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Study of two bacteriocins produced by *Enterococcus casseliflavus* and *Ent. faecalis*

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ABSTRACT

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Aims: The antimicrobial activity of two plasmid-borne bacteriocins produced by *Enterococcus casseliflavus* IM 416K1 and *Ent. faecalis* IM 388C and their mating transferability were studied.

Methods and Results: Both bacteriocins showed antibacterial activity against taxonomically related micro-organisms and *Listeria monocytogenes* but differ for heat sensitivity, antimicrobial titre, molecular size and class of affiliation. The transferability by mating of the antibacterial properties from producers to *Enterococcus faecalis* JH2-2 revealed that the bacteriocin-phenotype was linked in both strains to genes located on a 34 MDa plasmid. This result was confirmed by loss of antibacterial activity and immunity after curing treatment.

Conclusions: Restriction analysis has shown a different profile of the two conjugative plasmids. Enterocin 416K1 and Enterocin 388C could represent natural antilisterial agents to use in food technology.

Significance and Impact of the Study: The transferability of the 34 MDa conjugative plasmids might be considered a possibility for the study of bacteriocins expression in bacterial hosts different from the native strains.

Keywords: conjugation, enterocins, enterococci, *Listeria monocytogenes*, plasmids.

INTRODUCTION

Bacteriocins are ribosomally synthesized anti-microbial compounds produced by many bacterial species (Jack *et al.* 1995). Although bacteriocins may be found in numerous Gram-positive and Gram-negative bacteria, the most representative producers include members of the lactic acid bacteria (LAB) group, especially within the *Lactococcus*, *Pediococcus*, *Leuconostoc*, *Carnobacterium*, *Lactobacillus* and *Enterococcus* genera. LAB bacteriocins are commonly divided into four main groups based on observed common characteristics, mainly structural (Klaenhammer 1993). Class I bacteriocins are characterized by their unusual amino acids, such as lanthionine, methyl-lanthionine, dehydrobutyrine and dehydroalanine. These are generally small bacteriocins composed of one or two peptides of *ca* 3 kDa, recently subdivided into six subgroups (Twomey *et al.* 2002).

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Class II bacteriocins do not undergo post-translational changes, with the exception of cleavage of the leader or signal peptide and, in some cases, formation of disulfide bridges. These are generally small unmodified peptides of <5 kDa, and can be further subdivided into three groups (IIa, IIb and IIc), on the basis of their primary structure (Nes *et al.* 1996). A third class, class III, for which much less information is available, has been proposed (Klaenhammer 1993) and it includes large (>30 kDa) heat-labile antimicrobials. A fourth class contains proteins complexed with lipid or carbohydrate, but these are not well characterized (Nes *et al.* 1996).

According to many authors class IIa bacteriocins (pediocin-like peptides) are the most interesting substances to use as natural preservative antilisterial agents in food technology because they: (i) are highly active against *L. monocytogenes* (Ennahar *et al.* 2000), (ii) usually do not kill starter cultures (O'Sullivan *et al.* 2002), (iii) appear to have potential applications against other spoilage and food-borne pathogenic micro-organisms (Cintas *et al.* 1997). Although the narrow spectrum and heat-instability, limit their use as a food preservative, class III bacteriocins too may contribute

to the hosts ability to compete in complex dairy ecosystems (Rodríguez *et al.* 2003).

With regard to enterococci, bacteriocin production seems to be a common trait among strains associated with food systems and most of these substances (Enterocins), have been included in the pediocin-like peptides (Cintas *et al.* 1997). As the bacteriocin phenotype in enterococci is frequently associated with pheromone-responsive conjugative plasmids (Martínez-Bueno *et al.* 1994) their transferability in plasmid-free recipient strains could simplify the following genetical analysis and allow the study of the bacteriocin expression in hosts differing from the native micro-organism. In addition, this process could be used for the production of hybrid bacteriocins with improved properties obtained by protein engineering for an application employment.

In this work enterococci isolated from seasoned Italian sausages, were tested for bacteriocin-like substance production and the biological characteristics of the two best bacteriocins were studied. Genetic techniques were used to characterize and compare the plasmid profiles in the producers and to value the bacteriocins expression in bacterial hosts different from the native strains.

MATERIAL AND METHODS

Antibacterial activity evaluation

A total of 118 enterococci isolated from naturally fermented Italian sausages and identified on the basis of their biochemical properties (Api 20 Strep; bioMerieux, Mary-l'Etoile, France), were cultured as usual in tryptic soya broth (TSB; Oxoid, Milano, Italy) and incubated at 37°C for 24 h. Bacteriocin-like substance production (BLS) was screened by the deferred antagonism method (Kekessy and Piguet 1970), using as indicators the same enterococci. To eliminate inhibition because of hydrogen peroxide production, a first incubation was performed anaerobically.

Cell-free supernatant fluid (CFSF) of the best producers (*Ent. casseliflavus* IM 416K1 and *Ent. faecalis* IM 388C), was tested by agar well diffusion assay (Rogers and Montville 1991) against: (i) enterococci found to be particularly sensitive in the initial screening (zone of inhibition >5 mm), (ii) Gram-positive and Gram-negative belonging to different genera (Table 1). The indicator organisms were cultured in TSB at incubation temperatures of 30 or 37°C for 24 h, depending on the organisms.

The following evaluations, carried out to compare the physicochemical properties of the bacteriocins produced by *Ent. casseliflavus* IM 416K1 and *Ent. faecalis* IM 388C, were performed by the agar well diffusion assay using *L. monocytogenes* NCTC 10888 and *Ent. faecalis* IM C1, respectively, as indicators.

Sensitivity to chemicophysical parameters

To determine the effect of temperature on bacteriocin production, cultures in TSB of *Ent. casseliflavus* IM 416K1 and *Ent. faecalis* IM 388C were incubated at 18, 30, 37 and 40°C for 48 h and the bacteriocin activity of the CFSFs was assayed.

To evaluate the sensitivity to heat and protease, CFSFs were: (i) heated at 80 and 90°C for increasing periods of time (15, 30, 60 min) and autoclaved (121°C, 15 min), ii) treated with 0·1 mg ml^{-1} protease K (20 mg ml^{-1}) or 0·1 mg ml^{-1} pepsin or trypsin (all from Sigma Chemical Co, St Louis, MO, USA).

To test the stability to different pH values, CFSFs was adjusted to various pH values between 3·0 and 9·0 with 1 mol l^{-1} HCl or NaOH. After incubation at 37°C for 4 h the pH was readjusted to 6·0 and the residual antimicrobial activity determined.

To test the stability during refrigerated storage, CFSFs stored at 4°C up to 6 months, were collected at different intervals of time and the residual antibacterial activity was determined.

Antibacterial activity titration

To evaluate the minimal inhibitory concentration (MIC) of the crude bacteriocin preparation serial twofold dilutions of CFSFs were assayed for antibacterial activity. The antimicrobial titre was defined as the reciprocal of the highest dilution showing a distinct inhibition of the indicator lawn and expressed in terms of arbitrary units per millilitre (AU ml^{-1}) (Mayr-Harting *et al.* 1972).

Mode of action

To investigate the mode of action of Enterocins 416K1 and 388C the respective indicator strains, *L. monocytogenes* NCTC 10888 and *Ent. faecalis* IM C1, was grown at 37°C for 18 h in TSB. Cells were then harvested by centrifugation (10 000 *g* for 10 min at 4°C) and resuspended in a sterile 50 mmol l^{-1} phosphate buffer (pH 6·5) to give $8{\cdot}0 \times 10^8$ CFU ml^{-1}. Assays were performed by adding 320 AU ml^{-1} as final concentration of both bacteriocins to the samples, and incubating the tubes at 37°C. As control, samples without inhibitory substance were used. Sensitive cells were removed and the viable counts on tryptic soya agar plates (TSA; Oxoid, Milan, Italy) and optical density was detected over time.

Estimation of the molecular size

Cell-free supernatant fluid of both enterococci was collected by centrifugation (12 000 *g* for 30 min), dialysed against

Table 1 Antibacterial activity detected in cell-free supernatant fluid (CFSF) from *Enterococcus casseliflavus* IM 416K1 and *Ent. faecalis* IM 388C by agar well diffusion assay*

Producer	416K1	388C
Indicator strains		
Lactic acid bacteria		
Ent. faecalis IM *388* C	+++	–
Ent. faecalis IM *388* D	+++	–
Ent. faecalis IM *388* K	+++	–
Ent. faecalis IM *388* KE	+++	–
Ent. faecalis JH2-2	+	–
Ent. faecalis ATCC 29212	–	–
Ent. faecalis IM C1	–	+++
Ent. faecalis IM C2	++	++
Ent. faecalis IM C3	–	+++
Ent. faecium IM C4	++	+
Ent. casseliflavus IM 430K1	–	+++
Ent. casseliflavus IM 416K1	–	+++
Ent. casseliflavus IM C5	–	+++
Lactobacillus spp. IM (6)	–	–
Lact. plantarum IM 35D	–	–
Lact. plantarum IM 391	–	–
Lact. curvatus IM 766	–	–
Lact. fermentum IM E62	–	–
Gram-positive cocci		
Staphylococcus spp. IM (15)	–	–
Staph. aureus ATCC 25923	–	–
Staph. aureus ATCC 6538	–	–
Staph. aureus ATCC 29213	–	–
Staph. aureus IM TIEN 5	–	–
Staph. aureus IM TIEN 12	–	–
Streptococcus agalactiae IM 12	–	–
Strep. agalactiae IM 32	–	–
Gram-positive rods		
B. subtilis ATCC 44633	–	–
B. pumilus ATCC 10337	–	–
L. monocytogenes NCTC 10888 +++	+	
L. monocytogenes NCTC 10890 +++	+	
L. monocytogenes NCTC 04883 +++	–	
L. monocytogenes NCTC 07973	+++	–
L. monocytogenes NCTC 05214	++	–
L. monocytogenes NCTC 10528	++	–
L. monocytogenes NCTC 05105	++	–
L. monocytogenes IM LV1	++	–
L. monocytogenes IM LV2	++	–
L. monocytogenes IM 1	+++	–
L. monocytogenes IM 3	++	–
L. monocytogenes IM 4	+++	–
L. monocytogenes IM 5	+++	–
L. monocytogenes IM 6	+++	–
L. monocytogenes IM 9	+++	–
L. monocytogenes IM 10	+++	–
L. monocytogenes IM 12	+++	+
L. monocytogenes IM 13	++	++
L. monocytogenes IM 14	+++	+
L. ivanovii NCTC 11846	+	+
L. ivanovii NCTC 13932	+++	++
L. seeligeri NCTC 11856	+	+
L. welshimeri NCTC 11857	+	++
L. innocua NCTC 11288	+	–
Gram-negative rods		
Escherichia coli ATCC 13762	–	–
E. coli ATCC 25992	–	–
Klebsiella ozoenae IM 6	–	–
Salmonella spp. IM 300	–	–
Salmonella spp. IM 301	–	–
Yersinia enterocolitica IM 71	–	–
Y. enterocolitica IM 23	–	–
Pseudomonas aeruginosa ATCC 27853	–	–
Aeromanas hydrophila IM 24	–	–
Aer. hydrophila IM 104	–	–
Aer. hydrophila IM 106	–	–

*Isolates with the IM label were from our collection.
–, no zone of inhibition; +, 5 mm < zone < 10 mm; ++, 10 < zone < 15 mm; +++, zone > 15 mm. In parentheses are reported the number of strains used as indicators.

30 mmol l^{-1} sodium acetate buffer (pH 5·3) and filter sterilized (0·45 μm-pore-size filter; Millipore Corp, Bedfort, MA, USA). Dialysed CFSFs were filtered sequentially through 30, 10, 5 kDa mol with exclusion membranes (Diaflo Ultrafiltration Membranes; Amicon Corporation, Beverly, MA, USA). Inhibitory activity was determined in both filtrates and retentates at each stage.

Selection of bacteriocin-deficient mutants

Enterococcus casseliflavus IM 416K1 and *Ent. faecalis* IM 388C were individually inoculated in TSB with increasing concentrations (1–128 μg ml^{-1}) of curing agent (ethidium bromide; Sigma Chemical Co, St Louis, MO, USA) and incubated at 37°C for 18 h. Cultures that grew at the highest concentration of ethidium bromide were serially diluted and plated onto TSA to obtain isolated colonies (30–60 per plate). After 24 h incubation at 37°C the colonies were replica-plated on TSA, incubated at 37°C for an additional 24 h, and examined by the deferred method for bacteriocin production. Bac+ and Bac– colonies were visually differentiated according to the presence or absence of a distinct inhibition of the indicator lawn. Immunity of nonproducer variants to the antimicrobial substances was

examined by agar well diffusion assay (Rogers and Montville 1991).

Conjugation experiments

Mating was performed by the Jacob and Hobbs (1974) modified method. BLS producers, nitrofurantoin and rifampicin sensitive (Bac+, Nit^s, Rif^s), were inoculated individually in TSB with the plasmid-free recipient strain *Ent. faecalis* JH2-2 nitrofurantoin and rifampicin resistant (Bac−, Nit^r, Rif^r), using an initial ratio of one donor per nine recipients. The mixed cultures were incubated for 6 h at 37°C and added to rifampicin (100 μg ml^{-1}) and nitrofurantoin (25 μg ml^{-1}). After a further incubation (8 h at 37°C), to promote the survival of recipient and putative transconjugant cells, dilutions were plated on TSA containing the above selective agents. After 24 h of incubation at 37°C the Rif^r Nit^r colonies were replica-plated onto TSA, incubated at 37°C for 24 h, and examined for bacteriocin production by the deferred method. The putative transconjugants were scored by the presence of an inhibition zone around the colony of indicators.

Plasmid isolation

Small-scale plasmid isolation was performed as described by O'Sullivan and Klaenhammer (1993) to compare the plasmid profile of the parental strains with the Bac-derivatives after curing treatment, and with the putative transconjugants. The plasmid DNA was analysed in 0·7% agarose gel electrophoresis. Purified plasmids of *Escherichia coli* V517 (Macrina *et al.* 1978), were used as a size reference plasmid.

Restriction analysis

DNA plasmid of the transconjugants carrying the bacteriocin genetic information, was isolated by the lyses method (O'Sullivan and Klaenhammer 1993) and successively digested with *Eco*RI according to the supplier's instruction (Promega, Madison, WI, USA). Lambda phage DNA (Roche Applied Science, Indianapolis, IN, USA) digested with *Hin*dIII and *Eco*RI (Boehringer, Mannheim, Germany) was used as molecular size markers. Digests were separated by electrophoresis through 1% (w/v) agarose gel in Tris–acetate buffer at pH 8 at 30 V for 16 h.

RESULTS

Antibacterial activity evaluation

Among the strains isolated from fermented sausages and screened by the deferred antagonism method for BLS production, *Ent. casseliflavus* IM 416K1 and *Ent. faecalis* IM 388C presented the highest antibacterial activity against taxonomically related micro-organisms used as indicators (data nonreported). When the CFSF of the two producer was tested by agar well diffusion assay, the inhibitory spectra against the closely related micro-organisms were confirmed and, in addition, antagonism towards *Listeria* spp. was observed. Against *L. monocytogenes*, in particular, this activity was very strong for *Ent. casseliflavus* IM 416K1 and lower for *Ent. faecalis* IM 388C (Table 1). Enterocin 416K1 (Sabia *et al.* 2002) and Enterocin 388C were produced on solid medium and liquid culture.

Sensitivity to chemicophysical parameters

Both enterocins were sensitive to the proteolytic enzymes used and maintained their activity over a wide range of pH and during refrigerated storage up to 6 months. As concern heat treatment they showed a different sensitivity: whereas Enterocin 388C appears heat-labile, Enterocin 416K1 maintains the antibacterial activity even at autoclaving temperature (121°C for 15 min).

Antibacterial activity titration

The antibacterial activity against the respective indicators, translated as arbitrary units of inhibition (AU ml^{-1}), showed that activity was retained in 100 μl up to dilutions of 1/128 for Enterocin 416K1 and 1/62 for Enterocin 388C, corresponding to 1280 and 620 AU ml^{-1}, respectively.

Mode of action

The addition of Enterocin 416K1 and 388C to cell suspensions of indicators led to a marked decrease in the number of viable cells. In particular, as compared with controls without bacteriocin, bacterial count with Enterocin 416K1 was 1 log cycle lower after about 6 h and 3 log cycles lower after 24 h. For Enterocin 388C bacterial count was 1 log cycle lower after about 8 h and 3 log cycles lower after 16 h. This finding supports the bactericidal mode of action for both bacteriocins up to the end of exposure. No decrease in optical density of the cell suspension was observed over the experiments, indicating that activity of two bacteriocins was bactericidal without concomitant cell lyses.

Estimation of the molecular size

When CFSF of producers was filtered sequentially through a cellulose membrane with 30–10–5 kDa cut-off, the antibacterial activity was still found in both retentate and ultrafiltrate for *Ent. casseliflavus* IM 416K1 CFSF and in retentate only for *Ent. faecalis* IM 388C CFSF. This suggests that the crude activity was linked to a compound

with an apparent molecular weight smaller than 5 kDa for Enterocin 416K1 and greater than 30 kDa for Enterocin 388C.

Conjugation experiments

Transconjugants BLS producers were generated when viable cells of *Ent. casseliflavus* IM 416K1 and *Ent. faecalis* IM 388C were mated in separate experiments with the recipient *Ent. faecalis* JH2-2. No colonies were obtained in selective media with donor only, used as control. The expression of the bacteriocins in the transconjugants differed from that of the parental strains. In particular the antilisterial activity in *Ent. faecalis* JH2-416K was always overexpressed, whereas it results unchanged in *Ent. faecalis* JH2-388C.

Plasmid isolation and restriction analysis

In Fig. 1 the plasmid profiles of the original strains and the respective Bac-derivatives after curing treatment were compared. In both the original strains four plasmids with different molecular weights were present: one large plasmid of 34 MDa and three plasmids of *ca* 11, 7 and 3·3 MDa. All Bac-variants showed the loss of only the 34 MDa plasmid and were sensitive to the bacteriocin of the original strain. Therefore, bacteriocin phenotype seems to be linked to genes located on the 34 MDa plasmid for both producers.

In Fig. 2 the plasmid profile of the parental strains and the respective transconjugants were compared. Unlike the parental strains the transconjugants presented the 34 MDa plasmid only, responsible for the bacteriocin genetic information.

Figure 3 shows restriction analysis of the 34 MDa plasmid of the transconjugants. *Eco*RI cuts the two plasmids in 20 and 19 fragments, and in particular, the restriction profiles differ for the absence of the fragment of 1915 bp in *Ent. faecalis* JH2-388C.

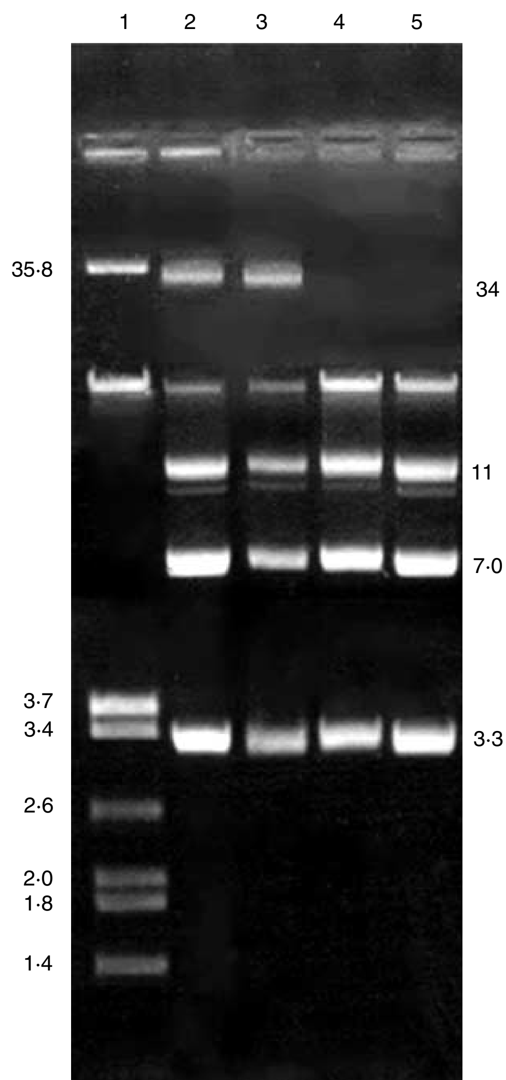

Fig. 1 Plasmid profiles of *Enterococcus casseliflavus* IM 416K1 and *Ent. faecalis* IM 388C, and respective Bac-derivatives after curing with ethidium bromide. Lane 1: molecular size markers prepared from *Escherichia coli* V517 (35·8, 3·7, 3·4, 2·6, 2, 1·8 and 1·4 MDa); lanes 2 and 3: parental strains (34, 11, 7 and 3·3 MDa.); lanes 4 and 5: variant strains *Ent. casseliflavus* IM 416K1 Bac− and *Ent. faecalis* IM 388C Bac− (11, 7 and 3·3 MDa)

DISCUSSION

In this work we studied the biological characters of two plasmid-encoded bacteriocins, Enterocin 416K1 and Enterocin 388C, produced by enterococci isolated from a same lot of Italian sausages and their transferability by mating. Both bacteriocins had antibacterial activity against taxonomically related micro-organisms and *Listeria* spp., but antagonism against *L. monocytogenes* was very strong for Enterocin 416K1 and lower for Enterocin 388C. Bacteriocins had the same bactericidal mode of action and a similar behaviour to pH and protease, but different antagonistic spectra, antibacterial activity titres, heat sensitivities and molecular sizes. According to Klaenhammer (1993), Enterocin 416K1, a small heat-stable nonlantibiobiotic bacteriocin, belongs to the class IIa pediocin-like group, and Enterocin 388C, a large heat-labile antimicrobials, to the class III bacteriocins.

Although plasmid analysis of the bacteriocin producers revealed the presence of four bands, the bacteriocin-phenotype was linked in both strains to genes located on a same plasmid, as reported for other bacteriocins (Martínez-Bueno *et al.* 1994; Cintas *et al.* 1997). The activity and immunity phenotypes in the transconjugants resulted correlated with

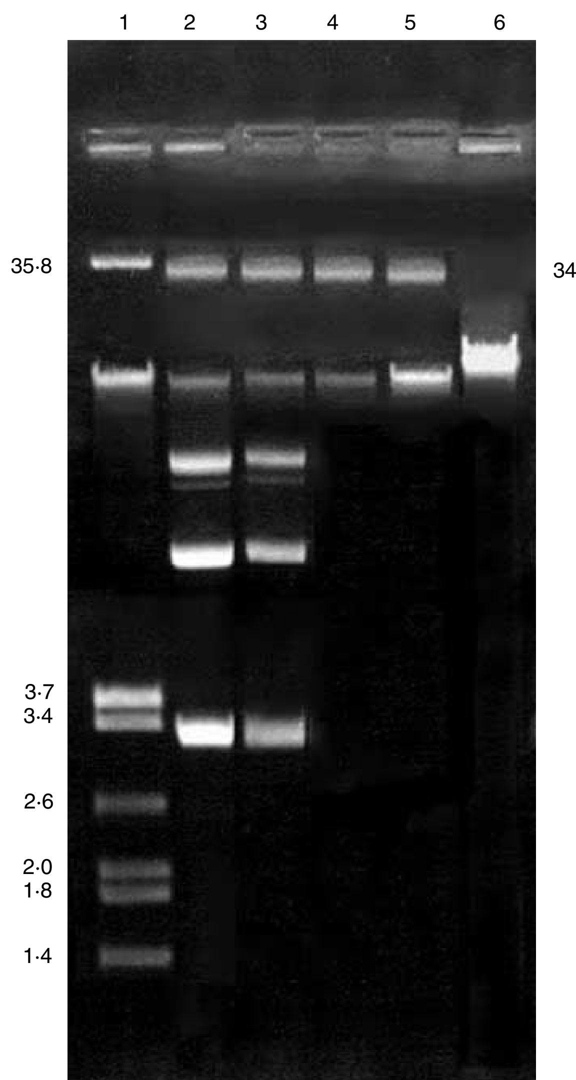

Fig. 2 Plasmid profiles of *Enterococcus casseliflavus* IM 416K1 and *Ent. faecalis* IM 388C and respective transconjugants. Lane 1: molecular size markers prepared from *Escherichia coli* V517 (35·8, 3·7, 3·4, 2·6, 2, 1·8, 1·4 MDa); lanes 2 and 3: parental strains (34, 11, 7 and 3·3 MDa.); lane 4: *Ent. faecalis* JH2-416K1 transconjugant (34 MDa); lane 5: *Ent. faecalis* JH2-388C transconjugant (34 MDa); lane 6: *Ent. faecalis* JH2-2 recipient strain

the 34 MDa plasmid, and the Bac-variants were sensitive to parental strain bacteriocins.

Restriction analysis of the 34 MDa plasmids gives different results in the transconjugants because of the lack of the 1915 bp fragment in *Ent. faecalis* JH2-388C, and this could justify the different biological properties of the two bacteriocins. As the plasmid profiles of the original strains are apparently similar, one may suppose that the extra-chromosomal equipment originates from a same parental micro-organism. The differences in biological characters that

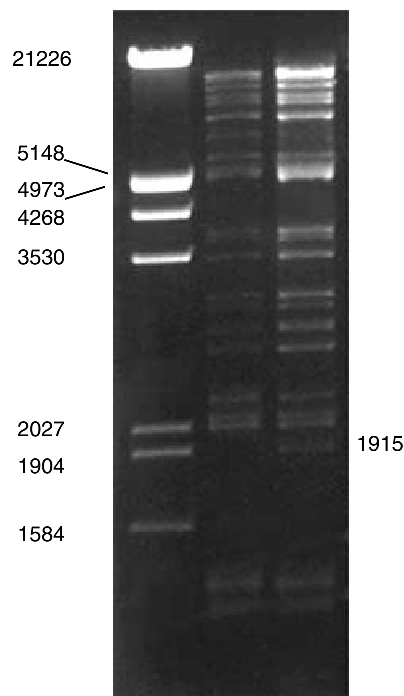

Fig. 3 Restriction analysis of the 34 MDa plasmid of *Enterococcus faecalis* JH2-416K and *Ent. faecalis* JH2-388C transconjugants. *Eco*RI cuts the two plasmids in 20 and 19 fragments, respectively. The restriction profiles differ for the absence of the fragment of 1915 bp in *Ent. faecalis* JH2-388C. Lane 1: molecular size markers derived from Lambda phage DNA digested with *Hin*dIII and *Eco*RI (21226, 5148, 4973, 4268, 3530, 2027, 1904 and 1586 bp); lanes 2 and 3: 34 MDa plasmids of *Ent. faecalis* JH2-416K and *Ent. faecalis* JH2-388C digested with *Eco*RI

emerge could be the result of a small rearrangement that occurred at recombination (Spellerberg *et al.* 2000).

The transferability by mating of the 34 MDa plasmids might be considered an interesting possibility for the study of heterologous expression of bacteriocins. This natural mechanism of recombination can be employed to construct multibacteriocinogenic strains with levels of production higher than those of the native sources (Makrides 1996) or to obtain a strain endowed with an amplified activity spectrum against food-borne pathogens and spoilage bacteria (Rodríguez *et al.* 2003). Even our results have shown that the phenotypic expression of the plasmid-encoded bacteriocins can differently be express in hosts distinct from the native strains. As Enterocin 416K1 and Enterocin 388C possess a different activity spectrum, a synergism might be obtained by their combined use.

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