GAATTC* GTCACACTATTCAATAC 3' (*EcoR*I)	This work
SalDEL-2	5' CGCG *GTCGAC* GATAGTTGGAGTAGGG 3' (*Sal* I)	This work
KpnPlnF-rev	5' CGCG *GGTACC* GGGGGAGATCAACAATTATG 3' (*Kpn*I)	This work
erm-EcoRI	5' CGCG *GAATTC* GAAACAGCAAAGAATGG 3' (*EcoR*I)	This work
erm-HindIII	5' CGCG *AAGCTT* TAGTAACGTGTAACTTTCC 3' *(Hind*III)	This work

Plasmid	Size; Marker	Features	Reference
pUC18	2.7 Kb; Ap^R	*E. coli* cloning vector	Stratagene
pBSII-KS+	2.9 Kb; Ap^R	*E. coli* 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 *Sau*3A insert obtained from pIL252 which includes the *erm*AM gene from pIL252	This laboratory (unpublished)
pSIG313	3.8 Kb; Ap^R	pBSIIKS+ containing an 0.9 kb *Bcl*I-*EcoR*I insert including the *plnP* gene of *L. plantarum* NC8	This work
pSIG314	3.8 Kb; Ap^R	pUC18 containing an 1 kb*Sal* I-*Pst*I insert including the *plnI* gene of *L. plantarum* NC8	This work
pSIG315	5.0 Kb; Ap^R Em^R	pSIG313 containing an 1.1 *EcoR*I-*Sal*I insert from pSIG227 which includes the *erm*AM gene from pIL252	This work
pSIG316	5.8 Kb; Ap^R Em^R	pSIG314 containing an 2.1 kb *Sac*I-*Sal*I insert from pSIG315 This vector harbors the *plnP-erm*AM-*plnI* gene fusion.	This work

[1]Nucleotide 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

Lactobacillus plantarum 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]	-	+

[1]For 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. [4]Inoculum size for the broth culture used to obtain the corresponding CFS.

Figure

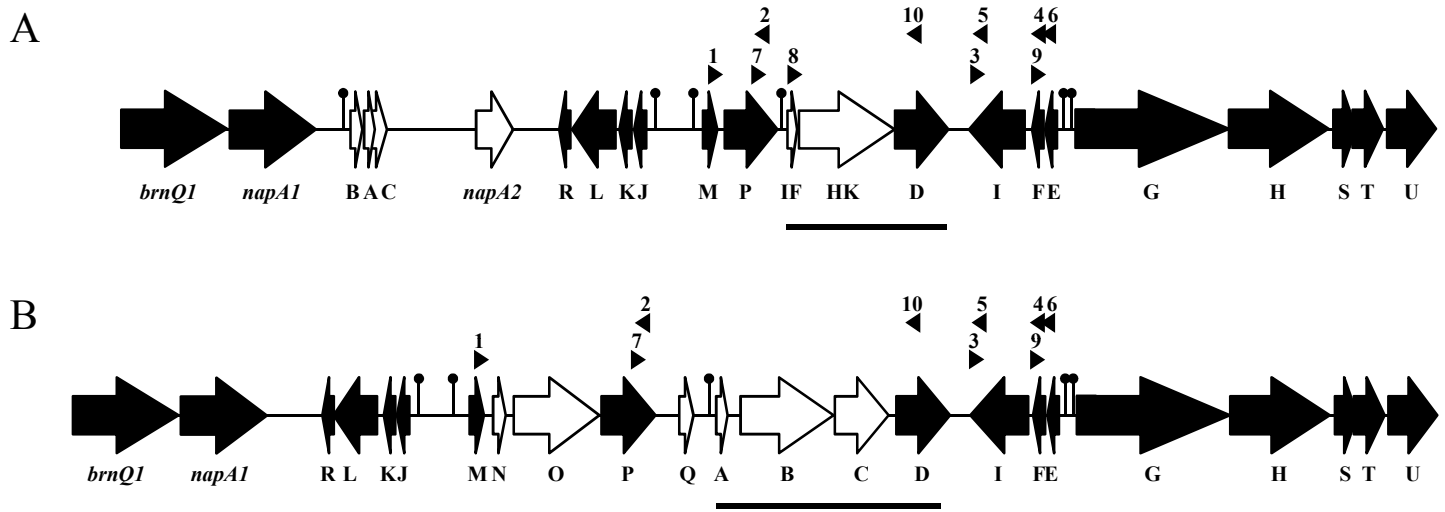

Figure 1, Maldonado-Barragán, Ruiz-Barba, and Jiménez-Díaz

Figure

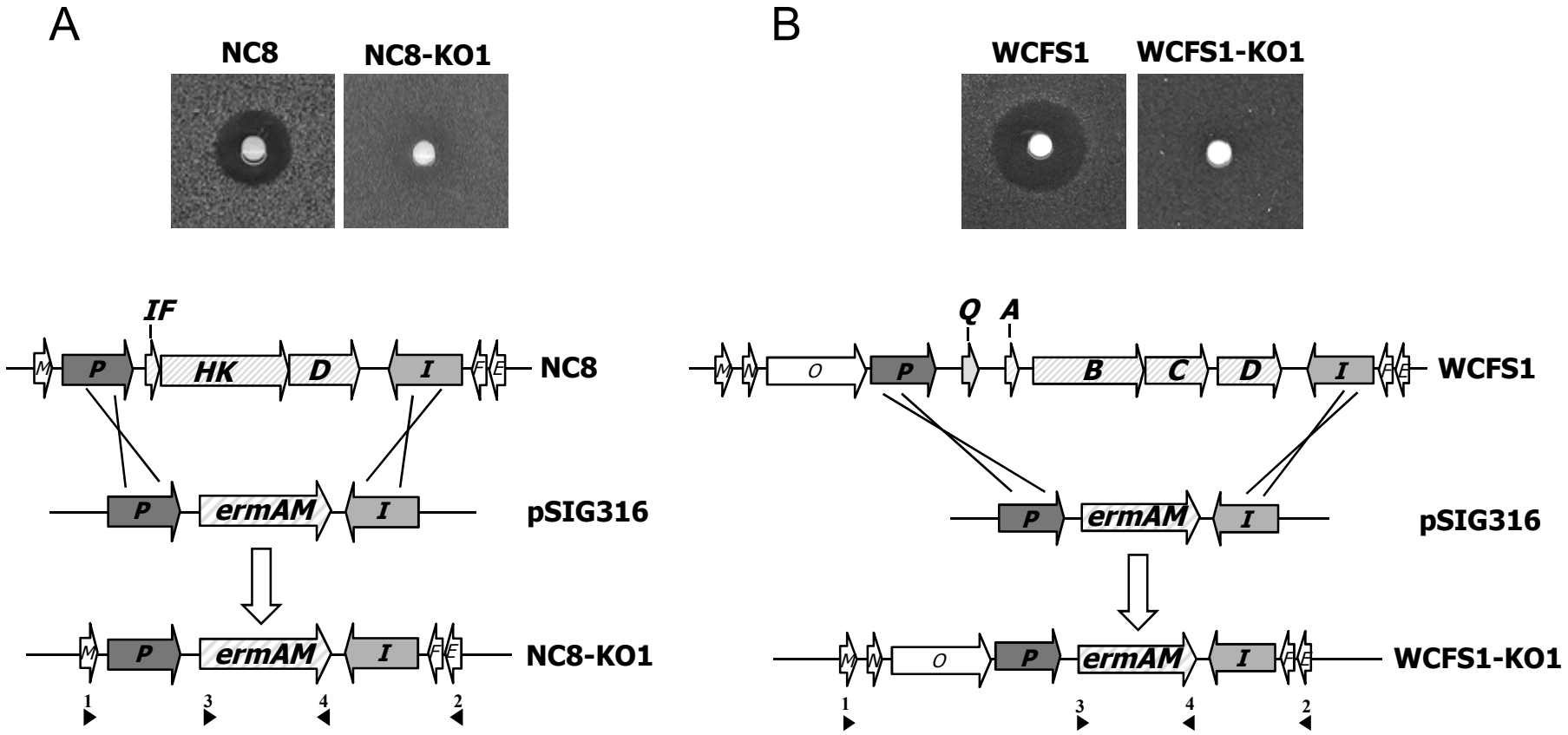

Figure 2, Maldonado-Barragán, Ruiz-Barba, and Jiménez-Díaz