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Biochimica et Biophysica Acta 1373 (1998) 47–58

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

# A novel bacteriocin with a YGNGV motif from vegetable-associated *Enterococcus mundtii*: full characterization and interaction with target organisms

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Received 6 May 1998; accepted 14 May 1998

**Abstract**

A novel broad-spectrum antimicrobial peptide produced by vegetable-associated *Enterococcus mundtii* was purified and characterized, and designated mundticin. To our knowledge, this is the first report on bacteriocin production by this organism. The elucidation of the full primary amino acid sequence of mundticin (KYYGNGVSCNKKGCSVDWGKAIGIIGNNSAANLATGGAAGWSK) revealed that this antimicrobial peptide belongs to the class IIa bacteriocins of lactic acid bacteria which share a highly conserved N-terminal 'YGNGV' motif. Data obtained by computer modelling indicated an oblique orientation of the α-helical regions of mundticin and homologous class IIa bacteriocins at a hydrophobic–hydrophilic interface, which may play a role in the destabilization of phospholipid bilayers. The average mass of mundticin, as determined by electron spray mass spectrometry, was found to be 4287.21 ± 0.59 Da. With respect to its biological activity, mundticin was shown to inhibit the growth of *Listeria monocytogenes*, *Clostridium botulinum* and a variety of lactic acid bacteria. Moreover, it was demonstrated to have a bactericidal effect on *L. monocytogenes* as a result of the dissipation of the membrane potential, and a loss of intracellular ATP in absence of ATP leakage. Its good solubility in water, and its stability over a wide pH and temperature range indicate the potential of this broad spectrum bacteriocin as a natural preservation agent for foods. 

*Keywords:* Bacteriocin; YGNGV motif; Oblique peptide; Lactic acid bacterium; (*Enterococcus mundtii*)

## 1. Introduction

Bacteriocins are antimicrobial peptides and proteins that are ribosomally synthesized by bacteria [1]. These compounds can inhibit or eliminate the growth of target organisms by affecting the membrane permeability [2,3], or by interfering with essential cell functions such as DNA replication [4] and translation [5]. Traditionally, bacteriocin research

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0005-2736/98/$19.00 
PII: S0005-2736(98)00086-8

has focussed on proteins of Gram-negative bacteria [6]. However, bacteriocins produced by Gram-positive organisms, particularly those from lactic acid bacteria (LAB), have recently provoked a great deal of interest for their potential as non-toxic preservatives in the food and feed industry [7]. Four classes of LAB bacteriocins can be distinguished [8], with the class I and II bacteriocins being by far the most abundant and best characterized. Members of these two classes are small, heat-stable peptides of a cationic and hydrophobic nature. Their antimicrobial activity generally seems to be related to the formation of pores in the cytoplasmic membrane of target cells, which is thought to involve binding of monomers to the membrane, followed by insertion into the membrane and aggregation of monomers to form water-filled pores. This process explains the death of target organisms due to the leakage of small intracellular components and the dissipation of the proton motive force [9–11].

The molecular mechanisms by which class I and II bacteriocins of LAB act, appear to differ considerably. Detailed studies of class I bacteriocins, which typically contain post-translationally modified amino acids (lanthionines), demonstrated that these compounds act in a membrane potential dependent way in absence of a protein-receptor [3,12]. By contrast, class II bacteriocins, which do not contain modified amino acids, are believed to interact with membrane receptor proteins prior to insertion into the cytoplasmic membrane in a voltage-independent way [11,13]. Within the latter class of bacteriocins, particular attention has been paid to members of the subclass IIa, mainly because of their activity toward the pathogen *Listeria monocytogenes*. The class IIa bacteriocins are characterized by the presence of a N-terminal consensus sequence 'Tyr-Gly-Asn-Gly-Val' (YGNGV) [8,14]. This highly conserved N-terminal region has been suggested to form part of a recognition sequence for a putative membrane-bound protein receptor [15,16]. However, it was recently demonstrated that a prominent member of this class, pediocin PA-1, can function in absence of a protein receptor [17].

The initial steps in pore formation by bacteriocins involve: (1) electrostatic interactions between the positively charged and polar residues of the peptide and the head groups of the phospholipids in the bilayer; and (2) hydrophobic interactions between the hydrophobic residues of the peptide and the lipid acyl chains [16,18,19]. Importantly, class IIa bacteriocins are predicted to contain α-helical regions with a varying amount of hydrophobicity [8,10], which are thought to play a key role in the interaction with the lipid bilayer [16]. Experimental evidence for the presence of an α-helical region in lipophilic environments was recently reported for the class IIa bacteriocin leucocin A, upon elucidation of its three-dimensional structure [20]. However, the structural and functional properties of complexes of the α-helical regions of the class IIa bacteriocins and lipid bilayers are unknown.

A recent classification of lipid-associating α-helices distinguishes between lipid-associated peptides with a constant hydrophobicity, and peptides with a hydrophobic gradient along the axis of their α-helix [18,21]. Peptides of the former category have been found to interact with lipid bilayers through helical structures which are positioned parallel or perpendicular to the hydrophobic–hydrophilic interface. Conversely, oblique peptides with a hydrophobicity gradient along their axis insert at a 30–60° angle into hydrophobic/hydrophilic interfaces. Such peptides have been identified in processes in which membrane perturbation underlies the action of the peptide (recently reviewed in [18]). These processes include a wide range of biological phenomena, such as cell signalling events [22], fusion events that are induced by viral peptides [23,24] or the Alzheimer β-amyloid peptide [25], and processes in lipid metabolism [18].

The objective of this study was to characterize the compound that is responsible for the antimicrobial activity of a vegetable-associated strain of *Enterococcus mundtii*. This compound was identified as the novel bacteriocin mundticin, and its action on the cytoplasmic membrane of sensitive cells was demonstrated. We furthermore examined the orientation of the α-helical region of mundticin and homologous bacteriocins at a hydrophobic–hydrophilic interface by means of computer modelling.

## 2. Materials and methods

### 2.1. Bacterial strains and culturing conditions

In a search for bacteriocinogenic LAB from minimally processed vegetables, we obtained three bacteriocin-producing LAB with activity toward *L. monocytogenes*, out of a total of 900 isolates as described previously [26]. Two strains were identified as *Pediococcus parvulus* [26]. The third strain was isolated from fresh chicory endive and preliminarily identified as *Enterococcus* strain ATO6 by the use of API Rapid CH fermentation strips (BioMerieux, Marcy d'Etoile, France). Full characterization by the services of the Deutsche Sammlung von Mikroorganismen (DSM, Braunschweig, Germany) identified this strain as *E. mundtii* on the basis of fermentation patterns and 16S rRNA analysis. *Lactobacillus sake* DSM 20017 was used as an indicator strain for the monitoring of bacteriocin activity. *E. mundtii* ATO6 and *L. sake* DSM 20017 were routinely grown in MRS medium (Oxoid, Basingstoke, UK). *L. monocytogenes* LDCD81-861 [27] (kindly provided by Dr. Nguyen-The, Institut Nationale de la Recherche Agronomique, France) was grown in brain heart infusion (BHI) broth (Oxoid) supplemented with 0.5% (w/v) glucose. All cultivations were started with 1% inocula and performed at 30°C.

### 2.2. Bacteriocin assays, antimicrobial spectrum and stability

The antimicrobial activity of mundticin was determined in a microtiter assay as previously described [26]. Hereby, the activity was expressed in bacteriocin units (BU) per ml, which was calculated from the reciprocal of the highest dilution of a sample that reduced the $OD_{660}$ of the indicator organism by 50% after 8 h of incubation at 30°C. This assay was also used to determine the minimally inhibitory concentration (MIC) of mundticin against *L. monocytogenes* LDCD81-861, starting from a purified bacteriocin solution with a known bacteriocin concentration.

To determine the nature of the inhibitory substance, the supernatant of *E. mundtii* ATO6 was treated with proteolytic enzymes, i.e. trypsin, α-chymotrypsin and proteinase K (1 mg/ml) (Boehringer, Mannheim, Germany) for 2 h at 37°C. The pH stability of the purified bacteriocin during storage at 4°C for 14 h was determined in the range of pH 1–12 with 1 pH unit intervals. Following incubation, the antimicrobial activity of the samples was determined by the use of the microtiter assay as described above. The heat resistance was assessed by boiling a bacteriocin solution which was adjusted to pH 6.0 for 1, 5, 10, 15, 30 or 60 min. Again, the remaining bacteriocin activity was measured by the use of the microtiter assay.

Growth inhibition of a panel of 65 bacteria and fungi (listed in [26]) in response to culture supernatant of *E. mundtii* was determined by the use of a well diffusion assay and a microtiter assay as described previously [26]. To establish whether the action of mundticin was bacteriostatic or bactericidal, late-exponential-phase cells of *L. monocytogenes* LCDC81-681 in BHI broth were exposed to this bacteriocin (final concentrations 0.1 and 1.0 μg/ml) and after dilution of the cells, the viable counts were determined at regular time intervals during 5 h at 30°C.

### 2.3. Purification of mundticin

The bacteriocin produced by *E. mundtii* ATO6 was isolated from 2 l of a 22 h static culture (grown in MRS broth (Oxoid) at 30°C). The cells were removed by centrifugation, and proteins were subsequently concentrated from the supernatants by a two-step ammonium sulfate precipitation (0–25% and 25–70% saturation). The fraction obtained from the second step of this precipitation was dissolved in 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer, pH 5.5, containing 0.85 M ammonium sulfate. Aliquots (3 ml) were loaded on a 24-ml phenyl Sepharose CL4B column (Pharmacia, Uppsala, Sweden) which was equilibrated with 0.42 M ammonium sulfate in 50 mM MES buffer, pH 5.5. The elution was performed by a 140 min linear gradient from 0.42 to 0.0 M ammonium sulfate in 50 mM MES buffer, pH 5.5, at 2 ml/min. The most active fractions were pooled, diluted five-fold in 10 mM formic acid–10% EtOH (solution A), and loaded on a 1 ml Resource S cation-exchange column (Pharmacia) which was equilibrated with solution A. The column was eluted with a 30 min linear gradient from 0.0 to 1.0 M NaCl in 10 mM formic acid–

10% EtOH at 5 ml/min. The active fractions were pooled, and further purification was accomplished by loading 50 μl samples on a Superdex Peptide PC3.2/30 gel filtration column (Smartsystem, Pharmacia), followed by elution in 0.15 M NaCl–0.1% trifluoroacetic acid (TFA)–20% EtOH at 80 μl/min (monitoring at 214, 254, and 280 nm). The homogeneity of the peaks was confirmed by reversed phase chromatography with a $C_2$–$C_{18}$ μRPC 3.2/30 column (Pharmacia) using a 30 min linear gradient from 20 to 95% EtOH in 0.1% TFA (100 μl/min, monitoring at 214 nm).

### 2.4. Enzymatic cleavage and alkylation of mundticin

Purified mundticin (40 μg/ml in 50 mM Tris–HCl, pH 7.4) was incubated with the sequence grade endoprotease Asp-N (Boehringer) at an enzyme to substrate ratio of 1:20 (w/w). After incubation for 7 h at 37°C, the reaction was stopped by the addition of trichloroacetic acid (TCA) (5% final concentration). The peptide fragments were immediately separated by injection of 50 μl samples on a Superdex Peptide PC3.2/30 gel filtration column (Pharmacia), using the same conditions as described above. Reduction and alkylation of purified mundticin were performed by dissolving the peptide in 0.2 M Tris–6 M guanidine–HCl buffer, pH 8.4, containing 20 mM DTT. After incubation for 1 h at 20°C, 1 μl of 4-vinylpyridine was added. This reaction mixture was further incubated for 1 h at 20°C, after which the sample was diluted (1:1 in water) and sequenced.

### 2.5. N-terminal amino acid sequence analysis and mass spectrometry

Purified peptides were analyzed by the Sequence Center Utrecht (University of Utrecht, Utrecht, The Netherlands). The N-terminal sequences were obtained by Edman degradation on an automated gas phase sequencer (Applied Biosystems model 476A) with on-line phenylthiohydantoin derivate identification by reversed phase chromatography on HPLC. Electronspray-mass spectrometry (ES-MS) was performed at the Institute for Animal Science and Health (ID-DLO, The Netherlands). The mass spectrum of purified peptides was determined by direct injection (1.2 μg/μl in 0.1% TFA–50% EtOH) on a Quattro IISQ instrument (Micromass, Manchester, UK).

### 2.6. Measurement of the membrane potential with fluorescent probe

Cells of *L. monocytogenes* LDCD81-861 were harvested in the exponential phase of growth ($OD_{660}$ 0.6) by centrifugation (5000×*g*, 15 min) at 4°C, washed twice in 50 mM potassium *N*-2-Hydroxyethylpiperazine-*N*′-2-ethane-sulfonic acid (K-HEPES) buffer (pH 7.0), resuspended in this buffer (to approximately 1/50 of the original culture volume), and stored on ice until use. Membrane potential ($\Delta\psi$) measurements were performed by using the fluorescent probe 3,3-dipropylthiocarbocyanine ($DiSC_35$). Cell suspensions were diluted in the K-HEPES buffer to a density of 0.06 mg bacterial protein/ml. Reactions were performed at 20°C in a Perkin Elmer LS 50B spectrofluorometer, and the $\Delta\psi$ was monitored with $DiSC_35$ (excitation wavelength, 643 nm; emission wavelength, 666 nm) at a final concentration of 5 μM. The was generated after the addition of glucose in the presence of the $H^+/K^+$ exchanger nigericin (final concentrations of 0.2% w/v and 5 μM, respectively). After reaching a steady-state $\Delta\psi$, the purified mundticin was added to the cells. The $K^+$ ionophore valinomycin (final concentration 2 μM) was used as a control for the absence of a membrane potential. Protein concentrations of bacteriocin preparations were determined with the NanoOrange Protein Quantitation kit (Molecular Probes Europe), using bovine serum albumin as a standard.

### 2.7. Measurement of ATP concentrations

To determine the influence of mundticin on the intracellular and extracellular ATP concentrations of *L. monocytogenes* LDCD81-681, cell suspensions were prepared essentially as described above for the $\Delta\psi$ measurements, however, with the use of 50 mM potassium phosphate ($KP_i$) buffer (pH 7.0). Cell suspensions were diluted in this $KP_i$ buffer (0.10 mg bacterial protein/ml) and incubated for 6 min at 30°C with glucose (final concentration of 0.5%, w/v), prior to the addition of purified mundticin (final concentrations of 0.14 μg/ml). At regular time intervals, 0.2 ml samples of the suspensions were re-

moved, and the cells were immediately separated from the external medium by spinning them through a layer of silicon oil, which was placed on top of a layer of 50 μl 10% (w/v) TCA with 2 mM EDTA [28]. Aliquots (5 μl) of both aqueous layers were used to determine the ATP content using the firefly luciferase assay as described previously [29]. Luminescence was recorded using a BIO-Orbit 1250 luminometer (Turku, Finland). Protein concentrations of bacterial cell suspensions were determined and by the method of Lowry et al. [30], using bovine serum albumin as a standard.

### 2.8. Identification of an oblique-orientated peptide and molecular modelling of peptide insertion into the lipid bilayer environment

The search for an oblique-orientated helical peptide in mundticin and other class IIa bacteriocins was carried out by scanning the entire bacteriocin sequences according to the procedure of Rahman et al. [31]. The hydrophobicity gradient for a 12–13 residue peptide window along the different bacteriocin sequences was calculated according to the method of Jähnig [32], taking into account the mean hydrophobicity ($H_o$) of the peptide and its hydrophobic moment ($\mu_H$), which is a measure for the amphiphilic nature of the peptide, using the Eisenberg's hydrophobicity scale [33]. Any helical peptide with an $H_o$ of $\geq 0.2$ and a hydrophobicity gradient along the axis of the helical peptide between the C-terminal and N-terminal end was considered as an acceptable candidate. Modelling of all peptides was carried out as described previously [21] and the method used is that applied to the study of amphiphilic molecule conformation [34]. The method used for the prediction of the conformational structure of the peptides accounted for the contribution of classic energy (Van der Waals, electrostatic and hydrophobic binding energy) as well as the lipid–water interface properties, including the concomitant variation of the dielectric constant and the transfer energy of atoms from a hydrophobic to a hydrophilic environment [35]. The structure, mode of insertion, and orientation of the peptides were predicted as in a hydrophobic–hydrophilic interface. In this model, the interaction energy was calculated and minimized until the lowest energy state of the entire peptide–lipid aggregate was reached. All calculations were performed on a Pentium Pro processor station, by using PC-TAMMO+ (Theoretical Analysis of Molecular Membrane Organization) and PC-PROT+ (Protein Plus Analysis) software. Graphs were drawn with the WINMGM program.

### 2.9. Accession number

The amino acid sequence of mundticin determined in this study has been deposited in the SwissProt database under accession number P80925.

## 3. Results

### 3.1. Characterization and purification of mundticin

A lactic acid bacterium with a broad spectrum of antimicrobial activity was isolated from fresh chicory endive and identified as *E. mundtii* ATO6. The supernatant of *E. mundtii* ATO6 cultures exhibited strong antimicrobial activity against the indicator strain *L.*

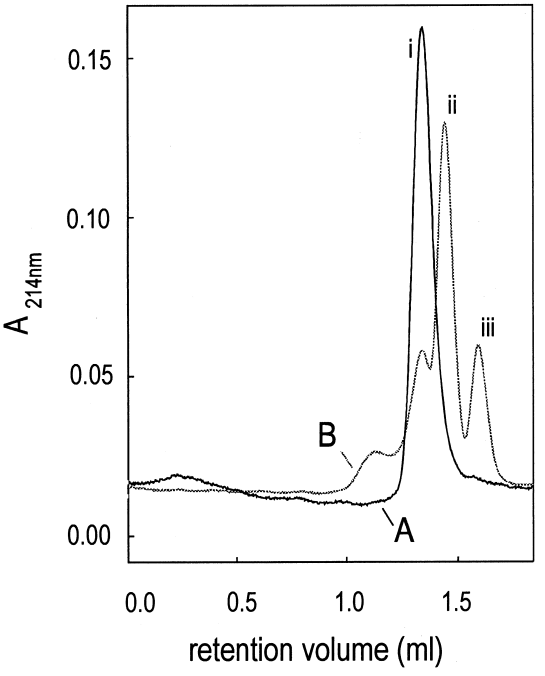


Fig. 1. Gel filtration elution profile of (A) purified mundticin and (B) mundticin cleaved with Asp-N on a Superdex Peptide PC3.2/30 column. Peak i, amino acids 1–43 (full length mundticin); peak ii, amino acids 17–43 of mundticin; peak iii, amino acids 1–16 of mundticin.

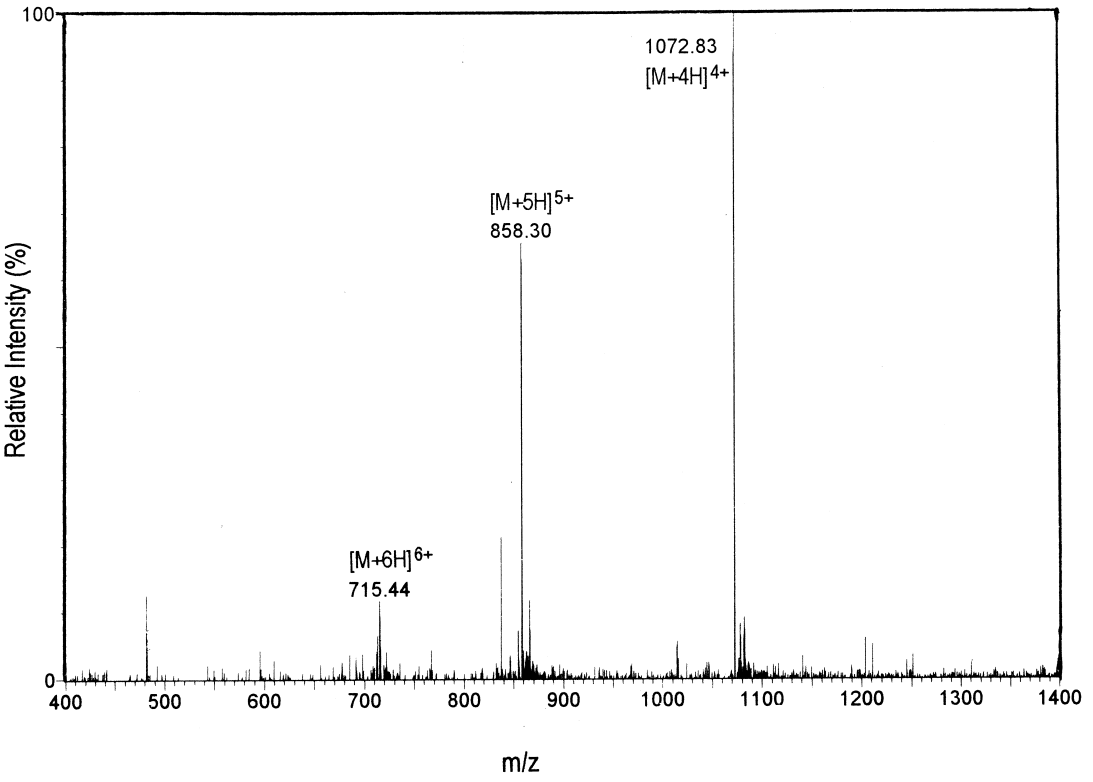


Fig. 2. Electronspray mass spectrum of mundticin with multiple charged molecular ions.

*sake* DSM 20017, which reached a maximum after approximately 22 h of growth, yielding $2.6 \times 10^6$ BU/l culture fluid. Proteolytic treatment of the supernatant resulted in total loss of this activity, indicating the proteinaceous nature of the antimicrobial compound. After a two-step ammonium sulfate precipi-

Table 1
Multiple sequence alignment of mundticin to bacteriocins produced by lactic acid bacteria containing a YGNGV amino acid motif

| Bacteriocin | Sequence | % ident | % homol | Swiss Prot accession number |
|---|---|---|---|---|
| Mundticin | KYYGNGVSCNKKGCSVDWGKA----IGIIGNNSAANLATGGAAGWSK- | 100 | 100 | P80925 |
| Piscicolin 126/Piscicocin Via | KYYGNGVSCNKNGCTVDWSKA----IGIIGNNAAANLTTGGAAGWNKG | 74 | 82 | P80569 |
| Sakacin P/674 | KYYGNGVHCGKHSCTVDWGTA----IGNIGNNAAANWATGGNAGWNK- | 65 | 75 | P35618 |
| Pediocin PA-1/AcH | KYYGNGVTCGKHSCSVDWGKA----TTCIINNGAMAWATGGHQGNHKC | 55 | 75 | P29430 |
| Leucocin A | KYYGNGVHCTKSGCSVNWGEA----FS----AGVHRLANGGNGFW--- | 50 | 71 | P34034 |
| Mesentericin Y105 | KYYGNGVHCTKSGCSVNWGEA----AS----AGIHRLANGGNGFW--- | 50 | 71 | P38577 |
| Carnobacteriocin B2 | VNYGNGVSCSKTKCSVNWGQAFQERYTAGINSFVSGVASGAGSIGRRP | 31 | 53 | P38580 |
| Carnobacteriocin BM1 | AISYGNGVYCNKEKCWVNKAENKQ----AITGIVIGGWASSLAGMGH-- | 26 | 47 | P38579 |
| Curvacin A, Sakacin A | ARSYGNGVYCNNKKCWVNRGEATQ----SIIGGMISGWASGLAGM---- | 34 | 49 | reference [41] |
| | ***** *.. * *. . .  ... | | | |

Multiple sequence alignments were performed using the Clustal program with a fixed gap penalty. Gaps (-) were introduced to optimize alignments. The percentages of identity and homology are calculated by taking the inserted gaps into account. Identical (*) and (·) similar residues are indicated. The homology score and similarity score were calculated according to Higgins and Sharp [42].

tation of the supernatant, with near total recovery of bacteriocin activity (99%) in the second step, the antimicrobial compound was purified to homogeneity by chromatographic techniques. Hydrophobic interaction chromatography and subsequent cationic exchange chromatography gave 60 and 55% recovery of the initial activity, respectively. A single peak eluted from the gel filtration column (95% yield of activity) (Fig. 1), which homogeneity was confirmed by reversed phase chromatography. The specific activity of the purified peptide against *L. sake* was 1500 BU/μg, and the final yield of the peptide from the culture supernatant was 0.5 mg/l. The antimicrobial activity of the peptide was not affected by heating for 15 min at 100°C, but prolonged heating for 1 h at 100°C resulted in a 50% loss of activity. The peptide was stable from pH 1 to 10 for 14 h at 4°C.

N-Terminal amino acid sequence analysis of the purified bacteriocin revealed a 43 amino acid (aa) residue peptide, which was designated mundticin. Initial sequence analysis did not identify the residues at positions 9 and 14 and the residues at positions 37 and 40–43 could not unequivocally be appointed. To fully elucidate the primary structure of the bacteriocin, the peptide was cleaved with Asp-N specific endoprotease. The two resulting fragments were separated by gel filtration chromatography, yielding two clear absorbency peaks as shown in Fig. 1. The full sequence of the C-terminal region was obtained after N-terminal amino acid sequencing of the larger fragment (aa 17–43). Following reduction and alkylation with 4-vinyl pyridine of the uncleaved peptide, the residues at positions 9 and 14 were identified as pyridethylated cysteinyl residues, rendering the complete primary structure of a novel bacteriocin, which was designated mundticin: KYYGNGVSCNKKGCSVDWGKAIGIIGNNSAANLATGGAAGWSK. A homology search for the entire sequence of mundticin through the SwissProt database rendered eight other bacteriocins of LAB with a highly conserved YGNGV motif near the N-terminus (see Table 1). This motif indicated that mundticin belongs to the class IIa bacteriocins of LAB [8].

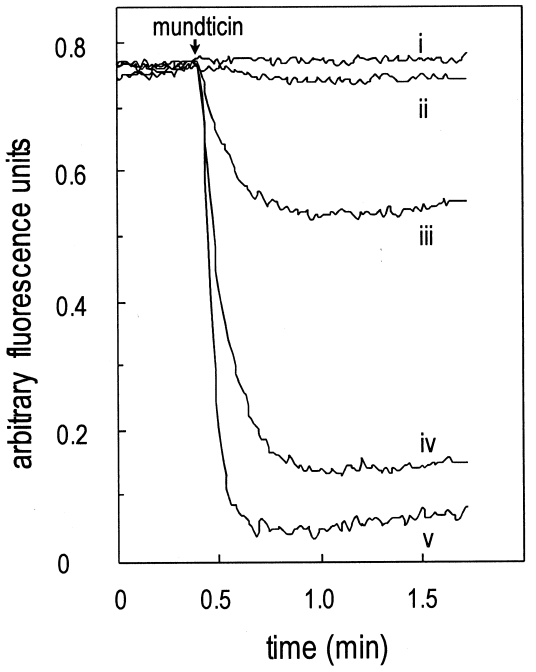


Fig. 3. Effect of mundticin on the membrane potential (Δψ) of energized, nigericin-treated whole cells of *L. monocytogenes* LDCD81-861 in absence or presence of purified mundticin: i, 0; ii, $1.4\times10^{-3}$; iii, $2.8\times10^{-3}$; iv, $1.4\times10^{-2}$; v, 0.14 μg mundticin/ml. The Δψ was measured with the fluorescent probe DiS-$C_3$5. The absence of the Δψ was established at 0.05 arbitrary fluorescence units by the addition of valinomycin (2 μM).

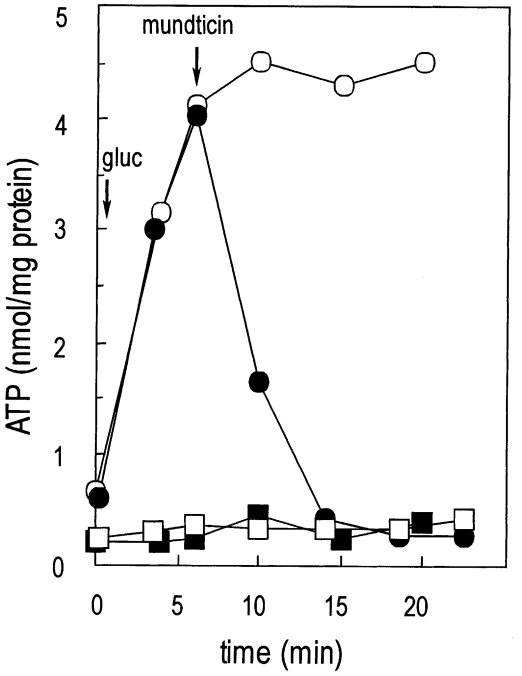


Fig. 4. Intracellular ATP concentrations (dots) or extracellular ATP concentrations (squares) of *L. monocytogenes* LDCD81-861 cells after the addition of glucose at $t=0$ (gluc; final concentration 0.5% w/v), in the absence (○□, control) or presence (●■) of mundticin (0.14 μg/ml). The values represent the average of duplicate determinations.

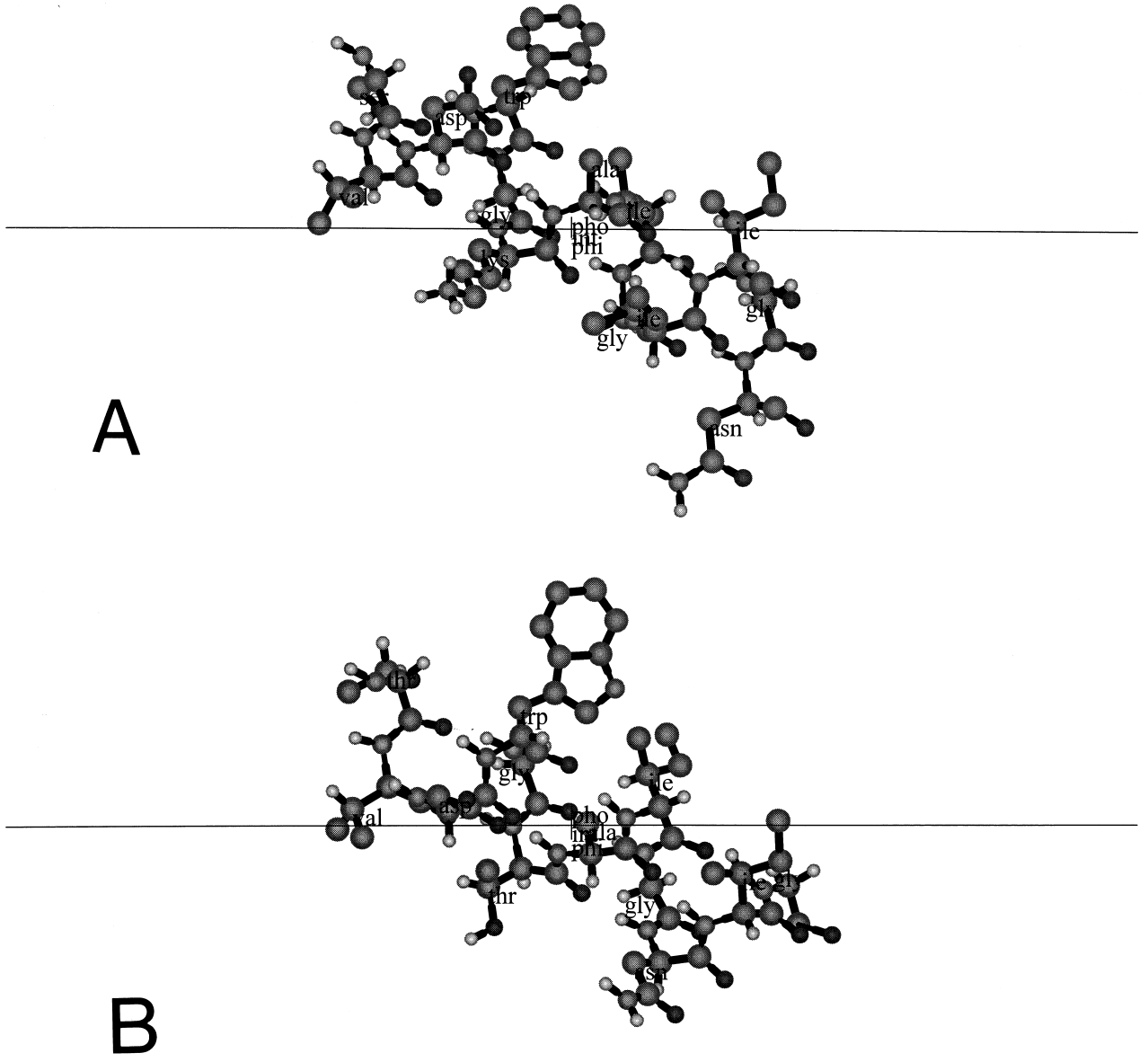


Fig. 5. Steric representation of the tilted peptide in the bacteriocins (A) mundticin (residues 15–27) and (B) sakacin P (residues 15–26) at a lipid (upper)/water (lower) interface. The peptides are orientated at an angle of 40° and 45°, respectively.

The average mass of mundticin as determined by electronspray mass spectrometry was 4287.21 ± 0.59 Da (Fig. 2), which fully agrees with a calculated mass of 4287.8 in the presence of one disulfide bridge, linking the cysteine residues at positions 9 and 14.

### 3.2. Antimicrobial spectrum of mundticin

Mundticin was found to inhibit the growth of a wide range of Gram-positive bacteria at nanomolar concentrations. Specifically, this bacteriocin caused a 10 000-fold reduction of viable counts of the pathogen *L. monocytogenes* within 25 or 150 min at a concentration of 1.0 or 0.1 μg/ml, respectively, which demonstrated its vigorous bactericidal action. Furthermore, mundticin prevented the outgrowth of spores and vegetative cells of toxin-producing strains of *Clostridium botulinum* and inhibited the growth of different species of LAB (*Lactobacillus salivarius*, *L. sake*, *Leuconostoc paramesenteroides*, *Leuconostoc mesenteroides*, *Carnobacterium piscicola*, *Pediococcus dextrinicus*, *P. pentosaseus*, *Enterococcus faecalis*, *E.*

Table 2
Sequence and properties of the oblique helical peptides identified in class IIa bacteriocins

| Bacteriocin[a] | Sequence of α-helical region | Residues | % Homology | % Identity | $H_o$ | $\mu_H$ | Angle of insertion (°) |
|---|---|---|---|---|---|---|---|
| Mundticin | SVDWGKAIGIIGN | 15–27 | 100 | 100 | 0.37 | 0.37 | 40 |
| Piscicolin 126 | TVDWSKAIGIIGN | 15–27 | 100 | 85 | 0.33 | 0.33 | 45 |
| Sakacin P | TVDWGTAIGNIG | 15–26 | 83 | 75 | 0.42 | 0.41 | 35 |
| Pediocin PA-1 | SVDWGKATTCIIN | 15–27 | 77 | 69 | 0.20 | 0.20 | 30 |
| Leucocin A | SVNWGEAFSAGVH | 15–27 | 69 | 37 | 0.32 | 0.26 | 30 |
| Mesentericin Y105 | SVNWGEAASAGIH | 15–27 | 69 | 37 | 0.30 | 0.23 | 35 |
| Carnobacteriocin B2 | SVNWGQAFQERYT | 15–27 | 54 | 38 | −0.11 | 0.25 | 42 |
| Carnobacteriocin BM1 | WVNKAENKQAITG | 16–28 | 46 | 15 | −0.01 | 0.15 | 53 |
| Sakacin A, Curvacin A | WVNRGEATQSIIG | 16–28 | 38 | 31 | 0.09 | 0.19 | 32 |

The mean hydrophobicity ($H_o$) and the hydrophobic moment ($\mu_H$) of the peptide was computed using the Eisenberg consensus scale [33]. The angle of insertion in a lipid bilayer was calculated according to Brasseur [21]. The amphiphilic α-helical peptides of the three bacteriocins with the lowest homology to mundticin were calculated to have an oblique orientation; however, their $H_o$ was low.
[a]SwissProt accession numbers are listed in Table 1.

*hirae*, and *Listeria inocua*). We did not observe activity against Gram-negative bacteria and fungi.

### 3.3. Mundticin targets the cytoplasmic membrane and dissipates the $\Delta\psi$

To investigate the effect of mundticin on the cytoplasmic membrane, the membrane potential ($\Delta\psi$) of whole cells of *L. monocytogenes* LDCD81-861 was measured after exposure to the purified bacteriocin. At increasing bacteriocin concentrations, a more rapid $\Delta\psi$ dissipation was observed in conjunction with a lower final membrane potential (Fig. 3). The addition of mundticin to energized cells of the same strain at a concentration that caused the complete dissipation of the $\Delta\psi$ resulted in an almost complete loss of intracellular ATP within 10 min, in absence of ATP leakage (Fig. 4). The elimination of the growth of *L. monocytogenes* was achieved at 7 ng/ml (=MIC), which is a lower concentration than required for full dissipation of the $\Delta\psi$.

### 3.4. Identification of the oblique-orientated peptides in class IIa bacteriocins

The sequence of mundticin and homologous bacteriocins was scanned to identify a peptide meeting the criteria of an oblique-orientated α-helical peptide (see Section 2). This analysis identified oblique-orientated peptides in these bacteriocins, which were predicted to insert into the hydrophobic–hydrophilic interface at an approximate angle of 40° relative to a lipid–water interface, due to the hydrophobicity gradient between the N-terminal and the C-terminal end of the peptide (Table 2). Fig. 5 shows the most probable conformation of the bacteriocin helical peptides at a hydrophobic/hydrophilic interface for mundticin (residues 15–27) and sakacin P (residues 15–26), and illustrates how these regions can perturb the regular parallel orientation of phospholipids.

We also verified the presence of oblique helical peptides in hybrid class IIa bacteriocins as described by Fimland et al. [15]. These investigators constructed hybrid class IIa bacteriocins by combining the N-terminal parts (residues 1–21) of pediocin PA-1, sakacin P and curvacin A (sakacin A) with the C-terminal parts (residues 22 to end) of these bacteriocins, rendering Ped-Sak, Sak-Ped, Cur-Sak and Sak-Cur. Each of the hybrid bacteriocins had an antimicrobial spectrum similar to the parent C-terminal bacteriocin. However, only Ped-Sak was as potent as the parent bacteriocin, whereas the other hybrids had significantly lower activities (Table 3 and [15]). Interestingly, the construction of these hybrid bacteriocins involved the regions that encompassed their predicted α-helical domains. Our analysis revealed that only Ped-Sak had a hydrophobic oblique peptide with an angle of insertion of 45°, which could explain retained activity. The hydrophobic α-helical regions of Sak-Ped and Sak-Cur had angles of insertion of 25 and 0°, respectively, indicating a near parallel or full parallel orientation to the hydrophobic–

Table 3
Sequence and properties of the oblique helical peptides identified in hybrid class IIa bacteriocins reported by Fimland et al. [15]

| Hybrid bacteriocin[a] | Sequence of α-helical region | $H_o$ | $\mu_H$ | Angle of insertion (°) | Activity of hybrid bacteriocin compared to native bacteriocins[a] |
|---|---|---|---|---|---|
| **Ped**–Sak | **SVDWGKA**IGNIGN | 0.2 | 0.45 | 45 | 100% |
| **Sak**–Ped | **TVDWGTA**TTCIIN | 0.32 | 0.23 | 25 | < 1% |
| **Sak**–Cur | **TVDWGTA**TQSIIG | 0.32 | 0.2 | 0 | < 1% |
| **Cur**–Sak | **WVNRGEA**IGNIGN | 0.09 | 0.31 | 70 | < 2% |

[a]The abbreviations of the hybrid bacteriocins are described in [15] and Section 3. The full sequence of these hybrid bacteriocins and their exact activities against various indicator strains are described in [15].

hydrophilic interface. In addition, Cur-Sak contained a helical peptide with an angle of insertion of 70° that resembles a perpendicular orientation (Table 3).

## 4. Discussion

This report describes the isolation and identification of a novel antimicrobial peptide from the Gram-positive bacterium *E. mundtii*. To our knowledge, this is the first report on bacteriocin production by this organism. The secreted antimicrobial compound, designated mundticin, belongs to the class IIa bacteriocins of LAB [8] and was shown to exhibit antimicrobial activity towards a broad range of Gram-positive bacteria, among which *C. botulinum* and *L. monocytogenes*. The action of mundticin on *L. monocytogenes* involves the dissipation of the membrane potential and a depletion of the intracellular ATP pools of this organism, in absence of ATP leakage. By computer modelling, we have studied the orientation of predicted α-helical regions of mundticin and homologous class IIa bacteriocins at a hydrophobic–hydrophilic interface.

Mundticin is a positively charged, hydrophobic, 43 amino acid peptide, with a highly conserved YGNGV motif at positions 3–7. Amino acid sequence analysis of mundticin in conjunction with its molecular mass indicated the presence of one disulfide bridge between $Cys^9$ and $Cys^{14}$, and the absence of further post-translational modifications. These two cysteines were also present in homologous class IIa bacteriocins. Although the presence of a disulfide bridge between these two cysteines was required for the activity of pediocin PA-1 and mesenterocin Y105 [13,16], it was reported not to be required for activity of leucocin A and carnobacteriocin B2 [14,36]. Except for residues 34–36, the sequence similarities between mundticin and the other class IIa bacteriocins were high at the N-terminal region, but low for the remaining 22 residues.

Mundticin was shown to inhibit the growth of a broad spectrum of bacteria, including the toxin producing *Clostridium botulinum* and *L. monocytogenes,* a Gram-positive bacterium that can cause severe infections of the central nervous system [27]. In addition to its broad antimicrobial spectrum, mundticin has a number of other favorable characteristics for application as a natural antimicrobial agent in foods, namely, its good solubility in water, and its stability over a wide pH and temperature range. However, the application of mundticin in foods will require detailed toxicity studies. Also, the safety of applying mundticin-producing *E. mundtii* in foods needs to be further investigated, since Enterococci do not have a generally recognized as safe (GRAS) status [37].

The bactericidal effect of mundticin on cells of *L. monocytogenes* was shown to be related to a rapid loss of the membrane potential, which indicated the dissipation of ionic gradients as a result of pore-formation. In absence of ATP leakage, the observed reduction of the ATP pool size can be explained by the accelerated consumption of ATP to regenerate the decreased PMF and/or by a shift in the ATP hydrolysis equilibrium resulting from phosphate efflux [38]. The absence of ATP efflux furthermore indicates that the size exclusion limit of mundticin-induced pores is smaller than 500 Da. The action of mundticin hereby resembles that of the class IIa bacteriocins pediocin PA-1 [39] and mesenterocin Y105 [40].

The results of our computer modelling studies indicate that mundticin and the other YGNGV bacteriocins contain obliquely orientated α-helical peptide regions. The presence of these oblique peptides may

contribute to destabilization of the phospholipid bilayers, thereby facilitating the insertion of bacteriocin molecules in the cytoplasmic membrane of the target organism. The suggested role of the oblique peptides in the activity of the class IIa bacteriocins was supported by our analysis of hybrid bacteriocins that were constructed by Fimland et al. [15]: only the hybrid molecule that had antimicrobial activity similar to the parent bacteriocins was predicted to contain an oblique peptide. However, experimental studies need to be performed to confirm the proposed relevance of oblique helical peptides in the action of YGNGV bacteriocins. To this end, rational design of molecules can be enabled by the presented computer analysis.

In conclusion, we here report the purification and identification of mundticin, a novel class IIa bacteriocin of LAB, and its action on the cytoplasmic membrane of target strains was demonstrated. Computer analysis indicated that members of this class contain α-helical regions with an oblique orientation at a hydrophobic–hydrophilic interface that may perturb phospholipid bilayers. Furthermore, mundticin was shown to have a broad spectrum of activity and properties that make it a good candidate for application as a natural antimicrobial compound in foods.

## Acknowledgements

The authors thank W. Schaaper and T. Postma for providing the mass spectra; F. van der Lecq for performing the N-terminal amino acid sequence analyses; S. Stringer for the tests with *C. botulinum*; and J.A. Kuivenhoven for critical reading of the manuscript. This research was supported by funds from the Commission of the European Union (Contract AIR1-CT92-0125) and by the Interuniversity Poles of Attraction Program–Belgian State, Prime Minister's Office–Federal Office for Scientific, Technical and Cultural affairs (Contract P4/03).

## References

[1] J.R. Tagg, A.S. Dajani, L.W. Wannamaker, Bacteriol. Rev. 40 (1976) 722–756.
[2] C.C. Yang, J. Konisky, J. Bacteriol. 158 (1984) 757–759.
[3] G. Jung, H.-G. Sahl, Nisin and Novel antibiotics, ESCOM, Leiden, The Netherlands, 1991.
[4] J.L. Vizán, C. Hernández-Chico, I. del Castillo, F. Moreno, EMBO J. 10 (1991) 467–476.
[5] J.F. García-Bustos, N. Pezzi, E. Mendez, Antimicrob. Agents Chemother. 27 (1985) 791–797.
[6] R. Kolter, F. Moreno, Annu. Rev. Microbiol. 46 (1992) 141–163.
[7] P.A. Vandenbergh, FEMS Microbiol. Rev. 12 (1993) 221–238.
[8] T.R. Klaenhammer, FEMS Microbiol. Rev. 12 (1993) 39–86.
[9] T. Abee, FEMS Microbiol. Lett. 129 (1995) 1–9.
[10] R.W. Jack, J.R. Tagg, B. Ray, Microbiol. Rev. 59 (1995) 171–200.
[11] K. Venema, G. Venema, J. Kok, Trends Microbiol. 3 (1995) 299–304.
[12] A.J.M. Driessen, H.W. Vandenhooven, W. Kuiper, M. Vandekamp, H.G. Sahl, R.N.H. Konings, W.N. Konings, Biochemistry 34 (1995) 1606–1614.
[13] M.L. Chikindas, M.J. Garcia Garcera, A.J.M. Driessen, A.M. Ledeboer, J. Nissen-Meyer, I.F. Nes, T. Abee, W.N. Konings, G. Venema, Appl. Environ. Microbiol. 59 (1993) 3577–3584.
[14] L.E.N. Quadri, M. Sailer, K.L. Roy, J.C. Vederas, M.E. Stiles, J. Biol. Chem. 269 (1994) 12204–12211.
[15] G. Fimland, O.R. Blingsmo, K. Sletten, G. Jung, I.F. Nes, J. Nissenmeyer, Appl. Environ. Microbiol. 62 (1996) 3313–3318.
[16] Y. Fleury, M.A. Dayem, J.J. Montagne, E. Chaboisseau, J.P. Le Caer, P. Nicolas, A. Delfour, J. Biol. Chem. 271 (1996) 14421–14429.
[17] Y. Chen, R. Shapira, M. Eisenstein, T.J. Montville, Appl. Environ. Microbiol. 63 (1997) 524–531.
[18] R. Brasseur, T. Pillot, L. Lins, J. Vandekerckhove, M. Rosseneu, Trends Biochem. Sci. 22 (1997) 167–171.
[19] Y. Chen, R.D. Ludescher, T.J. Montville, Appl. Biochem. Biotechnol. 63 (1997) 4770–4777.
[20] N.L. Fregeau Gallagher, M. Sailer, W.P. Niemczura, T.T. Nakashima, M.E. Stiles, J.C. Vederas, Biochemistry 36 (1997) 15062–15072.
[21] R. Brasseur, J. Biol. Chem. 266 (1991) 16120–16127.
[22] P. Talmud, L. Lins, R. Brasseur, Protein Eng. 9 (1996) 317–321.
[23] M. Horth, B. Lambrecht, M.C.L. Khim, F. Bex, C. Thiriart, J.M. Ruysschaert, A. Burny, R. Brasseur, EMBO J. 10 (1991) 2747–2755.
[24] V. Voneche, D. Portelle, R. Kettmann, L. Willems, K. Limbach, E. Paoletti, J.M. Ruysschaert, A. Burny, R. Brasseur, Proc. Natl. Acad. Sci. USA 89 (1992) 3810–3814.
[25] T. Pillot, M. Goethals, B. Vanloo, C. Talussot, R. Brasseur, J. Vandekerckhove, M. Rosseneu, L. Lins, J. Biol. Chem. 271 (1996) 28757–28765.
[26] M.H.J. Bennik, E.J. Smid, L.G.M. Gorris, Appl. Environ. Microbiol. 63 (1997) 2074–2076.
[27] W.F. Schlech, P.M. Lavigne, R.A. Bortolussi, A.C. Allen, E.V. Haldane, A.J. Wort, A.W. Hightower, S.E. Johnson,

S.H. King, E.S. Nicholls, C.V. Broome, New Engl. J. Med. 308 (1983) 203–206.
[28] B. Ten Brink, R. Otto, U.-P. Hansen, W.N. Konings, J. Bacteriol. 162 (1985) 383–390.
[29] A. Lundin, A. Thore, Anal. Biochem. 66 (1975) 47–63.
[30] O.H. Lowry, N.J. Rosebourg, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265–275.
[31] M. Rahman, L. Lins, A. Soumarmon, R. Brasseur, J. Mol. Model. 3 (1997) 203–215.
[32] F. Jähnig, Trends Biochem. Sci. 15 (1990) 93–95.
[33] D. Eisenberg, R.M. Weiss, T.C. Terwilliger, Nature 299 (1982) 371–374.
[34] R. Brasseur, J.M. Ruysschaert, Biochem. J. 238 (1986) 1–11.
[35] R. Brasseur, B. Vanloo, R. Deleys, L. Lins, C. Labeur, J. Taveirne, J.M. Ruysschaert, M. Rosseneu, Biochim. Biophys. Acta 1170 (1993) 1–7.
[36] J.W. Hastings, M. Sailer, K. Johnson, K.L. Roy, J.C. Vederas, M.E. Stiles, J. Bacteriol. 173 (1991) 7491–7500.
[37] G. Giraffa, D. Carminati, E. Neviani, J. Food Prot. 60 (1997) 732–738.
[38] G. Guihard, H. Benedetti, M. Besnard, L. Letellier, J. Biol. Chem. 268 (1993) 17775–17780.
[39] Y. Chen, T.J. Montville, J. Appl. Bacteriol. 79 (1995) 684–690.
[40] A. Maftah, D. Renault, C. Vignoles, Y. Hechard, P. Bressolier, M.H. Ratinaud, Y. Cenatiempo, R. Julien, J. Bacteriol. 175 (1993) 3232–3235.
[41] P.S. Tichaczek, R.F. Vogel, W.P. Hammes, Microbiology 140 (1994) 361–367.
[42] D.G. Higgins, P.M. Sharp, Gene 73 (1988) 237–244.