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PII: S0378-1135(08)00184-3
DOI: doi:10.1016/j.vetmic.2008.05.001
Reference: VETMIC 4032

To appear in: *VETMIC*

Received date: 23-1-2008
Revised date: 1-5-2008
Accepted date: 5-5-2008

Please cite this article as: Strompfová, V., Lauková, A., Simonová, M., Marciňáková, M., Occurrence of the structural enterocin A, P, B, L50B genes in enterococci of different origin, *Veterinary Microbiology* (2007), doi:10.1016/j.vetmic.2008.05.001

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Abstract

Enterococci are well known producers of antimicrobial peptides – bacteriocins (enterocins) and the number of characterized enterocins has been significantly increased. Recently, enterocins are of great interest for their potential as biopreservatives in food or feed while research on enterocins as alternative antimicrobials in humans and animals is only at the beginning. The present study provides a survey about the occurrence of enterocin structural genes A, P, B, L50B in a target of 427 strains of *Enterococcus faecium* (368) and *E. faecalis* (59) species from different sources (animal isolates, food and feed) performed by PCR method. Based on our results, 234 strains possessed one or more enterocin structural gene(s). The genes of enterocin P and enterocin A were the most frequently detected structural genes among the PCR positive strains (170 and 155 strains, respectively). Different frequency of the enterocin genes occurrence was detected in strains according to their origin; the strains from horses and silage showed the highest frequency of enterocin genes presence. All possible combinations of the tested genes occurred at least twice except the combination of the gene of enterocin B and L50B which possessed neither strain. The gene of enterocin A was exclusively detected among *E. faecium* strains, while the gene of enterocin P, B and L50B were detected in strains of both species *E. faecium* and *E. faecalis*. In conclusion, a high-frequency and variability of enterocin structural genes exists among enterococci of different origin what offers a big possibility to find effective bacteriocin-producing strains for their application in veterinary medicine.

Keywords: Bacteriocin; Bacteriocin structural genes; *Enterococcus* sp.; Enterocin; PCR

1. Introduction

The ability of enterococci to produce ribosomally synthesized, extracellularly released antimicrobial peptides - bacteriocins is well known (Nes at al., 1996). According to the classification scheme of Franz et al. (2007), enterocins belong to class I (lantibiotic enterocins), class II (small, nonlantibiotic peptides), class III (cyclic enterocins) and class IV (large proteins). Class II is subdivided in three subclasses: II. 1, enterocins of the pediocin family, II. 2, enterocins synthesized without a leader peptide and II. 3, other linear, non-pediocin type enterocins. Up to now, the best characterized enterocins are enterocin A (Aymerich et al., 1996), P (Cintas et al., 1997), B (Casaus et al., 1997), L50A and L50B (Cintas et al., 1998), I (Floriano et al., 1998), Q (Cintas et al., 2000). Moreover, the information on enterocin M was already published (Mareková et al., 2007). Enterocins are most frequently produced by *E. faecium* strains, however, other species of enterococci have also been found to produce bacteriocins e.g. *E. faecalis, E. hirae, E. mundtii, E. durans, E. avium, E. gallinarum, E. casseliflavus* and *E. columbae* (Jennes et al., 2000; Sabia et al., 2004; Sánchez et al., 2007).

Although, the efficiency of enterocins against food spoilage and pathogenic bacteria (*Listeria* sp., *Staphylococcus* sp., *Bacillus* sp., *Clostridium* sp., *Escherichia coli*) in various food systems is well demonstrated (Aymerich et al., 2000; Lauková and Czikková, 2001), only little information is available on the role of bacteriocins in the animal ecosystem. It is assumed that bacteriocin production is a bacterial defense mechanism, which gives the producer strain a competitive advantage towards non-producer and bacteriocin-sensitive strains in the same niche (Chen and Hoover, 2003). Several studies indicated applicability of enterocins or enterocin-producing strains to control/reduce pathogenic bacteria in the gastrointestinal tract of animals (*Salmonella* sp., *Campylobacter* sp., *E. coli*; Lauková et al., 2003; Cole et al., 2006). In addition, bacteriocins of other bacterial genera showed potential to

prevent bovine mastitis (Meaney et al., 2001) and gingivitis (Howell et al., 1993), but also to modulate the immune system (De Pablo et al., 1999) or the rumen fermentation (Callaway et al., 1997). Furthermore, the antitumorous activity was reported (Hill and Farkas-Himsley, 1991).

This study provides data on the occurrence of enterocin structural genes A, P, B, L50B (belong to firstly characterized enterocins in detail) as well as on the presence of various genes combinations in a target of 427 enterococci isolated from different sources. It compares the distribution of these genes in the three main environments – animals, food and feed.

2. Materials and Methods

2.1. Bacterial strains

A collection of 427 *Enterococcus* strains were isolated using M-*Enterococcus* agar (Becton and Dickinson, Cockeysville, USA). Species identification of the strains (mostly isolates of Laboratory of Animal Microbiology, Institute of Animal Physiology, Slovak Academy of Sciences, Košice, Slovakia) was performed by PCR method as well as by tDNA-PCR (Welsh and McClelland, 1991; Baele et al. 2001) and strains were allotted to the species *E. faecium* (368) and *E. faecalis* (59) (Lauková and Mareková, 2001; Strompfová et al., 2004; Marciňáková et al., 2005; Simonová, 2006). Strains were collected from the following animals: chicken (wall and content of crop, ileum and caecum; n=15), dog (faeces, n=10), horse (faeces, n=12), pig (rectal swabs, n=12), rabbit (faeces, n=15), rodent (intestinal content of *Clethrionomys glareolus*, *Microtus oeconomus* P., n=9, provided by Dr. I. Swiecicka, Poland), ruminants domestic (rumen content, faeces of calf n=8, lamb n=6, goat n=10) and from wild ruminants (rumen content of *Rupicapra rupicapra tatrica* n=8, *Cervus elaphus carpaticus* n=8). The strains of food origin were isolated from fermented meat products (French, Italian and Slovak traditional fermented sausages), French and Italian strains were provided by the partners of EU project QLK1-CT-2002-02240. The source of strains collected

from feed was grass silage (n=10) and commercial granulated feed for dogs (n=12).

2.2. PCR detection of enterocin structural genes

DNA was isolated by rapid alkaline lysis method as described by Baele et al. (2001). Following sequences of primers were used: 5'-GAGATTTATCTCCATAATCT-3' and 5'-GTACCACTCATAGTGGAA-3' for enterocin A (Aymerich et al., 1996), 5'-ATGAGAAAAAAATTATTTAGTTT-3' and 5'-TTAATGTCCCATACCTGCCAAACC-3' for enterocin P (Cintas et al., 1997), 5'-GAAAATGATCACAGAATGCCTA-3' and -5'-GTTGCATTTAGAGTATACATTTG-3' for enterocin B (Casaus et al., 1997), 5'-ATGGGAGCAATCGCAAAATTA-3' and 5'-TAGCCATTTTTCAATTTGATC-3' for enterocin L50B (Cintas et al., 1998). PCR was performed using Techgene KRD thermocycler (Techne, United Kingdom). All the PCR reactions were carried out in a 50 μL volume that consisted of 39.25 μL of water, 5 μL of a 10 x reaction buffer, 1.5 μL of $MgCl_2$ (50 mM), 1 μL of dNTPs (10 mM), 0.5 μL of primer (50 mM), 0.25 μL of Taq DNA polymerase (1 U), and 2 μL of template DNA. The reaction conditions, for enterocin A were 5 min. denaturation at 95 ºC, followed by 30 cycles of 30 s at 95 ºC, 30 s at 58 ºC, and 30 s at 72 ºC; this followed by 5 min. at 72 ºC and a cool down to 4 ºC. For enterocin B, P and L50B, 56 ºC instead of 58 ºC was used as annealing temperature. The PCR products (10 μL of each) were separated by electrophoresis in 2 % agarose gels (Sigma, Germany) buffered with 1xTAE (Merck, Germany) containing 1 μg/mL ethidium bromide (Sigma) and visualised by UV light. The strains *E. faecium* EK13 (CCM 7419), *E. faecium* AL41 (isolates of Dr. Lauková, IAP SAS, Košice) and *E. faecium* L50 (kindly provided by Dr. Cintas, FVUC, Madrid, Spain) were used as the positive control strains. *E. faecium* A3 (isolate from Italian fermented meat product provided by Italian partners of EU project) was used as the negative control.

2.3. Bacteriocin production assay

Cell-free neutralized supernatant (of selected gene positive strains) was used to test

bacteriocin production and was prepared by centrifuging of 18 h culture (MRS, Merck) of tested strains (10 000xg for 30 min.). Bacteriocin activity was tested by agar spot test according to De Vuyst et al. (1996) by using of BHI agar (Biomark, India) against principal indicator strains - *E. avium* EA5, *E. faecium* EK13, *L. monocytogenes* 255 (provided by IHE, Germany), *L. innocua* LMG 13568 (kindly provided by Prof. De Vuyst, University of Brussel, Belgium).

3. Results

The presence of one or more enterocin structural gene(s) was detected in 234 strains (54.8 %) from a target of 427 strains of *E. faecium* and *E. faecalis*. Among the enterocin gene positive strains (100 %; further referred to as PCR^+ strains), the structural gene of enterocin P was the most frequently occurred gene (in 170 strains, 72.6 %; Table 1). The gene of enterocin P dominated over other enterocin genes in 7 (chicken, pig, domestic ruminants, French and Slovak meat products, canine feed, silage) of 13 tested ecosystems; this gene was detected in enterococci isolated from silage, pig and domestic ruminants (cattle, goat, sheep) with the highest frequency (80.0-56.3 % of strains). Only strains isolated from rodents did not possess the gene of enterocin P. The second most frequently detected enterocin gene was the structural gene of enterocin A (in 155 strains, 66.2 % of PCR^+ strains). The enterocin A gene dominated over other genes in the groups of enterococci isolated from wild ruminants and from Italian meat products. The strains isolated from horses and wild ruminants showed the most frequent occurrence of the gene of enterocin A (88.9-70.6 % of strains). No gene of enterocin A was detected in strains isolated from rodents (similar to the case of enterocin P gene occurence). The less abundant structural genes were the genes of enterocin L50B (in 113 strains, 48.3 % of PCR^+ strains) and B (in 70 strains, 29.9 %). The gene of enterocin L50B was more frequently detected in strains from silage and pig (60.0-50.0 %). The only source of strains isolation in which the gene of enterocin L50B predominated was rabbit's and rodent's

ecosystem. The gene of enterocin B was absent in 3 of the tested ecosystems (rodents, rabbits, Italian meat products). On the other hand, the highest frequency of this gene occurrence was detected in strains isolated from horses (88.9 %).

In summary, the ecosystems with the highest frequency of the enterocin genes occurrence (containing at least one enterocin gene) were horses (100.0 % of PCR^+ strains) followed by silage (90.0 %), wild ruminants (76.5 %). On the contrary, the ecosystems of rodents (5.9 %) and rabbits (30.9 %) showed the lowest frequency of the structural enterocin genes occurrence. Finally, the general comparison of strains according 3 main ecosystems (animals, food and feed) showed that strains of feed origin possessed some of the tested structural enterocin genes with the highest frequency (70.0 % on average), followed by strains of food origin (55.8 %) and strains of animal origin (53.6 %).

Concerning the occurrence of various genes combinations, the presence of one single enterocin gene was observed in 83 strains (35.5 % of PCR^+ strains; Table 2). The combination of two different enterocin genes was observed in 62 strains (26.5 %). Three different genes were detected in 55 strains (23.5 %) and all four different genes were present in 34 strains (14.5 %). The elaboration of various combinations of structural genes showed that the combination of the genes of enterocin A, P, L50B and the combination of all tested enterocin genes (A, P, B, L50B) were the most commonly detected combinations. All possible combinations of the tested genes were occurred at least twice except the combination of the gene of enterocin B and L50B which possessed neither strain. The gene of enterocin A was exclusively detected among *E. faecium* strains, while the gene of enterocin P was detected also in *E. faecalis* species (6 strains) similar as the gene of enterocin B (2 strains) and L50B (3 strains).

Eighty-four strains with the presence of enterocin structural genes were selected for screening of bacteriocin production. Fifty-two strains (61.9 %) showed inhibitory activity

against indicator bacteria used (at least against one indicator).

4. Discussion

The interest on enterococci is raised in the last decades and most of *in vitro* studies deal with ability of enterococci to produce bacteriocins (spectrum of inhibitory activity, physicochemical properties of bacteriocin, their application in food products etc.). However, the information on distribution of genes encoding for enterocins in a target of strains from different sources is scarce. According to our results, occurrence of enterocin structural genes seems to be widespread among enterococcal isolates because in all tested ecosystems was detected at least one type of genes tested. Furthermore, the relatively high-frequency of the occurrence of enterocin structural genes (more then 50 % of tested strains contained at least one gene) was observed. Despite the lack of information, Pangallo et al. (2004) investigated a collection of 61 enterococci isolated from environmental sources (surface and waste waters, sheep manure) and similar frequency was reported (57.4 % of enterocin structural gene positive strains). The wide enterocin genes distribution may be due to remarkable ability of enterococci to disseminate and receive genetic material between strains but also between genera (e.g. between staphylococci and enterococci; Murray et al., 1986). Possibility of this ability supports also similar sequence of changed material (e.g. enterolysin A and enterocin L50 have sequence similarity to staphylococcal lysostaphin and staphylococcal peptide toxins, respectively; Cintas et al., 1998; Nilsen et al., 2003). Well-described gene transfer mechanisms in enterococci include conjugative (pheromone responsive with high-frequency of transfer) and non-conjugative plasmids, as well as conjugative transposons (Clewell, 1990).

Analysis of the gene frequency in enterococci according to their origin showed different results (ranging from 5.9 to 100 %). Whether it is really origin depending property, it is needing to screen a bigger collection of strains (also from geographically different locations) and to compare independent studies. Although the detection of enterocin structural genes

must not necessarily correspond with the production of the bacteriocin, it can be speculated that various environments (ecosystems) require various level of bacterial defense mechanism (thus also the presence of bacteriocin genes as the basis for bacteriocin production). This hypothesis is supported by the findings that a bacterial cell will produce a lower amount of antimicrobial compounds when grown in a rich environment or without a competing microbiota (Kim et al., 1997). On the other hand, environmental conditions are frequently changed also in the frame of ecosystem and a widespread prevalence of enterococci making it difficult to trace back the origin of a strain. The structural genes of enterocin P and enterocin A were shown to be the most abundant genes in this study. Enterocins A and P are grouped in the class II. 1 bacteriocins of the pediocin family, which are apparently very effective in preventing the growth of listeriae (Eijsink et al., 1998). On the contrary, the structural gene of enterocin B classified in the subgroup II. 3 (other linear, non-pediocin type enterocins) was shown to be the least frequent enterocin gene. Among enterocin gene positive strains, the screening indicated that the presence of one single enterocin gene was the most frequently detected case compared to the combinations of more enterocin genes in one strain. The same result observed De Vuyst et al. (2003), including descending tendency of occurrence frequency of two, three or four different enterocin genes. However, it was shown that not all enterocin genes must be expressed at the same time, multiple bacteriocin production in single strains has been demonstrated (Casaus et al., 1997; Cintas et al., 1998). Among the possible combinations of two enterocin genes, the combination of enterocin A and P was the most frequently found what agree with result of another screening study (De Vuyst et al., 2003). The presence of all four enterocin genes in 34 strains in our study indicates the high genetic potential of some strains to produce various bacteriocins.

Concerning species differences in distribution of enterocin structural genes, only 13.8 % of *E. faecalis* strains compared to 86.2 % *E. faecium* strains were enterocin A, P, B or L50B

gene positive. The result was expected because the tested genes occurred predominantly in *E. faecium* strains (Franz et al., 2007). However, the genes of enterocin P, B, L50A/B and A were detected in *E. faecalis* strains also by Pangallo et al. (2004).

A prescreening for bacteriocin production in enterocin structural gene positive strains showed that almost 70 % of strains were inhibitory active. Of course, the detection of enterocin structural genes does not necessarily means the production of the corresponding enterocin and lack of detectable antimicrobial activity does not necessarily means the genes involved in bacteriocin production are defective. In most cases, bacteriocin production appears regulated and is consequently produced only under suitable growth conditions, which are not clear up to now. Some bacteriocins are produced on solid growth media but not in liquid cultures (Qi et al., 2001). Growth temperatures have also been shown to influence bacteriocin production in *E. faecium*, e.g. strain L50 produces various bacteriocins at different temperatures (Cintas et al., 2000). Therefore, the frequency of the occurrence of silent enterocin structural genes can be concluded finally after involving of an ideal method for bacteriocin production, an optimal production conditions, and a large collection of susceptible indicators.

In conclusion, the PCR screening of enterocin structural genes showed the high-frequency and variability of the enterocin determinants among enterococci from different sources. In overall, more than half of tested strains possessed at least one enterocin gene. The wide distribution of enterocin structural genes together with almost all possible combinations of their occurrence offers great possibilities for isolation of effective bacteriocin-producing strains suitable for their use in the food industry or as probiotic cultures in human and animal health promotion.

Acknowledgements

Part of results concerning the occurrence of enterocin genes in enterococci isolated from silage and rabbits are included in previous publications: Marciňáková et al., 2005. Bull. Vet. Inst. Pulawy, 49, 387-391; Simonová et al., 2007. Vet. Res. Commun., 31, 143-152. The enterococci isolated from French and Italian fermented meat products provided partners of the project EU QLK1-CT-2002-02240. This work was supported by the project No. 2/0008/08 of the Slovak Scientific Agency VEGA and partially by the EU project former mentioned.

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Table 1
Occurrence of enterocin structural genes among enterococci according their origin

	Strain		Structural genes for production of enterocin:			
	Source/origin	Number	ent A	ent P	ent B	L50B
Animal	Chicken	27[a]/14[b]	13[c] (48.1)[d]	14 (51.9)	7 (25.9)	8 (29.6)
		22[e]/5[f]	13/0	14/0	7/0	8/0
	Dog	25/11	9 (36.0)	10 (40.0)	2 (8.0)	10 (40.0)
		21/4	9/0	10/0	2/0	10/0
	Horse	18/18	16 (88.9)	9 (50.0)	16 (88.9)	8 (44.4)
		17/1	16/0	8/1	15/1	8/0
	Pig	28/16	10 (35.7)	16 (57.1)	2 (7.1)	14 (50.0)
		26/2	10/0	16/0	2/0	15/0
	Rabbit	42/13	9 (21.4)	6 (14.3)	0 (0.0)	10 (23.8)
		31/11	9/0	6/0	0/0	9/1
	Rodent	17/1	0 (0.0)	0 (0.0)	0 (0.0)	1 (5.9)
		0/17	0/0	0/0	0/0	0/1
	Ruminats domestic	32/20	13 (40.6)	18 (56.3)	15 (46.9)	5 (15.6)
		31/1	13/0	18/0	15/0	5/0
	Ruminants wild	17/13	12(70.6)	7 (41.2)	6 (35.3)	8 (47.1)
		16/1	12/0	7/0	6/0	8/0
Food	Meat products:					
	French	44/27	17 (38.6)	24 (54.5)	9 (20.5)	12 (27.3)
		41/3	17/0	24/0	9/0	12/0
	Italian	63/31	26 (41.3)	7 (11.1)	0 (0.0)	4 (6.3)
		63/0	26/0	7/0	0/0	4/0
	Slovak	74/42	14 (18.9)	33 (44.6)	10 (13.5)	16 (21.6)
		69/5	14/0	33/0	9/1	16/0
Feed	Canine feed	20/10	8 (38.1)	10 (47.6)	2 (9.5)	5 (23.8)
		18/2	8/0	10/0	2/0	5/0
	Silage	20/18	8 (40.0)	16 (80.0)	1 (5.0)	12 (60.0)
		13/7	8/0	11/5	1/0	11/1
Summary		427/234	155 (36.3)	170 (39.8)	70 (16.4)	113 (26.5)
		368/59	155/0	164/6	68/2	111/3

[a] Number of tested strains

[b] Number of strains possessing at least one gene

[c] Number of strains possessing the respective genes

[d] Percentage of strains possessing the respective genes

[e] Number of tested *E. faecium* strains

[f] Number of tested *E. faecalis* strains

Table 2
Occurrence of various combinations of enterocin structural genes among enterococci according their origin

		Number of strains with various combination of structural genes:															
	Source/origin	0[a]	A	P	B	L50B	A,P	A,B	A,L50B	P,B	P,L50B	B,L50B	A,P,B	A,P,L50B	A,B,L50B	P,B,L50B	A,P,B,L50B
Animal	Chicken	13	0	1	0	0	5	0	0	0	0	0	0	1	0	0	7
	Dog	14	1	0	0	0	0	0	0	0	2	0	0	6	0	0	2
	Horse	0	1	0	1	0	1	6	0	1	0	0	0	0	1	0	7
	Pig	12	0	0	0	0	0	0	0	1	5	0	1	9	0	0	0
	Rabbit	29	3	0	0	4	0	0	0	0	0	0	0	6	0	0	0
	Rodent	16	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
	Ruminants:																
	wild	4	5	0	0	1	0	0	0	0	0	0	0	1	0	0	6
	domestic	12	2	0	0	0	1	0	0	7	0	0	5	2	0	0	3
Food	Meat products:																
	French	17	2	7	1	0	3	0	0	0	2	0	2	4	0	0	6
	Italian	32	21	4	0	1	2	0	2	0	0	0	0	1	0	0	0
	Slovak	32	0	12	3	4	7	1	0	2	6	0	1	3	1	1	1
Feed	Canine feed	10	0	1	0	0	4	0	0	0	0	0	0	3	0	1	1
	Silage	2	0	6	0	1	0	0	1	0	3	0	0	6	0	0	1
Summary		193	35	31	5	12	23	7	3	11	18	0	9	42	2	2	34

[a] Number of negative strains (without tested genes)