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Original article

Transfer by conjugation and method of selection of the food-grade large lactococcal phage resistance plasmid pPF66.0 into a commercial starter strain

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Summary — Transfer by conjugation and selection of the food-grade plasmid pPF66.0 is reported. This 66-kbp plasmid confers resistance to phages (Rap^+), to bacteriocins (Bac^R), is Tra^+ and is not involved in the secretion of bacteriocins (Bac^-). It is derived from the Bac^+-pPF66 plasmid. Following conjugation, the first step of selection was carried out using phage ∅ML19, active on industrial recipient *Lactococcus lactis* subsp *cremoris* FL388. A second step of selection was conducted with bacteriocins in order to eliminate spontaneous phage-resistant mutants.

conjugation / *Lactococcus* / selection / plasmid / phage resistance

Résumé — Transfert par conjugaison et mise au point de la méthode de sélection du plasmide de qualité alimentaire pPF66.0 dans une souche de levain industrielle. *Ce plasmide pPF66.0, d'une taille de 66 kpb, confère la résistance aux phages (Rap^+), aux bactériocines (Bac^R) et Tra^+ et n'est pas impliqué dans la sécrétion de bactériocines (Bac^-). Il est dérivé du plasmide pPF66 Bac^+. Après la conjugaison, on utilise pour la première étape de sélection le phage ∅ML19 actif sur la souche industrielle réceptrice* Lactococcus lactis *subsp* cremoris *FL388. Une seconde étape de sélection a lieu pour éliminer les mutants spontanés résistant aux phages.*

conjugaison* / Lactococcus / *sélection* / *plasmide* / *résistance aux phages

INTRODUCTION

Lactococcus lactis used in cheese production is susceptible to bacteriophage infection (Prévots et al, 1990; Jarvis et al, 1991). To prevent this problem, many authors decided to study natural lactococcal phage resistance mechanisms. Several phage-resistance genes were subcloned (Klaenhammer and Fitzgerald, 1994; Prévots et al, 1994). Introduction of these genes into industrial lactococcal strains will provide strains with increased phage resistance. Several ways to introduce genes include conjugation, transduction, transformation or protoplast fusion (Gasson and Fitzgerald, 1994). Conjugation systems in

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lactococci are very common (Gasson and Fitzgerald, 1994), but often the difficulty is to find an efficient method for selection of transconjugants. Plasmid pPF66.0 is a Bac$^-$ plasmid derived from pPF66 encoding a phage resistance (Prévots et al, 1994). In the present study, we report the transfer of this plasmid to an industrial strain, and an easy way to select the transconjugants.

MATERIALS AND METHODS

Bacterial strains and phages

All bacterial strains were *Lactococcus lactis*. The donor strain constructed and used in conjugal matings was FL401, a strain derived from S45-114-1 (Prévots et al, 1994). This strain contained pPF66.0, a Bac$^-$ plasmid spontaneously derived from pPF66, and no lactose plasmid. This strain has retained the ability to resist bacteriocins produced by S45-114-1 and phage resistance to phages ∅53 and ∅59 (respectively groups I and III; Prévots et al, 1990). The recipient industrial strain used in this study is *Lactococcus lactis* subsp *cremoris* FL388. This strain is sensitive to the phage ∅ML19 (group III of homology; Prévots et al, 1990). Two other industrial strains were used, *L lactis* subsp *cremoris* FL368 and *L lactis* subsp *lactis* var *diacetylactis* FL363. Both are sensitive to a group III phage.

Media

All strains were grown in M17 broth (Terzaghi and Sandine, 1975) at 30 °C. M17 broth was supplemented with 0.4% glucose for S45 and FL401. For phage stocks, M17 agar plates were supplemented with 10 mmol L^{-1} $CaCl_2$. Elli-1 medium was used for conjugal matings (Prévots et al, 1994). Bac-Elli-1 agar plates are Elli-1 agar plates supplemented with 2.5% bacteriocin stock (v/v) and 0.005% BCP.

Phage production and determination of bacteriophage resistance

They were performed as previously described (Trautwetter et al, 1986; Prévots et al, 1994).

Conjugal matings

Conjugal matings were performed on milk (5% w/v) agar supplemented with 0.4% glucose, as previously described (McKay et al, 1980), with modifications:

Five mL 0.4% glucose supplemented and non-supplemented M17 medium were incubated at 30 °C until OD at 600 nm reached 0.5. Volumes of 133 µL donor and 66 µL recipient strain were mixed, spread onto 10% milk 0.5% glucose agar plates, and incubated overnight at 30 °C. One mL M17 broth was spread over the surface, and the cells were harvested. 100 µL of this mixture and 100 µL of a fresh phage stock (> 10^9 pfu mL^{-1}) active on the recipient strain were gently mixed. After 10 min of incubation at 30 °C, the mixture was spread onto Elli-1 agar plates (Prévots et al, 1994) supplemented with 0.005% bromocresol purple and 10 mmol L^{-1} $CaCl_2$. Following overnight incubation at 30 °C, 1 mL M17 broth was spread over the surface, and cells were harvested. Dilutions were prepared and spread onto Bac-Elli-1 plates supplemented with 10 mmol L^{-1} $CaCl_2$. Plates were incubated at 30 °C overnight. Yellow colonies were isolated and tested.

Pulsed-field gel electrophoresis (PFGE), plasmid curing by novobiocin and bacteriocin activity assay

They were performed as described earlier (Van Belkum et al, 1989; Prévots et al, 1994).

Bacteriocin production

Bacteriocin stock was obtained as follows: 45 L M17 medium inoculated at 1% with an overnight culture of S45-114-1 were incubated overnight at 30 °C. Cells were pelleted and supernatant filtered through a 3 kDa membrane (Amicon ultrafilter ten square feet) to reduce the volume to 1.2 L. This solution was filtered through a 0.45 µm membrane to eliminate the residual cells, aliquoted and conserved at -20 °C.

Activity testing and bacteriophage screening

Basic activity test

11.5% pasteurised non-fat dry milk (NDM) was inoculated at 2% (v/v) from overnight strain

growth in M17. After incubating 18 h at 22 °C, pH was taken.

Temperature activity test

11.5% pasteurised NDM was inoculated at 1% (v/v) from overnight strain growth in M17. After incubation for 70 min at 32 °C, the culture was transferred to a 38 °C water bath for 190 min, then transferred back to 32 °C for 40 min, and finally to 4 °C; pH was then taken.

Bacteriophage screening

Above procedures were carried out through five cycles. From a phage stock, 0.2 mL (10^9 PFU mL^{-1}) was added at the beginning of each cycle, or only the first cycle. After the fifth cycle, 10^{-2}, 10^{-4} and 10^{-6} dilutions of filtered whey were spotted onto an M17-$CaCl_2$ agar plate seeded with the test strain in order to check for bacteriophage.

RESULTS AND DISCUSSION

Often, the efficiency of transfer of a large plasmid by homologous conjugation systems in lactococcal strains is very low (Prévots et al, 1994). Preliminary work showed that transfer by conjugation of pPF66 or pPF66.0 from donors S45-114-1 or FL401 to recipient *L lactis* subsp *cremoris* FL388 was, respectively, 4.0×10^{-8} and 4.4×10^{-8}. If there is a need to select, after conjugation, transconjugants with one of these plasmids, selection must be efficient to distinguish between donors and recipients.

Donor strains S45-114-1 and FL401 can be easily detected because they are Lac^-. In Elli-1 medium with BCP (Prévots et al, 1994), these strains exhibit a white colour in contrast with a Lac^+ strain, such as FL388, which exhibits a yellow colour. The distinction must be made between recipient FL388 with and without pPF66 or pPF66.0 plasmids. Strains containing pPF66 or pPF66.0 are Tra^+, Rap^+, Bac^R, and respectively Bac^+ or Bac^-. In this case, selection is only possible with bacteriocin resistance and phage resistance. A phage stock of ∅ML19, active on FL388, was used for phage selection. This industrial strain was chosen because of the large number of spontaneous phage-resistant mutants found with ∅ML19 (3.6×10^{-6}). When tested on Bac-Elli-1 agar plates, 3.2×10^{-5} spontaneous bacteriocin resistant mutants were found. When tested on Bac-Elli-1 agar plates supplemented with 10 mmol L^{-1} $CaCl_2$, no double mutant was found in the conditions of the experiments ($< 5.2 \times 10^{-9}$). The transconjugants, which are Bac^R and Rap^+, must be selected in this manner.

However, first attempts to select transconjugants after conjugation with this type of phage and bacteriocin selection were unsuccessful. Clones obtained were Bac^S and Rap^+. This is probably due to the fact that FL401 is present in the selective medium and adsorbed bacteriocins; the location of the immunity proteins, at least for the well characterised bacteriocins lactococcin A and B, is probably the cytoplasmic membrane (Van Belkum et al, 1991; Venema et al, 1993; Dodd and Gasson, 1994). To avoid this problem, it was decided to carry out a two-step selection. The first step consisted of phage selection, which resulted in an increase of phage resistant cells: spontaneous mutants and transconjugants. This step decreases the ratio of donor cells divided by phage- resistant cells. The second step involved bacteriocin selection (table I). Under these experimental conditions, 20 yellow clones were selected, reisolated and tested for the phenotypes Bac^R, Rap^+, Lac^+ together with the presence of the pPF66.0 plasmid by PFGE (fig 1). Novobiocin was used to cure pPF66.0: all clones were transconjugants. Preliminary results showed that, using the same protocol, pPF66.0 was successfully introduced into two other industrial strains, FL363 and FL368, sensitive to bacteriocins added to the solid selective medium. This demonstrated that this approach can be applied to many commercial starter strains. When conducting activity tests and measurements of growth kinetics, it was shown that all transconjugants from the four indus-

Table I. Evaluation of pPF66.0 transfer into FL388.
Évaluation du transfert du plasmide pPF66.0 dans la souche FL388.

Mating pair		*Frequency*		
Donor	*Recipient*	*Lac$^+$ Rap$^+$*	*Lac$^+$ Bac^R*	*Lac$^+$ Rap$^+$ Bac^R*
S45-114-1 (pPF66)	FL388	3.7×10^{-6}	2.7×10^{-5}	4.9×10^{-8}
FL401 (pPF66.0)	FL388	3.4×10^{-6}	3×10^{-5}	4.7×10^{-8}
	FL388	3.6×10^{-6}	3.2×10^{-5}	$< 5.2 \times 10^{-9}$

FL388 alone was used as a control. Lac$^+$: able to ferment lactose. Bac^R: immunity to bacteriocin. Rap$^+$: resistance to phages Ø53 and Ø59.

trial strains retained the industrially important traits of the recipient, such as growth rate and acid production. Following five cycles of activity tests in the presence of phage, it was shown that the level of phage was < 1 per mL.

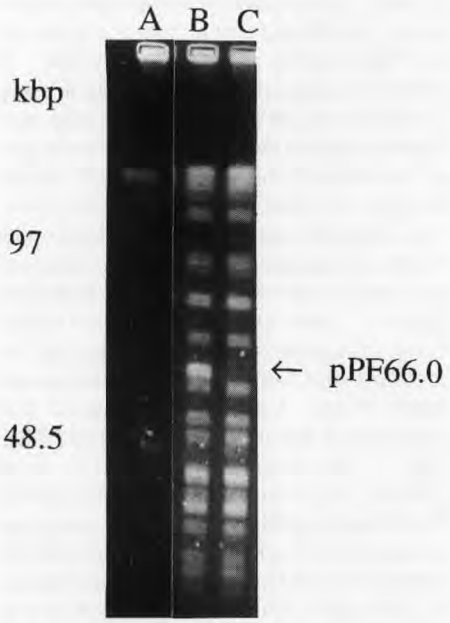

Fig 1. *Apa*I-digested genomic DNA separated by pulsed-field electrophoresis. Lanes: A, lambda DNA oligomers; B, transconjugant FL388 (pPF66.0); C, FL388.
ADN génomique digéré par ApaI *séparé par électrophorèse en champs pulsés.*

Authors reported the use of nisin and phage for selection (Harrington and Hill, 1991). However, they used two phages instead of one; under industrial conditions, infrequently is a commercial strain sensitive to two phages. Secondly, they used nisin in broth because the strains were not sensitive to nisin added to the solid selective medium. Preliminary results showed that, on 20 industrial lactococcal strains tested, 11 were sensitive to bacteriocins under the conditions of the experiments (data not shown).

Sanders et al (1986) also reported conjugal transfer of pTR2030 plasmid from a lactose-negative donor to lactose-positive recipient strains, using phage alone for selection. In this case, however, the level of phage-resistant mutants has to be lower than the level of phage-resistant colonies due to pTR2030. In the case of a high level of phage-resistant mutants, such as with FL388, this method cannot be used.

Several additional selection markers can be used with other plasmids, such as lactose-fermenting ability or nisin resistance (Murphy et al, 1988; Klaenhammer and Fitzgerald, 1994). In order to introduce a lactose plasmid to use lactose utilisation as a selection marker, it is necessary to eliminate the resident lactose plasmid of the industrial strain. In many cases, this disturbs

phenotypic characteristics of the strains (data not shown).

Another method of selection is to introduce two plasmids with two different phage resistance mechanisms: the selection can be achieved solely through the use of phage (Klaenhammer and Fitzgerald, 1994).

Using this strategy, the next step would be to integrate different phage resistance genes into pPF66.0. These complementary mechanisms would allow us to perform a single selection with phages without relying on bacteriocin selection for a large number of industrial strains.

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REFERENCES

Dodd HM, Gasson MJ (1994) Bacteriocins of lactic acid bacteria. *In: Genetics and Biotechnology of Lactic Acid Bacteria* (Gasson MJ, de Vos WM, eds) Blackie Academic & Professional, Chapman & Hall, London, 211–251

Gasson MJ, Fitzgerald GF (1994) Gene transfer systems and transposition *In: Genetics and biotechnology of lactic acid bacteria* (Gasson MJ, de Vos WM, eds), Blackie Academic & Professional, Chapman & Hall, London, 1–51

Harrington A, Hill C (1991) Construction of a bacteriophage-resistant derivative of *Lactococcus lactis* subsp *lactis* 425A by using the conjugal plasmid pNP40. *Appl Environ Microbiol* 57, 3405–3409

Jarvis AW, Fitzgerald GF, Mata M, Mercenier A, Neve H, Powell IB, Ronda C, Saxelin M, Teuber M (1991) Species and types phages of lactococcal bacteriophages. *Intervirology* 32, 2–9

Klaenhammer TR, Fitzgerald GF (1994) Bacteriophages and bacteriophage resistance *In: Genetics and Biotechnology of Lactic Acid Bacteria* (Gasson MJ, de Vos WM, eds), Blackie Academic & Professional, Chapman & Hall, London, 106–168

McKay LL, Baldwin KA, Walsh PM (1980) Conjugal transfer of genetic information in group N streptococci. *Appl Environ Microbiol* 40, 84–91

Murphy MC, Steele JL, Daly C, McKay LL (1988) Concomitant conjugal transfer of reduced-bacteriophage-sensitivity mechanisms with lactose- and sucrose-fermenting ability in lactic streptococci. *Appl Environ Microbiol* 54, 1951–1956

Prévots F, Mata M, Ritzenthaler P (1990) Taxonomic differentiation of 101 lactococcal bacteriophages and characterization of bacteriophages with unusually large genomes. *Appl Environ Microbiol* 56, 2180–2185

Prévots F, Rémy E, Mata M, Ritzenthaler P (1994) Isolation and characterization of large lactococcal phage resistance plasmids by pulsed-field gel electrophoresis. *FEMS Microbiol Lett* 117, 7–14

Sanders ME, Leonhard PJ, Sing WD, Klaenhammer TR (1986) Conjugal strategy for construction of fast acid-producing, bacteriophage-resistant lactic streptococci for use in dairy fermentations. *Appl Environ Microbiol* 52, 1001–1007

Terzaghi BE, Sandine WE (1975) Improved medium for lactic streptococci and their bacteriophages. *Appl Environ Microbiol* 29, 807–813

Trautwetter A, Ritzenthaler P, Alatossava T, Mata-Gilsinger M (1986) Physical and genetic characterization of the genome of *Lactobacillus lactis* bacteriophage LL-H. *J Virol* 59, 551–555

Van Belkum MJ, Hayema BJ, Geis A, Kok J, Venema G (1989) Cloning of two bacteriocin genes from a lactococcal bacteriocin plasmid. *Appl Environ Microbiol* 55, 1187–1191

Van Belkum MJ, Kok J, Venema G, Holo H, Nes IF, Konings WN, Abee T (1991) The bacteriocin lactococcin A specifically increases permeability of lactococcal cytoplasmic membranes in a voltage-independent, protein-mediated manner. *J Bacteriol* 173, 7934–7941

Venema K, Abee T, Haandrikman AJ, Leenhouts KJ, Kok J, Konings WN, Venema G (1993) Mode of action of lactococcin B, a thiol-activated bacteriocin from *Lactococcus lactis*. *Appl Environ Microbiol* 59, 1041-1048