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Milk Protein Fragments Induce the Biosynthesis of Macedocin, the Lantibiotic Produced by *Streptococcus macedonicus* ACA-DC 198▽

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The aim of the present work was to study the mode of the induction of the biosynthesis of macedocin, the lantibiotic produced by *Streptococcus macedonicus* ACA-DC 198. Macedocin was produced when the strain was grown in milk but not in MRS or M17 broth. No autoinduction mechanism was observed. Production did not depend on the presence of lactose or galactose in the culture medium or on a coculture of the producer strain with macedocin-sensitive or macedocin-resistant strains. Induction seemed to depend on the presence of one or more heat-stable protein components produced when *S. macedonicus* ACA-DC 198 was grown in milk. The partial purification of the induction factor was performed by a combination of chromatography methods, and its activity was confirmed by a reverse transcription-PCR approach (RT-PCR). Mass spectrometric (MS) and tandem mass spectrometric (MS/MS) analyses of an induction-active fraction showed the presence of several peptides of low molecular mass corresponding to fragments of α_{S1}- and β-casein as well as β-lactoglobulin. The chemically synthesized α_{S1}-casein fragment 37-55 (2,253.65 Da) was proven to be able to induce macedocin biosynthesis. This is the first time that milk protein degradation fragments are reported to exhibit a bacteriocin induction activity.

Lactic acid bacteria (LAB) have been used for centuries in the fermentation of foods not only for flavor and texture development but also because of their ability to produce antimicrobial compounds that prevent the growth of spoilage and pathogenic microorganisms (33). Among these antimicrobial compounds, bacteriocins have been defined as proteinaceous compounds that kill closely related bacteria, but it has become apparent that some have a broad host range, inhibiting many different species (38). In particular, the lantibiotics have attracted much attention in recent decades because of the success of the well-characterized lantibiotic nisin as a food preservative (11). Numerous other lantibiotics have been identified and extensively characterized, resulting in an advanced understanding of these molecules at both structural and mechanistic levels (16, 38).

Streptococcus macedonicus ACA-DC 198 (56) produces the food-grade lantibiotic macedocin (20, 21), which is identical to the lantibiotics streptococcin A-FF22 (SA-FF22) and streptococcin A-M49 (SA-M49), both isolated from the pathogen *S. pyogenes* (26, 27, 29). Recently, a 15,171-bp region in the *S. macedonicus* ACA-DC 198 genome, containing the biosynthetic gene cluster of macedocin, has been sequenced (42). The region contains 10 open reading frames, which correspond to the genes (*mcdKRAA′A1MTFEG*) involved in macedocin biosynthesis, regulation, and immunity. The macedocin gene cluster is organized in two operons (regulatory and biosynthetic/immunity) instead of the three operons (regulatory, biosynthetic, and immunity) present in the SA-FF22 gene cluster. It contains an additional structural gene (*mcdA′*) as well as a putative transposase gene (*tnp*) located upstream of *mcdR*. The deduced product of this gene is 99% identical to a transposase of *S. thermophilus*, which is part of the mobile element IS*1191* (42).

The production of bacteriocins is not always constitutive, and particular growth conditions or external factors, such as medium composition (41, 44), temperature (15, 23, 44), osmotic stress (50), and the pH of the growth medium (25, 44), may be required to induce bacteriocin production. A combination of these factors is needed to produce some bacteriocins (10). The production of SA-FF22 is affected by various growth conditions, such as temperature, pH, and growth medium (28). As for many lantibiotics, its production is controlled by a typical two-component signal transduction system comprised of a receptor-histidine kinase (LanK) and a transcriptional response regulator (LanR). Moreover, SA-FF22, nisin, subtilin, salivaricin A, and mersacidin (9, 52, 60) have been shown to serve as the sensing molecules that trigger the transcription of their prepeptides through an extracellular autoregulatory mechanism (30, 31, 54). The biosynthesis of other lantibiotics, however, seems to be governed by additional regulatory sys-

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tems; e.g., mutacin is dependent on a competence-stimulating peptide (CSP) signaling system encoded by the *com* genes (59). Indeed, in several cases, in addition to the two-component signal transduction pathway, a third component has been identified that is encoded by an induction peptide gene. In these three-component systems, a peptide secreted by the producing strain itself serves as the extracellular signal causing the transcription of the genes necessary for bacteriocin production (40). Genes encoding induction peptides have been found in gene clusters of several bacteriocin-producing LAB (57), such as clusters of enterocins A and B (40), sakacin P (17), and the two-peptide plantaricins EF and JK (2). In the case of lactacin B, which is produced by *Lactobacillus acidophilus* N2, induction is triggered by a protein produced by the sensitive *Lactobacillus delbrueckii* subsp. *lactis* ATCC 4797 strain (3). Also, it has been reported that bacteriocin production by *Ruminococcus gnavus* E1 is controlled through a complex signaling mechanism involving the proteolytic processing of a putative extracellular inducer peptide by trypsin, a specific environmental cue of the digestive ecosystem (22). Finally, an antimicrobial peptide is produced by *Propionibacterium jensenii* LMG 3032 through the extracellular processing of a noninhibitory protein from this strain (18).

Interestingly, macedocin is produced only when *S. macedonicus* ACA-DC 198 is grown in milk (21). Thus, the aim of the present work was to study the mode of the induction of macedocin biosynthesis and to elucidate the role of milk in this biosynthesis process.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. macedonicus* ACA-DC 198, a strain isolated from Greek Kasseri cheese (ACA-DC Collection of the Laboratory of Dairy Research, Agricultural University of Athens, Athens, Greece) was used throughout this study. Before experimental use, the strain was subcultured twice (inoculum of 1%, vol/vol) in sterile skim milk (10%, wt/vol; Oxoid, Basingstoke, Hampshire, United Kingdom) containing yeast extract (0.3%, wt/vol; Merck, Darmstadt, Germany) at 37°C for 24 h unless otherwise stated. Growth experiments were performed under the same conditions.

Lactococcus lactis subsp. *lactis* LMG 6890^T and *Clostridium tyrobutyricum* LMG 1285^T, which were used as routine target strains, were cultured aerobically in M17 broth (Biokar Diagnostics, Beauvais, France) at 30°C and anaerobically (GasPak EZ anaerobe container system; Becton Dickinson & Co., Sparks, MD) in RCM broth (Oxoid) at 37°C, respectively.

All other lactococcal, streptococcal, and enterococcal strains used (see below) were cultured in M17 broth at 30 or 37°C, lactobacilli in De Man, Rogosa, Sharpe broth (MRS; Biokar Diagnostics) (12) at 37°C, and *Listeria* strains in brain heart infusion broth (BHI; Biokar Diagnostics) at 30°C. Strains were stored at −80°C in the respective growth medium supplemented with 20% (vol/vol) glycerol.

Determination of the antimicrobial activity. The well diffusion assay (WDA) was used for the determination of bacteriocin activity (55). Briefly, *L. lactis* subsp. *lactis* LMG 6890^T was subcultured twice in M17 broth, and finally a 16-h culture (10^8 CFU/ml) was used to inoculate (0.1%, vol/vol) M17 agar, which was poured into petri dishes, and wells of 5 mm in diameter were made in the solidified medium. The wells were filled with 50 μl of serial 2-fold dilutions (in 50 mM sodium phosphate buffer, pH 6.5) of samples (cell-free culture supernatants or chromatography fractions), and inhibition zones were recorded after incubation at 30°C for 20 to 24 h. The activity was expressed in arbitrary units (AU) per ml, corresponding to the reciprocal value of the highest dilution causing a clear zone multiplied by 20. The method was performed similarly with *C. tyrobutyricum* LMG 1285^T, except that RCM agar (Oxoid) and 2% (vol/vol) inoculum were used.

Determination of the induction activity (induction assay [IA]). Induction activity was assayed according to Eijsink et al. (17). All samples (cell-free culture supernatants, chromatography fractions, or synthetic peptide solutions; 50 μl) and their 2-fold dilutions in sterile water were put in wells of sterile microtiter plates. M17 broth (150 μl), inoculated with an overnight culture of *S. macedonicus* ACA-DC 198 (1%, vol/vol), was added, and plates were incubated at 37°C for 24 h. Samples then were centrifuged at 10,000 × *g* and 4°C for 10 min, and 50-μl aliquots of the supernatants were assayed for antimicrobial activity using the WDA as described above. In all cases, appropriate controls were performed (e.g., samples [50 μl] with plain noninoculated M17 broth [150 μl] or M17 broth inoculated with *S. macedonicus* [200 μl]). One induction unit (IU) ml^{-1} was defined as the minimum concentration of induction factor causing detectable bacteriocin production in a WDA (40).

Autoinduction assay. Macedocin, either crude (160 AU/ml) or purified (5,120 AU/ml), was added (0.1 or 1.0%, vol/vol) to *S. macedonicus* liquid cultures in MRS or M17 at various stages of growth, namely, lag (3 h), exponential (6 h), and stationary (12 h) phases. After overnight growth (20 h), macedocin production was detected by the WDA as described above.

Also, autoinduction was tested in the respective solid medium using the multilayer agar petri dish system (14). Briefly, the system consisted of (i) a supporting layer of 10 ml M17 agar (1.5% agar, wt/vol) and (ii) a layer of 10 ml M17, MRS, or skim milk (without yeast extract) soft agar (0.7% agar, wt/vol) containing *S. macedonicus* (100 to 150 cells per plate) and macedocin at the same concentrations as those described above (induction layer). The petri dish was incubated at 37°C for 20 to 24 h to allow the growth of *S. macedonicus* and the induction of macedocin, and then it was overlaid with a third layer of 10 ml M17 soft agar containing the indicator organism *L. lactis* subsp. *lactis* LMG 6890^T (optical density at 600 nm of 0.1 to 0.2). Inhibition zones were perceived after further incubation at 30°C for 20 to 24 h.

Effect of growth medium and growth conditions on macedocin production. The production of macedocin in the presence of other organisms was investigated with cocultures in M17 broth of *S. macedonicus* with other strains, namely, either macedocin-sensitive (*L. delbrueckii* subsp. *delbrueckii* ACA-DC 81, *L. lactis* subsp. *lactis* LMG 6890^T, *L. lactis* subsp. *cremoris* LMG 6897^T, *S. thermophilus* ACA-DC 9, *Enterococcus faecium* LMG 11423^T, and *Listeria innocua* LMG 11387^T) or macedocin-resistant strains (*Lactobacillus paracasei* subsp. *paracasei* ACA-DC 116, *L. lactis* subsp. *lactis* ACA-DC 46, *S. thermophilus* ACA-DC 79, *S. macedonicus* ACA-DC 206^T, *Enterococcus faecalis* LMG 7937^T, and *L. innocua* LMG 13568). Also, we examined the effect on macedocin production of the addition of lactose, galactose, milk proteins, or supernatants to MRS or M17 broth and the precipitates of overnight milk cultures of two macedocin-negative but proteolytic *S. macedonicus* strains, namely, ACA-DC 194 and 199. Finally, the impact of the inoculum derived from cultures in M17 broth, MRS broth, or milk supplemented with yeast extract on the maintenance of macedocin production in M17 broth was investigated (Table 1).

Macedocin purification. Macedocin purification was performed as described by Georgalaki et al. (21).

Induction factor purification. All purification steps were performed at room temperature using the fast protein liquid chromatography (FPLC) system of Waters (650E advanced protein purification system) and an Ettan LC system from Amersham Biosciences (Uppsala, Sweden). FPLC columns were purchased from Amersham, and the Alltima HP hydrophilic interaction liquid chromatography (HILIC) column was purchased from Grace Davison Discovery Sciences (2.1 by 150 mm; Deerfield, IL).

Skim milk (10%, wt/vol; 1,000 ml) supplemented with yeast extract (0.3%, wt/vol) was inoculated with *S. macedonicus* ACA-DC 198 (inoculum of 1%, vol/vol) and incubated at 37°C for 24 h. The cells were removed by centrifugation at 15,000 × *g* and 4°C for 30 min. The supernatant was adjusted to pH 6.5 with 5 N NaOH and saturated up to 50% with ammonium sulfate. After overnight stirring at 4°C, proteins were pelleted by centrifugation at 15,000 × *g* and 4°C for 30 min and dissolved in 20 mM phosphate buffer, pH 5.5. The sample then was applied to a Resource S column (16 mm [inside diameter] by 30 mm) equilibrated with the same buffer. Elution was performed at a flow rate of 2 ml/min with a linear gradient of 0 to 1.0 M NaCl in the same buffer. The nonretained, induction activity-containing fraction, after ammonium sulfate precipitation (50% saturation), was diluted in double-distilled water (ddH_2O) containing 0.1% (vol/vol) trifluoroacetic acid (TFA) and applied to a Resource reverse-phase chromatography (RPC) column (6.4 mm [inside diameter] by 100 mm) equilibrated with the same solvent. Elution was performed at a flow rate of 2 ml/min with a linear gradient of 0 to 100% acetonitrile (ACN) containing 0.1% (vol/vol) TFA.

The fraction containing the highest induction activity was applied to an HILIC column equilibrated with 95% solvent A (100% ACN containing 0.1% HCOOH) and 5% solvent B (100% high-performance liquid chromatography [HPLC]-grade water containing 0.1% HCOOH) at a flow rate of 100 μl/min. After an isocratic run of 20 min with 5% solvent B, a linear gradient of 5 to 100% solvent B for 30 min was used. During the separation gradient, the eluate was fraction-

TABLE 1. Effects of coculturing, sugars, milk proteins, growth medium of the inoculum, and *S. macedonicus* proteolysis products on macedocin production by *S. macedonicus* ACA-DC 198

Growth medium[a] (liquid)	Antimicrobial activity (AU/ml)
Skim milk (10%, wt/vol) supplemented with YE (0.3%, wt/vol)	160
M17 cocultures of *S. macedonicus* with macedocin-sensitive or -resistant strains[b]	0
MRS or M17 supplemented with lactose (1.0–5.0%, wt/vol)	0
MRS or M17 supplemented with galactose (0.5–2.5%, wt/vol)	0
MRS supplemented with whole milk (2%, wt/vol)	80
MRS supplemented with milk caseins (2%, wt/vol)	80
M17 supplemented with milk caseins (2%, wt/vol) ± YE (0.3%, wt/vol)	40 (−YE), 80 (+YE)
M17 supplemented with milk whey proteins (0–0.75%, wt/vol) ± YE (0.3%, wt/vol)	40 (−YE), 80 (+YE)
M17 inoculated with *S. macedonicus* grown in milk (10%, wt/vol) with YE (0.3%, wt/vol)	40
M17 inoculated with *S. macedonicus* grown in M17	0
M17 inoculated with *S. macedonicus* grown in MRS	0
MRS supplemented with a culture supernatant (1 or 10%, vol/vol) or a cell precipitate of a 5-ml *S. macedonicus* ACA-DC 194 or 199 culture grown in milk (10%, wt/vol) supplemented with YE (0.3%, wt/vol)	0

[a] YE, yeast extract. Milk was from Oxoid (Basingstoke, Hampshire, United Kingdom); yeast extract, lactose, and galactose were from Merck (Darmstadt, Germany); MRS broth and M17 broth were from Biokar Diagnostics (Beauvais, France); milk whey proteins and milk caseins were prepared in the Laboratory of Dairy Research of the Agricultural University of Athens by acidification or the addition of rennet, respectively (Golfo Moatsou).

[b] Macedocin-sensitive strains were *L. delbrueckii* subsp. *delbrueckii* ACA-DC 81, *L. lactis* subsp. *lactis* LMG 6890^T, *L. lactis* subsp. *cremoris* LMG 6897^T, *S. thermophilus* ACA-DC 9, *E. faecium* LMG 11423^T, and *L. innocua* LMG 11387^T. Macedocin-resistant strains were *L. paracasei* subsp. *paracasei* ACA-DC 116, *L. lactis* subsp. *lactis* ACA-DC 46, *S. thermophilus* ACA-DC 79, *S. macedonicus* ACA-DC 206^T, *E. faecalis* LMG 7937^T, and *L. innocua* LMG 13568.

ated in the 96-well plate at a volume of 250 μl using a Frac-950 fraction collector (Amersham).

Protein determination. Protein concentrations were determined according to the method of Bradford (6) using the Bio-Rad Bradford assay (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as a standard.

MS and MS/MS analysis. Mass spectrometric (MS) analyses were carried out on a 4700 Proteomics Analyzer (Applied Biosystems). HPLC fractions were dried and resuspended in 10 μl of 50% acetonitrile–0.01% TFA. One μl of the solution was mixed with an equal volume of 10 mg/ml matrix solution (alpha-cyanohydroxycinnamic acid) in 50% ACN–0.1% TFA and spotted onto the matrix-assisted laser desorption ionization (MALDI) plate. MS/MS was performed using 1 kV of collision energy using the no-gas option. The identification of the peptides from the MS/MS fragmentation patterns was performed using MASCOT (Matrix Science) against the nonredundant NCBI database.

Apart from the induction-positive fractions derived from the last step of the induction factor purification procedure, the above-described analysis was performed for the following control samples, namely, the ammonium sulfate precipitates (50%) of (i) noninoculated milk containing yeast extract and incubated overnight, (ii) overnight milk culture of *S. macedonicus* ACA-DC 198, and (iii) overnight milk cultures of the two macedocin-negative but proteolytic *S. macedonicus* strains ACA-DC 194 and 199.

Peptide synthesis. The synthesis of the peptide RFFVAPFPEVFGKEKVNEL (2,253.65 Da) was performed by Invitrogen (Carlsbad, CA). It was dissolved in 60% ddH_2O–40% ACN containing 0.1% HCOOH and further used in the induction assay mentioned above at a final concentration range of 2.5 mg/ml to 0.25 ng/ml. Appropriate blanks were performed containing 50 μl of the peptide solvent. The starting population of the sensitive strain used in the WDA ranged from 10^3 to 10^5 CFU/ml.

Effect of heat and protease treatment. Induction activity-containing fractions as well as samples containing the induced bacteriocin activity were boiled for 10 min. The residual induction and bacteriocin activities were determined as described above.

Moreover, induction activity-containing fractions (50 μl) as well as samples containing the induced bacteriocin activity were incubated at 37°C for 2 h in the presence of *Streptomyces griseus* protease (50 μl; 10 mg/ml in 50 mM Tris, pH 8.5; Sigma). The residual induction and bacteriocin activities were determined as described above. Fractions in buffer without enzyme, enzyme/buffer solutions, and buffers only were used as controls.

RT-PCR. The reverse transcriptase PCR (RT-PCR) approach was used to confirm macedocin biosynthesis during the induction assay. *S. macedonicus* ACA-DC 198 was grown at 37°C for 24 h in M17 broth containing the RPC induction-active fraction 18 or the chemically synthesized peptide dissolved in 60% ddH_2O–40% ACN, containing 0.1% HCOOH, at a final concentration range of 2.5 mg/ml to 0.25 ng/ml. The optical density was measured at 610 nm. Cells were harvested (5 min at 6,000 × *g* and 4°C) at various time intervals of incubation (4, 6, and 8 h) and used for RT-PCR experiments, whereas the corresponding supernatants were used in a WDA for the detection of induced macedocin activity. Appropriate blanks also were performed containing 50 μl of the solvent of the fraction 18 or the chemically synthesized peptide. RNA isolation from harvested cells was performed using the RNeasy Mini kit (Qiagen GmbH, Hilden, Germany), and 100 ng of each RNA sample was used as the template for separate cDNA synthesis reactions. cDNA synthesis was performed using Superscript reverse transcriptase (Invitrogen) with the primer pRA′ (5′-CATATTAGACACTTAGTCATCC-3′). This primer is located 4 bp downstream of the *mcdA′* structural gene of macedocin and corresponds to the bp 5,900 to 5,921 region of the GenBank complete sequence of the macedocin gene cluster (accession no. DQ8353494) (42). The cDNA products were used as the template in PCRs using the primers pFA and pRA′. pFA (5′-ATTTCTTGCT ACATGTTGCTCATAA-3′) is located at the 3′ end of the *mcdA* structural gene of macedocin and corresponds to the bp 5,687 to 5,711 region of the DQ8353494 GenBank sequence. The expected size of the amplification product was 234 bp.

RESULTS

Autoinduction experiments. When macedocin, both in crude and purified forms, was added to *S. macedonicus* ACA-DC 198 liquid cultures in MRS or M17 broth, no induction of macedocin production was observed. This was also the case for the respective solid medium.

Effect of growth medium and growth conditions on macedocin production. When *S. macedonicus* was cocultured with macedocin-sensitive or -resistant strains, no production of macedocin was observed (Table 1). The addition of lactose or galactose to MRS or M17 broth did not cause macedocin production. On the other hand, when the same media were supplemented with plain milk, milk caseins, or milk whey proteins, macedocin activity was evident. Moreover, macedocin was produced in M17 broth when this was inoculated from an overnight culture of *S. macedonicus* in milk; this was not the case when the inoculum was derived from either an MRS or M17 broth culture. When MRS broth was fortified by the use of supernatants and precipitates of overnight milk cultures of two macedocin-negative but proteolytic *S. macedonicus* strains, namely, ACA-DC 194 and 199, no macedocin production was observed.

TABLE 2. Partial purification of *Streptococcus macedonicus* ACA-DC 198 induction factor[a]

Purification step	Vol (ml)	Act (IU/ml)	Protein concn (mg/ml)	Total act (IU)	Sp act (IU/mg)	Fold purification	Yield (%)
Crude	850	1,280	0.30	1,088,000	4,266	1.0	100
Crude-ASP	260	1,280	0.82	332,800	1,561	0.4	31
Resource S (pH 5.5)	131	1,280	0.49	167,680	2,612	0.6	15
Resource S-ASP	92	640	0.83	58,880	771	0.2	5
Resource RPC	22	1,280	1.18	28,160	1,084	0.3	3

[a] ASP, ammonium sulfate precipitation; IU, induction units (1 IU/ml is defined as the minimum concentration of induction factor causing detectable bacteriocin production in a WDA).

Purification of the induction factor. A combination of ammonium sulfate precipitation (ASP), strong cation exchange (SCX), reverse-phase chromatography (RPC), and HILIC was applied for the purification of the induction factor (IF). Initially, IF precipitated together with macedocin at 50% ammonium sulfate saturation of a milk culture supernatant of *S. macedonicus* ACA-DC 198. Afterwards, IF was eluted in the nonretained fraction of the SCX column (pH 5.5), whereas it was retained on the RPC column and eluted with 40% (vol/vol) acetonitrile (fractions 11 to 21 in a 0 to 100% acetonitrile gradient). The recovery and degree of purification of the IF up to the RPC step are summarized in Table 2.

The RPC IF-active fraction 18 (the highest induction activity-containing fraction) was applied on the HILIC column, where it was retained and eluted as a peak at 52 to 54% acetonitrile (Fig. 1). The IF-active fractions (C11 to D3) were used further for the MS and MS/MS analysis.

MS and MS/MS analysis. Acetonitrile HILIC fractions exhibiting induction activity (C11 to D3) were subjected to MS and MS/MS analysis (Fig. 2). In all fractions several peaks were detected. The results are summarized in Table 3. All peptides identified by tandem mass spectrometric peptide sequencing correspond to bovine milk protein fragments and, more precisely, to the C terminus of α_{S1}- and β-casein, as well as to internal fragments of α_{S1}-casein and β-lactoglobulin. Some peaks were modified. Despite the fact that we could unambiguously identify the peptide sequence, we could not reveal the nature of the modifications.

Interestingly, three of these peptides, deriving from α_{S1}-casein, namely, RFFVAPFPEVFGKEKVNEL (2,253.65 Da) and RFFVAPFPEVFGKEKVNELSKDIG (2,752.7 Da), as well as β-lactoglobulin, namely, DEALEKFDKALKALPM HIRL (2,338 Da), also were detected in the ammonium sulfate precipitate of *S. macedonicus* ACA-DC 198, while they were

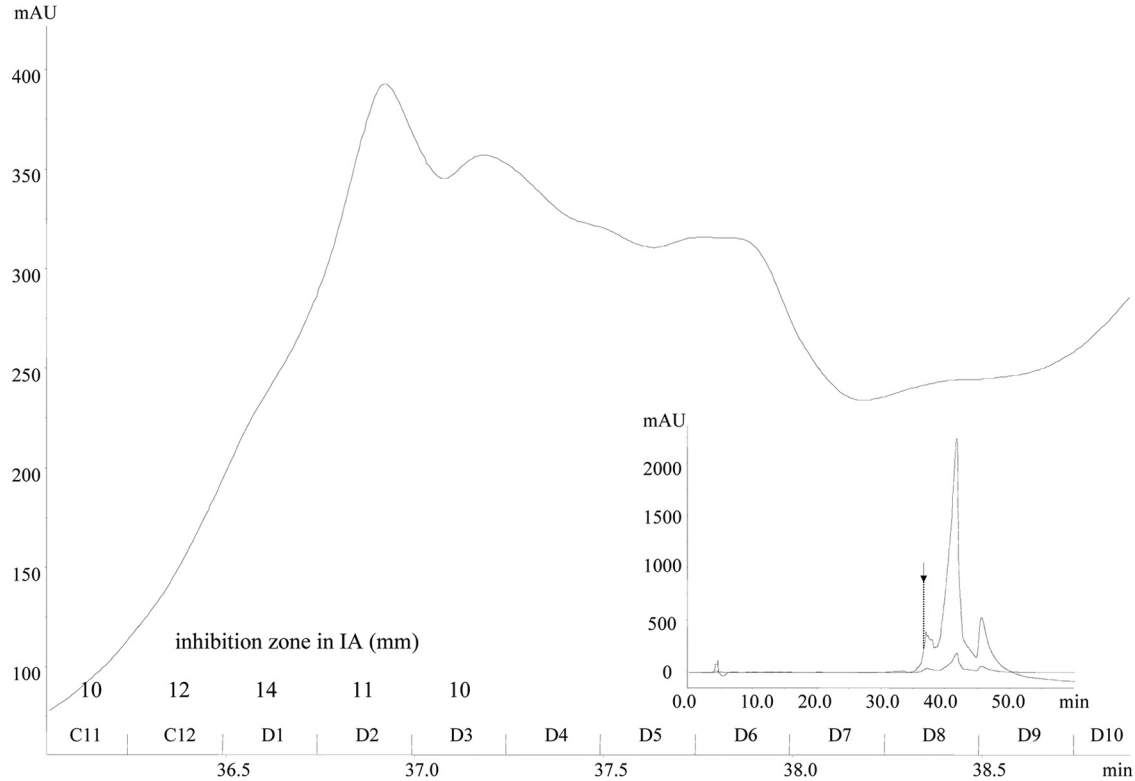

FIG. 1. Elution profile of the macedocin induction factor (fraction 18 of the FPLC-RPC column) from the HILIC column showing fractions C11 to D10 and their induction activity (IA) in mm of inhibition zone. The insert represents the complete elution profile of the same chromatographic separation, of which the arrow shows the peak containing the macedocin induction factor.

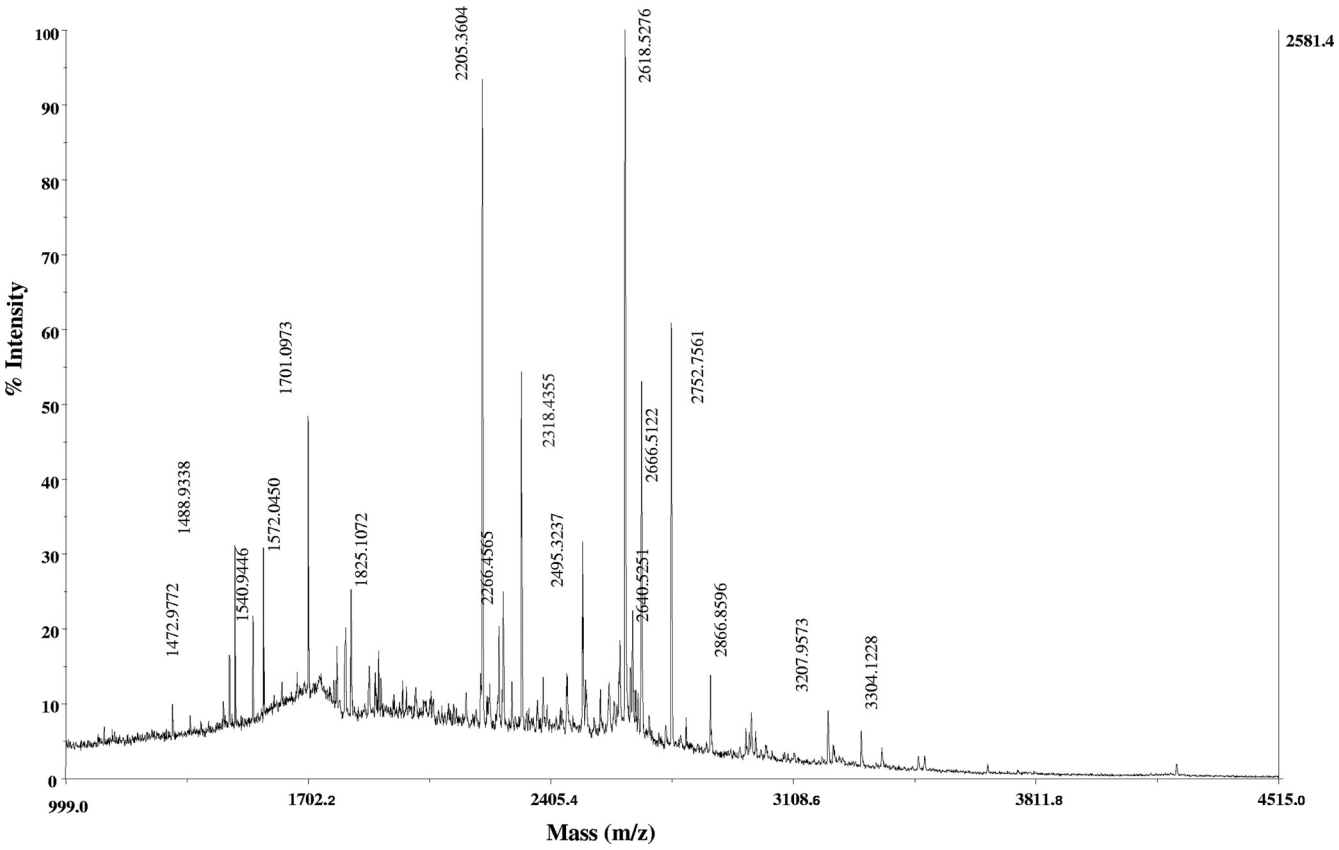

FIG. 2. MALDI-MS analysis of the induction-active fraction D1.

not present in the respective ammonium sulfate precipitates of milk or milk cultures of the macedocin-negative but proteolytic *S. macedonicus* strains ACA-DC 194 and 199. This was considered a strong indication that these peptides, deriving from the proteolytic action of *S. macedonicus* ACA-DC 198 on milk proteins, were the most probable candidates for being the induction factor of macedocin biosynthesis.

Synthesis of the induction peptide. Because two of the three above-mentioned peptides were α_{S1}-casein fragments having the same amino acid sequence (but with the second peptide being longer by 5 residues at the C terminus) and only one peptide was derived from β-lactoglobulin, the shorter α_{S1}-casein fragment, namely, RFFVAPFPEVFGKEKVNEL (2,253.65 Da), was chemically synthesized and used in the induction assay at a final concentration range of 2.5 mg/ml to 0.25 ng/ml. Clear haloes in the WDA (with a starting population of the sensitive strain ranging from 10^3 to 10^5 CFU/ml) were observed for concentrations of >0.625 mg/ml, indicating that this peptide exhibits macedocin induction activity. The appropriate blanks performed only with the peptide's solvent were negative.

Effect of heat treatment and protease on the induction activity. The induction activity of the RPC fractions was fully retained after heat treatment at 100°C, indicating that it was due to a heat-stable molecule. Furthermore, proteolytic treatment did not result in the loss of induction activity, indicating that this corresponded either to a nonproteinaceous compound or to a proteinaceous compound of low molecular mass. Moreover, heat-treated induction assay samples fully retained their antimicrobial activity against both *L. lactis* subsp. *lactis* and *C. tyrobutyricum* target strains, while activity was lost in protease-treated samples, confirming that the induced antibacterial activity corresponded to a heat-stable and protease-labile molecule, typical properties of macedocin.

RT-PCR experiments. Using either the RPC IF-active fraction 18 or the synthetic peptide (2,253.65 Da) in the induction assay, macedocin production was confirmed by the RT-PCR approach. The expected product, corresponding to the 234-bp region of the macedocin structural gene *mcdA′* and confirmed by nucleotide sequence analysis, was amplified from mRNA derived from *S. macedonicus* ACA-DC 198 cells growing in the presence of either the IF-active fraction samples or the synthetic peptide samples after 4, 6, and 8 h of growth (Fig. 3). Using a WDA, the activity of macedocin was evident throughout the exponential phase of growth in both cases.

DISCUSSION

Previous studies already have shown that *S. macedonicus* ACA-DC 198 does not produce macedocin when cultured in various liquid media, such as MRS and M17 broth, despite the good growth of the strain under such culturing conditions (21). Macedocin is produced only when the strain is grown in skim milk. This observation led to the hypothesis that milk components play an important role in macedocin production.

According to the results obtained in the present study, au-

TABLE 3. Peptides detected in the induction-active HPLC fractions

Peptide sequence	HPLC–IF-positive fraction(s)	Calculated molecular mass (Da)	Exptl molecular mass (Da)	Amino acid position in milk protein	Bioactivity referred to in literature for peptide or fragment[d]
EPVLGPVRGPFPILV	C12, D1	1,589.93	1,572.88	β-Casein (210–224)[a]	Antimicrobial (5, 39), immunomodulatory (49)
QEPVLGPVRGPFPILV	C11, C12, D1	1,717.99	1,701.10	β-Casein (209–224)[a]	ACE-IA (7, 45, 53), antioxidant (53)
(*QE*)PVLGPVRGPFPILV[e]	C11, C12	1,717.99	1,785.09	β-Casein (209–224)[a]	Detected in Colostrinin, antimutagenic (32), detected in cheese extracts (35)
LYQEPVLGPVRGPFPILV	C12, D1, D2, D3	1,994.14	2,205.36	β-Casein (207–224)[a]	Modulators of the bitter taste receptors (34)
LLYQEPVLGPVRGPFPILV	C11, C12, D1	2,107.22	2,318.43	β-Casein (206–224)[a]	
RFFVAPFPEVFGKEKVNEL	C11	2,253.20	2,253.30	α_{S1}-Casein (37–55)[b]	Antimicrobial (47), ACE-IA (7, 39)
RFFVAPFPEVFGKEKVNELSKDIG	C12, D1	2,753.46	2,752.75	α_{S1}-Casein (37–60)[b]	Detected in colostrinin, antimutagenic (32), detected in cheese extracts (35)
KHPIKHQGLPQEVLNENLLRFF	D2, D3	2,657.46	2,657.58	α_{S1}-Casein (18–39)[b]	Antimicrobial (5), ACE-IA (53), detected in cheese extracts (35), detected in colostrinin, antimutagenic (32)
APSFSDIPNPIGSENSEKTTMPLW	C12, D1, D2, D3	2,618.24	2,618.53	α_{S1}-Casein (191–214)[b]	Antimicrobial (24), detected in colostrinin, antimutagenic (32)
DEALEKFDKALKALPMHIRL	D2, D3	2,338.29	2,338.40	β-Lactoglobulin (146–165)[c]	ACE-IA (36)

[a] According to sequence NP 851351 of β-casein from *Bos taurus*.
[b] According to sequence ACG63494 of α_{S1}-casein from *Bos taurus*.
[c] According to sequence ACG59280 of β-lactoglobulin from *Bos taurus*.
[d] ACE, angiotensin-converting enzyme-inhibiting activity. Colostrinin is a proline-rich polypeptide complex produced from colostrum with nutraceutical qualities.
[e] (*QE*), unknown modification at N-terminal QE.

toinduction, which is a very common regulatory mechanism of bacteriocin biosynthesis (46, 51, 52), does not occur in the case of macedocin, as the addition of either crude or purified macedocin to MRS or M17 (liquid or solid) medium did not cause any macedocin production. Moreover, the presence of sugars or coculturing conditions, which have been reported for other bacteriocins (8, 37, 48), do not seem to have an induction effect on macedocin biosynthesis.

Interestingly, macedocin production was evident when *S. macedonicus* was grown in MRS or M17 broth supplemented with milk or milk protein fractions, as well as when M17 broth was inoculated from an overnight culture of *S. macedonicus* in milk. These results support the initial hypothesis about the role of milk components, and more precisely of milk proteins, in the induction of macedocin biosynthesis. Moreover, the absence of macedocin production in MRS, fortified with supernatants or precipitates of overnight milk cultures of bacteriocin-negative but proteolytic *S. macedonicus* strains, was a strong indication that the induction molecule is a product deriving from the proteolytic degradation of milk proteins by the macedocin producer strain itself.

An induction factor of peptidic nature, not sensitive to heat or proteases, was isolated through chromatographic purification from the cell-free culture supernatant of *S. macedonicus* ACA-DC 198 grown in milk. When the highly induction-active RPC fraction 18 was used in the RT-PCR experiments, a 234-bp band, corresponding to the macedocin structural gene *mcdA′*, was revealed, confirming the presence of an induction molecule. The amplification of the expected RT-PCR product from cells taken at 4, 6, and 8 h of growth indicated that the transcripts of macedocin genes are produced throughout the exponential phase of growth of *S. macedonicus* ACA-DC 198.

Interestingly, the highly induction-active RPC fraction 18 consisted of milk protein fragments and, more precisely, of α_{S1}- and β-casein, as well as β-lactoglobulin. The peptides detected by MS/MS, or fragments of them, often have been reported in literature as bioactive, with remarkably diverse functionalities (Table 3). Indeed, several milk-derived peptides reveal multifunctional properties; e.g., specific peptide sequences have two or more different physiological activities. Some regions in the primary structure of caseins contain overlapping peptide sequences that exert different activities. These regions have been considered strategic zones that are partially protected from further breakdown (19). It should be stressed,

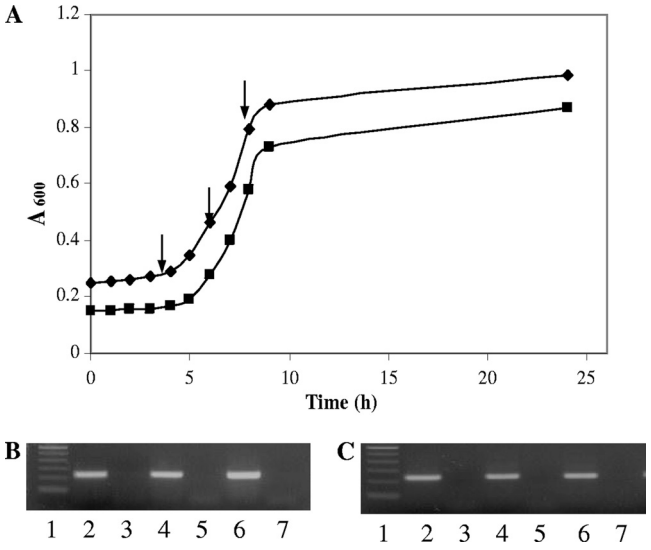

FIG. 3. (A) Growth curve of *S. macedonicus* ACA-DC 198 in the presence of the RPC induction-active fraction 18 (◆) or the chemically synthesized peptide RFFVAPFPEVFGKEKVNEL (final concentration of 2.5 mg/ml [■]). (B and C) RT-PCR analyses of the *mcd* biosynthetic/immunity operon of *S. macedonicus*. cDNA was synthesized using primer pRA′ and amplified using primers pFA and pRA′. Lane 1, DNA molecular size marker; lanes 2, 4, and 6, PCR products using a cDNA template originating from RNA obtained from *S. macedonicus* ACA-DC 198 grown for 4, 6, and 8 h, respectively, in the presence of the induction-active fraction 18 (B) or of the chemically synthesized peptide (C); lanes 3, 5, and 7, the respective PCR products in the absence of the induction-active fraction (B) or of the chemically synthesized peptide (C).

however, that this is the first time that these peptides are reported to act as bacteriocin induction molecules.

Three of the peptides detected in the highly induction-active RPC fraction 18, corresponding to fragments of α_{S1}-casein or β-lactoglobulin, were found to be unique in the overnight milk culture of *S. macedonicus* ACA-DC 198 compared to the respective milk cultures of two bacteriocin-negative but proteolytic *S. macedonicus* strains or the noninoculated milk itself. This was considered a strong indication that these peptides, originating from the degradation of milk proteins by *S. macedonicus* ACA-DC 198, were the most probable candidates for being the induction factor of macedocin biosynthesis. We chose one of them, namely, the α_{S1}-casein fragment RFFVA PFPEVFGKEKVNEL (2,253.65 Da), which was chemically synthesized, and tested it for induction activity. Indeed, both the WDA and the RT-PCR proved that the peptide was able to induce macedocin biosynthesis at a final concentration of >0.625 mg/ml (0.28 mM). Similarly to the samples of the induction-active fraction, transcription takes place throughout the exponential phase of the growth of *S. macedonicus* ACA-DC 198. The concentration required for induction (0.28 mM) seems to be high compared to the concentrations reported in the literature for other induction factors. Enterocins A and B can be induced by 10^{-17} M synthetic inducer, while threshold levels of about 10^{-10} M have been reported for plantaricin A, sakacin P, nisin, and carnobacteriocin B2 and BM1 systems (40).

The fact that this peptide was detected only in the induction-active fraction C11, whereas fractions C12, D1, D2, and D3 also were induction positive, indicates that the other two peptides with molecular masses of 2,752.7 (fractions C12 and D1) and 2,338 Da (fractions D2 and D3) also could act as macedocin inducers. Furthermore, the peptide of 2,752.7 Da has the same amino acid sequence as the tested one (2,253.65 Da), only being longer by five residues at the C terminus. Additionally, the fact that peptides deriving from the C terminus of β-casein are present in all induction-positive fractions suggests that such peptides act as macedocin biosynthesis inducers. The above-described hypothesis of the involvement of more than one peptide in the macedocin induction mode may justify our findings concerning the unusually high concentration of the synthetic peptide, which was necessary for the macedocin induction.

In conclusion, the results confirm our initial hypothesis that milk proteins serve as induction factors for macedocin biosynthesis. This was proven in the present study for the α_{S1}-casein 37-55 fragment (2,253.65 Da), while other fragments of milk protein degradation by *S. macedonicus* ACA-DC 198 cannot be excluded and thus need further investigation. To our knowledge, this is the first report of the induction of lantibiotic biosynthesis by peptides derived from milk protein degradation by the lantibiotic-producing LAB strain.

Besides this major finding, during the course of the present study, several additional functionalities about *S. macedonicus* ACA-DC 198 have been revealed that complement the protective profile of the strain, which has been described previously (1, 13, 58). These functionalities include the release of bioactive peptides from milk proteins through its proteolytic action, and this certainly deserves further study. Moreover, it is worth mentioning that during the purification procedure of the induction factor of macedocin, two further antimicrobial activities were detected. First, from milk cultures of *S. macedonicus* ACA-DC 198 an antimicrobial activity against *L. lactis* subsp. *lactis* LMG 6890^T was detected that did not correspond to macedocin, as this was eluted separately during the SCX chromatography, and, in contrast to the heat-stable macedocin, it was inactivated by heat treatment (data not shown). Second, a heat- and protease-labile antimicrobial activity against *C. tyrobutyricum* LMG 1285^T was observed when *S. macedonicus* ACA-DC 198 was grown in M17 broth (data not shown). This antimicrobial activity may be attributed to an antimicrobial molecule other than macedocin. The production of multiple bacteriocins by lactic acid bacteria has been reported before (4, 43, 60), and it may contribute to their dominance in certain ecosystems.

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REFERENCES

1. **Anastasiou, R., M. Georgalaki, E. Manolopoulou, I. Kandarakis, L. De Vuyst, and E. Tsakalidou.** 2007. The performance of *Streptococcus macedonicus* ACA-DC 198 as a protective starter in Kasseri cheese production. Int. Dairy J. **17:**208–217.
2. **Anderssen, E. L., D. B. Diep, I. F. Nes, V. G. H. Eijsink, and J. Nissen-Meyer.**

1998. Antagonistic activity of *Lactobacillus plantarum* C11: two new two-peptide bacteriocins, plantaricins EF and JK, and the induction factor plantaricin A. Appl. Environ. Microbiol. **64:**2269–2272.
3. **Barefoot, S. F., Y.-R. Chen, T. A. Hughes, A. B. Bodine, M. Y. Shearer, and M. D. Hughes.** 1994. Identification and purification of a protein that induces production of the *Lactobacillus acidophilus* bacteriocin lactacin B. Appl. Environ. Microbiol. **60:**3522–3528.
4. **Basanta, A., J. Sánchez, B. Gómez-Sala, C. Herranz, P. E. Hernández, and L. M. Cintas.** 2008. Antimicrobial activity of *Enterococcus faecium* L50, a strain producing enterocins L50 (L50A and L50B), P and Q, against beer-spoilage lactic acid bacteria in broth, wort (hopped and unhopped), and alcoholic and non-alcoholic lager beers. Int. J. Food Microbiol. **31:**293–307.
5. **Birkemo, G. A., P. O'Connor, C. Hill, and R. P. Ross.** 2006. Characterisation of three antimicrobial peptides produced in bovine colostrum, poster A09, p. 54. 1st Int. Symp. Antimicrob. Peptides. Food, Veteterinary, and Medical Applications, Nantes, France.
6. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
7. **Cadee, J. A., and L. F. Mallee.** 2007. Peptides inhibiting angiotensin-converting enzyme. Publication no. WO/2007/004876, international application no. PCT/NL2006/050155.
8. **Chandrapati, S., and D. J. O'Sullivan.** 1999. Nisin independent induction of the *nisA* promoter in *Lactococcus lactis* during growth in lactose or galactose. FEMS Microbiol. Lett. **170:**191–198.
9. **Chatterjee, C., M. Paul, L. Xie, and W. A. van der Donk.** 2005. Biosynthesis and mode of action of lantibiotics. Chem. Rev. **105:**633–684.
10. **Delgado, A., D. Brito, C. Peres, F. Noe-Arroyo, and A. Garrido-Fernandez.** 2005. Bacteriocin production by *Lactobacillus pentosus* B96 can be expressed as a function of temperature and NaCl concentration. Food Microbiol. **22:**521–528.
11. **Delves-Broughton, J., P. Blackburn, R. J. Evans, and J. Hugenholtz.** 1996. Applications of the bacteriocin nisin. Antonie Van Leeuwenhoek **69:**193–202.
12. **De Man, J. C., M. Rogosa, and M. E. Sharpe.** 1960. A medium for the cultivation of lactobacilli. J. Appl. Bacteriol. **23:**130–135.
13. **De Vuyst, L., and E. Tsakalidou.** 2008. *Streptococcus macedonicus*, a multifunctional and promising species for dairy fermentations. Int. Dairy J. **18:**476–485.
14. **Diep, D. B., L. S. Håvarstein, and I. F. Nes.** 1995. A bacteriocin-like peptide induces bacteriocin synthesis in *Lactobacillus plantarum* C11. Mol. Microbiol. **18:**631–639.
15. **Diep, D. B., L. Axelsson, C. Grefsli, and I. F. Nes.** 2000. The synthesis of the bacteriocin sakacin A is a temperature-sensitive process regulated by a pheromone peptide through a three-component regulatory system. Microbiology **146:**2155–2160.
16. **Dufour, A., T. Hindré, D. Haras, and J.-P. Le Pennec.** 2007. The biology of lantibiotics from the 481 group is coming of age. FEMS Microbiol. Rev. **31:**134–167.
17. **Eijsink, V. G. H., M. B. Brurberg, P. Hans Middelhoven, and I. F. Nes.** 1996. Induction of bacteriocin production in *Lactobacillus sake* by a secreted peptide. J. Bacteriol. **178:**2232–2237.
18. **Faye, T., D. A. Brede, T. Langsrud, I. F. Nes, and H. Holo.** 2002. An antimicrobial peptide is produced by extracellular processing of a protein from *Propionibacterium jensenii*. J. Bacteriol. **184:**3649–3656.
19. **Fiat, A. M., D. Miglore-Samour, P. Jollès, L. Drouet, C. B. D. Sollier, and J. Caen.** 1993. Biologically active peptides from milk proteins with emphasis on two examples concerning antithrombotic and immunomodulating activities. J. Dairy Sci. **76:**301–310.
20. **Georgalaki, M., P. Sarantinopoulos, E. S. Ferreira, L. De Vuyst, G. Kalantzopoulos, and E. Tsakalidou.** 2000. Metabolic properties of *Streptococcus macedonicus* strains isolated from Greek Kasseri cheese. J. Appl. Microbiol. **88:**817–825.
21. **Georgalaki, M., E. Van den Berghe, D. Kritikos, B. Devreese, J. Van Beeumen, G. Kalantzopoulos, L. De Vuyst, and E. Tsakalidou.** 2002. Macedocin: a food grade lantibiotic produced by *Streptococcus macedonicus* ACA-DC 198. Appl. Environ. Microbiol. **68:**5891–5903.
22. **Gomez, A., M. Ladiré, F. Marcille, and M. Fons.** 2002. Trypsin mediates growth phase-dependent transcriptional regulation of genes involved in biosynthesis of ruminococcin A, a lantibiotic produced by a *Ruminococcus gnavus* strain from human intestinal microbiota. J. Bacteriol. **184:**18–28.
23. **Gursky, L. J., N. I. Martin, D. J. Derksen, M. J. van Belkum, K. Kaur, J. C. Vederas, M. E. Stiles, and L. M. McMullen.** 2006. Production of piscicolin 126 by *Carnobacterium maltaromaticum* UAL26 is controlled by temperature and induction peptide concentration. Arch. Microbiol. **186:**317–325.
24. **Hayes, M., R. P. Ross, G. F. Fitzerald, C. Hill, and C. Stanton.** 2006. Casein-derived antimicrobial peptides generated by *Lactobacillus acidophilus* DPC6026. Appl. Environ. Microbiol. **72:**2260–2264.
25. **Hindré, T., J.-P. Le Pennec, D. Haras, and A. Dufour.** 2004. Regulation of lantibiotic lacticin 481 production at the transcriptional level by acid pH. FEMS Microbiol. Lett. **231:**291–298.
26. **Hynes, W. L., J. J. Ferretti, and J. R. Tagg.** 1993. Cloning of the gene encoding streptococcin A-FF22, a novel lantibiotic produced by *Streptococcus pyogenes*, and determination of its nucleotide sequence. Appl. Environ. Microbiol. **59:**1969–1971.
27. **Hynes, W. L., V. L. Friend, and J. J. Ferretti.** 1994. Duplication of the lantibiotic structural gene in M-type 49 group A *Streptococcus* strains producing streptococcin A-M49. Appl. Environ. Microbiol. **60:**4207–4209.
28. **Jack, R. W., and J. R. Tagg.** 1992. Factors affecting production of the group A *Streptococcus* bacteriocin SA-FF22. J. Med. Microbiol. **36:**132–138.
29. **Jack, R. W., A. Carne, J. Metzger, S. Stefanovic, H.-G. Sahl, G. Jung, and J. R. Tagg.** 1994. Elucidation of the structure of SA-FF22, a lanthionine-containing antibacterial peptide produced by *Streptococcus pyogenes* strain FF22. Eur. J. Biochem. **220:**455–462.
30. **Kleerebezem, M., and L. E. Quadri.** 2001. Peptide pheromone-dependent regulation of antimicrobial peptide production in Gram-positive bacteria: a case of multicellular behavior. Peptides **22:**1579–1596.
31. **Kleerebezem, M.** 2004. Quorum sensing control of lantibiotic production; nisin and subtilin autoregulate their own biosynthesis. Peptides **25:**1405–1414.
32. **Kruzel, M., and I. Boldogh.** February 2008. Use of colostrinin, constituent peptides thereof, and analogs as anti-mutagenic agents. Publication no. WO/2008/016604, international application no. PCT/US2007/017112.
33. **Leroy, F., and L. De Vuyst.** 2004. Functional lactic acid bacteria starter cultures for the food fermentation industry. Trends Food Sci. Technol. **15:**67–78.
34. **Li, X., X. Hong, L. Qing, T. Huixian, and A. Pronin.** February 2008. Identification of bitter ligands that specifically activate human T2R receptors and related assays for identifying human bitter taste modulators. U.S. patent 20,080,038,739.
35. **Losito, I., T. Carbonara, M. Domenica De Bari, M. Gobbetti, F. Palmisano, C. G. Rizzello, and P. G. Zambonin.** 2006. Identification of peptides in antimicrobial fractions of cheese extracts by electrospray ionization ion trap mass spectrometry coupled to a two-dimensional liquid chromatographic separation. Rapid Commun. Mass Spectrom. **20:**447–455.
36. **Maes, W., J. V. Camp, V. Vermeirssen, M. Hemeryck, J. M. Ketelslegers, J. Schrezenmeir, P. Van Oostveldt, and A. Huyghebaert.** 2004. Influence of the lactokinin Ala-Leu-Pro-Met-His-Ile-Arg (ALPMHIR) on the release of endothelin-1 by endothelial cells. Regulatory Peptides **118:**105–109.
37. **Maldonado, A., R. Jiménez-Díaz, and J. L. Ruiz-Barba.** 2004. Induction of plantaricin production in *Lactobacillus plantarum* NC8 after coculture with specific Gram-positive bacteria is mediated by an autoinduction mechanism. J. Bacteriol. **186:**1556–1564.
38. **McAuliffe, O., R. Paul Ross, and C. Hill.** 2001. Lantibiotics: structure, biosynthesis and mode of action. FEMS Microbiol. Rev. **25:**285–308.
39. **Minervini, F., F. Algaron, C. G. Rizzello, P. F. Fox, V. Monnet, and M. Gobbletti.** 2003. Angiotensin I-converting-enzyme-inhibitory and antibacterial peptides from *Lactobacillus helveticus* PR4 proteinase-hydrolyzed caseins of milk from six species. Appl. Environ. Microbiol. **69:**5297–5305.
40. **Nilsen, T., I. F. Nes, and H. Holo.** 1998. An exported inducer peptide regulates bacteriocin production in *Enterococcus faecium* CTC492. J. Bacteriol. **180:**1848–1854.
41. **Nilsson, L., M. K. Nielsen, Y. Ng, and L. Gram.** 2002. Role of acetate in production of an autoinducible class IIa bacteriocin in *Carnobacterium piscicola* A9b. Appl. Environ. Microbiol. **68:**2251–2260.
42. **Papadelli, M., A. Karsioti, R. Anastasiou, M. Georgalaki, and E. Tsakalidou.** 2007. Characterization of the gene cluster involved in the biosynthesis of macedocin, the lantibiotic produced by *Streptococcus macedonicus*. FEMS Microbiol. Lett. **272:**75–82.
43. **Papathanasopoulos, M. A., F. Krier, A.-M. Revol-Junelles, G. Lefebvre, J. P. Le Caer, A. Von Holy, and J. W. Hastings.** 1997. Multiple bacteriocin production by *Leuconostoc mesenteroides* TA33a and other *Leuconostoc*/*Weissella* strains. Curr. Microbiol. **35:**331–335.
44. **Parente, E., and C. Hill.** 1992. A combination of factors affecting the production of two bacteriocins from lactic acid bacteria. J. Appl. Bacteriol. **73:**290–298.
45. **Quirós, A., B. Hernández-Ledesma, M. Ramos, L. Amigo, and I. Recio.** 2005. Angiotensin-converting enzyme inhibitory activity of peptides derived from caprine kefir. J. Dairy Sci. **88:**3480–3487.
46. **Ra, S. R., M. Qiao, T. Immonen, I. Pujana, and P. E. J. Saris.** 1996. Genes responsible for nisin synthesis, regulation and immunity form a regulon of two operons and are induced by nisin in *Lactococcus lactis* N8. Microbiology **142:**1281–1288.
47. **Rizzello, C. G., I. Losito, M. Gobbetti, and T. Carbonara.** 2005. Antibacterial activities of peptides from the water-soluble extracts of Italian cheese varieties. J. Dairy Sci. **88:**2348–2360.
48. **Rojo-Bezares, B., Y. Sáenz, L. Navarro, M. Zarazaga, F. Ruiz-Larrea, and C. Torres.** 2007. Coculture-inducible bacteriocin activity of *Lactobacillus plantarum* strain J23 isolated from grape must. Food Microbiol. **24:**482–491.
49. **Sandré, C., A. Gleizes, F. Forestier, R. Gorges-Kergot, S. Chilmonczyk, J. Léonil, M. C. Moreau, and C. Labarre.** 2001. A peptide derived from bovine beta-casein modulates functional properties of bone marrow-derived macrophages from germfree and human flora-associated mice. J. Nutr. **131:**2936–2942.

50. **Sashihara, T., M. Dan, H. Kimura, H. Matsuaki, K. Sonomoto, and A. Ishizaki.** 2001. The effect of osmotic stress on the production of nukacin ISK-1 from *Staphylococcus warneri* ISK-1. Appl. Microbiol. **56:**496–501.
51. **Saucier, L., A. Poon, and M. E. Stiles.** 1995. Induction of bacteriocin in *Carnobacterium piscicola* LV17. J. Appl. Bacteriol. **78:**684–690.
52. **Schmitz, S., A. Hoffmann, C. Szekat, B. Rudd, and G. Bierbaum.** 2006. The lantibiotic mersacidin is an autoinducing peptide. Appl. Environ. Microbiol. **72:**7270–7277.
53. **Silva, S. V., A. Pihlanto, and F. X. Malcata.** 2006. Bioactive peptides in ovine and caprine cheeselike systems prepared with proteases from *Cynara cardunculus*. J. Dairy Sci. **89:**3336–3344.
54. **Sturme, M. H. J., M. Kleerebezem, J. Nakayama, A. D. L. Akkermans, E. E. Vaughan, and W. M. de Vos.** 2002. Cell to cell communication by autoinducing peptides in Gram-positive bacteria. Antonie Van Leeuwenhoek **81:** 233–243.
55. **Tagg, J. R., and A. R. McGiven.** 1971. Assay system for bacteriocins. Appl. Microbiol. **21:**943–946.
56. **Tsakalidou, E., E. Zoidou, B. Pot, L. Wassil, W. Ludwig, L. A. Devriese, G. Kalantzopoulos, K. H. Schleifer, and K. Kersters.** 1998. Identification of streptococci from Greek Kasseri cheese and description of *Streptococcus macedonicus* sp. nov. Int. J. Syst. Bacteriol. **48:**519–527.
57. **Van Belkum, M. J., and M. E. Stiles.** 2000. Nonlantibiotic antibacterial peptides from lactic acid bacteria. Nat. Prod. Rep. **17:**323–335.
58. **Van den Berghe, E., G. Skourtas, E. Tsakalidou, and L. De Vuyst.** 2006. *Streptococcus macedonicus* ACA-DC 198 produces the lantibiotic, macedocin, at temperature and pH conditions that prevail during cheese manufacture. Int. J. Food Microbiol. **107:**138–147.
59. **Wang, B.-Y., and H. K. Kuramitsu.** 2005. Interactions between oral bacteria: inhibition of *Streptococcus mutans* bacteriocin production by *Streptococcus gordonii*. Appl. Environ. Microbiol. **71:**354–362.
60. **Wescombe, P. A., M. Upton, K. P. Dierksen, N. L. Ragland, S. Sivabalan, R. E. Wirawan, M. E. Inglis, C. J. Moore, G. V. Walker, C. N. Chilcott, H. F. Jenkinson, and J. R. Tagg.** 2006. Production of the lantibiotic salivaricin A and its variants by oral streptococci and use of a specific induction assay to detect their presence in human saliva. Appl. Environ. Microbiol. **72:**1459–1466.