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ORIGINAL ARTICLE

The identification of a low molecular mass bacteriocin, rhamnosin A, produced by *Lactobacillus rhamnosus* strain 68

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bacteriocin, class II, isolation, *Lactobacillus rhamnosus*, rhamnosin A.

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Abstract

Aims: This study focuses on the isolation and characterization of a peptide with bacteriocin-like properties isolated from *Lactobacillus rhamnosus* strain 68, previously identified by 16S rRNA gene sequencing and originating from human gastrointestinal flora.

Methods and Results: The peptide was isolated from a supernatant of bacteria maintained under restrictive conditions by a combination of ethanol precipitation and reversed-phase chromatography. The molecular mass of the peptide as assessed by mass spectrometry was 6433·8 Da. An isoelectric point of 9·8 was determined by 2D-PAGE. The peptide designated rhamnosin A inhibited *Micrococcus lysodeikticus* ATCC 4698 but did not inhibit *Lactobacillus plantarum* 8014 or *Lact. plantarum* 39268. Inhibitory activity against *M. lysodeikticus* at concentrations used in this study was shown to be bacteriostatic rather than bacteriolytic or bactericidal. Rhamnosin A retained biological activity after heat treatment (95°C, 30 min) but was sensitive to proteolytic activity of pepsin and trypsin.

Conclusions: The N-terminal sequence of rhamnosin A, as determined by Edman degradation and in more detail by BLAST analysis, did not show identity with any currently available *Lact. rhamnosus* HN001-translated protein sequences, nor any significant similarity with other sequences in the nonredundant protein sequence database. Being a small, heat-stable, nonlanthionine-containing peptide, rhamnosin A should be categorized as a class II bacteriocin.

Significance and Impact of the Study: This study describes a partial bacteriocin sequence isolated from *Lact. rhamnosus* 68 and broadens our understanding of bacteriocins.

Introduction

Strains of lactic acid bacteria (LAB) are commonly used in fermentation processes, in food preservation and in dried form as probiotic pills for human and animal consumption. Probiotics are defined as live microbial food supplements with beneficial effects on human health (Salminen *et al.* 1998). Major groups of compounds produced by LAB to which inhibitory properties are attributed include lactic and volatile acids, hydrogen peroxide and other antibiotic-like compounds (Gibbs 1987). Bacteriocins are antimicrobial peptides synthesized by ribosomes of bacteria which have the property of inhibiting other bacteria, either of the same species (narrow spectrum) or across genera (Cotter *et al.* 2005).

Among the bacteriocins isolated from LAB, a distinction can be made between the following: first, class I lantibiotics or small heat-stable lanthionine-containing single- and two-peptide bacteriocins; second, class II peptide bacteriocins or small heat-stable nonlanthionine-containing bacteriocins; lastly, bacteriolysins or large heat-labile proteins.

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Class II includes pediocin-like or *Listeria*-active bacteriocins (subclass IIa), two-peptide bacteriocins (subclass IIb), circular bacteriocins (subclass IIc) and nonpediocin single linear peptides (subclass IId) (Cotter *et al.* 2005).

Bacteriocin production is often associated with probiotic strains (Ghosh *et al.* 2004). The increased interest in LAB bacteriocins stems from their potential to modify and control the microbial ecosystems of foodstuffs. The first bacteriocin to appear on both the European food additive list and the United States FDA list was nisin, it was intended for use in the production of pasteurized processed cheese (Federal Register 1988).

Besides their applications in the food industry and their relevance in the improvement of food quality and safety, the increased interest in bacteriocinogenic LAB strains has been ascribed to their potential to modulate the immune system.

It has been suggested that some as yet unidentified molecules from probiotic LAB can cross the epithelial layer and inhibit the lipopolysaccharide-induced secretion of tumour necrosis factor-α by monocytic action (Kaiserlian *et al.* 2005). *In vivo* studies have documented the ability of probiotics to modulate T cell-mediated allergic responses (Kaiserlian *et al.* 2005). The production of bacteriocin by *Lactobacillus salivarius* UCC118 *in vivo* has been shown to afford mice protection against infection with the invasive foodborne pathogen *Listeria monocytogenes* (Corr *et al.* 2007). Lactobacilli are found in normal gastrointestinal and genitourinary flora (Aguirre and Collins 1993). The existence of the species *Lactobacillus rhamnosus* has been reported in habitats as varied as sucuk (Çon and Gökalp 2000), boza (Todorov and Dicks 2004) and in healthy vaginal microflora (Aroutcheva *et al.* 2001). Because of its immunomodulatory efficacy in the prevention of infantile atopic diseases (Majamaa and Isolauri 1997; Kalliomaki *et al.* 2001), one of the most studied probiotic strains is *Lact. rhamnosus* GG (originally isolated from human intestinal flora). Concerns were raised about the incidence of *Lact. rhamnosus* GG-induced bacteremia in immunocompromised patients following its use in Finland as a probiotic. Such a causative link has now been dismissed (Salminen *et al.* 2002).

Although probiotic LAB have important benefits to health, caution should be exercised when considering their administration to immunocompromised subjects (Avlami *et al.* 2001; Land *et al.* 2005; Z'Graggen *et al.* 2005; Brahimi *et al.* 2008; Tommasi *et al.* 2008).

The *Lact. rhamnosus* 68 used in this study was isolated from human gastrointestinal flora and has been characterized by 16S rRNA gene sequencing and subsequent sequence analysis (NCIMB Ltd, Aberdeen, UK; assigned strain name NCSQ 1872). There was a sequence similarity of 99·4% when compared with the *Lact. rhamnosus* species, sufficient to categorize this strain as belonging to the species *Lact. rhamnosus* (Janda and Abbott 2007). The aim of this study has been to isolate and characterize a bacteriocin produced by *Lact. rhamnosus* strain 68.

Materials and methods

Bacterial strains and growth conditions

Lactobacillus rhamnosus 68 and the other *Lactobacillus* species used (*Lactobacillus plantarum* 8014 and 39268) were grown in MRS broth (De Man *et al.* 1960) at 36°C without agitation. *Lactobacillus rhamnosus* 68 was also kept without agitation in a chemically defined medium (CDM) consisting of 0·85% sodium chloride and 0·5% glucose. *Micrococcus lysodeikticus* ATCC 4698 was grown in nutrient broth (NB) (Institute Torlak, Belgrade, Serbia) at 30°C with constant agitation. All bacteria were kept in 15% glycerol stock at −80°C and were transferred to new media at least three times before use.

Production and isolation of bacteriocin

MRS broth (1·5 l) was inoculated with one per cent (v/v) of an overnight culture of *Lact. rhamnosus* 68 and was incubated for 16 h at 37°C without agitation. Bacteria were centrifuged (1500 ***g***, 20 min), and the supernatant was removed. After washing with sterile physiological solution and recentrifugation, the bacterial pellet was transferred into the same starting volume (1·5 l) of CDM. Bacterial growth was monitored by optical density at 610 nm. After 12 h of incubation at 36°C, the bacteria were centrifuged (20 000 ***g***, 2 min) to obtain a clear supernatant, free of cell debris. The supernatant (1 l) was concentrated to a volume of 30 ml by lyophilization. The concentrate was subjected to ethanol precipitation using one volume of the sample to nine volumes of 96% ethanol previously cooled at −20°C. After centrifugation (1500 ***g***, 5 min), the pellet obtained was air-dried to remove any residual ethanol and then dissolved in two volumes of distilled water. Separation of bacteriocin was performed using a reversed-phase C5 column (RPC) (Supelco, Bellefonte, PA, USA) on an Äkta purifier (Pharmacia Amersham, Uppsala, Sweden). Solution A consisted of distilled water with 0·1% (v/v) trifluoroacetic acid (TFA). Solution B consisted of 0·1% TFA (v/v) and 80% (v/v) acetonitrile in distilled water. The column was eluted with 20 column volumes in a linear gradient of solutions A and B. Fractions eluted from the column were dried on the concentrator 5301 (Eppendorf system) and dissolved in

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100 μl of phosphate-buffered saline (PBS) for further analysis. All fractions were analysed with 16% Tricine–SDS-PAGE (Schagger and Von Jagow 1987). Protein concentration was determined using the Lowry assay (Lowry *et al.* 1951).

Biochemical characterization of bacteriocin

The isoelectric point of the peptide was assessed by 2D-PAGE according to Bollag *et al.* (1996). Initial separation was achieved by electrofocusing in the first dimension (5% PAGE, gradient of ampholytes pH 3·5–10; Pharmacia) and there then followed a period of incubation (30 min) in the equilibration buffer [62·5 mmol l^{-1} Tris–Cl pH 6·8, 5% (v/v) β-mercaptoethanol, 2·3% SDS (w/v) and 10% (v/v) glycerol]. The second dimension was then run using 16% Tricine–SDS-PAGE. Gel was stained with CBB-R250 according to Walker (2002). Digestion of bacteriocin (2·5 mg) was performed overnight using either a final concentration of 1 mg ml^{-1} of pepsin (Sigma-Aldrich, St Louis, MO, USA) or trypsin (Sigma-Aldrich) according to the method described by Yagami *et al.* (2000).

Mass analysis

Mass analysis of bacteriocin was carried out using a 6210 Time-of-Flight (TOF) LC/MS system (G1969A; Agilent Technologies, Santa Clara, CA, USA). Mobile phase was a 50 : 50 mixture of solvent A (0·2% formic acid in water) and solvent B (acetonitrile) at a flow rate of 0·2 ml min^{-1}. Mass spectrometer was run in positive electron spray ionization (ESI) mode with mass/charge (*m/z*) ratio in the range of 100–3200 *m/z*. The sample was introduced via 1200 series HPLC system (Agilent Technologies). Agilent MassHunter Workstation Software was used for data acquisition, and Agilent MassHunter Workstation Software and Analyst QS were used for data processing.

Amino acid sequencing

Following separation by Tricine–SDS-PAGE and electrotransfer to a PVDF membrane (Serva, Heidelberg, Germany), the bacteriocin-containing membrane fragment was washed in double-distilled water and then stained with 0·1% Coomassie Blue-G (Serva) in 50% methanol, destained in 50% methanol and air-dried. Peptide band was excised and subjected to N-terminal sequencing. The first 13 amino acids were determined by Edman degradation using a Procise protein sequencer connected to an online phenylthiohydantoin amino acid analyser (PE Biosystems, Weiterstadt, Germany).

Biological activity assay

For the biological activity assay, *M. lysodeikticus* ATCC 4698 and *Lact. plantarum* 8014 and 39268 were used. The procedure used was a modification of the previously described microtitre plate assay system (Geis *et al.* 1983). All fractions obtained by RPC were evaporated using Concentrator 5301 and dissolved in 0·1-ml PBS, pH 7·2. All the test strains were grown overnight. The cultures were diluted to 10^6 CFU ml^{-1} for the biological activity assay, with NB for *M. lysodeikticus* ATCC 4698 and with MRS for *Lact. plantarum* 8014 and 39268. A bacterial strain (100 μl) was incubated with different fractions each obtained by RPC (100 μl), with solvent controls; agitation was initiated at the appropriate temperatures. After 16 h, samples were transferred to a microtitre 96-well plate (Sarstedt, Germany). Optical density at 610 nm and absorbance at 450 nm were measured with the ELISA reader (LKB Micro plate reader 5060-006; LKB, Austria). To determine thermal stability of bacteriocin, biological activity was measured following heat treatment at 95°C (15–30 min).

Tricin-PAGE overlaying assay was then performed to assess the inhibitory activity of the isolated bacteriocin and the extent to which inhibition was modified by the presence of proteolytic enzymes. The peptide (0·2 mg ml^{-1}) was digested overnight with pepsin or tripsin (1 mg ml^{-1}) at 37°C. The same starting amount of bacteriocin was added to all three lanes. The gel was washed with sterile distilled water, fixated in 70% ethanol (10 min), then rinsed several times with sterile distilled water (2 h) and finally washed with PBS (10 min). The gel was further overlaid with NB supplemented with 0·7% agarose and *M. lysodeikticus* was added to 10^6 CFU ml^{-1}.

To test the effects of rhamnosin A on *M. lysodeikticus* cells, a bacteriocin sample (or PBS as a control) was added to a 1000-times diluted overnight culture of *M. lysodeikticus* cells in NB to a concentration of 0·1 mg ml^{-1}. At fixed intervals, an aliquot (1000-times diluted) was plated onto nutrient agar and incubated (48 h) at 37°C, when clearly distinctive yellow colonies were visualized and counted. This experiment was repeated twice, and each time point was plated in triplicate.

Results

Isolation and biochemical characterization of the bacteriocin

A novel bacteriocin was isolated from the medium in which *Lact. rhamnosus* 68 was incubated under restrictive conditions. The peptide was isolated by a combination of ethanol precipitation and reversed-phase chromatography.

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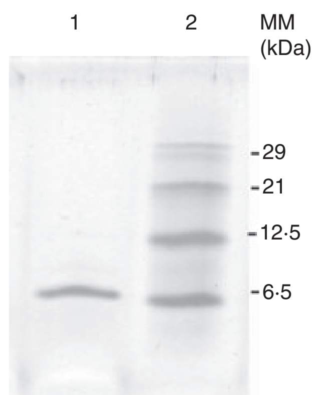

Figure 1 CBB-R250-stained Tricine–SDS-PAGE (16%) performed under reducing conditions. Lane (1) rhamnosin A; lane (2) molecular mass markers (6·5, 12·5, 21, 29 kDa; SERVA).

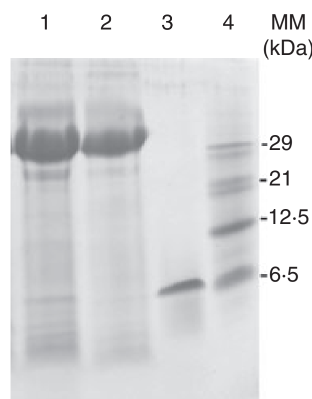

Figure 3 CBB-R250-stained Tricine–SDS-PAGE (16%). Lane (1) pepsin digestion of rhamnosin A after 5 min, lane (2) pepsin digestion of rhamnosin A after 12 h, lane (3) rhamnosin A, lane (4) molecular mass markers (6·5, 12·5, 21, 29 kDa).

Approx. 4 mg of the peptide were obtained from 1 l of medium. Purification of the peptide was monitored by Tricine–SDS-PAGE. Bacteriocin was eluted using RPC C5 with 35% acetonitrile. Using a 1D Tricin-PAGE, the mobility of the isolated bacteriocin was slightly above 6·5 kDa (Fig. 1). Following a 2D-PAGE, the isolated bacteriocin revealed an isoelectric point of 9·8. By ESI-TOF mass spectrometry, the determined molecular mass was 6434 Da (Fig. 2). The isolated peptide revealed the following N-terminal sequence: AV(K)P(T)AVRKTNETLD. Both proteolytic enzymes (trypsin and pepsin) digested the peptide rhamnosin A after an overnight incubation at 37°C (Fig. 3).

Bacteriocin activity against *Micrococcus lysodeikticus* ATCC 4698

In biological assays, the peptide produced by *Lact. rhamnosus* 68 showed inhibitory activity against *M. lysodeikticus* ATCC 4698. Biological activity was evaluated by measuring the optical density of the bacterial growth of *M. lysodeikticus* (10^6 CFU ml^{-1} bacterial dilution in NB) in the presence or absence of the isolated bacteriocin (Fig. 4). An initial rhamnosin A concentration of 0·25 mg ml^{-1} was sufficient to inhibit the indicator strain. Inhibition was diminished to 50% by a ten-fold sample dilution. Inhibitory activity was verified by

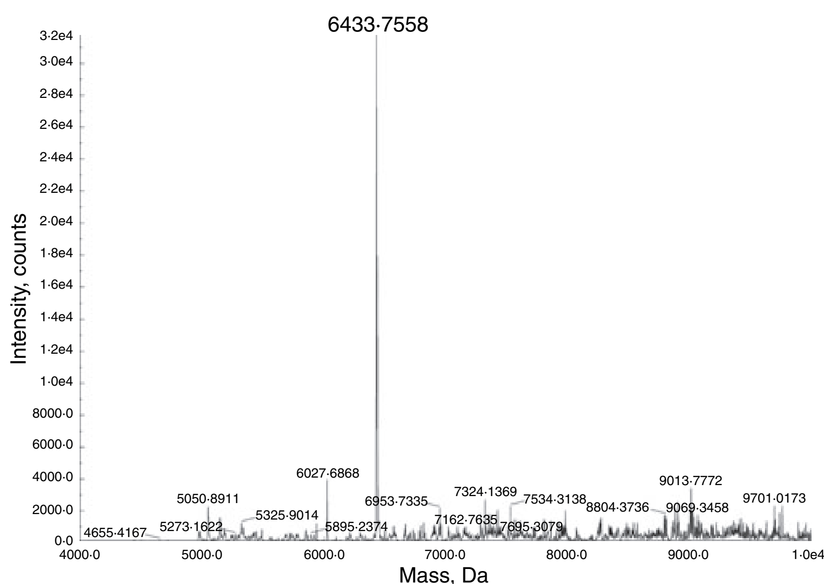

Figure 2 Mass reconstruction of the active fraction containing rhamnosin A (6210 Time-of-Flight LC/MS). Deconvulsion was performed with AnalystQS program, ions selected from the interval 1100–2000 *m/z*.

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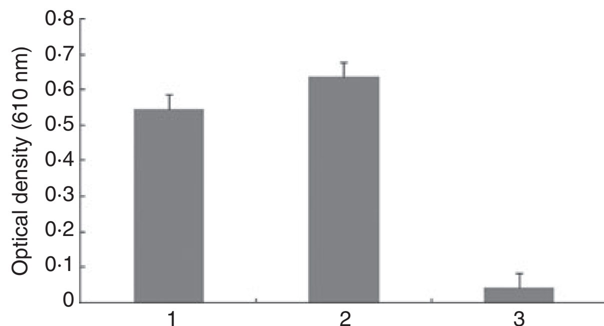

Figure 4 Growth inhibition of *Micrococcus lysodeikticus* ATCC 4698 by rhamnosin A monitored by optical density at 610 nm. Growth medium: (1) 50% nutrient broth (NB) in phosphate-buffered saline (PBS), (2) NB, (3) rhamnosin A (0·25 mg ml^{-1}) in 50% NB, in PBS.

seeding the indicator bacteria (following 12 h of incubation with bacteriocin) onto plates with nutrient agar. The number of *M. lysodeikticus* ATCC 4698 colonies was considerably diminished after 12 h of incubation with bacteriocin in comparison with the control (incubated with PBS).

The kinetics of *M. lysodeikticus* CFU mean that this diminution was not caused by bacteriolytic action. There was no rapid decrease in CFU (Fig. 5). Heat exposure at 95°C for 30 min had no influence on the biological activity of bacteriocin (Figure not shown). Exposure to proteolytic enzymes diminished bacteriocin action (Fig. 6).

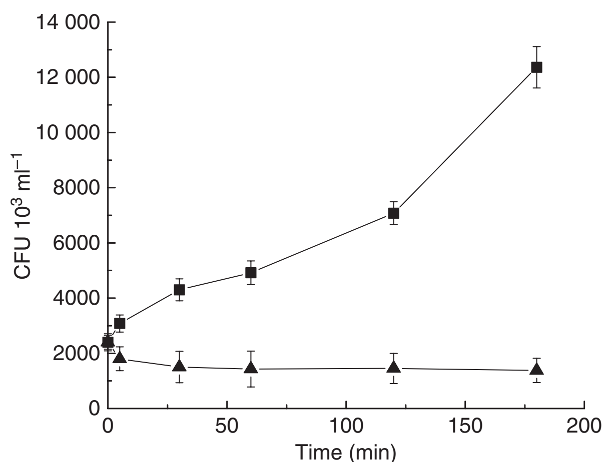

Figure 5 Effect of rhamnosin A on *Micrococcus lysodeikticus* cells, measured by the number of viable cells of *M. lysodeikticus* per millilitre. (■) Control, no rhamnosin A added and (▲) rhamnosin A added to a concentration of 0·1 mg ml^{-1}. Mean values, with standard deviations, of a discrete experiment carried out in triplicate are shown.

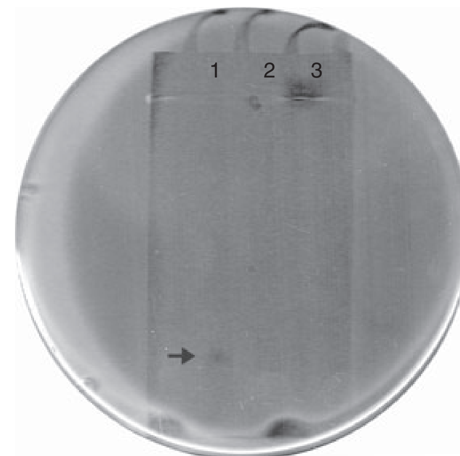

Figure 6 Washed Tricin–SDS-PAGE overlaid with 10^6 CFU ml^{-1} of *Micrococcus lysodeikticus* cells in nutrient broth supplemented with 0·7% agarose. Lane (1) rhamnosin A 0·2 mg ml^{-1}, lane (2) rhamnosin A 0·2 mg ml^{-1} pepsin digestion, lane (3) rhamnosin A 0·2 mg ml^{-1} trypsin digestion. The arrow indicates the inhibition zone.

Discussion

Different methods have been employed for the purification of bacteriocins produced by LAB. These are mostly based on the 'salting out' of bacteriocins from the medium by ammonium sulfate precipitation and further separation according to charge, size or hydrophobic interactions (Mortvedt *et al.* 1991; Parente and Riccardi 1999). This study describes the isolation and characterization of a novel bacteriocin from *Lact. rhamnosus* 68. Cultivation conditions for rhamosin A production by *Lact. rhamnosus* 68 were designed to keep bacterial cells alive in starvation conditions. The CDM was chosen for two reasons: first, it has been noted that in certain instances bacterial growth is not a prerequisite for bacteriocin production (ten Brink *et al.* 1994; Aroutcheva *et al.* 2001); second, use of this medium minimizes the risk of the preparation being contaminated by peptides originating from MRS. Rhamnosin A obtained from freeze-dried CDM was purified from a highly concentrated salt and glucose solution by ethanol precipitation. The precipitated peptides were dissolved in distilled water and subsequently resolved by RPC using a C5 column. An increase in the gradient length of reverse phase separation resulted in improved peptide purification.

In conformity with other class II bacteriocins, rhamnosin A revealed a highly basic pI suggestive of a cationic surface topography. Rhamnosin A is a heat-stable bacteriocin which is digestible by proteolytic enzymes present in the gastrointestinal tract. Based on structural characteristics obtained from this study (molecular mass, pI, N-terminal sequence), rhamnosin A should be considered

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a class II bacteriocin. Although most bacteriocins are bactericidal or bacteriolytic in nature, rhamnosin A at the concentration used has a bacteriostatic effect. A bacteriostatic effect was previously reported for other bacteriocins such as lactocin 27 (Upreti and Hinsdill 1975), leuconocin S (Lewus *et al.* 1992), lactacin 3147 (McAuliffe *et al.* 1998) and plantaricin C19 (Atrih *et al.* 2001). Bacteriocins act either bactericidally or bacteriostatically. Factors, which may affect their mode of action include dose, the degree of purification, the growth phase of the indicator cells and overall experimental conditions (Deraz *et al.* 2007). The activity of any single bacteriocin may differ with differing bacterial strains (González *et al.* 1994). Little has been published concerning *Lact. rhamnosus* bacteriocins. *Lactobacillus rhamnosus* GG produces no bacteriocin in MRS broth (Avonts *et al.* 2004). *Lactobacillus rhamnosus* strain ST461BZ produces a bacteriocin of 2·8 kDa, and strain ST462BZ produces a bacteriocin of approx. 8 kDa in MRS broth with a broad range of activity (Todorov and Dicks 2004). *Lactobacillus rhamnosus* 160 produces a bacteriocin with a molecular mass of around 3·8 kDa with an inhibitory activity towards *Micrococcus luteus* ATCC 10420 (Li *et al.* 2005). Our search (8 August 2009) for bacteriocins among translated sequences from *Lact. rhamnosus* HN001 at URL http://www.ncbi.nlm.nih.gov/sites/entrez?db=protein returned several results. One such was the predicted sequence of an ABC-type bacteriocin/lantibiotic exporter, containing an N-terminal double-glycine peptidase domain (accession number EDY98323). This data suggests that the bacteriocin produced by *Lact. rhamnosus* HN001 is of the class IIa pediocin type.

Another sequence found among the search results returned was designated as a prebacteriocin (accession number ZP_03212312). Scanning of this sequence for conserved motifs as described at http://pfam.sanger.ac.uk suggested that its N-terminal region belongs to the Enterocin A immunity family [EntA_Immun (PF08951)]. This is another indication that *Lact. rhamnosus* HN001 may contain a pediocin-like molecule whose presence is as yet unelucidated; no significant similarity with pediocin PA-1 has been observed within the currently available translated sequences. Because only thirteen N-terminal amino acids have been identified, no sequence similarities could be determined; further subclassification must therefore rely upon a process of elimination. Rhamnosin A does have a specific biological activity as a single- and not a two-peptide bacteriocin; amino acid sequencing of its amino terminus suggests that it is not a class IIc circular bacteriocin; for these two reasons, we believe that Rhamnosin A is a class IId bacteriocin of the type recently proposed and described. Categorizing rhamnosin A as a class IId bacteriocin and simultaneously postulating that *Lact. rhamnosus* HN001 contains a pediocin-like bacteriocin is clearly paradoxical. However, such paradoxes are not uncommon as shown by the fact that different strains of *Enterococcus faecium* produce bacteriocins of different class II subclasses (Franz and Holzapfel 2004). We note that bacteriocin production varies both among different strains of the same bacterial species and in different media (Todorov and Dicks 2004). We believe that we have identified a novel bacteriocin and our future work will focus on a more thorough characterization of this bacteriocin, including a characterization of its inhibitory spectra and its mechanisms of action. Several mechanisms of bacteriocin activity are now postulated; both modified and unmodified peptides exert activity through interaction with the bacterial membrane. The majority of class II bacteriocins analysed dissipate the proton motive force of the target cell via pore formation. There is a nascent awareness that specific membrane molecules are targeted by these class II bacteriocins (Héchard and Sahl 2002).

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