Title: Enhancement of nisin production in milk by conjugal transfer of the protease-lactose plasmid pLP712 to the wild strain *Lactococcus lactis* UQ2.

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Running title: pLP712 conjugal transfer to the nisin A producer *L. lactis* UQ2

ABSTRACT

Lactococcus lactis UQ2 is a wild nisin A producer isolated from a Mexican cheese that grows poorly in milk. Conjugal matings with *L. lactis* NCDO712 to transfer the Lac+ Prt+ plasmid pLP712 and selection with nisin and lactose yielded *L. lactis* NCDO712 NisA+. Naturally rifampicin resistant *L. lactis* UQ2Rif was isolated to provide an additional selective marker. The identity of a transconjugant *L. lactis* UQ2Rif Lac+ was confirmed by RAPD-PCR fingerprinting, *nisA* PCR amplification, nisin production, presence of pLP712 and phospho-β-galactosidase activity. This strain performed well in milk and synthesized 200 IU/mL nisin, 40 times more than the original strain.

Keywords Dairy starter, Nisin, *Lactococcus lactis*, pLP712, conjugation

INTRODUCTION

Food biopreservation relies on the use of microorganisms or their metabolites to inhibit the growth of food spoilage or pathogenic microorganisms. Lactic acid bacteria (LAB) have been used for centuries in food fermentations, not only to promote flavor and texture properties, but also seeking the ability of starter-derived inhibitors to maintain microbial food safety (Stiles 1996). Many of these antimicrobial substances are thought to have potential applications as natural food preservatives. The antimicrobial peptide nisin, produced by several *Lactococcus lactis* ssp. *lactis* strains, shows inhibitory effect on spoilage and foodborne pathogenic microorganisms, it is widely used as biopreservative in the food industry and, currently, it is the only bacteriocin with a GRAS status in the USA (Federal Register 1988).

An alternative strategy to bacteriocin supplementation is the incorporation of bacteriocin-producing starter strains. This has led to enhanced protection against undesirable microorganisms in a wide variety of food matrices (reviewed by Gálvez *et al*. 2007). Furthermore, use of bacteriocin producers isolated from traditional fermented products also contributes to maintain their typical organoleptic properties, highly demanded by today´s consumer. For instance, nisin Z producing strains such as *L. lactis* ssp. *lactis* IPLA 729, isolated from a raw milk cheese, efficiently inhibited *Staphylococcus aureus* and *Clostridium tyrobutyricum* while enhancing the sensory attributes of pasteurized milk cheeses (Rilla *et al.* 2002; Rilla *et al.* 2004).

The native strain *L. lactis* UQ2 has been isolated from a fresh Mexican style cheese, made from raw milk, and has been shown to synthesize nisin A (García-Almendárez *et al.* 2008). However, *L. lactis* UQ2 growth and nisin production in milk were rather low, reaching a maximum population of 10^7 cfu/mL and producing only 3 to 5 IU/mL of nisin (García-Parra *et al*. 2009). As a result, pH was never low enough to promote casein coagulation and curd formation, preventing the use of this strain as an autochthonous nisin-producing starter for

traditional Mexican style cheese manufacture using pasteurized milk. Poor growth in milk is usually associated to the lack of the lactose fermenting ability and/or poor proteolytic activity. *L. lactis* NCDO712 contains the 56.5 kb pLP712 plasmid encoding the genes for lactose and protein utilization (Gasson 1983). This plasmid has been transferred by conjugation at relatively high frequencies to other *L. lactis* strains which were unable to utilize lactose (Lac-) or degrade casein (Prt-) from milk (O'Sullivan *et al.* 1998).

Considering that gene transfer by conjugation is generally accepted to obtain food-grade modified strains (Toomey *et al.* 2009), the objective of this study was to improve growth of *L. lactis* UQ2 and nisin production in milk by conjugal matings with the Lac+ Prt+ *L. lactis* NCDO712 in order to encourage the use of this native strain as an autochthonous starter for traditional cheese manufacture.

MATERIALS AND METHODS

Microbial strains and culture conditions

Strains used in this study are summarized in Table 1. All bacterial strains were grown at 30 ºC and stored at -80 ºC with 10% (v/v) glycerol. *Lactococcus lactis* UQ2, a native Mexican strain isolated from fresh Mexican style cheese made from unpasteurized milk, and *L. lactis* MG1614 were routinely grown in M17 broth (Oxoid, Basingstoke, England) supplemented with 0.5% (w/v) glucose (GM17). The other Lac+ *Lactococcus* strains shown in Table 1 were grown in M17 with 0.5% (w/v) lactose (LM17). *Micrococcus luteus* NCIB 8166 was grown in assay broth, containing (w/v): 1% bacteriological peptone (Oxoid), 0.3% meat extract (Bioxon, Cuautitlan, Mexico), 0.03% NaCl (Merck, Darmstadt, Germany), 0.4% yeast extract (Bioxon), 0.1% raw cane sugar (Dilis, Técnica Mexicana de Alimentación, Mexico). Lactose or glucose agar medium containing bromocresol purple dye (BCP-agar) was prepared by adding (w/v) 0.5% tryptone (Oxoid), 0.3% meat extract, 1% lactose or glucose, 0.004%

bromocresol purple (Merck) and 2% agar. When required, rifampicin or nisin were added at indicated concentrations. Rifampicin was purchased from Sigma and nisin from MP Biomedicals (Solon, OH, USA), with 2.5% (w/w) purity, where 1 μg = 1 international unit (IU). Commercial skim milk (Central Lechera Asturiana Light, Asturias, Spain) was used as model food, and was heat sterilized at 118 °C for 12 min before growth experiments.

Selection of spontaneous rifampicin-resistant *L. lactis* UQ2

One ml from an overnight culture was plated onto GM17 plates with 200 μg/mL of rifampicin, and incubated at 32°C for 24 h. Ten-fold dilutions were also plated onto GM17 without antibiotic to estimate the frequency of Rif^R mutants. The frequency was defined as the population (colony forming units (cfu) per mL) in GM17Rif divided by that in GM17. Colonies capable of growing in GM17Rif were deemed as rifampicin resistant. A single colony was further streaked onto GM17 to obtain *L. lactis* UQ2Rif.

Conjugation experiments

The direct plate conjugation technique was used. Samples of 0.5 mL of overnight cultures of *L. lactis* UQ2Rif (receptor) and *L. lactis* NCDO712 (donor) in GM17 and LM17 broths, respectively, were centrifuged. Cells were re-suspended in GM17 broth, and mixed in 1:1 (v/v) ratio in a final volume of 1 mL. This mixture (100 μL) was spread plated on GM17, and incubated at 30°C for 24 h. Cells were harvested with 2 mL of quarter-strength Ringer solution (Oxoid). Appropriate ten-fold dilutions were plated onto lactose-BCP agar with rifampicin (transconjugants selection), glucose-BCP plus rifampicin (receptor) and lactose-BCP agar (donor). Strong yellow colonies on lactose-BCP were considered as lactose fermenting strains (Lac+). Transfer frequencies were expressed as the number of transconjugants per donor cells.

115

116 **Plasmid isolation and stability of pLP712**

117 Plasmids were isolated according to O´Sullivan & Klaenhammer (1993). To evaluate the
118 stability of the lactose pLP712 plasmid, *L. lactis* UQ2Rif Lac+ was sequentially inoculated in
119 GM17 broth at 0.1% and incubated at 30°C for 16 h. After 10 and 20 passages, approximately
120 100 and 200 generations, in the absence of lactose, an average of 135 colonies were streaked
121 onto lactose-BCP and checked for their lactose fermentation ability (yellow color
122 development) after incubation for 24 h at 30ºC.

123

124 **Growth of *L. lactis* UQ2 and *L. lactis* UQ2Rif Lac+ in skim milk**

125 Overnight cultures of *L. lactis* UQ2 in GM17 and *L. lactis* UQ2Rif Lac+ in LM17 were
126 centrifuged for 2 min, resuspended in the same volume of Ringer solution, and adjusted to,
127 approximately, 3.0 x 10^9 cfu/mL. Sterile skim milk (100 mL) was inoculated at 1.5% (v/v)
128 and statically incubated at 30 °C. Ten mL samples were taken at 3 h intervals during 12 h, and
129 a final sample was taken at 24 h. Lactic acid, lactose, cell counts, pH, and nisin activity were
130 determined for every sample. Experiments were conducted in duplicate. pH was measured
131 using a MicropH 2001 pH meter (Crison, Barcelona, Spain). Lactose and lactic acid
132 concentrations were determined by HPLC as described by Cárcoba *et al.* (2000).

133

134 **Detection and quantification of nisin**

135 Nisin was extracted from milk samples by mixing with HCl 0.02 N in a 1:1 (v/v) ratio. The
136 mixture was then centrifuged at 10,000 x *g* for 10 min at 4°C. The supernatant was boiled for
137 5 min, followed by centrifugation for 2 min, and the resulting supernatant was adjusted to pH
138 6.5, with NaOH 0.1 N, and filter-sterilized using 0.45 µm pore size Millipore (Ireland)
139 membranes. Nisin concentration (IU/mL) was determined according to the British Standard

4020 (BS 1974), using *M. luteus* NCIB8166 as indicator strain. A standard curve using nisin at 0, 5, 10, 25, 50, 100, and 200 IU/mL, dissolved in skim milk and extracted as described above, established a linear relationship between log (nisin concentration) and diameter of the inhibition halo (in mm) with a determination coefficient (R^2) of 0.95. For quick detection of nisin production, transconjugant cells were replicated onto GM17 plates freshly inoculated with 10^5 cfu/mL of *L. lactis* MG1614. Inhibition halos surrounding the colony were observed after overnight incubation at 30ºC.

PCR

DNA extracts were obtained from fresh single colonies (Ruiz-Barba *et al.* 2005). PCR was carried out with PuRe Taq Ready-to-go PCR Beads (GE Healthcare, Buckinghamshire, UK). The forward *nis*A-F (5' GAGTACAAAAGATTTTAACTTGGATTTGG 3') and the reverse *nis*A-R (5' TTGGTTATTTGCTTACGTGAATACTAC 3') primers were used to amplify the *nis*A gene. An initial denaturation step at 94 ºC/5 min was followed by 30 cycles of 94 ºC/0.5 min, 58 ºC/0.5 min and 72 ºC/0.5 min and a final extension step at 72 °C for 10 min. Random Amplification of Polymorphic DNA (RAPD-PCR) was carried out with the primer OPL5 (5' ACGCAGGCAC 3') with the following conditions: one cycle of 94 °C/3 min, 15 cycles of 94 °C/0.5 min, 30 °C/0.5 min, 72 °C/1 min, and 25 cycles of 94 °C/0.5 min, 30 °C/0.5 min, 72 °C/1 minplus 15 s increment per cycle. PCR products were resolved by electrophoresis in 2% (w/v) agarose gels.

Phospho-β-galactosidase activity

Protein extracts were obtained from mid-log cultures of *L. lactis* UQ2, *L. lactis* UQ2Rif Lac+ and *L. lactis* NCDO712. After centrifugation at 10,000 x *g* for 15 min, cells were washed using Z-buffer (100 mM sodium phosphate buffer, pH 6.8, 10 mM KCl, and 1 mM

MgSO4·7H_2O) and re-suspended in 1/10 of the initial volume. Cells were broken by two passages in a one-shot cell disruptor (Constant Systems, UK) at 255 Mpa. The cell lysate was centrifuged for 10 min at 12,000 x *g* to remove cell debris and unbroken cells. Protein concentration was determined using the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA), and bovine serum albumin as standard. The enzymatic reaction was conducted in microtiter plates using 50 µL of the protein extract (or dilutions in Z-buffer) and *o*-nitrophenyl-galactopyranoside phosphate (ONPG-P) (Sigma) at 0.5 mM final concentration, in a total volume of 200 µL. The reaction was incubated at 37°C, and absorbance (A) was measured at 420 nm for 15 min, in a Benchmark Plus microplate reader (BioRad). Specific activity was calculated as ΔA_{420}/[(min) (µg protein)]. Measurements for each strain were carried out in triplicate.

RESULTS

Growth of *L. lactis* UQ2 in milk supplemented with glucose and/or yeast extract

We initially proceeded to determine the reason behind the low performance of *L. lactis* UQ2 in milk previously reported (García-Parra *et al*. 2009). Poor growth in milk seemed to be mainly linked to the lack of lactose fermenting ability as well as to low proteolytic activity, as shown in Table 2. Compared to the control milk, and milk supplemented with either glucose or yeast extract, cultures with both supplements yielded higher nisin activity and lowered the pH low enough to coagulate milk. Since both metabolic activities are encoded in the plasmid pLP712, we attempted to transfer this plasmid by conjugation to *L. lactis* UQ2 in order to improve growth performance in milk and, presumably, reach higher nisin production levels.

Conjugal matings

Prior to proceeding with conjugal matings, the nisin susceptibility of the donor strain *L. lactis* NCDO712 was determined to establish the suitable conditions to select *L. lactis* UQ2 Lac+ transconjugants and inhibit growth of the donor cells. A total of 4.8 x 10^7 cfu from an overnight culture of *L. lactis* NCDO712 were plated onto LM17 agar plates containing increasing nisin concentrations of 0, 5, 10, 20, and 40 IU/mL. After overnight incubation at 30ºC, only 0.01% of the cells survived in the presence of 20 IU/mL, while complete inhibition was observed at 40 UI/ mL of nisin (data not shown). As expected, this nisin concentration did not have any inhibitory effect on the NisA+ recipient strain *L. lactis* UQ2 and, therefore, 40 IU/mL of nisin were added to the selection plates. Initial conjugation experiments using lactose-BCP plus nisin yielded Lac+ NisR transconjugants at frequencies of 1.1 x 10^{-6} per donor cells. Inhibition zones on the nisin sensitive *L. lactis* MG1614 were detected with 225 colonies, indicating the likely production of nisin by these transconjugants. Thirty three of these colonies were randomly selected and presence of the *nisA* gen was confirmed by PCR. However, according to the band pattern obtained by RAPD-PCR, these transconjugant colonies were identified as *L. lactis* NCDO712 (Figure1 and data not shown). Therefore, instead of transferring the Lac+ phenotype to *L. lactis* UQ2, it was *L. lactis* NCDO712 which acquired the Nis+ phenotype.

To have an additional selection marker and avoid the recurrent isolation of *L. lactis* NCDO712 Nis+, a spontaneous rifampicin resistant (Rif^R) *L. lactis* UQ2Rif was selected to provide an additional selection marker for *L. lactis* UQ2 transconjugants. Rif^R mutants were isolated at a frequency of 6.6 x 10^{-5}. A single colony was randomly selected and used as receptor in conjugal matings. The selection was performed in the presence of lactose plus 100 µg/mL rifampicin. A frequency of Lac+ Rif^R transconjugants of 9.6 x 10^{-10} per donor cell was obtained. Control plates with only *L. lactis* NCDO712 donor cells yielded no colonies at all

(data not shown). Therefore, we presumed that all the Lac+ colonies on the selection plates should be *L. lactis* UQ2 transconjugants.

Molecular characterization of *L. lactis* UQ2Rif Lac+ transconjugant

A single colony from the selection lactose-BCP rifampicin plates was colony purified on LM17. RAPD-PCR fingerprinting demonstrated the identity of this transconjugant as *L. lactis* UQ2Rif Lac+ with a nearly identical band profile to the parental strain *L. lactis* UQ2 (Figure 1a). There was only an extra DNA band, also present in *L. lactis* NCDO712, which might be associated to the newly acquired DNA. The presence of the *nisA* gene was confirmed by PCR as well (Figure 1b). An additional plasmid band corresponding to the expected size of pLP712 was also present in the transconjugant *L. lactis* UQ2Rif Lac+ confirming the conjugation event (Figure 1c).

The plasmid pLP712 was relatively stable in *L. lactis* UQ2Rif. After 100 generations growing in glucose, approximately 14% of the cells had lost the Lac+ phenotype. However, after 200 generations only 3% retained the lactose fermenting ability (data not shown).

The presence and functionality of the phospho-ß-galactosidase gene (*lacG)* encoded by the lactose plasmid pLP712 of *L. lactis* NCDO712 (Maeda and Gasson 1985) in *L. lactis* UQ2Rif Lac+ was also tested. Phospho-ß-galactosidase activity from both *L. lactis* UQ2Rif Lac+ and *L. lactis* NCDO712 cell free extracts was very similar, as shown in Table 3, while the activity of *L. lactis* UQ2 was undetectable.

Growth performance of ***L. lactis*** UQ2Rif **and the transconjugant *L. lactis* UQ2Rif Lac+ in milk**

To assess if the newly lactose fermenting ability acquired by the transconjungant *L. lactis* UQ2Rif Lac+ implied a better growth performance in milk than its parent, viable counts, pH,

lactose consumption and lactic acid production in milk were monitored and displayed in Figure 2. Both strains reached the stationary phase after 12 h of incubation at 30 ºC. Notably, the Lac+ strain population (1.6 x 10^9 cfu/mL) doubled that of the native *L. lactis* UQ2Rif (8 x 10^8 cfu/mL) (Figure 2a). Lactose consumption, as well as the concomitant increase of lactic acid, was only detected in the *L. lactis* UQ2Rif Lac+ cultures (Figure 2b). Accordingly, the fermented milk reached a pH of 4.75 after 12 h, while hardly any pH decrease was recorded in the *L. lactis* UQ2Rif cultures (Figure 2a). These results further confirmed that the lack of lactose fermenting ability and the low protease activity of *L lactis* UQ2 clearly hindered proper performance in milk.

Nisin production in milk

The presence of nisin was also followed along the growth of *L. lactis* UQ2Rif Lac+ in milk and compared to the parental strain *L. lactis* UQ2Rif as shown in Figure 3. Maximal nisin production was detected at the beginning of the stationary phase and was kept during 24 h in both strains. However, nearly 200 IU/mL of nisin were synthesized by the transconjugant in contrast to 5-7 IU/mL obtained by *L. lactis* UQ2Rif. Therefore, nisin production was improved by a factor of 40.

DISCUSSION

Lactose and casein are, respectively, the main carbon and nitrogen sources present in milk. Hence, the ability to ferment lactose and degrade casein are two essential attributes of dairy cheese starters in order to produce enough lactic acid and reach the pH that leads to milk clotting. Our preliminary growth experiments in milk clearly indicated that the nisin producing *L. lactis* UQ2 strain was unable to metabolize lactose and had a low proteinase activity. The pH hardly changed unless milk was supplemented with glucose and yeast

264 extract. The Lac- phenotype was further confirmed by the absence of phospho-ß-galactosidase
265 activity in *L. lactis* UQ2 cell free extracts. On the other hand, based on the fact that nisin
266 production is growth-associated (De Vuyst and Vandamme 1992), it could be anticipated that
267 poor growth in milk would result in low nisin production by *L. lactis* UQ2. According to this,
268 milk supplementation also increased nisin activity. These results indicated that in order to use
269 this strain as an autochthonous nisin producing starter, metabolic traits such as lactose
270 catabolism and proteolytic activity had to be gained.

271 An alternative to improve growth of *L. lactis* UQ2 in milk was to transfer the plasmid
272 pLP712 by conjugation. The presence of this plasmid was already proved to be sufficient for
273 proper growth of *L. lactis* in milk (Gasson 1983), and conjugal transfer of this plasmid has
274 already been shown, although at low frequencies (Gasson and Davies 1980; O'Sullivan et al.,
275 1998). Moreover, conjugation is a food-grade event accepted to genetically enhance starter
276 strains used in the food industry. The first conjugation matings yielded, at least at higher
277 frequency, *L. lactis* NCDO712 Nis+ when using nisin and lactose as selection markers. These
278 transconjugants were able to grow in the presence of 40 IU/mL of nisin which was proved to
279 be inhibitory for the donor strain. They also produced inhibition halos on *L. lactis* MG1614,
280 the *nisA* gene was amplified by PCR and the RAPD-PCR profile was the same as *L. lactis*
281 NCDO712. These results revealed *L. lactis* UQ2 as a good donor of the nisin biosynthesis
282 machinery. The nisin A cluster is located in a 70 kb transposon, which belongs to conjugative
283 class I (Rauch and de Vos 1992; Rauch *et al*. 1994). Conjugation frequency of the nisin
284 cluster from *L. lactis* UQ2 was 10^{-6}, which is similar to that previously reported (Blaiotta *et*
285 *al*. 2000; Broadbent and Kondo 1991). These authors accomplished nisin transfer from *L.*
286 *lactis* ssp. *lactis* ATCC11454 to other *L. lactis* strains, at a frequency ranging 10^{-9}-10^{-6}.
287 Nevertheless, results may also vary depending on the mating technique (Toomey *et al*. 2009).

Since the use of nisin as selection marker was not effective, spontaneous mutants resistant to rifampicin were selected. Resistance to rifampicin is due to point mutations in *rpoB*, the gene that codes for the β subunit of the RNA polymerase (Telenti *et al*. 1993), and the possibility of horizontal transmission is very low. Nevertheless, this resistance could preclude the use of this strain for certain food applications. The use of rifampicin in the selection plates facilitated the isolation of the *L. lactis* UQ2Rif Lac+ transconjugant. The conjugation frequency of the plasmid pLP712 (10^{-9}) was very low compared to that reported for the pLP712 plasmid using other *Lactococcus* strains as recipients (Gasson and Davies 1980). Among all the variables involved in efficient DNA transfer, it should be considered that *L. lactis* UQ2 is a wild isolate that carries several cryptic plasmids which may interfere with pLP712 replication, unless there is a strong selection pressure. In fact, the plasmid was lost in most of the cells after 200 generations in the absence of lactose as a carbon source.

Transfer of pLP712 resulted in the strain *L. lactis* UQ2Rif Lac+ able to multiply efficiently in milk, decreasing the pH to values resembling those reported by other *L. lactis* used as starter cultures in the manufacture of traditional Afuega'l Pitu cheese (Cárcoba *et al*. 2000). Low pH occurred concomitantly with lactose consumption and production of lactic acid. Growth of the parent strain *L. lactis* UQ2Rif in milk occurred likely at expense of small amounts of glucose present in milk (0.06 mg/mL). Glucose was not detected after3 h in *L. lactis* UQ2Rif cultures while glucose consumption by *L. lactis* UQ2Rif Lac+ was slower (data not shown). Nevertheless, the available glucose was clearly not enough to support high nisin production levels like those reached by the transconjugant. Our results showed that transfer of pLP712 to *L. lactis* UQ2Rif enhanced nisin production by a factor of 40. Based on the many variables involved in bacteriocin production (Parente and Ricciardi 1999), it should still be possible to further optimize and increase nisin production. Jozala *et al*. (2005) reported up to 16,320 IU/mL of nisin by *L. lactis* ATCC11454 growing in milk as substrate. This is among

313 the highest nisin levels using milk as substrate. Other strategies encompass genetic
314 engineering on the nisin biosynthetic cluster increasing the copy number of the immunity and
315 regulation genes (Kim *et al.* 1998; Cheigh *et al.* 2005).

316 Producers of traditional fresh Mexican cheese prefer the organoleptic quality of those
317 produced using raw over pasteurized milk (Renye *et al.* 2007). Fresh cheese made from
318 pasteurized milk may benefit from the use of *L. lactis* UQ2Rif Lac+ as starter or adjunct
319 culture to help preserving the traditional characteristics of raw milk cheeses, while assuring
320 the safety of these products. Therefore, this strain may have a promising future as a dairy
321 starter for traditional Mexican cheese manufacture. There are, however, other relevant
322 features which should be better characterized, such as phage resistance, production of volatile
323 compounds and the possible inhibitory effect on other dairy starters when used in mixed
324 starter cultures.

325

326 CONCLUSIONS

327 This study has shown that the low nisin production levels in milk by the wild isolate *L. lactis*
328 UQ2 was due to the lack of the lactose fermenting and protein degradation phenotype. Nisin
329 production in milk could be enhanced by improving growth performance in milk which was
330 achieved by conjugal transfer of the Lac+ Prt+ plasmid pLP712. The transconjugant was able
331 to acidify milk properly and nisin production was increased 40 times. On the other hand, *L.*
332 *lactis* UQ2 turned out to be an efficient donor of the nisin transposon and might be used to
333 transfer the NisA+ phenotype to other dairy strains of interest.

334

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339

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FIGURE LEGENDS

Figure 1. Molecular characterization of *Lactococcus lactis* strains. (a) RAPD-PCR profile. (b) *nisA* PCR amplification. (c) Plasmid profile. The arrows point to the expected 168 bp PCR product of the *nisA* gene (in b) and the conjugative plasmid pLP712 (in c). *L. lactis* UQ2 (lane 1), *L. lactis* UQ2Rif (lane 2), *L. lactis* UQ2Rif Lac+ (lane 3), *L. lactis* NCDO712 (lane 4). M: Molecular weight marker, EZ Load 500 bp (in a), EZ Load 100 bp (in b) (BioRad), and Bac-tracker supercoiled DNA ladder (in c) (Epicentre, Madison, USA).

Figure 2. Growth performance in milk at 30ºC of *Lactococcus lactis* UQ2Rif (closed symbols) and its transconjugant *L. lactis* UQ2Rif Lac+ (open symbols). (a) Colony forming units (squares) and pH evolution (circles). (b) Lactose consumption (triangles) and lactic acid production (diamonds).

Figure 3. Nisin production by *L. lactis* UQ2Rif (closed circles) and its transconjugant *L. lactis* UQ2Rif Lac+ (open circles) growing in milk at 30ºC.

434 **Table 1.** Bacterial strains used in this study.

Strain	Observations	Reference
Lactococcus lactis UQ2	Wild isolate Lac- Prt- NisA+	García-Almendárez *et al.* (2008)
L. lactis NCDO712	Lac+ Prt+, donor of pLP712	Gasson and Davies (1980)
L. lactis UQ2Rif	Spontaneous rifampicin resistant *L. lactis* UQ2	This study
L. lactis UQ2Rif Lac+	Transconjugant NisA+Lac+	This study
L. lactis MG1614	Nisin sensitive	Gasson (1983)
Micrococcus luteus NCIB8166	Indicator for nisin quantification	BS 4020 (1974)

435 NisA: nisin A production; Lac: lactose fermenting strain; Prt: proteolytic activity

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437

438 **Table 2.** Nisin production and pH of *L. lactis* UQ2 in supplemented milk incubated at 30ºC
439 for 12 h.

Skim milk (+ supplement)	pH	Inhibition halo (mm)
Control (no supplement)	6.3	ND
+ Glucose 0.5%	5.5	11
+ Yeast extract 0.25%	6.3	7
+ Glucose 0.5% + Yeast extract 0.25%	4.6	14

440 Inhibitory activity was determined by the agar diffusion test using *L. lactis* MG1614 as
441 indicator. ND: not detected.

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443

444 **Table 3.** Phospho-β-galactosidase activity of *L. lactis* UQ2, *L. lactis* UQ2Rif Lac+, and *L.*

445 *lactis* NCDO712. Mean values of three replicates ± standard deviation.

Strain	Phospho β-galactosidase activity (ΔA_{420nm}/min μg protein)
L. lactis UQ2	ND
L. lactis UQ2Rif Lac+	4.33 ± 0.78
L. lactis NCDO712	4.50 ± 1.58

446 ND: Not detected.

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449 Figure 1
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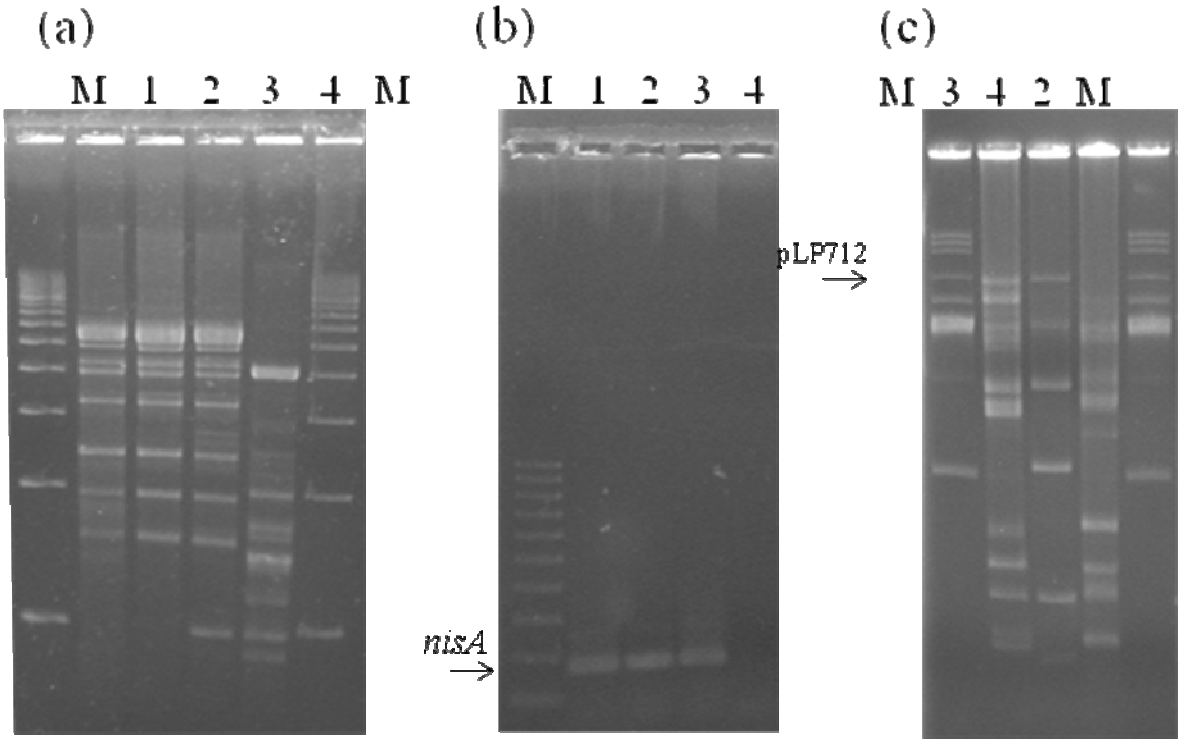

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454 Figure 2.
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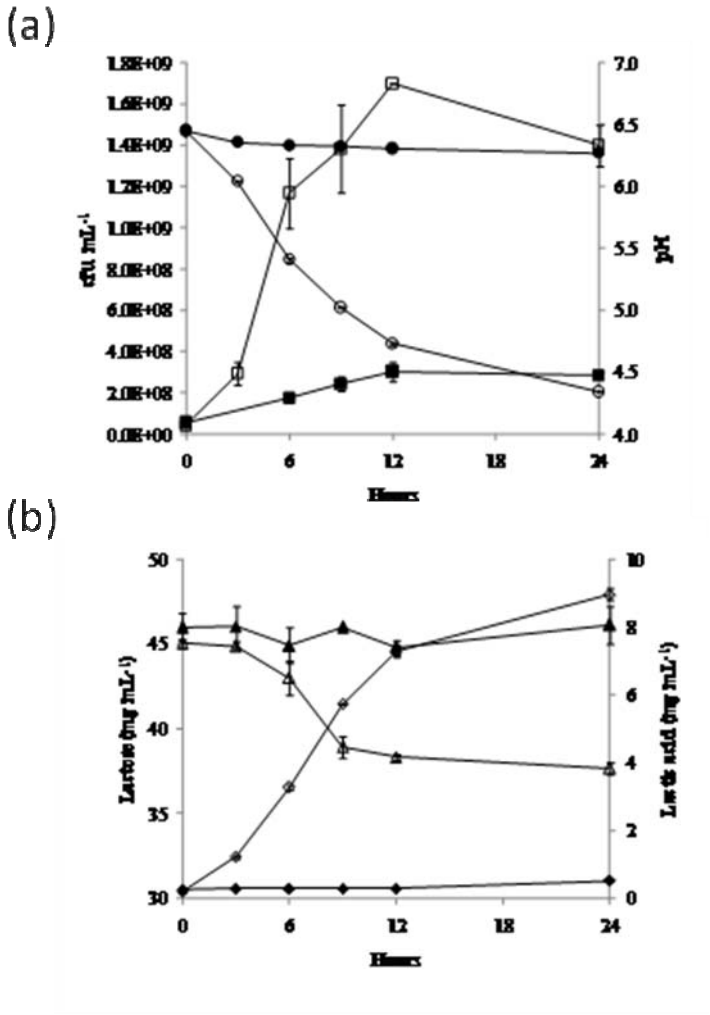

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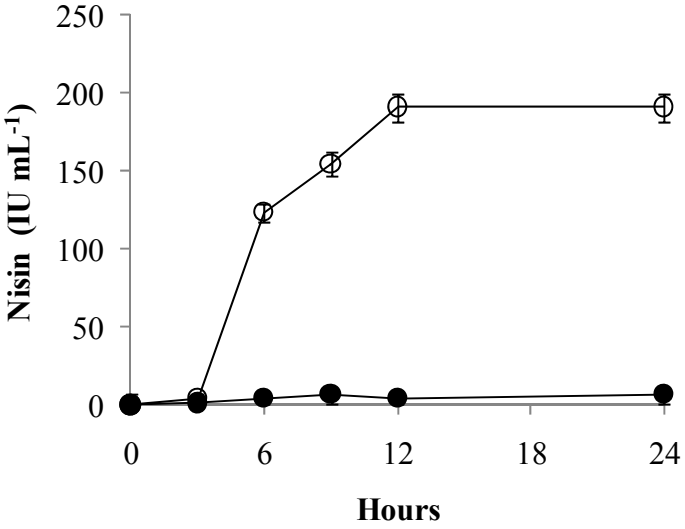

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