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Short communication

Enterocin 416K1, an antilisterial bacteriocin produced by *Enterococcus casseliflavus* IM 416K1 isolated from Italian sausages

C. Sabia, G. Manicardi, P. Messi, S. de Niederhäusern, M. Bondi*

Department of Biomedical Sciences, University of Modena and Reggio E., Via Campi 287, 41100 Modena, Italy

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Abstract

Enterococci (118) from Italian sausages were tested for the production of antimicrobial substances. Of these, 7.6% showed antibacterial activity against one or several closely related microorganisms used as indicators. *Enterococcus casseliflavus* IM 416K1 in particular produced a bacteriocin (Enterocin 416K1) with strong anti-listerial antagonistic activity. The bacteriocin withstood heating at 90 °C for 120 min and storage at 4 °C for 6 months. The mode of action was identified as bactericidal. The crude activity of Enterocin 416K1 was linked to a molecule with an apparent molecular weight smaller than 5 kDa. Plasmid analysis of *E. casseliflavus* IM 416K1 revealed the presence of four plasmids with different molecular weights (34, 11, 7 and 3.3 MDa). All the Bac − variants produced by curing experiments showed loss of the single plasmid of 34 MDa. Bacteriocin activity and immunity production may be linked to genes located on that same plasmid.

Keywords: Lactic acid bacteria; Enterocin; *Listeria monocytogenes*

1. Introduction

The discovery of inhibitory substances active against pathogens have become of great interest in the past few years, in relation to the increase of food-borne illness caused by microbial contamination (Gravani, 1987; Klaenhammer, 1993; Jack et al., 1995). Among the lactic acid bacteria (LAB), representatives of all genera have been reported to produce bacteriocins (Jack et al., 1995). Because LAB strains are generally recognised as safe (GRAS) in food production (Schillinger et al., 1996), use of either their bacteriocins or the bacteriocin-producing LAB starter cultures for food preservation has received a special attention (Holzapfel et al., 1995; Ennahar et al., 1999). With regard to enterococci, bacteriocin production seems to be a common trait among strains associated with food. These antibacterial substances are called enterocins. They generally belong to class II bacteriocins and have the potential to inhibit the growth of a narrow range of strains closely related to the producer microorganism (Tagg et al., 1976; Klaenhammer, 1993); some are also active against Gram-positive food-borne pathogens and spoilage bacteria (Klaenhammer, 1988; Farías et al., 1994; Lyon et al., 1995; Cintas et al.,1997; Ennahar et al., 1998; Ennahar

* Corresponding author. Tel.: +39-59-2055469; fax: +39-59-2055483.

E-mail address: bondi.moreno@unimo.it (M. Bondi).

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and Deschamps, 2000; Ohmomo et al., 2000), and Gram negative species (Galvez et al., 1989; Martinez-Bueno et al., 1990; Lewus et al., 1991; Jennes et al., 2000).

In 1975, Krämer and Brandis reported that Enterocin E1A, a well-characterized bacteriocin from *E. faecium*, was active against *Listeria* spp. Now, according to a number of recent reports, the ability to inhibit *Listeria monocytogenes* is widespread among enterococci (Arihara et al., 1991; Parente and Hill, 1992; Arihara et al., 1993; Villani et al., 1993; Vlaemynck et al., 1994; Giraffa et al., 1995; Maisnier-Patin et al., 1996). This activity may be explained by the close phylogeny of enterococci and listeriae (Stackebrandt and Teuber, 1988; Devriese and Pot, 1995). Bacteriocin-producing *Enterococcus* strains with strong anti-*Listeria* activity have been isolated from dairy products (Nuñez et al., 1997; Ennahar et al.,1998), fermented sausages (Lyon et al., 1995; Aymerich et al., 1996; Cintas et al., 1997, 1998), fish (Ben Embarek et al., 1994), vegetables (Villani et al., 1993; Bennik et al., 1998; Floriano et al., 1998), fermented olives (Franz et al., 1996) and silages (Kato et al., 1994). Because enterococci are common in various food systems and their technological and probiotic benefits are widely recognised (Giraffa et al., 1997), these microorganisms could be good candidates for potential application of bacteriocin-mediated antagonism against *L. monocytogenes* in foods (Muriana, 1996). In a previous study (Messi et al., 2001), we reported the detection and preliminary characterization of a bacteriocin produced by *L. plantarum*, endowed with a wide range of inhibitory spectrum. In the present work, 118 *Enterococcus* strains, isolated from Italian sausages, were tested for bacteriocin production to obtain a compound with a specific anti-*L. monocytogenes* activity. In particular, among the bacteriocin-like substances observed, Enterocin 416K1 was studied and partially characterized with the aim of assessing its use as ''natural preservative'' for application in food production.

2. Material and methods

2.1. 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, bio-Merieux France), were cultured as usual in MRS broth (Oxoid) and incubated at 37 °C for 24 h. All strains were maintained in the same media containing 20% (w/v) glycerol at − 80 °C. Production of bacteriocin-like substances was screened by the deferred antagonism method according to Kekessy and Piguet (1970).

Crude filtrate supernatant fluid (CFSF) of the best producer, *Enterococcus casseliflavus* IM 416K1, 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 indicator bacteria belonging to different genera (Table 1). The indicator organisms were cultured in Tryptic Soy Broth or Agar (TSB or TSA, Difco Laboratories, Detroit, MI), at incubation temperatures of 30 or 37 °C for 24 h depending on the organisms.

2.2. Kinetic of growth and bacteriocin biosynthesis

For these studies, 250 ml of MRS broth was inoculated with 100 μl of an 18-h culture of *E. casseliflavus* IM 416K1 at an initial cell density of 10^5 cfu ml^{-1} and incubated at 37 °C without agitation. At appropriate intervals, samples were removed for measurement of biomass by absorbance at A_{630} nm, cell growth by viable plate counts (cfu ml^{-1}) in MRS agar (Oxoid), pH value and bacteriocin production. The antibacterial activity was evaluated assaying serial twofold dilutions of *E. casseliflavus* IM 416K1 CFSF against *L. monocytogenes* NCTC 10888. The antimicrobial titre was defined as the reciprocal of the highest dilution producing a distinct inhibition of the indicator lawn and expressed in terms of arbitrary units per millilitre (AU ml^{-1}) according to Mayr-Harting et al. (1972).

2.3. Mode of action

To investigate the effect of *E. casseliflavus* IM 416K1 CFSF on the indicator organism, *L. monocytogenes* NCTC 10888 was grown at 30 °C for 18 h in TSB. Cells were then harvested by centrifugation ($10\,000 \times g$ for 10 min at 4 °C) and resuspended in a sterile 50 mmol l^{-1} phosphate buffer (pH 6.5) to give 8×10^8 cfu ml^{-1}. Assays were performed by adding CFSF to the sample to obtain a final concen-

Table 1
Antibacterial activity detected in crude filtrate supernatant fluid (CFSF) from *E. casseliflavus* IM 416K1 by agar well diffusion assay[a]

Indicator strains			
Lactic acid bacteria		Gram-positive rods	
E. casseliflavus IM 430K1	–[b]	*L. monocytogenes* NCTC 10888	+++
E. casseliflavus IM 416KE	–	*L. monocytogenes* NCTC 10890	+++
E. casseliflavus IM 430K	–	*L. monocytogenes* NCTC 04883	+++
E. casseliflavus IM 430K2	–	*L. monocytogenes* NCTC 07973	+++
E. casseliflavus ATCC 14432	+	*L. monocytogenes* NCTC 05214	++
E. faecalis IM 388C	+++	*L. monocytogenes* NCTC 10528	++
E. faecalis IM 388K	+++	*L. monocytogenes* NCTC 05105	++
E. faecalis IM 388KE	+++	*L. monocytogenes* IM LV1	++
E. faecalis IM 388D	+++	*L. monocytogenes* IM LV2	++
E. faecalis JH2-2	+	*L. ivanovii* NCTC 11846	+
E. faecalis ATCC 29212	–	*L. seeligeri* NCTC 11856	+
E. faecalis ATCC 51299	–	*L. welshimeri* NCTC 11857	+
E. faecium ATCC 700221	–	*L. innocua* NCTC 11288	+
L. plantarum IM 35D	–	*B. subtilis* ATCC 44633	–
L. plantarum IM 391	–	*B. pumilus* ATCC 10337	–
L. curvatus IM 766	–	Gram-negative rods	
L. fermentum IM E62	–	*E. coli* ATCC 13762	–
Gram positive cocci		*E. coli* ATCC 25992	–
S. aureus ATCC 25923	–	*P. aeruginosa* ATCC 27853	–
S. aureus ATCC 6538	–	*A. hydrophila* IM 24	–
S. aureus ATCC 29213	–	*A. hydrophila* IM 104	–
S. aureus IM TIEN 5	–	*A. hydrophila* IM 106	–
S. aureus IM TIEN 12	–	*Y. enterocolitica* IM 71	–
		Y. enterocolitica IM 23	–
		K. ozoenae IM 6	–
		Salmonella sp. IM 300	–
		Salmonella sp. IM 301	–

[a] Isolates with the IM label were from our collection.
[b] –, no zone of inhibition; +, 5 mm < zone < 10 mm; ++, 10 mm < zone < 15 mm; +++, zone > 15 mm.

tration of 640 AU ml^{-1} and culturing the tube at 30 °C. As control, a sample without inhibitor was used. The viable counts on TSA plates and optical density were determined over time.

2.4. Sensitivity to physico-chemical parameters

To determine the effect of temperature on bacteriocin production, cultures in MRS broth of *E. casseliflavus* IM 416K1 were incubated at 18, 25, 30, 37 and 40 °C for 48 h. At the end of each incubation period, the bacteriocin activity of cell-free supernatant fluid was assayed.

To evaluate the sensitivity to heat and protease, *E. casseliflavus* IM 416K1 CFSF was: (i) heated at 60, 70, 80 and 90 °C for increasing periods of time (15, 30, 60 and 120 min); (ii) treated with 0.1 mg ml^{-1} of protease K (20 mg ml^{-1}) or 0.1 mg ml^{-1} pepsin or trypsin (all from Sigma, St. Louis, MO, USA).

To test the stability to different pH values, CFSF was adjusted to various pH values between 3.0 and 9.0 with 1 mol l^{-1} HCl or 1 mol l^{-1} NaOH. After incubation at 30 °C for 4 h, the pH was readjusted to 6.0 and the residual antimicrobial activity determined.

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

2.5. Estimation of the molecular size of Enterocin 416K1

Crude filtrate supernatant fluid (CFSF) from a culture of *E. casseliflavus* IM 416K1 in MRS broth

grown at 37 °C for 24 h was collected by centrifugation (12 000 × *g* for 30 min), dialyzed against 30 mmol $^{-1}$ sodium acetate buffer (pH 5.3) and filter-sterilized (0.45-μm-pore size filter; Millipore, Bedfort, MA). Dialyzed CFSF was ultrafiltered sequentially through 10 kDa, 5 kDa mol with exclusion membranes (Diaflo Ultrafiltration Membranes, Amicron, USA). Inhibitory activity was determined for both retentate and ultrafiltrate against *L. monocytogenes* NCTC 10888 as the indicator organism.

2.6. Selection of bacteriocin-deficient mutants

E. casseliflavus IM 416K1 was inoculated in MRS broth with various concentration of curing agent (1 to 32 μg ml $^{-1}$ ethidium bromide; Sigma) and incubated for 18 h at 37 °C. Those cultures that displayed growth 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 of incubation at 37 °C, the colonies were replicated and the original plate overlaid with semi-solid agar seeded with *L. monocytogenes* NCTC 10888 as indicator strain. After 24 h of an additional incubation at 30 °C, the colonies were checked for loss of antibacterial activity, i.e. the absence of zone inhibition, indicating phenotypic loss of the Bac+ trait. Immunity of nonproducer variants was examined by agar well diffusion assay (Rogers and Montville, 1991).

Plasmid DNA in *E. casseliflavus* IM 416K1 and in non-bacteriocin-producing colonies (Bac −) was isolated by the lysis method (O'Sullivan and Klaenhammer, 1993). Electrophoresis was conducted on 0.7% agarose, horizontal slab gels, in Tris acetate buffer at pH 8.0, using a steady voltage of 75 V for 120 min. The purified plasmids of *Escherichia coli* V517, described by Macrina et al. (1978), was used as source of size reference plasmid for molecular weight determinations. After staining in 0.5 mg ml $^{-1}$ ethidium bromide, the gels were photographed.

3. Results and discussions

3.1. Antibacterial activity evaluation

Nine (7.6%) of enterococci isolated from fermented sausages over the study period and screened by the deferred antagonism method for bacteriocin-like substance production showed antibacterial activity against one or more taxonomically related microorganisms (data not reported). Among the producers, *E. casseliflavus* IM 416K1 had the highest antibacterial activity to the closely related microorganisms used as indicators. *E. casseliflavus* IM 416K1 CFSF, in addition, showed the strongest antagonistic activity against *L. monocytogenes* among the indicator strains examined (Table 1). We called this substance produced by *E. casseliflavus* IM 416K1 on both solid medium or liquid culture Enterocin 416K1. This bacteriocin, given its narrow spectrum of action and anti-listerial activity, resembles enterocins E1A and E1B (Kramer and Brandis, 1975), Enterocin EL1 (Lyon et al., 1995), Enterocin 81 (Ennahar et al., 1998), Enterocin A (Ennahar and Deschamps, 2000) and Enterocin ON-157, (Ohmomo et al., 2000). Enterocin 416K1 differs from Enterocins A and B (Casaus et al., 1997), Enterocin P (Cintas et al., 1997), Enterocin L50A and L50B (Cintas et al., 1998), Enterocin I (Floriano et al., 1998), which were inhibitory to a wide spectrum of Gram positive bacteria, including spoilage and food-borne pathogens, and Enterocin 012 (Jennes et al., 2000), which is also active against *Pseudomonas aeruginosa* and *Salmonella typhimurium*.

3.2. Growth kinetics and bacteriocin biosynthesis

As shown in other studies on enterococci, production of Enterocin 416K1 in MRS broth was dependent on the bacterial growth phase (Fig. 1). *E. casseliflavus* IM 416K1 started to produce bacteriocin (about 80 AU ml $^{-1}$) at 4 h during the early logarithmic growth phase when biomass absorbance was 0.059, at a cell count of 4×10^5 cfu ml $^{-1}$, as reported for Enterocin 226 (Villani et al., 1993) and Enterocin 012 (Jennes et al., 2000). Enterocin 416K1 concentration reached a maximum (1280 AU ml $^{-1}$) after 14 h of incubation (early stationary phase), at an absorbance of 1.9 and a cell count of 1.2×10^9 cfu ml $^{-1}$, as observed for other bacteriocins (Du Toit et al., 2000). Bacteriocin titre remained constant after 60 h of incubation, unlike for Enterocin 226 (Villani et al., 1993) whose activity decreased after 10 h of incubation of the producer strain. In the same period the bacterial count did not change, like pH and absorbance (results not shown). Inhibitory activity was detected at the same level (1280 AU ml $^{-1}$) when the

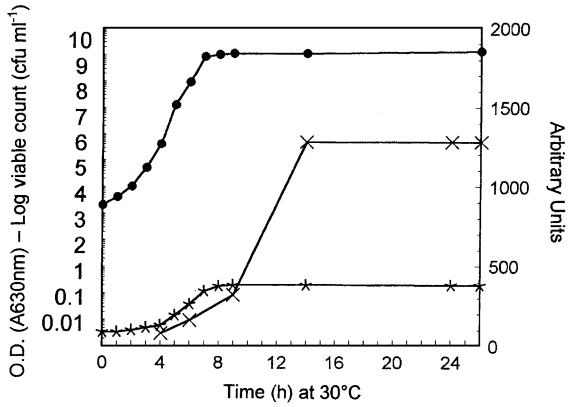

Fig. 1. Production of Enterocin 416K1 by *E. casseliflavus* IM 416K1 in MRS broth at 30 °C. At appropriate time intervals, samples were taken and log viable count (cfu ml^{-1}) (●), absorbance at A_{630} nm (∗), and bacteriocin activity (AU ml^{-1}) (×) were determined.

producer strain was incubated at 18, 22, 37 and 45 °C, as Enterocin P produced during growth in MRS broth from 16 to 45 °C (Cintas et al., 1997).

3.3. Mode of action

The addition of 640 AU ml^{-1} Enterocin 416K1 CFSF to cell suspensions of *L. monocytogenes* NCTC 10888, led to a marked decrease in the number of viable cells (Fig. 2). Bacterial count was 1 log cycle lower after about 6 h and 3 log cycles lower after 24 h than controls without bacteriocin. This finding supports the bactericidal mode of action of Enterocin 416K1. No decrease in optical density of the cell suspension was observed (results not shown) over the experiments, indicating that activity of Enterocin 416K1 against *L. monocytogenes* was bactericidal without concomitant cell lysis. A bactericidal mode of action without concomitant lytic effect has also been described for Enterocin 1146 (Parente and Hill, 1992), Enterocin 226NWC (Villani et al., 1993), Enterocin EL1 (Lyon et al., 1995), Enterocin A and B (Casaus et al., 1997), Enterocin P (Cintas et al., 1997), an enterocin produced by *E. faecium* WHE81 (Ennahar et al., 1998), Enterocin ON-157 (Ohomomo et al., 2000), and Enterocin A (Ennahar and Deschamps, 2000). Enterocin 416K1 differs form Enterocin L50A and L50B (Cintas et al., 1998) and Enterocin 012 (Jennes et al., 2000), which caused cell lysis.

3.4. Sensitivity to proteolytic enzymes and physico-chemical parameters

Enterocin 416K1 was sensitive to the proteolytic enzymes used and active after heat treatment at 90 °C for 120 min (results not shown). Activity was maintained over a wide range of pH and during storage at refrigeration up to 6 months (results not shown), as reported for Enterocin P (Cintas et al., 1997).

3.5. Estimation of the molecular size of Enterocin 416K1

When Enterocin 416K1 CFSF was dialyzed through a cellulose membrane with 5–10 kDa cutoff, the antibacterial activity evaluated in dialyzed and ultrafiltered CFSF was still found in both retentate and ultrafiltrate (results not shown). This suggested that the crude activity of Enterocin 416K1 was linked to a molecule with an apparent molecular weight smaller than 5 kDa as reported for Enterocin EL1 (Lyon et al., 1995), Enterocin A (Aymerich et al., 2000), Enterocin 012 (Jennes et al., 2000) and Enterocin ON-157 (Ohmomo et al., 2000).

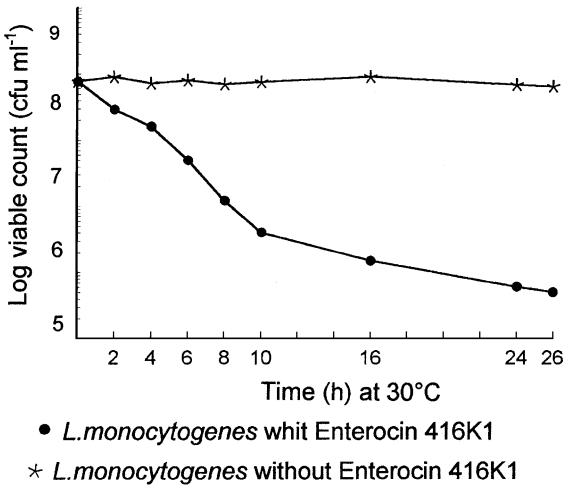

Fig. 2. Effect of Enterocin 416K1 addition (640 AU ml^{-1}) on the viability of *L. monocytogenes* NCTC 10888 suspended in 50 mmol l^{-1} phosphate buffer (pH 6.5). The initial cell number was 8×10^8 ml^{-1}. Data points represent the average of at least four experiments run in duplicate.

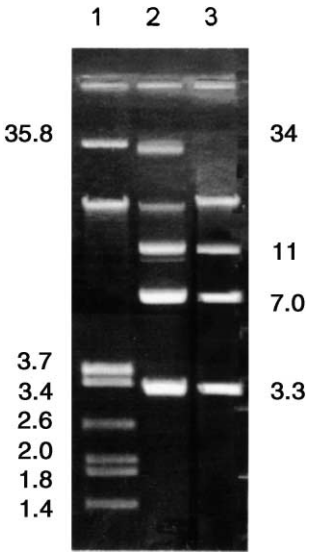

Fig. 3. Plasmid profile of *E. casseliflavus* IM 416K1. Lane 1: molecular size markers prepared from *E. coli* V517 (35.8, 3.7, 3.4, 2.6, 2.0, 1.8 and 1.4 MDa); lane 2: parental strain (34, 11, 7 and 3.3 MDa); lane 3: variant strain IM 416K1b Bac −, after curing with ethidium bromide (11, 7 and 3.3 MDa).

3.6. Selection of bacteriocin-deficient mutants

Fig. 3 shows the plasmid profiles of the parental strain *E. casseliflavus* IM 416K1 and an example of a Bac − derivative obtained by curing treatment with ethidium bromide. Plasmid analysis of original strain revealed the presence of four plasmids with different molecular weights: one large plasmid of 34 MDa and three plasmids of approximately 11, 7 and 3.3 MDa. All the variants showed loss of a single plasmid of 34 MDa. The Bac − isolates were also sensitive to the bacteriocin produced by the parental strain. These findings suggest that bacteriocin activity and immunity production may be linked to genes located on the same plasmid as reported by other Authors (Martinez-Bueno et al., 1990; Floriano et al., 1998; Balla et al., 2000; Ohmomo et al., 2000).

Taking into account its bactericidal activity, proteinaceous nature, heat resistance, and low molecular weight, Enterocin 416K1 can be classified as a small, heat-stable *listeria*-active peptide, presumably belonging to class IIa pediocin-like bacteriocin according to the definition given by Klaenhammer (1993). However, based on our results, it could not be determined whether Enterocin 416K1 is a novel bacteriocin. As all class IIa bacteriocins, Enterocin 416K1 exhibited a relatively narrow spectrum of activity and was predominantly a *Listeria* inhibitor, as strains of *L. monocytogenes* were the most sensitive. Enterocin 416K1 is besides thermostable, and thus can be used in pasteurized products. It is also stable over a wide range of pH and may be used in acid as well as nonacid foods. Further, it is also stable at low temperatures for long periods of time and so can be employed in refrigerated foods against *L. monocytogenes*, and the control of this microorganism is a major concern in food safety since this pathogen can be present in many food products (Muriana, 1996). From an application point of view, Enterocin 416K1, a bacteriocin that is active against undesired bacteria with no effects on useful starter and nonstarter LAB, would be an ideal food preservative (Ennahar et al., 1999; Ennahar and Deschamps, 2000).

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