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ORIGINAL PAPER

Isolation and characterization of a new bacteriocin, termed enterocin M, produced by environmental isolate *Enterococcus faecium* AL41

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Abstract The new bacteriocin, termed enterocin M, produced by *Enterococcus faecium* AL 41 showed a wide spectrum of inhibitory activity against the indicator organisms from different sources. It was purified by $(NH_4)_2SO_4$ precipitation, cation-exchange chromatography and reverse phase chromatography (FPLC). The purified peptide was sequenced by N-terminal amino acid Edman degradation and a mass spectrometry analysis was performed. By combining the data obtained from amino acid sequence (39 N-terminal amino acid residues was determined) and the molecular weight (determined to be 4 628 Da) it was concluded that the purified enterocin M is a new bacteriocin, which is very similar to enterocin P. However, its molecular weight is different from enterocin P (4 701.25). Of the first 39 N-terminal residues of enterocin M, valine was found in position 20 and a lysine in position 35, while enterocin P has tryptophane residues in these positions.

Keywords Enterocin · *E. faecium* · Characterization

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Introduction

The genus *Enterococcus* belongs to a group of microorganisms known as lactic acid bacteria [9, 33]. Enterococci are widely distributed not only in warm-blooded animals [8, 19] and in some human outbreaks, but they also occur in soil, surface waters, plants and vegetables [29, 33, 34, 36] and they can be isolated from various ecological niches including municipal sewage [20, 21, 32]. Enterococci are often encountered as the dominating flora in lactic acid fermented food, in particular, in Mediterranean countries [12]. Enterococci (in addition to coliform) are also regarded as indicators of hygiene conditions. Although, the European Union established a maximum limit for the presence of coliforms including *Escherichia coli* in the year 1992, no limit was set for the enterococci [1, 14]. Enterococci have the ability to produce bacteriocins, small peptides with antimicrobial activity towards more or less related bacteria [9, 31]. In general, the bacteriocins showed both complementary activity and are active against different bacteria; some of the bacteriocins have a narrow spectrum of activity while others inhibited a wide variety of bacteria [25, 28]. Bacteriocins, enterocins produced mostly by the species *Enterococcus faecium* are studied in detail [2, 4, 26, 27]. Till now, enterocins such as enterocin A, P, B, L50A, L50B, I, Q [2–7, 11] were purified and determined in detail. In contrast, many bacteriocins produced by *E. faecium* strains were not purified to homogeneity yet [18], whereas enterocins produced by the other species of the genus *Enterococcus* were characterized [17, 33]. Franz et al. [15] even reported a new classification scheme to group enterococcal bacteriocins. According to that scheme, enterocins are classified into four groups: Class I enterocins—lantibiotic enterocins, Class II enterocins subgrouped into enterocins of pediocin family, enterocins synthesized without a leader peptide, other

linear, nonpediocin-type enterocins, Class III enterocins—cyclic antibacterial peptides and Class IV enterocins. Although, Mareková et al. [26] reported environmental strain *E. faecium* EK13 (isolated in Laboratory of Animal Microbiology Slovak Academy of Sciences in Košice, Slovakia, LAM-IAPSAS, Lauková et al. [21]; deposited to Czech Collection of Microorganisms in Brno, Czech republic designated as CCM7419) producing enterocins A and P, the studies on bacteriocins related to the producers originating from sludge or sewage are rare. It is area which is new to look for antimicrobial compounds; because till now the most determined enterocins as well as the more frequent field to apply bacteriocins (in general) is food to eliminate food spoilage and/or food-borne pathogens [22–24]. In this paper, isolation, purification and characterization of a bacteriocin produced by environmental strain *E. faecium* AL41 is reported. The specificity of the producer strain is its origin as it was isolated from sewage sludge [21].

Materials and methods

Bacterial cultures, media and growth conditions

Enterococcus faecium AL41 strain was isolated from the sewage sludge using M-Enterococcus agar (Becton & Dickinson, Cockeysville, USA). First, the strain was identified by the commercial API test (Bio Merioux, L'Etoile, France) followed by analysis of its 16S rRNA DNA sequence [34]. *E. faecium* AL41 produces mainly lactic acid from glucose (0.914 mol/l), it is a strain with low urease activity (7.23 nkat/ml) and a bacteriocin producer. *E. faecium* AL41 as well as the indicator bacteria (listed in Table 1) were cultivated in MRS broth, Trypticase soy broth (Difco) inoculated with 10 µl of a frozen stock (−80°C) and cultivated at 30 and 37°C for overnight or for 48 h and in CO_2 atmosphaere for clostridiae. The indicator bacteria (Gram-positive and Gram-negative) were originated from different sources such as faeces of deer, chamois, piglets, rabbits, from caecum of rabbits, from mastitis milk, from rumen content, from dairy products, from human clinical samples as well as from rabbit meat. They represent own isolates (LAM-IAPSAS), or isolates obtained from IHE—Institute of Hygiene, University of Tuebingen (Germany), University of Brussels and Ghent (Belgium), University of Ljubljana (Slovenia) and LMGT of NLH in As (Norway). During purification of bacteriocin produced by the AL41 strain, following strains were used as indicators: *E. faecium* T136 (LMG 2384), *E. faecium* P13 (LMG 2773) and *Carnobacterium piscicola* (LMG 2332)—Laboratory of Microbial Gene Technology, NLH As, Norway. The microorganisms used for the bacteriocin screening were propagated in media appropriate for species used.

Table 1 Antimicrobial activity of *Enterococcus faecium* AL41 strain

Indicator strains	Tested strains[a]	Inhibited strain[b] (%)	Activity[c]
Enterococcus faecium	36	36	400–25,800
E. faecalis	4	4	400
E. casseliflavus	8	8	800–3,200
E. avium	4	4	3,200–12,800
Enterococcus sp.	8	2	800–12,800
Staphylococcus aureus	10	10	3,200
S. vitulinus	2	1	800
S. xylosus	4	4	400
S. chromogenes	2	1	3,200
S. lentus	5	2	400–1,600
S. saprophyticus	1	1	3,200
S. capitis	1	1	3,200
S. gallinarum	1	1	100
Staphylococcus sp.	47	7	100
Micrococcus luteus	2	1	12,800
Lactobacillus plantarum	2	1	14[d]
L. fermentum	2	0	0
L. salivarius	1	0	0
L. curvatus	1	1	10[d]
L. sakei	1	1	4,000
Leuconostoc sp.	1	0	0
Pediococcus pentosaceus	2	0	0
Pediococcus acidilactici	1	0	0
Carnobacterium piscicola	1	1	20[d]
Bacillus cereus	12	17	100–3,200
Listeria innocua	3	1	1,600–51,200
Listeria monocytogenes	1	1	64,000
Clostridium-like spp.	60	32	100
Escherichia coli	42	6	100
Pseudomonas spp.	36	0	0

[a] The counts of the strains tested

[b] The count of inhibited strains

[c] Activity is expressed in Arbitrary unit per ml (AU/ml)

[d] Activity expressed the size of the inhibitory zones in mm

Antimicrobial activity of bacteriocin produced by *E. faecium* AL41

The bacteriocin activity was determined by three methods; by detection of clear zone around indicator organisms [35], by critical dilution method on micro titer plates [16] and by the agar spot test [10]. One bacteriocin unit was defined as the amount of bacteriocin causing 50% growth inhibition (50% of the turbidity of the control culture without bacteriocin) or in AU/ml (arbitrary units per ml—the reciprocal of the highest twofold dilution demonstrating complete inhibitory activity of the indicator strain).

Heat treatment and effect of enzymes

Samples of bacteriocin were exposed to heat under different temperatures (60, 80 and 100°C) for 10, 30 min and 1 h. Bacteriocin was also stored at different temperatures (4, −20, −80°C) for 1 week, 1 month and 1–2 years. The enzymes (pronase P, Sigma, USA; trypsin and chymotrypsin, Serva, Germany) were added to the concentrated bacteriocin 500 µl (after precipitation with 40% ammonium sulphate) at a final concentration 0.5 mg/ml. Samples with and without enzymes were incubated at 37°C for 60 min before the remaining activities were determined which were tested using the indicator strain *E. avium* EA5.

Bacteriocin purification

A 14–18 h culture (2 l) of *E. faecium* AL41 in MRS broth (Difco) was centrifuged for 30 min at 10,000×*g* in order to remove the cells. Ammonium sulphate (to obtain 40% saturation) and/or Amberlite XAD116 were gently added to the supernatant, and the mixture was stirred at 4°C for 2 h. After centrifugation at 10,000×*g* for 30 min, the resulting pellet was re-suspended in 10 mmol/l sodium phosphate buffer (pH 5.0); and loaded on a column on PD10. In the next step, cation exchange chromatography on SP-Sepharose (Pharmacia-LKB, Sweden) equilibrated with 10 mmol/l sodium phosphate buffer (pH 5.0) was used. The bacteriocin was eluted with 10 ml of the same buffer containing 1 mol/l NaCl (natrium chloride). The active fraction was applied to a C2/C18 reverse phase FPLC column (Pep RPC HR 5/5, Pharmacia-LKB, Sweden) equilibrated with 0.1% tri-fluoro-acetic acid (TFA) in distilled water. The bacteriocin was eluted from the reverse-phase column by using a linear gradient of 2-propanol in aqueous 0.1% (v/v) tri-fluoro-acetic acid (TFA) at a flow rate of 1 ml/min. Fractions with high bacteriocin activity were mixed and re-chromatographed on the reverse-phase column (Table 2). Purified bacteriocin was stored at −20°C after evaporation. Their protein content was estimated by absorbance measuring at 280 nm and activity was tested after each step.

Amino acid sequence analyses and molecular mass determination

Amino acid sequencing was performed by automated Edman degradation with a model 477A sequencer/model 120A phenyl-thiohydantion analyzer (Applied Biosystems, Foster City, California) at University of Oslo, Norway by Dr. K Sletten. The mass was determined with a Voyager-DE RP matrix-assisted laser desorption ionization-time of mass spectrometer (Perseptive Biosystems, G. Finland, University of Oslo) Norway.

Table 2 Purification of the enterocin M from *E. faecium* AL41

	Vol (ml)	Total activity (AU ml^{-1})	Yield (%)[a]
Culture supernatant	2,000	1,490,000	100
Ammonium sulphate precip.	150	970,000	65
SP-sepharose	10	450,000	30
1-FPLC	1.5	260,000	17
2-FPLC	1	170,000	9

[a] 100% yield is defined as total bacteriocin units measured in the cell-free growth medium

Results and discussion

Enterococcus faecium AL41 strain was allotted to the species *E. faecium* by both, the API commercial kit as well as by 16S rRNA DNA sequencing (data not shown). This strain produces an antimicrobial substance, which inhibits the growth of 144 indicator bacteria among 305 strains tested (Table 1). The antimicrobial activity depends on the sensitivity of the indicator strains, and the activity was spanning between 100 AU/ml using the least sensitive indicator strain and 64,000 AU/ml when the most sensitive indicator strain was used (by the micro-titer assays). The inhibitory zones varied between 10 and 20 mm when the antimicrobial potency was tested on agar plates by Skalka [35]. The antimicrobial activity produced by the AL41 strain was lost after the treatment by proteases, which strongly suggested that the activity was due to the proteina-

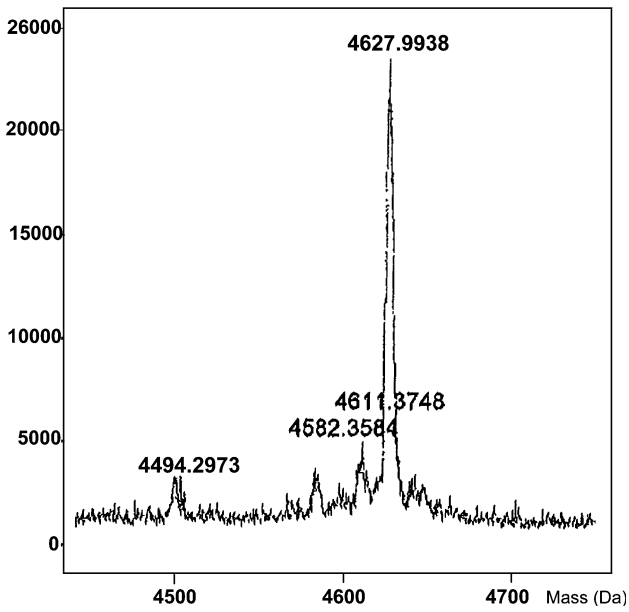

Fig. 1 Determination of molecular mass of enterocin M produced by *Enterococcus faecium* AL41

Fig. 2 Comparison of enterocin M with enterocin P

Enterocin M A T R S Y G N G V Y C N N S K **C** W - N **V** G E A K E N I A G I V I S G **K** A S G L
Enterocin P A T R S Y G N G V Y C N N S K C W V N **W** G E A K E N I A G I V I S G **W** A S G L A G M G H

ceous substance, most probably a bacteriocin, and consequently that substance was purified and characterized. The antimicrobial substance produced by *E. faecium* AL41 appeared to be heat-stable. Neither heat treatments of the bacteriocin sample at 60, 80 and 100°C for 1 h nor long-term storage at 4 or −20°C affect the activity. The bacteriocin activity was obtained from 14–18 h culture in MRS medium at pH 5.0 and concentrated by ammonium sulphate precipitation followed by cation-exchange chromatography using SP-Sepharose (Pharmacia). The recovery was 30% of the initial activity. The final step in the purification was reverse-phase chromatography (FPLC), which was repeated twice to obtain pure bacteriocin (Table 2). This bacteriocin resulted in only one major absorbance peak, coinciding with the antimicrobial activity. Mass spectrometry analysis of the activity fraction from the last FPLC column identified a major peak of mass being 4,627.99 Da (Fig. 1). This work was followed up by N-terminal amino acid sequencing by Edman degradation. One peptide sequence was found and the identified N-terminal sequence was: Ala–Thr–Arg–Ser–Tyr–Gly–Asn–Gly–Val–Tyr–Xaa–Asn–Asn–Ser–Lys–Xaa–Trp–Val–Asn–Trp–Gly–Glu–Ala–Lys–Glu–Asn–Ile–Ala–Gly–Ile–Val–Ile–Ser–Gly–Trp–Ala–Ser–Gly–Leu. The amino acid sequencing by Edman degradation was not completed to the very C-terminal end of the peptide, the sequencing had to stop after 39 steps because it was impossible to identify the residues beyond that step. However, by comparing the amino acid sequence obtained with the determined mass 5–6 of the C-terminal residues should be missing in the peptide. It could be concluded taking into account the amino acid sequence information that the bacteriocin detected is very similar to enterocin P (Fig. 2). In amino acid positions 11 and 16 the Edman degradation gave blank signals that are always observed when cystein residues are sequenced and this fact justifies our assumption that these two positions contain cysteins. With the exception of position 20 and 35 where both contain tryptophane in enterocin P [4] while valine and lysine are found in the respective position in the enterocin M, the two bacteriocins are identical in their 36 sequenced residues. It seems that, a novel bacteriocin has been found and that belongs to the strong anti-listerial bacteriocins which are grouped according to Nes et al. [30, 31] to the un-modified Class II a Pediocin-like bacteriocins.

From molecular weight estimations one can conclude that additional differences between the two bacteriocins have to be present in the non-sequenced part of enterocin M. The molecular weight of enterocin P is calculated to 4,701.25, which was wrongly calculated in the original paper describing enterocin P [4]. An estimate of enterocin M mass by assuming that the five C-terminal amino acid residues are identical to that of enterocin P should be 4,556 which is 72 Da below the determined value. This observation strongly suggests that additional differences in the sequences of the two bacteriocins. Industrial use of bacteriocin-producing lactic acid bacteria as well as their bacteriocin may have future in fighting antibiotic resistant bacteria. Numerous studies have shown that bacteriocins and bacteriocin-producing bacteria are able to inhibit and/or reduce effectively food and feed spoilage bacteria and food and feed-borne pathogenic bacteria [3, 12, 13, 22–24]. Our study increases recent knowledge concerning the bacteriocins, especially enterocins produced by the species of *E. faecium*, the isolate originated from a new environment, not commonly investigated.

Conclusion

A novel bacteriocin, enterocin M was determined, possessing a molecular mass of 4,628 Da with a broad antimicrobial spectrum and strong anti-listerial effect.

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