RESEARCH LETTER

Purified sakacin A shows a dual mechanism of action against *Listeria* spp: proton motive force dissipation and cell wall breakdown

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Received 7 June 2012; revised 12 June 2012; accepted 24 June 2012.

DOI: 10.1111/j.1574-6968.2012.02630.x

Editor: Anthony George

Keywords
sakacin A; dual mechanisms; *Listeria* spp.

Abstract

Sakacin A was purified to homogeneity through simple chromatographic procedures from cultures of *Lactobacillus sakei* DSMZ 6333 grown on a low-cost medium. The highly purified protein dissipated both transmembrane potential ($\Delta\Psi$) and transmembrane pH gradient (ΔpH) in *Listeria* cells in a very intense, rapid, and energy-dependent fashion. On a slower timescale, purified sakacin A also showed a lytic activity toward isolated cell walls of *Listeria*. Mass spectrometry was used to analyze the products of sakacin A action on cell walls, evidencing that sakacin A acts on various types of bonds within peptoglycans.

Introduction

Consumers are demanding high-quality foods, with minimal processing and low preservative levels (Batdorj *et al.*, 2007). Natural and safe substances may represent an alternative to chemicals for inhibiting the growth of undesirable microorganisms. Bacteriocins from lactic acid bacteria can protect food against spoilage or prevent growth of pathogenic bacteria (Cotter *et al.*, 2005) and are rapidly digested by humans (Deraz *et al.*, 2005). Class IIa bacteriocins are of the greatest interest, because of strong antimicrobial activity against *Listeria* spp.

This has stimulated investigation on rapid and cost-effective purification protocols and on functional characterization of these compounds. Standard purification methods include salt precipitation, followed by gel filtration, ion-exchange, and reverse-phase chromatography. These methods are time-consuming and low-yielding (Guyonnet *et al.*, 2000) and have been improved somewhat using cation-exchange chromatography (Berjeaud & Cenatiempo, 2004).

Sakacin A is a class IIa bacteriocin produced by *Lactobacillus sakei* DSMZ 6333, able to inhibit the growth of several lactic acid bacteria and of *Listeria monocytogenes*. This bacteriocin is a small heat-stable protein with no posttranslational modifications (Schillinger & Lucke, 1989). All class IIa bacteriocins have a highly conserved N-terminal domain with the consensus motif YGNGV responsible for activity against *Listeria* (Richard *et al.*, 2004). Upon exposure to these bacteriocins, leakage of ions and small molecules from sensitive cells accompanies dissipation of the proton motive force and depletion of intracellular ATP (Drider *et al.*, 2006).

We report here on: (1) purification of the bacteriocin produced by *L. sakei*, cultured in a low-cost medium (Trinetta *et al.*, 2008a) of potential industrial interest; (2) the mechanism of action of the purified bacteriocin on *Listeria* cells; and (3) some mechanistic

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aspects of the lytic activity of sakacin A toward *Listeria* cell walls.

Materials and methods

Bacterial strains and culture conditions

Lactobacillus sakei DSMZ 6333 (DSMZ, Braunschweig, Germany) was cultured in an inexpensive culture medium broth (Trinetta *et al.*, 2008a). *Listeria ivanovii* ATCC BAA-678 grown in Tryptic Soy Agar (Difco Laboratories, Sparks, MD) for 18 h at 37 °C was used as an indicator strain. Stocks were maintained at − 20 °C in appropriate liquid media containing 10% (w/v) glycerol and propagated twice before use.

Protein purification

Sakacin A was purified from 1 L cultures of *L. sakei*, grown at 30 °C for 18 h. Cells were centrifuged (10 000 ***g***, 35 min, 4 °C). The cell-free supernatant was made 50 mM in sodium acetate, and the pH was adjusted to 4.5 with acetic acid/NaOH. The resulting solution was loaded onto a SP-Sepharose fast flow cation exchange column (4 × 11.3 cm; Whatman). Proteins were eluted stepwise with 0.2 and 1 M NaCl, and fractions were assayed for antimicrobial activity (Batdorj *et al.*, 2007). The active fraction was applied on a 10 × 250 mm reversed phase (RP) C_{18} column (300 Â pores, 10 μm, Labservice; Analytica, Milan, Italy) run on a Waters HPLC (625 LC, Toronto, Canada) and equilibrated with 95% (v/v) solvent A [0.1% aqueous trifluoroacetic acid (TFA)] and 5% (v/v) solvent B (0.1% aqueous TFA, 80% acetonitrile). Stepwise elution by increasing acetonitrile concentration (to 30%, 50% and 80%) was carried out at a flow rate of 1.5 mL min^{-1}. The active fraction, eluted at 50% acetonitrile, was loaded on a Superdex Peptide column (Amersham Biosciences, Milan, Italy) equilibrated in aqueous 20% (v/v) acetonitrile containing 0.01% (v/v) TFA. The final chromatographic step was carried out on a 4.6 × 250 mm RP Symmetry C_{18} column (5 μm, 100 Å; Waters, Milan, Italy) equilibrated with 95% (v/v) solvent A and 5% (v/v) solvent B. Sakacin A was eluted with a linear gradient from 20% to 60% of solvent B for 20 min at a flow rate of 0.8 mL min^{-1}.

SDS-PAGE and mass spectrometry

Tricine SDS-PAGE was carried out in precast 12% acrylamide gels (NuPage®; Invitrogen, Milan, Italy). Markers covered the range from 3.5 to 260 kDa (Novex Sharp Pre-Stained Standard; Invitrogen). One half of the gel was stained with Coomassie Blue (Symply-Blue Safestain; Invitrogen), whereas the other half was washed with sterile water and overlaid with soft nutrient agar medium (10 mL) containing the indicator strain. Antimicrobial activity was assessed after incubation at 37 °C (Yamamoto *et al.*, 2003).

MALDI-TOF/MS (matrix-assisted laser desorption/ionisation-time of flight mass spectrometry) measurements were carried out on a Voyager DEPRO spectrometer (PerSeptive Biosystems, Framingham, MA) equipped with an N_2 laser (337 nm, 3 ns pulse width) operated in the positive reflector ion mode and using delay extraction. For assessing the purity and mass of sakacin A, the peptide was co-crystallized with 2,5-dihidroxybenzoic acid (DHB) and α-cyano-4-hydroxycinnamic acid (CHCA), both at 10 mg mL^{-1} in 50% acetonitrile (v/v)/0.1% (v/v) TFA. External mass calibration was performed with low-mass peptide standards (PerSeptive Biosystems).

For the characterization of products of cell wall breakdown, postsource decay (PSD) fragment ion spectra were obtained after isolation of the appropriate precursor using timed ion selection. Fragment ions were refocused onto the final detector by stepping the voltage applied to the reflector and individual segments combined using PERSEPTIVE BIOSYSTEMS software (De Simone *et al.*, 2009). CHCA was used in this study according to Boneca *et al.*, 2000. The sample (1 μL, in water) was loaded on the target, dried, and re-dissolved in CHCA (1 μL, 10 mg mL^{-1} in 0.1% TFA in 50% aqueous acetonitrile). For each sample, 200 laser pulses were accumulated.

Determination of protein concentration and bacteriocin activity (AU mL^{-1})

Concentration of purified sakacin A was calculated by assuming ε_{280} = 14 105 (mol^{-1} cm^{-1}; http://web.expasy.org/protparam/; Kelly *et al.*, 2005). The bacteriocin titer was determined by a serial dilution assay, activity being defined as the reciprocal of the last serial dilution that exhibited a clear zone of inhibition and being expressed as activity units (AU; De Kwaadsteniet *et al.*, 2005).

Measurement of transmembrane potential (ΔΨ)

Changes in the cell transmembrane electrical potential were measured by quenching of the potential-sensitive fluorescent probe 3, 3-dipropylthiadicarbocyanine iodide (diSC3; Molecular Probes Inc., Eugene, OR; Deraz *et al.*, 2005). Cells were suspended in 50 mM potassium-HEPES, pH 7, containing 0.2% glucose (final $OD_{600\,nm}$ = 0.4), to give glucose-energized cells. The probe (5 μM) and nigericin (1.5 μM) were mixed with the glucose-

energized cell suspension, and sakacin A (80 AU mL^{-1}) or valinomycin (1.5 μM) was added as appropriate. Fluorescence was measured at 30 °C in a spectrofluorometer (Model LS 50; PerkinElmer, Milan, Italy), with excitation at 643 nm and emission at 666 nm (Suzuki *et al.*, 2005).

Measurement of transmembrane pH (ΔpH)

Changes in the transmembrane pH gradient were measured with the pH-sensitive fluorescent probe 5 (and 6) carboxyfluorescein diacetate succinimidyl ester (cFDASE; Molecular Probes Inc.; McAuliffe *et al.*, 1998). The cells were concentrated threefold in 1.5 mL of 50 mM potassium-HEPES buffer, pH 8, and then incubated at 30 °C for 10 min with the probe (1 μM). Nonconjugated probe was eliminated by incubating the cells with 10 mM lactose at 30 °C for 30 min. The cells were washed twice, suspended in 50 mM potassium phosphate buffer at pH 7 and placed on ice until used. The intracellular pH was determined by diluting the lactose-loaded cells to a concentration of 10^7 CFU mL^{-1} in a 3-mL glass cuvette. Fluorescence was measured as reported earlier.

Preparation of cell walls and cell wall breakdown measurements

Bacterial cell walls were isolated according to Simelyte *et al.* (2000). Harvested *Listeria* cells (10^7 CFU mL^{-1}) were heat-treated at 80 °C for 30 min to inactivate autolytic enzymes, collected by centrifugation at 17 000 ***g*** for 10 min, and suspended in 3 mL of 10 mM phosphate buffer (pH 7). The suspension was disrupted with a MSK Cell Homogenizer (Braun Biotech, Goettingen, Germany) using glass beads (five 1-min cycles). A pellet collected by centrifugation at 20 000 ***g*** for 10 min at 4 °C was suspended as above and treated for 1 h at 37 °C with DNase (25 μL, 5 U μL^{-1}) and RNase A (25 μL, 100 mg mL^{-1}) and 1 h at 37 °C with trypsin (250 μg mL^{-1}). All hydrolytic enzymes were from Sigma-Aldrich (Milan, Italy). Following ultracentrifugation (100 000 ***g***, 60 min, 4 °C; Beckman L7-65 Instruments, Gagny, France), the pellet was suspended in 3 mL of phosphate buffer and sonicated for 10 min in an ice bath. Residual cell wall-associated proteins were removed by papain treatment (3 μL, 50 mg mL^{-1} solution; Sigma-Aldrich) for 1 h at 37 °C, followed by ultracentrifugation.

For the cell wall breakdown assay, the pellet from ultracentrifugation was suspended in 6 mL of 10 mM phosphate (pH 7) and divided into four aliquots that were treated with vancomycin (100 μg mL^{-1}), lysozyme (100 μg mL^{-1}), sakacin A (80 AU mL^{-1}, 100 μg mL^{-1}), or left untreated. The absorbance at 600 nm was measured after incubation at 30 °C (30 min and 24 h). Samples were frozen, lyophilized, and used as such for subsequent MS analysis of released products. In a separate set of experiments, aliquots of the same cell wall preparations were treated overnight (16 h) at 30 °C with increasing amounts of sakacin A (from 0 to 300 μg, equivalent to 0–240 AU) and analyzed by MS.

Statistical analysis

All experiments were performed in triplicate. Statistical analysis was carried out using a Tukey's multiple comparison test (Minitab 15v, State College, PA) and differences considered significant at $P < 0.05$.

Results

Purification and characterization of sakacin A

Sakacin A was purified through a sequence of chromatographic steps from *L. sakei* cultures propagated in an inexpensive broth (Trinetta *et al.*, 2008a). Sakacin A was eluted at *c.* 0.45 M NaCl from a cation exchanger at pH 4.5, confirming its cationic character and was further purified through RP and gel-permeation HPLC. A final RP-HPLC step eliminated a minor contaminant (Supporting Information, Fig. S1) and gave 1.7 mg of purified sakacin A L^{-1} of the original culture (Table S1).

The highly purified material showed a single band in SDS-PAGE, with a molecular mass of *c.* 4000 Da (Fig. 1a). The band retained antimicrobial activity against *L. ivanovii* (Fig. 1b), highlighting a peculiar resistance of the protein to denaturation as suggested also by activity

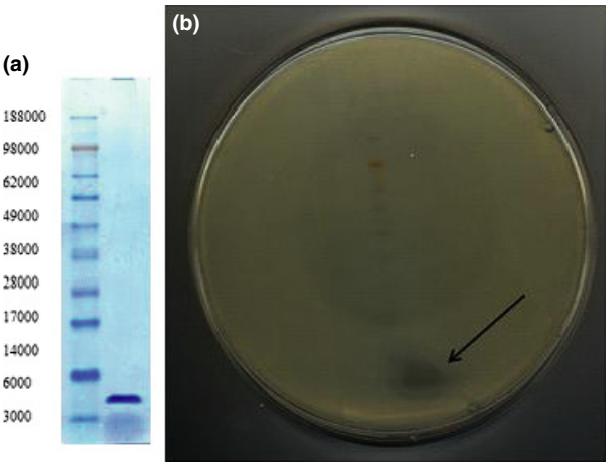

Fig. 1. Tricine-SDS-PAGE profiles of purified sakacin A and detection of antimicrobial activity on the gel. (a) Comassie Blue stained gel, the left lane represents the protein molecular mass marker and the other lane the purified sakacin A; (b) gel overlaid with *Listeria ivanovii* to determine the antimicrobial activity of the purified bacteriocin.

retention at the high acetonitrile concentrations used in RP-HPLC.

Purity and identity of the isolated material and correspondence to a published sequence (Holck *et al.*, 1992) were established by MALDI-TOF MS (Figure S2). The observed molecular mass (4302.36 Da) agrees with the sequence-calculated monoisotopic (4302.89 Da) and average isotopic (4306.89 Da) values.

Effect of sakacin A on *Listeria* cells: rapid dissipation of ΔΨ and ΔpH

The effects of sakacin A on the individual components of the proton motive force (PMF) (namely, ΔΨ and ΔpH) on *Listeria* cells were studied. In all the experiments described later, highly purified sakacin A was used at a concentration close to the MIC determined in previous studies (Trinetta *et al.*, 2008b).

The potential-sensitive fluorescent cyanine dye diSC3 (5) was used for assessing the sakacin A-induced dissipation of ΔΨ. By adding glucose to *Listeria* cells, a negative-inside ΔΨ was generated, resulting in the quenching of the probe fluorescence as a consequence of probe accumulation within the cells. As shown in Fig. 2, *Listeria* cells were able to maintain ΔΨ in the presence of nigericin (arrow 4) that dissipates transmembrane ΔpH.

When sakacin A was added to glucose-energized and nigericin-treated cells, the fluorescence of the probe increased, as a result of its release from the cell interior (arrow 5). This indicates a depolarization of the cytoplasmic membrane consequent to the addition of sakacin A. Figure 4 also makes it evident that the decrease in fluorescence induced by the addition of glucose has an amplitude very similar to the fluorescence increase ensuring from the addition of sakacin A. The ionophore valinomycin was used at the end of these experiments (arrow 6) to completely dissipate ΔΨ (McAuliffe *et al.*, 1998).

The pH-sensitive fluorescent probe cFDASE was used to assess the transmembrane ΔpH in *Listeria* cells. As also shown in Fig. 2, the fluorescence of the probe rapidly increased upon addition of lactose to cells (arrow 1), consequent to increased internal pH. When sakacin A was added (arrow 2), a rapid decrease in the signal was observed. No further signal increase was observed when nigericin was added (arrow 3), indicating that sakacin A completely dissipated the transmembrane ΔpH of *Listeria* cells.

Effects on the cell walls

The effects of sakacin A on isolated cell walls were studied by measuring the time course of turbidity decrease in cell wall suspensions at sakacin A concentration close to the MIC. As shown in Table 1, turbidity decreased by

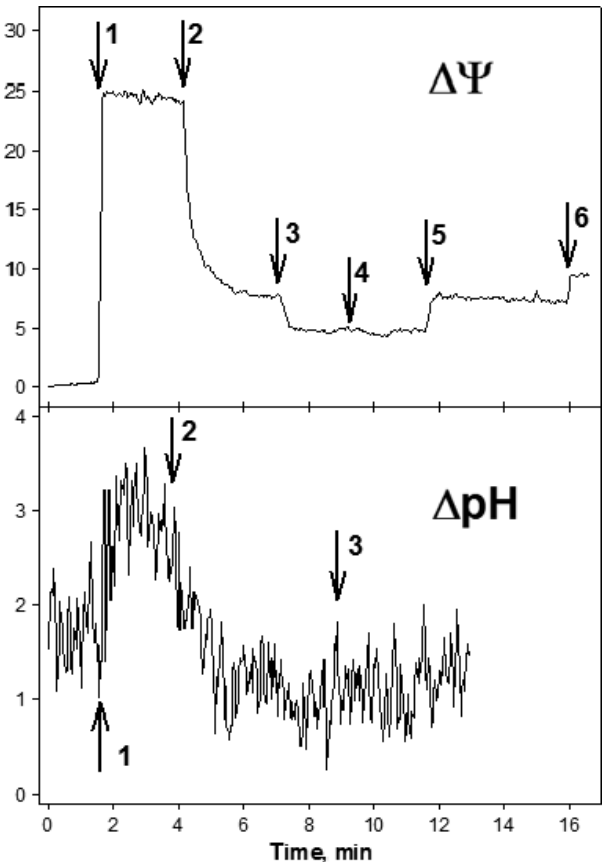

Fig. 2. Effect of sakacin A on the ΔΨ and ΔpH in *Listeria* cells as monitored through fluorescent probes. Top, ΔΨ. Arrows indicate the additions of the following: 1, diSC3(5) probe; 2, *Listeria* cells; 3, 10 mM glucose; 4, 1 mM nigericin; 5, 80 AU mL^{-1} sakacin A; 6, valinomycin. Bottom, ΔpH. The following additions to suspensions of *Listeria* cells containing the cFDASE probe are indicated by arrows: 1, lactose 10 mM; 2, 80 AU mL^{-1} sakacin A; 3, 1 mM nigericin. In both experiments, fluorescence was monitored at an excitation wavelength of 643 nm and an emission wavelength of 666 nm.

c. 20% within 30 min of sakacin A addition. After 24 h, the sample treated with sakacin A gave a turbidity decrease (38–40%) not significantly different ($P > 0.05$) from that obtained with lysozyme.

Isolated *Listeria* cell walls were exposed to various antimicrobials, and the solubilized material was analyzed by

Table 1. Optical density at 600 nm of *Listeria* cell wall isolates treated with lysozyme and sakacin A

Time (hours)	Control	Lysozyme	Sakacin A
0	0.527 ± 0.02[a]	0.540 ± 0.04[a]	0.534 ± 0.04[a]
0.5	0.501 ± 0.06[a]	0.499 ± 0.05[a]	0.436 ± 0.01[b]
24	0.509 ± 0.02[a]	0.335 ± 0.03[b]	0.330 ± 0.02[b]

Values are presented as means ± SD. In the same row, numbers with the same superscript are not significantly different ($P > 0.05$).

MALDI-TOF MS. The differences in the MS spectra in Fig. 3 indicate that individual antimicrobials had specific mechanisms of action and suggest that *Listeria* cell walls were broken down by sakacin A into fragments in the 1000–2500 Da range.

In separate set of experiments, isolated *Listeria* cell walls were treated for 24 h at 30 °C with increasing amounts of sakacin A, and the released fragments (Fig. 4) were sequenced by MS/MS. No fragments were released in the absence of sakacin A or with sakacin A concentrations

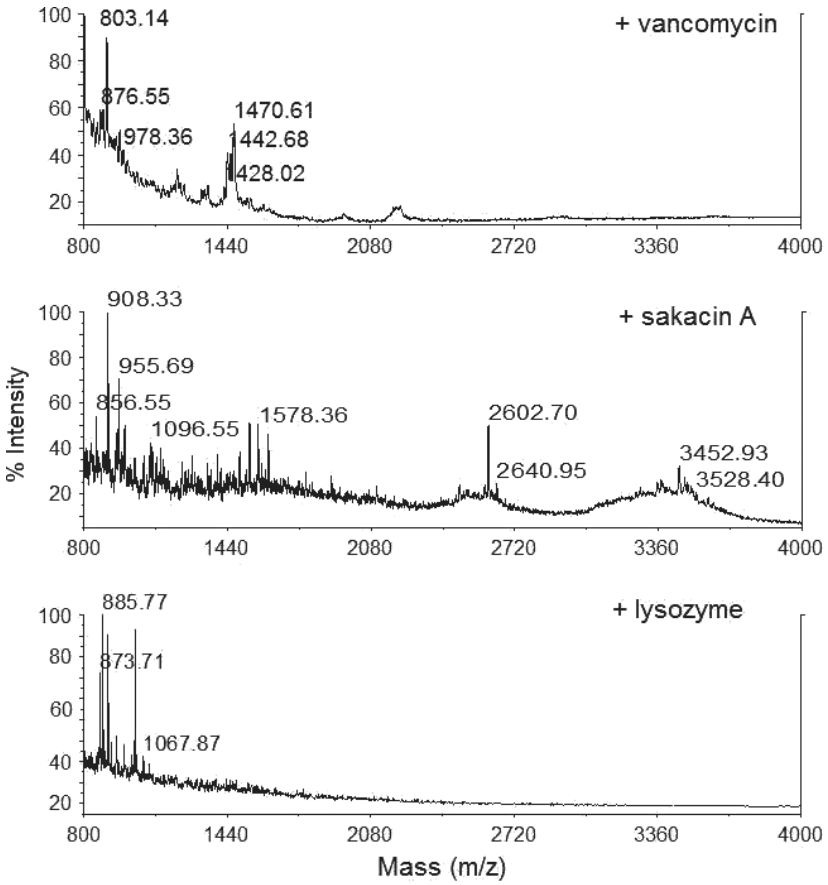

Fig. 3. MALDI-TOF profile of the water-soluble fraction from isolated cell walls incubated for 24 h at 30 °C in the presence of equivalent concentrations (0.1 mg mL^{-1}) of the given antimicrobials. (a) control samples, where vancomycin was present; (b) cell wall isolates incubated with sakacin A; (c) cell wall isolates incubated with lysozyme.

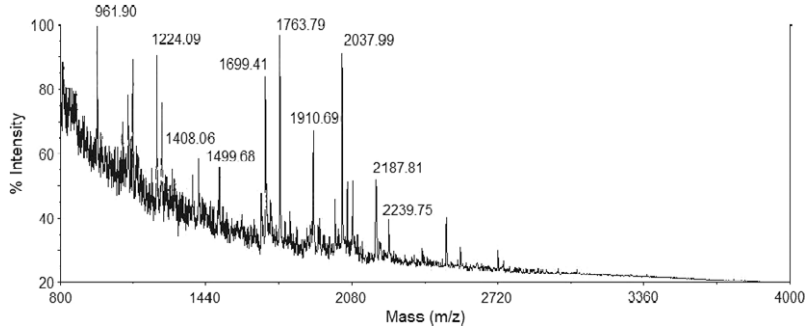

Fig. 4. MALDI-TOF profile of the water-soluble fraction from isolated cell walls incubated for 16 h at 30 °C with high concentrations of sakacin A (0.3 mg mL^{-1}).

lower than 0.1 mg mL^{-1}. As summarized in Table 2, products containing fragments from both the polysaccharide and the peptide components of the peptoglycan were evident at sakacin A concentrations of 0.1 mg mL^{-1}, and further breakdown products originating by former 'primary cuts' were evident only at higher sakacin A concentrations (Fig. 4).

PSD analysis of the fragments revealed the partial structures reported in Table 2. From the sequences of these products, sakacin A also seems to elicit proteolytic activity, with a preference for the bond formed by the *N*-acetyl muramic acid (NAM)-linked L-alanine residue nearest to the polysaccharide chain in the peptoglycan. Thus, the specific action of sakacin A on *Listeria* cell walls resulted in breakdown of the peptoglycan component in a fashion similar to lysozyme, but with a different specificity.

Discussion

The purification of sakacin A produced by *L. sakei* DSMZ 6333 from bacteria cultured in a low-cost media formulation, based on industrial ingredients and/or residuals from agro-food production (Trinetta *et al.*, 2008a), through the procedure reported here, compares favorably with protocols using higher-cost media and resulting in lower purification yields. The availability of significant amounts of purified sakacin A made it possible to investigate its mode of action.

We confirmed sakacin A as a membrane-active bacteriocin that kills *Listeria* cells by making their membranes permeable (Kaiser & Montville, 1996; Ennahar *et al.*, 1998). The cytoplasmic membrane seems the primary target of sakacin A, whose action is enhanced when cells are energized, possibly because transmembrane gradients favor the bacteriocin interaction with the membrane. The sakacin A action is straightforward and intense: both $\Delta\Psi$ and ΔpH are completely dissipated in seconds, resulting in leakage of cellular material (McAuliffe *et al.*, 1998).

One suggested mechanism of action for class IIa bacteriocins is the 'barrel-stave model' that implies an electrostatic binding step mediated by a membrane-bound receptor followed by a step involving hydrophobic interaction of an amphiphilic bacteriocin domain with the lipid acyl chains and in pore formation (Ennahar *et al.*, 1998; Drider *et al.*, 2006). However, other hypothetical mechanisms of action for class II bacteriocins imply a direct effect on cell walls (Kabuki *et al.*, 1997; Nielsen *et al.*, 2003).

Our observations, obtained with a highly purified bacteriocin preparation, support that cell walls are a target for sakacin A. A similar mode of action was shown by enterolysin A on *Listeria innocua* cell walls, where the activity was muralytic (Nielsen *et al.*, 2003). *Enterococcus mundtii* ST15 produced a bacteriocin active against Gram-positive and Gram-negative bacteria that displays a lytic action toward growing cells of *Lactobacillus casei* (De Kwaadsteniet *et al.*, 2005). El Ghachi *et al.* (2006) investigated the lytic action of colicin M on *Escherichia coli* cell walls by HPLC and MALDI-TOF MS analysis, similar to our study.

The data presented here confirm a slow hydrolytic action of sakacin A toward *Listeria* cell walls and suggest that sakacin A can break specific peptide bonds in the peptoglycan structure. This 'proteolytic' activity accompanies the capability of sakacin A to hydrolyze the β-1,4 glycosidic bond in NAM-NAG units and complements the effects on membrane structure discussed earlier, thus contributing to the efficacy (and selectivity) of this antimicrobial molecule.

In conclusion, purified sakacin A shows a dual mechanism of action: (1) it acts rapidly by changing the electrical charge distribution across the membrane and consequently dissipating the PMF; and (2) it slowly breaks down cell walls of sensitive bacteria, acting on both the polysaccharide and peptide components of the cell wall peptoglycan. This second activity might be useful to decrease the number of bacteria that compete for limiting nutrients in the same environment (Nielsen *et al.*, 2003).

The strong anti-*Listeria* activity of sakacin A, the high bacteriocin titer obtained at the end of the purification, and the application of a low-cost media formulation pave the use of this bacteriocin as an antimicrobial agent in food systems to prevent the growth of spoilage and pathogen bacteria and improve quality, safety, and food shelf life.

Table 2. Mass and structural data for products of cell wall hydrolysis by different amounts of sakacin A. Treatment of cell wall suspensions was performed overnight (*c.* 18 h) at 30 °C. Amino acids in the sequences are given as single letter symbols. Other abbreviations are as follows: DPM, diaminopimelic acid; NAG, *N*-acetyl glucosamine. Entries in italics are ion fragments from MS

Product mass			
Amount of sakacin A added to cell walls, mg			
0.1	0.2	0.3	Product sequence
2392	2392	2392	NAG-NAM-(A)-NAG-NAM-A-E-DPM-A-A-DPM-E-A-NAM-NAG
2188	2188	2188	NAG-NAM-(A)-NAG-NAM-A-E-DPM-A-A-DPM-E-A-NAM
	2258	2258	NAG-NAM-A-E-K-G-G-G-G-G-A-K-E-A-NAM-NAG
	2239	*2239*	*2258-H_2O*
1765 *1700*	*1763*	*1763*	*2258-(NAM, NAG, H_2O)* *(A)-NAG-NAM-A-E-DPM-A-A-DPM-E-A-NAM*
	1296	1296	NAG-NAM-A-A-E-DPM-A-DPM-E
	1167	1167	NAG-NAM-A-A-E-DPM-A-DPM

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Elution profile of sakacin A from a (RP) C18 column, with a linear gradient from 20 to 60% acetonitrile in water containing 0.1% TFA (dotted lines).
Fig. S2. MALDI-TOF mass spectrometric analysis of sakacin A.
Table S1. Purification of sakacin A.

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