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DIFFERENCES OF ANTIBACTERIAL ACTIVITY SPECTRA AND PROPERTIES OF BACTERIOCINS, PRODUCED BY *GEOBACILLUS* SP. BACTERIA ISOLATED FROM DIFFERENT ENVIRONMENTS

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ABSTRACT

Ability of bacteriocins to inhibit pathogenic bacteria may depend on the environment inhabited by the producing strain. However, there are just few reports on bacteriocin-producing thermophilic bacteria from unconventional environments. Therefore, in this work we investigated differences in antibacterial activity spectra of microorganisms isolated from two different environments: the oil wells (first collection) and surface soil above the oil pools (second collection).
All strains were Gram-positive, rod-shaped, thermophilic and endospore forming bacteria. Using spot-on-lawn method we have demonstrated that strains from the second collection possess higher and broader antibacterial activity spectra than first collection strains. Furthermore, all strains were active against at least 1 of 19 tested pathogenic bacteria. Analysis of antibacterial compounds has shown that all of them were stable in broad temperature and pH ranges, sensitive to proteolytic enzymes, proving their proteonaceus nature. They were all secreted during the exponential growth phase and active against closely related thermophilic bacteria, which suggest that these substances are most likely bacteriocins. Most of the bacteriocins had molecular masses under 20 kDa. Plasmid isolation and elimination experiments suggest that bacteriocins are most likely chromosome-encoded.
Our results confirm the differences between antibacterial spectra of bacteriocins produced by bacterial strains isolated from different environmental niches.

Keywords: Antibacterial activity spectrum, bacteriocin, endospore, growth analysis, thermophile

INTRODUCTION

Bacteriocins are naturally ribosomally synthesized antimicrobial peptides or proteins, usually possessing the narrow antibacterial activity spectrum against bacterial strains closely related to the producer (**Abriouel *et al.*, 2011**, **Gálvez *et al.*, 2008**). These proteonaceous molecules are produced by various Gram-positive and Gram-negative bacterial strains, often during the regulation of bacterial population size by the mechanism of quorum sensing or during the stressful conditions (**Fontaine and Hols, 2008**). The activity of this group of molecules against foodborne and pathogenic bacteria opens wide opportunities for bacteriocin application (**Anisimov and Amoako, 2006; Desriac *et al.*, 2010; Lohans and Vederas, 2012; Montesinos, 2007; Piper *et al.*, 2009; Settanni and Corsetti, 2008**).
Bacteriocins produced by Gram-positive bacteria are mainly classified by their size, structure and modifications, like the presence of disulphide bonds, carbohydrate or lipid moieties and modified or unusual amino acids (**Lohans and Vederas, 2012; Heng *et al.*, 2007; Nissen-Meyer *et al.*, 2010; Maqueda *et al.*, 2007; Lee and Kim, 2011**). Most of described bacteriocins are secreted by the lactic acid bacteria (LAB) (**Gálvez *et al.*, 2008**, **Khan *et al.*, 2008**). Little is known about bacteriocins, produced by Gram-positive thermophilic bacteria, although the interest in these microorganisms is increasing due to their resistance to high temperatures which may be relevant to the protection of heat-treated food products. The application of bacteriocins from thermostable microorganisms may help to reduce the usage of chemical substances and/or heat (**Sit and Vederas, 2008**). Although there are few reports about cytotoxicity of bacteriocins, produced by some streptococci, enterococci or *Escherichia coli* strains to mammalian cell lines, but usually only in higher concentrations than required for their biological activity against bacterial cells **(Paiva *et al.*, 2012; Lagos, 2007)**, most bacteriocins are not toxic for eukaryotic cells (**Gálvez *et al.*, 2008**), Therefore, bacteriocins of thermophilic bacteria can also be applied in medicine, agriculture and veterinary **(Abriouel *et al.*, 2011**; **Anisimov and Amoako, 2006; Desriac *et al.*, 2010; Lohans and Vederas, 2012; Mantovani *et.al.*, 2011; Montesinos, 2007)**.
Up to now bacteriocins from *Streptoccoccus thermophilus* strains constitute the majority of investigated thermophilic bacteria bacteriocins (**Fontaine and Hols, 2008; Aktypis *et al.*, 1998; Aktypis and Kalantzopoulos, 2003; Aktypis *et al.*, 2007; Ivanova *et al.*, 1998; Gilbreth and Somkuti, 2005; Kabuki *et al.*, 2007; Marciset *et al.*, 1997; Mathot *et al.*, 2003; Villani *et al.*, 1995**). Meanwhile there are only few described bacteriocins, produced by *Geobacillus* sp. bacteria: six bacteriocins of low molecular mass, secreted by *G. stearothermophilus* bacteria **(Yule and Barridge, 1976; Sharp *et al.*, 1979; Pokusaeva *et al.*, 2009)**; two bacteriocins of *G. thermoleovorans* strains **(Novotny and Perry, 1992)**; Toebicin, secreted by *G. toebii* HBB-218 strain **(Özdemir and Biyik, 2011)** and two lantibiotics, produced by *G. thermodenitrificans* NG80-2 strain **(Garg *et al.*, 2012)**. All these bacteriocins are characterized by the thermostability and resistance to pH changes.
Bacteriocins of thermophilic bacteria have many perspective qualities, as described above. Specific antibacterial activity spectrum is also a very important feature and may depend on the species habitat. Accordingly, in this work we investigated two collections of thermophilic bacteria, isolated from Lithuanian oil wells and the surface soil above the oil pools. The goal of this study was to evaluate the influence of antibacterial compounds secreted by thermophilic bacteria from two different environments on the antibacterial activity spectra. Antibacterial activity spectra and physicochemical properties of antibacterial substances produced by some of the isolates were determined in this work, as these criteria are the most important aiming to evaluate the possibilities of the bacteriocins' application in the food and other industries.

MATERIAL AND METHODS

Bacterial strains and culture conditions

In this work we used *Geobacillus* sp. bacterial strains from the culture collection of the Department of Microbiology and Biotechnology of Vilnius University (Vilnius, Lithuania). The strains of the first collection were isolated from Lithuanian oil wells, and strains of the second collection – from the surface soil above the oil pools. All isolated strains are listed in Table 1.

Mainly two media were used for cultivation of the bacterial strains: enriched Nutrient Broth (NB) and mineral modified M9 medium (mM9) (growth media were prepared as described at "Fermentas molecular biology catalog & product application guide", 2002-2003). Cultures were grown aerobically on agar media at 60°C, or in liquid media at 55°C, with 180 rpm agitation.

Table 1 Isolated strains from the first and second collections

Collection (number of strains)	Source	Strains
First collection (68)	Lithuanian oil wells	1K; 2; 3; 3K; 3N; 4; 4K; 5; 6; 7; 8; 9; 10; 11; 12; 13; 15; 16; 17; 18; 19; 21; 22; 22N; 23; 24; 25; 26; 27; 28; 29; 29A; 30; 31; 32A; 32B; 32C; 32D; 33; 34; 35A; 35B; 35C; 36A; 36B; 51; 62; 64; 65; 67; 69; 70; 71; 72; 73; 74; 75; 77; 78; 79; 80; 82; 84; 85; 86; 87; 88; 89
Second collection (68)	Surface soil above the oil pools	1.1; 1.2; 1.3; 1.4; 1.5; 1.7; 1.81; 1.82; 1.9; 1.10; 1.11; 1.12; 1.14; 1.16; 1.17; 2.3; 2.4; 2.7; 3.2; 4.1; 4.2; 4.3; 4.5; 4.7; 4.8; 4.81; 4.9; 4.10; 4.11; 4.12; 4.21; 4.22; 5.1; 5.2; 5.3; 5.6; 5.7; 5.8; 5.9; 5.10; 6.1; 6.2; 6.3; 6.4; 6.5; 6.10; 6.11; 6.12; 6.13; 6.14; 7.1; 7.2; 7.3; 7.41; 7.42; 7.6; 7.7; 7.13; 8.1; 8.2; 8.4; 9.1; 9.2; 9.3; 9.7; 9.8; 9.9; 9.10

Phenotypic analysis of isolated strains

Gram-Staining and KOH test were performed as described by Chandra and Mani **(Chandra *et al.*, 2011)**, except Safranin (Merck Millipore, Darmstand, Germany) was used instead of Carbol Fuchsin in Gram Staining. Formation of endospores of isolated strains was determined by Schaeffer-Fulton Stain **(Gerhardt *et al.*, 1981)**. Growth temperature range of the strains was determined by growing strains in liquid NB medium with agitation, at temperatures differing by 5°C. Optical Density (wavelength of 590 nm) of cultures was measured during the growth.

16 S rDNA sequence analysis

Genomic DNA of bacterial strains was extracted as described by **Sambrook and Russel (2001)**. PCR amplification of 16S rDNR genes was done using universal primers 27F (5'-AGATTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTCGACTT-3'). PCR products were purified from 1% agarose gel and sequenced at the Sequencing Center of the Institute of Biotechnology (Vilnius, Lithuania). Obtained sequences were aligned using Lasergene 6 Program package (DNAStar, USA). *in silico* data analysis was performed using the NCBI Blast program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Determination of antibacterial activity spectrum

Strains isolated in this work were tested against each other in order to determine their capability to inhibit the growth of closely related thermophilic bacteria. The antagonistic interactions of isolated strains were determined using the spot-on-lawn method **(Çadirci and Çitak, 2005; Kormin *et al.*, 2001; Moraes et.al., 2010)**. The tested strains were grown overnight at 60°C in the middle of Petri dish with an NB agar medium. Then the layer of NB agar media with inoculated other presumed sensitive strain was poured on the top of the grown culture. The agar media with inoculated strain was prepared by adding 20% (v/v) of culture inoculum ($OD_{590\ nm}$=1.8) into an appropriate agar medium. Plates were incubated at 60°C for 6-12 h.

Evaluation of antibacterial activity against closely related species was also determined by spot-on-lawn method, as described above. Determination of antibacterial activity against pathogenic bacteria was carried out by the same method at the National Public Health Surveillance Laboratory (NPHSL) in Lithuania. Closely related thermophilic bacteria species and pathogenic bacteria used for the evaluation of antibacterial activity spectrum are listed in Table 2.

Table 2 Thermophilic and pathogenic bacteria strains used for the determination of antibacterial activity spectrum of isolated strains

Thermophilic bacteria	Pathogenic bacteria
Geobacillus stearothermophilus NUB36187 (9A11) (BGSC)	*Streptococcus pyogenes* ATCC 19615
Geobacillus uzenensis VKM B-2229^T	*Steptococcus pneumoniae* ATCC 6305
Geobacillus subterraneus VKM B-2226^T	*Enterococcus faecalis* ATCC 2912
Geobacillus thermocatenulatus VKM B-1259^T	*Enterococcus faecium* 402-3/03
Geobacillus thermoleovorans DSM 5366^T	*Haemophilus influenzae* ATCC 10211
Geobacillus thermodenitrificans DSM 466^T	*Staphylococcus aureus* ATCC 25923
Geobacillus lituanicus N-3	*Staphylococcus haemolyticus* P903
	Staphylococcus epidermidis ATCC 12228
	Pseudomonas aeruginosa ATCC 27853
	Escherichia coli ATCC 25922
	Klebsiella pneumoniae DSM 30104
	Yersinia enterocolitica ATCC 9610
	Salmonella typhimurium ATCC 14028
	Salmonella enteritidis ATCC 13076
	Listeria monocytogenes ATCC 19117
	Listeria innocua ATCC 33090
	Bacillus cereus DSM 12001
	Bacillus subtilis ATCC 6633
	Clostridium perfringens ATCC 13124

Legend: ^{T} - indicates the type strain. Culture collections: ATCC – American Type Culture Collection, BGSC – *Bacillus* Genetic Stock Center (Ohio, USA); DSMZ - German Collection of Microorganisms and Cell Cultures (Braunschweig, *Germany)*; VKM – All-Russian Collections of Microorganisms (Moscow, Russia)

Evaluation of antibacterial activity

Antimicrobial activity of supernatants of tested cultures was determined by agar well diffusion assay. Agar medium with indicator strain was prepared adding 20% of indicator strain inoculums ($OD_{590\ nm}$=1.8) into the agar NB media. *Geobacillus stearothermophilus* NUB36187 (9A11) was used as indicator strain. 0.5 cm diameter wells were made using sterile plastic pipette tips in the agar media with inoculated sensitive strain. Culture samples were centrifuged and the serial two-fold dilutions of cell free supernatants were made, using 50 mM Tris-HCl (Sigma-Aldrich, Taufkirchen, Germany) (pH 7.5) buffer. The volume of 100 µl of every dilution was poured into the prepared wells and plates were incubated at 60°C temperature for 6-8 hours. The antibacterial activity was expressed in terms of arbitrary units (AU), as a maximum dilution which produced a clearly visible inhibition zone. The unit of antimicrobial activity (AU) was defined as the reciprocal of the highest level of dilution resulting in a clear zone of growth inhibition **(Hyronimus *et.al.*, 1998; Vera Pingitore *et al.*, 2007)**.

Sodium dodecyl sulfate polyakrylamide gel electrophoresis (SDS-PAGE) and zymogram preparation

Crude protein extracts of cultures with antimicrobial activity were gained after precipitation with ammonium sulfate (Roth, Karlsruhe, Germany) to 70 % saturation **(Rosenberg, 2005)**. These extracts were examined using SDS-PAGE method **(Vera Pingitore *et al.*, 2007)**. In order to determine the molecular mass

of antimicrobial compounds we prepared the zymogram: part of PAGE gel was fixed in fixing solution (20% isopropanol (Roth, Karlsruhe, Germany), 10% acetic acid (Merck Millipore, Darmstand, Germany), 70% water) for two hours, after that the gel was washed with distilled water for at least two hours on a shaker, changing water every 15-20 minutes. Then the gel was placed in a Petri dish and overlaid with agar NB media with indicator strain (media preparation is described above). Prepared zymogram was incubated at 60°C temperature overnight. Clear zone in the zymogram indicates the position of antibacterially active protein in the gel.

Physicochemical analyses of crude protein extracts

Enzymatic treatment

Crude protein extracts were treated with the following enzymes at final concentration of 1 mg ml^{-1}: proteinase K, trypsin and α-chymotrypsin (Merck Millipore, Darmstand, Germany); pronase E, pepsin, α-amylase and lipase (Sigma-Aldrich, Taufkirchen, Germany). All enzymes were dissolved in buffers as recommended by the suppliers. Sample and enzyme mixtures were incubated at 37°C for 2 hours. Remaining antibacterial activity was determined in terms of AU ml^{-1}. Untreated crude protein extracts and enzyme solutions were taken as respective controls **(Tiwari and Srivastava, 2008)**.

pH treatment

Aiming to determine bacteriocin stability within various pH range, crude protein extracts were mixed with series of 100 mM buffers (Sigma-Aldrich, Taufkirchen, Germany) with pH from 2.0 to 10.0 (ratio 1:1) and incubated at 37°C for two hours. Buffers were chosen according to pH value of the samples. pH was restored with 0.5 M Tris-HCl buffer, pH 7.5. The remaining activity in pH treated samples was determined by agar well diffusion assay **(Tiwari and Srivastava, 2008)**. Samples of crude protein extracts in Tris-HCl buffer (50 mM, pH 7.5) were taken as respective controls (K).

Sensitivity to various temperatures

In order to determine the impact of temperature changes on antibacterial activity of bacteriocins, crude protein extracts were incubated at various temperatures: 60, 70, 80, 90, 100 and 121°C for 30 minutes. The remaining antibacterial activity was evaluated by agar well diffusion assay and expressed in arbitrary units (AU). Samples were also kept at +4°C temperature for 2 weeks and 1 week at room temperature (20°C) to evaluate the stability of bacteriocin activity. The untreated samples were taken as respective controls (K) **(Lee *et al.*, 2001)**.

Ion exchange chromatography

Ion exchange chromatography was performed at the Institute of Biochemistry (Vilnius, Lithuania), using Resource Q ion exchange column and liquid chromatography system AKTA Purifier (GE Healthcare, Helsinki, Finland). Gradient was created using Tris-HCl (pH 7.5, 50 nM) buffer and the same buffer with 1 M NaCl.

Screening for presence of plasmids and plasmid elimination

Plasmid DNA extraction was performed by alkaline extraction method as described by **Sambrook and Russel (2001)**. The presence of plasmids and size of superspiralized small plasmids was determined by agarose gel electrophoresis **(Sambrook and Russel, 2001)**. For linearization of the detected plasmids we used 21 restriction endonucleases (RE): *Aat*II, *Acc*65I, *Bam*HI, *Bsp*143I, *Eco*88I, *Eco*RI, *Eco*33I, *Hinc*II, *Hin*dIII, *Nco*I, *Nde*I, *Nhe*I, *Not*I, *Pae*I, *Pst*I, *Pvu*I, *Sac*I, *Sal*I, *Sma*I, *Ssp*I, *Xba*I (Thermo Fisher Scientific, Vilnius, Lithuania). Size of linearized plasmids was determined by comparission of agarose gel electrophoresis **(Sambrook and Russel, 2001)** with DNA fragments standard GeneRuler™ DNA Ladder Mix (Thermo Fisher Scientific, Vilnius, Lithuania).
Elimination of plasmids was performed using 1 μg ml^{-1} concentration of Novobiocin (INC Biochemicals, California, USA) in the culture medium (NB) **(Hyronimus *et.al.*, 1998)**.

RESULTS

Phenotypic analysis of isolated strains

Results of Gram staining and KOH test have shown that all isolated strains from both collections are Gram-positive. Use of Schaeffer-Fulton stain has confirmed that all isolated strains also are endospore forming, rod-shaped bacteria. Analysis of the growth temperature range has shown that all strains from the first collection are obligate thermophiles with the growth range from 50°C to 80°C. Analysis of the growth of the second collection strains has revealed that from 68 strains 49 were obligate thermophiles (growth range from 50°C to 80°C) and 19 strains were facultative thermophiles (growth range from 30°C to 70°C).

16S rDNA sequence analysis

Strains 3, 11, 18, 32A and 36 A (**Pokusaeva *et al.*, 2009)** and strains 9, 17, 19, 28, 30 and 31 are identified by 16 rDNA analysis and DNA hybridization as *Geobacillus stearothermophilus*, also strain 22 identified as *G. vulcani* in previous studies (unpublished results). Results of 16S rDNA analysis of other isolated strains were inconclusive and require deeper investigation, such as DNA-DNA hybridization. Strain N-3 is a type strain of *Geobacillus lituanicus* species, described in our laboratory **(Kuisiene *et al.*, 2004)**. According to the results of 16 rDNA analysis, 12.1 strain belongs to *Geobacillus thermoglucosidasius* and 6.10 strain to *Geobacillus thermodenitrificans* species (unpublished results).

Antibacterial interactions between the isolated strains

For the analysis of antibacterial interactions between isolated strains we used spot-on-lawn method. Due to the enormous amounts of isolated strains and experiments required to analyze their antagonistic interactions, we were not able to test all of the isolated strains. From the first collection 43 strains were analyzed. Results of the analysis are presented in Figure 1.

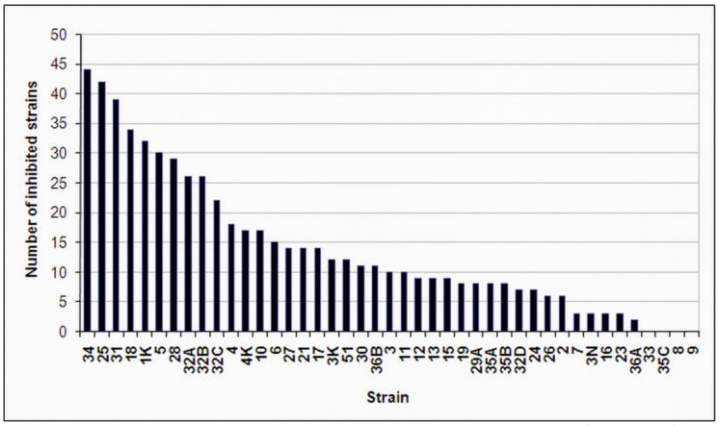

Figure 1 Antibacterial interactions between 43 isolated strains from the first collection. Symbols: - number of inhibited strains from the 43 strains of the first collection

We have determined that all tested strains were sensitive to at least one antibacterial compound, produced by the tested strains. 40 strains were secreting antibacterial substances. 10 strains, which constitute about 23% of the investigated strains of the first collection, were active against more than 50% of tested strains. However, 4 strains did not inhibit any of the tested strains (Fig 1).
Antibacterial interactions between 21 strains from the second collection were analyzed. Results are presented in Figure2.

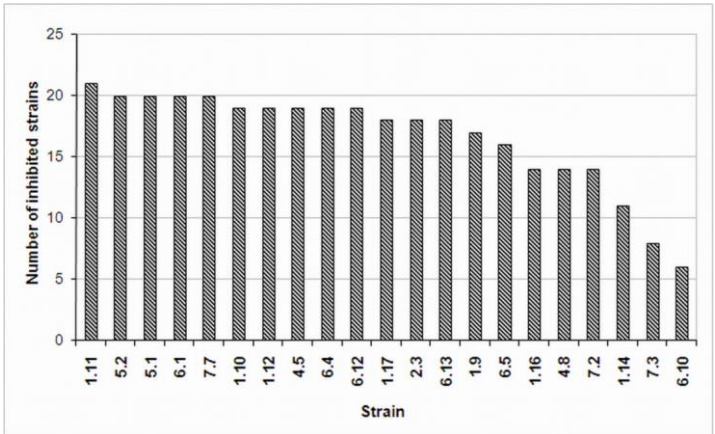

Figure 2 Antibacterial interactions between 21 strains from the second collection: - number of inhibited strains from the 21 strains of the second collection

It was shown that all tested strains were able to inhibit at least 6 other tested strains. 19 strains, which constitute about 90% of the investigated strains from the second collection, inhibited the growth of more than 50% of tested strains. It should be noted that three strains (1.11, 6.12 and 7.7) inhibited their own growth.

Determination of antibacterial activity spectrum

Antibacterial activity of isolated strains against closely related thermophilic bacteria species was also evaluated. It was shown that 52 of 68 strains (76.5%) from the second collection were active against *Geobacillus stearothermophilus*

NUB36187 (9A11) strain. From 43 tested strains of the first collection, 33 (76.7%) were also active against 9A11 strain. Two strains from the first collection (32A and 32B) were also tested against other *Geobacillus* sp. bacteria (Tab 2). 32A strain was active against *G. uzenensis* VKM B-2229^T, *G. subterraneus* VKM B-2226^T, *G. thermocatenulatus* VKM B-1259^T and *G. thermoleovorans* DSM 5366^T strains. *G. uzenensis* was the most sensitive to antibacterial compound, secreted by 32A strain, while *G. thermodenitrificans* DSM 466^T was resistant to it. 32B strain possessed the antibacterial activity against *G. lituanicus* N-3 strain.

101 isolated strains (56 strains from the first and 45 strains from the second collection) were also tested against 19 pathogenic bacteria strains (Tab 2). It was shown that all 101 tested strains were able to inhibit the growth of at least 1 pathogenic bacteria strain (Fig 3).

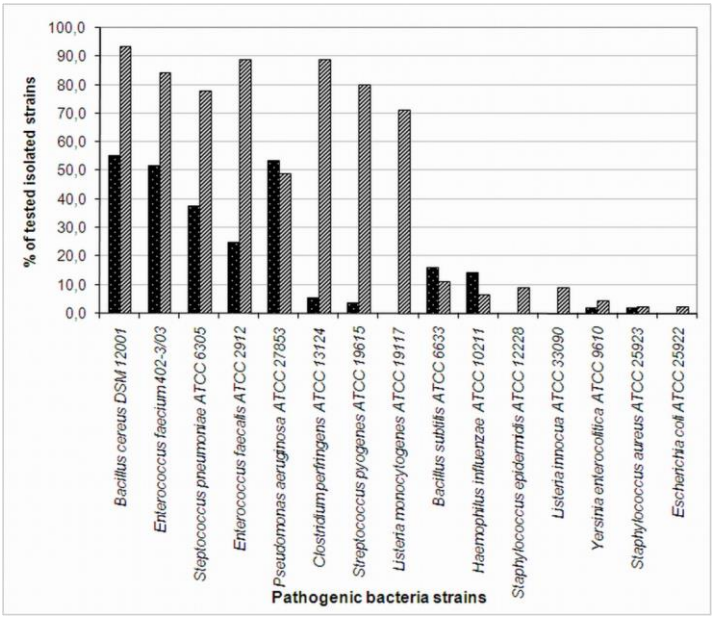

Figure 3 Antibacterial activity of 101 isolated strains against pathogenic bacteria: ■ - number of strains from the first collection, active against pathogenic bacteria; ▪ - number of strains from the second collection, active against pathogenic bacteria

More than 50% of the investigated strains were active against *B. cereus* DSM 12001 (72.3%), *E. faecium* 402-3/03 (66.3%), *S. pneumoniae* ATCC 6305 (55.4%), *E. faecalis* ATCC 2912 (53.5%) and *P. aeruginosa* ATCC 27853 (51.5%). 42.6, 37.6 and 31.7% of strains were active against *C. perfringens* ATCC 13124, *S. pyogenes* ATCC 19615 and *L. monocytogenes* ATCC 19117 respectively. None of the tested strains were able to inhibit 4 pathogenic bacteria strains (*S. haemolyticus* P903, *K. pneumoniae* DSM 30104, *S. typhimurium* ATCC 14028 and *S. enteritidis* ATCC 13076).

Strains from the second collection inhibited the growth of 15 pathogens, while strains of the first collection were active only against 11 pathogenic bacteria strains. Data presented in Figure 3. show that considerably more strains from the second collection were active against *B. cereus* DSM 12001 (55.6% of tested strains from the first collection and 93.3% of strains from the second collection) , *E. faecium* 402-3/03 (51.8% and 84.4%), *S. pneumoniae* ATCC 6305 (37.5% and 77.8 %), *E. faecalis* ATCC 2912 (25.0% and 88.9%), *C. perfringens* ATCC 13124 (5.4% and 88.9%) and *S. pyogenes* ATCC 19615 (3.6% and 80.0%). Moreover, only the strains from the second collection possessed the antibacterial activity against *L. monocytogenes* ATCC 19117, *S. epidermidis* ATCC 12228, *L. innocua* ATCC 3309 and *E. coli* ATCC 25922 (Fig 3).

Physicochemical analyses of secreted antibacterial compounds

According to the results of the antibacterial activity spectrum against other collection strains (Fig 1 and Fig 2) and pathogenic bacteria (Fig 3), 11 strains (17, 18, 22, 25, 30, 31, 32A, 32B, 1K, 4.7 and 9.1) were chosen for the more detailed investigation of their secreted antibacterial compounds. Crude protein extracts of investigated strains were used for the phenotypic analysis.

Crude protein extracts of 9 strains (17, 18, 22, 25, 30, 31, 32A, 32B and 1K) were treated with different pH values. Results, presented in Table 3, show that all examined antibacterially active substances are stable in the pH range from 3 to 10.

Table 3 Remaining antibacterial activity of the crude protein extracts after treatment with different pH values. Remaining antibacterial activity was determined by agar well diffusion assay

pH	Remaining antibacterial activity (%) of the crude protein extracts from isolated strains								
	17	**18**	**22**	**25**	**30**	**31**	**32A**	**32B**	**1K**
3	50	100	30	100	100	100	100	100	100
4	50	100	30	100	100	100	100	100	100
5	50	100	30	100	60	100	100	100	100
6	50	100	30	100	60	100	100	100	100
7	50	100	60	100	60	100	100	100	100
8	70	100	100	100	100	100	100	100	100
9	80	100	60	100	100	100	100	100	100
10	100	100	30	100	100	100	100	100	100
K	100	100	100	100	100	100	100	100	100

Legend: K – control

The influence of different temperatures on the antibacterial activity was investigated using crude protein extracts from 11 strains: 17, 18, 22, 25, 30, 31, 32A, 32B, 1K, 4.7 and 9.1. Results presented in Table 4 suggest that most of 11 antibacterial compounds are relatively thermostable and retain antibacterial activity in broad temperature range up to 100°C. These substances also remain completely stable after prolonged storage at +4 and 20°C. Crude protein extracts from 9 strains (17, 18, 22, 25, 30, 31, 32A, 32B and 1K) were treated with different proteolytic enzymes and α-amylase (Tab 5).

Table 4 Remaining antibacterial activity of the crude protein extracts after treatment with different temperatures. Remaining antibacterial activity was determined by agar well diffusion assay

Temperature (°C)	Remaining antibacterial activity (%) of the crude protein extracts from isolated strains										
	17	**18**	**22**	**25**	**30**	**31**	**32A**	**32B**	**1K**	**4.7**	**9.1**
4	100	100	100	100	100	100	100	100	100	100	100
20	100	100	100	100	100	100	100	100	100	100	100
60	100	100	100	100	100	100	100	100	100	100	100
70	25	100	100	100	100	80	75	50	100	100	100
80	25	75	100	100	90	80	60	25	50	100	100
90	25	50	100	0	90	70	60	13	50	100	100
100	25	25	100	0	60	40	50	0	0	0	0
121	0	0	0	0	0	0	0	0	0	0	0
K	100	100	100	100	100	100	100	100	100	100	100

Legend: K – control

Table 5 Remaining antibacterial activity of the crude protein extracts after treatment with different enzymes. Remaining antibacterial activity was determined by agar well diffusion assay

Enzyme	Remaining antibacterial activity (%) of the crude protein extracts from isolated strains								
	17	**18**	**22**	**25**	**30**	**31**	**32A**	**32B**	**1K**
Proteinase K	0	50	0	100	100	0	0	0	13
α-chymotrypsin	0	50	20	100	50	33	30	0	6
β-chymotrypsin	100	75	20	100	100	50	50	66	3
Papain	0	50	20	100	0	0	33	50	3
Tripsin	0	100	40	80	100	40	100	13	3
Ficin	0	100	66	100	100	100	50	100	6
α-amylase	0	75	66	100	100	100	100	13	13
K	100	100	100	100	100	100	100	100	100

Legend: K – control

All antibacterial compounds, except the ones secreted by strains 30 and 25, were sensitive to the treatment with proteinase K. Antibacterial substance of strain 30 was sensitive to papain and α-chymotrypsin, while the substance of the strain 25 was only slightly sensitive to trypsin. Three antibacterial substances, secreted by strains 17, 32B and 1K, were almost completely inactivated by the treatment with α-amylase. These results indicate that bacteriocins secreted by these three strains may contain carbohydrate moieties in their structure. Also, antibacterial compounds of 5 strains (17, 22, 30, 31, 32A) had lost their activity after treatment with DTT (Dithiothreitol) (data not shown), which means that disulphide bonds are necessary for the activity of the five investigated bacteriocins.

Determination of molecular masses of antibacterial compounds

Using SDS-PAGE and zymography methods we determined that most of the investigated bacteriocins had molecular masses between 5 and 20 kDa: strain 17 – 6.8 kDa, strain 22 – 6.2 kDa, strain 30 – 5.6 kDa; strain 31 – 7.1 kDa; strain 32A – 7.2 kDa, strain 32B – ~15 kDa; strain 35C – 12 and <10 kDa; strain 4.7 - ~7 kDa, strain 9.1 – 7 and 20 kDa. Two strains (9.1and 1K) secreted 2 bacteriocins each. Strain 18 secreted 3 antibacterial substances with molecular masses of 2, 12 and ~100 kDa. Only strains 18, 25 and 1K secreted antibacterial compounds or aggregates of high molecular mass of ~100, ~60 and ~200 kDa respectively. It should be noted that after ion-exchange chromatography of the strain 1K crude protein extract, two antibacterially active peaks were obtained, indicating that this strain produces two bacteriocins or a two-peptide bacteriocin, where each of the peptides possesses an antibacterial activity. This was partially confirmed by the fact that we have obtained two clear zones in the zymogram, compatible with 60 and 120 kDa molecular mass markers (data not shown). Antibacterial compound secreted by strain 1K may contain carbohydrate moieties as the compound is sensitive to the treatment with α-amylase. The example of such two-peptide bacteriocin could be thermophilin 580, produced by *Streptococcus thermophilus* 580 strain **(Mathot *et al.*, 2003)**.

Screening for the presence and curing of plasmids

Isolated strains were screened for the possession of plasmids. From 36 strains of the first collection, 12 strains (3, 3K, 4, 5, 9, 13, 18, 19, 22, 32B, 32D, 33) had one large (>50 kb) plasmid each. Since all detected plasmids were similar in size, three strains – 3, 18 and 19 – were randomly chosen for further analysis. Based on the results of 16S rDNA analysis and DNA-DNA hybridization from previous studies, these strains were assigned to *G. stearothermophilus* species **(Pokusaeva *et al.*, 2009)**. The plasmids of the chosen strains were named pGS3, pGS18 and pGS19 respectively. Sizes of the plasmids from strains 3, 18 and 19 were evaluated by determining the sizes of restriction fragments. Plasmids pGS3 and pGS19 were about 52 kb in size. Plasmid pGS18 had molecular mass of about 62 kb.

Plasmids were detected only in three strains from the second collection. One strain (1.12) had two plasmids: one small (1.5 kb), named pGTG5, and one larger (>12 kb). Two strains (6.10 and 7.7) had three plasmids each: one large (>12 kb) and to small (~3.5 and ~3.0 kb). Aiming to determine possible cytoplasmic heredity of bacteriocins, we performed plasmid elimination experiments. After the plasmid elimination, strains have retained antibacterial activity.

DISCUSSION

There is a growing interest in thermophilic bacteria bacteriocins as they are characterized by the better stability, which is a favorable quality in the food industry. As most of bacteriocins, the ones produced by thermophilic bacteria are characterized by specific antimicrobial activity spectra which may vary depending on the environment of the producing species. Accordingly, in this work we analyzed two collections of thermophilic bacteria, isolated from different environmental niches: the Lithuanian oil wells or the surface soil above the oil pools. Environmental sources of isolated strains differ in several aspects such as temperature and the main nutrition sources. The temperature in the oil well is about 80^{o}C, so it is not surprising that the results of growth dependence on temperature have shown all strains from the first collection being obligate thermophiles, while the second collection contained obligate and facultative thermophiles.

Type of the main nutritional substance in the environment determines the variety of microorganisms that can assimilate it and colonize the niche. The main nutritional carbon source in the oil well is hydrocarbonates, while in the surface soil the main source is carbohydrates. Hydrocarbonates are not such a favorable carbon source for most microorganisms as carbohydrates, so the variety of bacterial species in the oil well should be poorer than in the surface soil. This fact can explain the results of antibacterial interactions between isolated strains and against pathogenic bacteria, which have shown that strains, isolated from the surface soil above the oil pools (second collection), possess higher and broader antibacterial activity spectra against other strains of the collection as well as against pathogenic bacteria than strains isolated from the oil wells (first collection). The reason for this could be that strains from the surface soil (second collection) evolved broader antibacterial spectrum during the evolution due to the possibility of higher interaction rate with other bacterial species compared with the strains living deep in the oil pools where the number of different bacterial species is considerably lower.

Physicochemical analysis of antibacterial compounds secreted by 11 strains has shown that all analyzed substances were sensitive to proteolytic enzymes, which indicates their proteinaceous nature. They were all secreted during the exponential growth phase and active against closely related thermophilic bacteria species. These results suggest that the investigated antibacterial substances are most likely bacteriocins. Moreover, the investigated bacteriocins remain stable in broad temperature and pH ranges. These properties and specific antibacterial activity spectrum against pathogenic bacteria, makes them favorable for the application in the food industry, veterinary or medicine.

Aiming to determine the location of bacteriocin encoding genes, strains were screened for the presence of plasmids. It was shown than the investigated collections also differ in the frequency of plasmids and their sizes. 33% of strains from the first collection had large plasmids (>50 kb), which coincides with the results obtained by **Ståhl (1991)**, as three of our investigated strains had been previously identified as *G. stearothermophilus* **(Pokusaeva *et al.*, 2009)**. Meanwhile only in three strains from the second collection small plasmids were detected. However, it is not known if the number and size of plasmids may be dependent on the inhabitation environment of the strain. Moreover, strains have not lost their antibacterial activity after the elimination of plasmids, which suggests that bacteriocin encoding genes of *Geobacillus* sp. bacteria are more likely localized in the chromosome. It may be because more stable genes of bacteria living in higher temperatures are localized in the chromosome and plasmids of thermophilic bacteria more often encode genes associated with resistance to high temperature **(Ståhl, 1991)**.

The study revealed that after a long storage (a year or more) at -70^{o}C or -20^{o}C, the isolated strains lose part of their antibacterial activity (data not shown). For example, strains, active against all or more than a half of the collection strains, after year and a half storage at -70^{o}C were able to inhibit only one or few collection strains. Unfortunately, causes of this phenomenon are unknown. Considering the properties of the investigated bacteriocins, which are beneficial for their application, efforts will be made to examine the causes of the activity loss.

CONCLUSION

The differences in the antibacterial activity spectra, optimal growth temperature, frequency and size of plasmids among strains isolated from Lithuanian oil wells and the surface soil above the oil pools show that the environmental niche has an influence on the properties of bacteriocins. This fact highlights the niches where bacteriocin producers with even broader antibacterial activity spectra may be found. Perspective physicochemical properties and the spectra of inhibited pathogenic bacteria species makes bacteriocins produced by *Geobacillus* sp. bacteria even more suitable for the application in the food industry, veterinary or medicine.

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