Milk Protein Fragments Induce the Biosynthesis of Macedocin, the Lantibiotic Produced by *Streptococcus macedonicus* ACA-DC 198\(^\text{a}\)

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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 co-culture 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 \(\alpha_s\)-casein and \(\beta\)-casein as well as \(\beta\)-lactoglobulin. The chemically synthesized \(\alpha_s\)-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’1MTFEG*) 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 IS191 (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 bacteriocins, 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 transcrip-
tion 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 *Lactoba-
cillus delbrueckii* subsp. *lactis* ATCC 4797 strain (3). Also, it has been reported that bacteriocin production by *Ruminococcus gravis* E1 is controlled through a complex signaling mech-
anism involving the proteolytic processing of a putative extra-
cellular 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. macedon-
dicus* 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 Labora-
tory 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, Bas-
ingstoke, 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 6890T and *Clostridium tyrobutyricum* LMG 1285T, which were used as routine target strains, were cultured aerobically in M17 broth (Biokar Diagnostics, Beauvais, France) at 30°C and anaerobi-
cally (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 6890T was subcultured twice in M17 broth, and finally a 16-h culture (10 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 superna-
tants 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 1285T, except that RCM agar (Oxoid) and 2% (vol/vol) inoculum was used.

**Determination of the induction activity (induction assay [IA]).** Induction activity was assayed according to Eijink 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. macedoni-
cus* 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 mult-
tilter 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 concentra-
tions 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 6890T (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 investi-
gated 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 6890T, *L. lactis subsp. cremoris* LMG 6897T, *S. thermophilus* ACA-DC 9, Enterococcus lactis LMG 1143T, and *Listeria innocua* LMG 11387T) 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 206T, *Enterococcus faecalis* LMG 7937T, and *L. innocua* LMG 13588). Also, we examined the effect on macedocin pro-
duction 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 Georganaki et al. (21).

**Induction factor purification.** All purification steps were performed at room temperature using the fast protein liquid chromatography (FPLC) system of Waters (Milford, MA) equipped with a ProteinPak preevidced protein purification system) and an Ettan LC system from Amersham Biosciences (Uppsala, Sweden). FPLC columns were purchased from Amersham, and the Altima HP hydrophilic interaction liquid chromatography (HLIC) 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) equili-
brated 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 (ddH2O) 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) equili-
brated 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 (19).

The fraction containing the highest induction activity was applied to an HLIC 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 proteolytic products on macedocin production by S. macedonicus ACA-DC 198

<table>
<thead>
<tr>
<th>Growth medium (liquid)</th>
<th>Antimicrobial activity (AU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skim milk (10%, wt/vol) supplemented with YE (0.3%, wt/vol)</td>
<td>160</td>
</tr>
<tr>
<td>M17 cocultures of S. macedonicus with macedocin-sensitive or -resistant strains</td>
<td>0</td>
</tr>
<tr>
<td>MRS or M17 supplemented with lactose (1.0–2.0%, wt/vol)</td>
<td>0</td>
</tr>
<tr>
<td>MRS or M17 supplemented with galactose (0.5–2.5%, wt/vol)</td>
<td>0</td>
</tr>
<tr>
<td>MRS supplemented with whole milk (2%, wt/vol)</td>
<td>80</td>
</tr>
<tr>
<td>MRS supplemented with milk caseins (2%, wt/vol)</td>
<td>80</td>
</tr>
<tr>
<td>M17 supplemented with milk caseins (2%, wt/vol) ± YE (0.3%, wt/vol)</td>
<td>40 (–YE), 80 (+YE)</td>
</tr>
<tr>
<td>M17 supplemented with milk whey proteins (0–0.75%, wt/vol) ± YE (0.3%, wt/vol)</td>
<td>40 (–YE), 80 (+YE)</td>
</tr>
<tr>
<td>M17 inoculated with S. macedonicus grown in milk (10%, wt/vol) with YE (0.3%, wt/vol)</td>
<td>40</td>
</tr>
<tr>
<td>M17 inoculated with S. macedonicus grown in M17</td>
<td>0</td>
</tr>
<tr>
<td>M17 inoculated with S. macedonicus grown in MRS</td>
<td>0</td>
</tr>
<tr>
<td>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)</td>
<td>0</td>
</tr>
</tbody>
</table>

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 68907, L. lactis subsp. cremoris LMG 68977, S. thermophilus ACA-DC 9, E. faecalis LMG 11387T, and L. innocua LMG 11387. 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 2067, E. faecalis LMG 7937T, and L. innocua LMG 13568.

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 with 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.
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 $\alpha_S^1$- and $\beta$-casein, as well as to internal fragments of $\alpha_S^1$-casein and $\beta$-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 $\alpha_S^1$-casein, namely, RFFVAPFPEVFGKEKVNE (2,253.65 Da) and RFFVAPFPEVFGKEKVNEKDIG (2,752.7 Da), as well as $\beta$-lactoglobulin, namely, DEALEKFDKALKALPMHRL (2,338 Da), also were detected in the ammonium sulfate precipitate of S. macedonicus ACA-DC 198, while they were

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### TABLE 2. Partial purification of Streptococcus macedonicus ACA-DC 198 induction factor

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Vol (ml)</th>
<th>Act (IU/ml)</th>
<th>Protein concn (mg/ml)</th>
<th>Total act (IU)</th>
<th>Sp act (IU/mg)</th>
<th>Fold purification</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>850</td>
<td>1,280</td>
<td>0.30</td>
<td>1,088,000</td>
<td>4,266</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>Crude-ASP</td>
<td>260</td>
<td>1,280</td>
<td>0.82</td>
<td>332,800</td>
<td>1,561</td>
<td>0.4</td>
<td>31</td>
</tr>
<tr>
<td>Resource S (pH 5.5)</td>
<td>131</td>
<td>1,280</td>
<td>0.49</td>
<td>167,680</td>
<td>2,612</td>
<td>0.6</td>
<td>15</td>
</tr>
<tr>
<td>Resource S-ASP</td>
<td>92</td>
<td>640</td>
<td>0.83</td>
<td>38,880</td>
<td>771</td>
<td>0.2</td>
<td>5</td>
</tr>
<tr>
<td>Resource RPC</td>
<td>22</td>
<td>1,280</td>
<td>1.18</td>
<td>28,160</td>
<td>1,084</td>
<td>0.3</td>
<td>3</td>
</tr>
</tbody>
</table>

*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).
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 αs1-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 αs1-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 medA 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-
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 the macedocin producer strain itself.

An induction factor of peptidic nature, not sensitive to heat or proteolysis, 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 αs1-casein and β-casein, as well as β-lactoglobulin. The peptides detected by MS/MS, or fragments of them, often have been reported in literature for peptide bioactivities. However, 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.

Some regions in the primary structure of caseins contain overlapping peptide sequences that exert different activities. These functionalities (Table 3). Indeed, several milk-derived peptides reported in literature as bioactive, with remarkably diverse activities (49), detected in cheese extracts (35), were 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.

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A and B can be induced by 10 mM) seems to be high compared to the concentrations required for induction (0.28 M) of the exponential phase of the growth of S. macedonicus. The induction-active fraction, transcription takes place throughout the presence of the RPC 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).

Three of the peptides detected in the highly induction-active RPC fraction 18, corresponding to fragments of αs1-casein or β-lactoglobulin, were found to be unique in the overnight 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, were the most probable candidates for being the induction factor of macedocin biosynthesis. We chose one of them, namely, the αs1-casein fragment RFFVA PPFEVFGEKVNEL (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 10⁻⁷ M have been reported for plantaricin A, sakacin P, nisin, and carnobacteriocin B2 and BMI1 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 Da (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 αs1-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 6890⁷ 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 1285⁷ 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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